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Inhibitor Kappa B Kinase Beta Dependent Cytokine Upregulation in Nociceptive Neurons Contributes to Nociceptive Hypersensitivity After Sciatic Nerve Injury

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Abstract: Inhibitor kappa B kinase (IKK)-mediated nuclear factor-kappa B (NF-κB) activation is a major pathway for transcriptional control of various pro-inflammatory factors. We here assessed whether activation of this pathway specifically in primary nociceptive neurons of the dorsal root ganglia (DRG) contributes to the development of nociceptive hypersensitivity. Mice carrying a cre-loxP–mediated deletion of inhibitor kappa B kinase beta (IKKβ) in DRG neurons were protected from nerve injury–evoked allodynia and hyperalgesia. This effect was mimicked by systemic treatment with an IKKβ inhibitor but was not observed upon specific inhibition of IKKβ in the spinal cord, suggesting a specific role of IKKβ in the peripheral neurons. The deletion of IKKβ in DRG neurons did not affect constitutive neuronal NF-κB activity, but reduced nerve injury–evoked NF-κB stimulation in the DRG and was associated with reduced upregulation of interleukin-16, monocyte chemoattractant protein-1/chemokine (CC motif) ligand 2 (MCP-1/CCL2), and tumor necrosis factor alpha (TNFα) in the DRG. These cytokines evoked a rapid rise of intracellular calcium in subsets of primary DRG neurons. The results suggest that IKKβ-mediated NF-κB stimulation in injured primary sensory neurons promotes cytokine and chemokine production and contributes thereby to the development of chronic pain.

***Perspective:** Inhibitors of IKK that do not pass the blood-brain barrier and act only in the periphery might be useful for reduction of the pro-inflammatory response in peripheral DRG neurons and reduce thereby nerve injury-evoked pain without affecting neuroprotective effects of NF-κB in the central nervous system.*

***Key words:** NF-kappaB, dorsal root ganglia, neuropathic pain, tumor necrosis factor alpha, monocyte chemoattractant protein-1.*

Nuclear factor-kappa B (NF-κB) is a ubiquitously expressed transcription factor that activates inducible gene expression that is crucial for the regulation of inflammatory processes,[25,30] immunity,[44] and cell survival[16] and likely acts as an important mediator for the development of chronic pain.[34,51] Targeting of the NF-κB pathway is considered a potential novel approach in the treatment of chronic pain, mainly because of the stimulus-evoked pro-inflammatory role of NF-κB in immune cells. Classically, NF-κB activation is initiated by phosphorylation of the inhibitor kappa B (IκB) protein through the IκB kinase (IKK) complex.[17] This leads to degradation of IκB, allowing NF-κB to translocate into the nucleus and to initiate transcription of NF-κB-dependent genes. In neurons, IKK and NF-κB are also constitutively active[24] and may serve as signal transducers between neurites and nucleus and are considered to fulfill neuroprotective functions, presumably by transcriptional control of pro-survival genes.[24,32,39]

However, upon neuronal injury, the beta subunit of IKK (IKKβ) and NF-κB may also modify transcription of pro-inflammatory and pro-apoptotic genes in the neurons and may thereby contribute to the adaptations or maladaptations in nociceptive signaling pathways in response to ongoing stimulation or axonal injury. Hence, inhibition of IKK-mediated NF-κB activation in the

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treatment of pain may be a double-edged sword, because it may simultaneously suppress pro-inflammatory factors but also transcription of pro-survival genes. The idea of such duality is supported by findings in brain ischemia models where the outcome of IKK inhibition or deletion depended on the site and time course of IKK activation.[18,41,43]

To dissect out the site-specific functions of IKKβ-dependent NF-κB activation for chronic nerve injury–evoked nociception, we deleted IKKβ specifically in primary sensory neurons of the dorsal root ganglia (DRGs) and we blocked IKKβ pharmacologically. Analysis of the nociceptive behavior, production of cytokines in the dorsal root ganglia (DRG) and cytokine-evoked calcium fluxes in primary neurons reveal a pro-nociceptive role of IKKβ−NF-κB activation in primary sensory neurons upon axonal injury that overrides the baseline regulatory effects of constitutive IKKβ.

Methods

Animals

We generated mice deficient of IKKβ in peripheral primary sensory neurons (SNS-IKKβ$^{-/-}$) via cre-loxP–mediated recombination by mating mice carrying the IKKβ-flox allele (IKKβ$^{fl/fl}$) with a mouse line expressing cre recombinase under control of the Nav1.8 promoter (SNS-Cre).[1,6] The SNS-Cre mice enable gene recombination commencing at birth selectively in sensory neurons expressing the sodium channel Nav1.8, without affecting gene expression in the spinal cord, brain, or any other organ in the body.[1,2] Genotyping was done as described[1,38] and littermates were used for experiments. For pharmacological experiments we used male C57BL/6 mice (Charles River, Sulzfeld, Germany). Mice were housed in climate- and light-controlled quiet rooms, with free access to food and water. All experiments adhered to the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain. The local Ethics Committee for Animal Research (Darmstadt, Germany) approved the experimental protocol.

Treatments

An IKKβ inhibitor (Calbiochem IKK inhibitor VII) was used for systemic intraperitoneal delivery (1× or 2× daily, .75 mg/kg) and continuous intrathecal delivery (7.5 ng/g/hr) with chronically implanted spinal catheters and Alzet osmotic mini-pumps (DURECT Corporation, Cupertino, CA) (200 μL for 4 weeks). To enable intrathecal delivery at the level of lumbar spinal segments in mice, a polytetrafluoroethylene catheter (PTFE Sub-Lite Wall Tubing .05 mm inner diameter × .15 mm outer diameter; Braintree Scientific Inc., Braintree, MA) was stereotactically inserted under isoflurane anesthesia. At vertebra L4/5 the vertebral arch was removed with fine scissors and the catheter tip inserted and smoothly moved a few millimeters in rostral direction so that the tip reached a position above the lumbar spinal cord. The intrathecal catheter was attached to a silicone tube, which was connected to the outlet of the Alzet mini-pump. The Alzet pump was inserted into the subcutaneous space at the left flank. Correct positioning of the catheter tip was checked at the end of the treatment period by stereomicroscopic inspection and flushing of the catheter with a blue dye.

Behavioral Experiments

All tests were performed by an investigator (T.M.) blinded of the mouse genotype or treatment and included 8 to 10 mice per group as indicated in the respective figure legends. After habituation, we determined the latency for paw withdrawal using a Dynamic Plantar Aesthesiometer (Ugo Basile, Comerio, Italy) to assess the sensitivity to mechanical stimulation. The steel rod was pushed against the plantar paw with ascending force (0–5 g over a 10-second period, .5 g/second) and then maintained at 5 g until the paw was withdrawn. The paw withdrawal latency was the mean of 3 consecutive trials with at least 30-second intervals.

To assess the sensitivity to cold, we recorded the number of withdrawal reactions including shaking, lifting, or licking the paw after placing the mice onto a thermoelectric cold plate set at a constant temperature of 5°C (AHP-1200CPHC; Teca, Chicago, IL). Reactions were counted during a period of 90 seconds starting right after placing the mice onto the cold surface. To avoid cold stress for the mice, 2 tests were performed at each time point with an interval of 1 hour and averaged.

We assessed the sensitivity to painful heat stimuli by means of a hot plate at 52°C by recording the paw withdrawal latency with a stopwatch. The mean paw withdrawal latency of each 3 tests with at least .5- to 1-hour intervals was used for statistical analysis.

Behavioral tests were performed at baseline and then after spared nerve injury (SNI) at 3, 7, 14, 21, and 28 days, with somewhat higher testing frequency in experiments involving drug treatments. Exact time courses are shown in the respective figures.

Spared Nerve Injury

Surgery was performed under 1.5% to 2% isoflurane anesthesia. For the SNI model of neuropathic pain, 2 of the 3 peripheral branches of the sciatic nerve, the common peroneal and the tibial nerves, were ligated with silk (6-0) and distally transected, leaving the sural nerve intact.[10] Mechanical, heat, and cold withdrawal latencies were determined before and after SNI 2 to 3 times weekly up to 4 weeks after SNI.

Quantitative RT-PCR

Total RNA was extracted from mouse DRG tissue and reversely transcribed with random primers. Twenty nanograms DNA equivalent were subjected to real-time–polymerase chain reaction (RT-PCR) (AB 7500 Applied Biosystems; Life Technologies Corporation, Carlsbad, CA) using a FastStart Universal Master Mix (Roche Diagnostics, Mannheim, Germany) with Sybr Green fluorescence staining. Monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor alpha (TNFα),

and interleukin 16 (IL-16) mRNA were amplified with specific primers (Primer Express 2.0 software; Applied Biosystems) and normalized to 18S mRNA, which was detected with VIC-labeled 18S probe (Applied Biosystems). The comparative threshold cycle (C_T) method was used for quantification of relative mRNA expression. The following primer pairs were used:

TNFα FOR: 5′-GCTGAGCTCAAACCCTGGTA-3′
TNFα REV: 5′-CGGACTCCGCAAAGTCTAAG-3′
MCP-1/CCL2 FOR: 5′-CCAATGAGTAGGCTGGAGA-3′
MCP-1/CCL2 REV: 5′-TCTGGACCCATTCCTTCTG-3′
IL-16 FOR: 5′-GCCATTCAGCCTACACCAGT-3′
IL-16 REV: 5′-GACCAGAAAATCGTCCTCCA-3′

For IKKβ the primers 5′- GCAGTCTGTGCACGTCATTT -3′ and 5′- TATGTGTGAACGGTGCCTGT -3′ were used for RT-PCR analysis of IKKβ mRNA expression, with β-actin as housekeeping control gene. Riboprobes for p65 and p105/50 were obtained by in vitro transcription and labeling with digoxigenin (Dig-labeling kit, Roche Diagnostics). In situ hybridization was done as described.[6] Briefly, fresh frozen lumbar spinal cord was cut at 14 μm, fixed for 10 minutes in 4% paraformaldehyde in .1 M phosphate buffered saline (PBS), and acetylated. Sections were prehybridized for 2 hours at room temperature and hybridized at 70°C for 16 hours with 200 ng/mL of sense and antisense probes in the prehybridization mix (50% formamide, 5 × saline sodium citrate [SSC], 5 × Denhardt's solution, 500 μg/mL herring sperm DNA, 250 μg/mL yeast tRNA), washed in .2% SSC at 60°C, and incubated with anti-Dig-AP (1:1000; Roche Diagnostics) in .12 M maleic acid buffer with .15 M NaCl, pH 7.5, and 1% Blocking Reagent (Roche Diagnostics), washed in tris buffered solution, equilibrated in alkaline buffer (.1 M Tris-HCl, .1 M NaCl, .05 M MgCl2, pH 9.5, 2 mM levamisole) and developed with BM Purple AP substrate (Roche Diagnostics). Slides were embedded in glycerol/gelatin or postimmunostained.

Immunofluorescence

We perfused terminally anesthetized mice transcardially with .9 % saline followed by 4% paraformaldehyde in .1 M PBS (pH 7.4). The L4 and L5 spinal cord segments, DRG, and sciatic nerve were dissected and postfixed for 2 hours and then transferred into 20% sucrose in PBS for overnight cryoprotection at 4°C. The tissue was embedded in Tissue-Tek O.C.T. Compound (Science Services, Munich, Germany) and cut in transverse sections (10 μm for DRG, 14 μm for spinal cord) on a cryotome. Sections were permeabilized for 5 minutes in PBST (.1% Triton X-100 in .1 M PBS), blocked for 1 hour with 1% Blocking Reagent containing casein (Roche Diagnostics) in PBST, and incubated overnight at 4°C with primary antibodies dissolved in 1% Blocking Reagent in PBST. Antibodies directed against MCP-1, TNFα, and adenovirus were purchased from Abcam (Cambridge, UK) and antibodies-online GmbH (Aachen, Germany), respectively. After washes in PBS, we incubated the sections for 2 hours at room temperature with species-specific secondary antibodies conjugated with Alexa dyes (Invitrogen, Karlsruhe, Germany), subsequently rinsed in PBS, and cover-slipped in antifade medium. Fluorescent microscopic images were acquired using an inverted fluorescent microscope (Axiovert 200, Carl Zeiss, Jena, Germany) and analyzed with AxioVision 4.0 software (Carl Zeiss). For each animal, 3 midsections from the L5 DRG were analyzed and averaged. Three mice were analyzed per condition (naïve versus SNI) and genotype. The number of regions above the intensity threshold identifying immunoreactive neurons, mean intensity, pixel area, and the percentage of the area above threshold relative to the image area were calculated.

Western Blot Analysis

Tissue samples were homogenized in PhosphoSafe Buffer (Sigma, Steinheim, Germany) and protease inhibitor mixture (Complete, Roche Diagnostics). Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (30 μg/lane), transferred onto nitrocellulose membranes (Amersham Pharmacia Biotech, Freiburg, Germany) by wet-blotting and incubated with primary antibodies overnight at 4°C. We used heat shock protein of 90 kDa (HSP90) or beta-actin as loading control. Antibodies for IKKβ, phospho-IKKβ, IκBα, phospho-IκBα, p65, p105/50 were from Cell Signaling (NEB, Frankfurt, Germany) or Abcam. After incubation with the secondary antibody conjugated with IRDye 680 or 800 (1:10000; LI-COR Biosciences, Bad Homburg, Germany), blots were visualized and analyzed on the Odyssey Infrared Imaging System (LI-COR Biosciences). The ratio of the respective protein band to the control band was used for semiquantitative analysis.

NF-κB Activity

Nuclear extracts were prepared from DRG or spinal cord tissue using a Nuclear-Cytosol Fractionation Kit (PromoKine, Heidelberg, Germany). To assess NF-κB activity we analyzed nuclear extracts with the NF-κB Transcription factor ELISA kit from Active Motif (La Hulpe, Belgium) that allows for the detection and quantification of transcription factor activation by a combination of NF-κB specific oligonucleotide binding and subsequent detection of the p65 subunit with a specific antibody. The assay was performed as recommended by the manufacturer.

Cytokine/Chemokine Protein Array

A mouse cytokine array from R&D Systems (Wiesbaden, Germany) was performed according to the manufacturer's instructions. Briefly, the assay employs capture antibodies spotted in duplicate on nitrocellulose membranes. Protein extracts of DRG homogenates were mixed with biotinylated detection antibodies and then incubated with the array membranes. Complexes of the cytokine with the detection antibody were bound by the cognate immobilized capture antibodies and detected with streptavidin-HRP and chemiluminescent detection reagents. The analysis of the spot pixel density was done with ImageJ software. Pooled protein extracts

of the DRG of each 3 mice were used for duplicate array membranes.

Primary Neuron Cultures

Primary neuron-enriched cultures of DRG neurons were prepared by dissecting dorsal root ganglia of adult mice into Hank's Balanced Salt Solution (HBSS; Dulbecco) (Invitrogen) and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Invitrogen), followed by digestion with 5 mg/mL collagenase A and 1 mg/mL dispase II (Roche Diagnostics) prior to treatment with .25 % trypsin (Gibco-BRL, Karlsruhe, Germany). Triturated cells were centrifuged through a 10% bovine serum albumin solution, plated, and cultivated on poly-L-lysine and laminin coated cover slips in serum-free Neurobasal medium (Gibco-BRL) containing 2% (vol/vol) B27 supplement (Gibco-BRL), 50 μg/mL Pen-Strep (Sigma), 10 μM Ara-C (Sigma), 100 ng/mL NGF (Gibco-BRL), and 200 mM L-glutamine (Gibco-BRL) at 5% CO_2. Calcium imaging experiments were performed 24 to 36 hours after plating.

Calcium Imaging

Cultured adult DRG neurons were loaded for 1 hour with 10 μM fura-2 (Invitrogen) in Neurobasal medium with .02% Pluronic (Invitrogen) (wt/vol). For bath application, cover slips were transferred to a perfusion chamber on the stage of a fluorescence microscope (Axioskop 40, Carl Zeiss). Cells were illuminated with a xenon lamp and observed with a 10× Achroplan water-immersion objective lens (Carl Zeiss). Images were captured using a digital CCD camera (Photometrics, Roper Scientific, Ottobrunn, Germany) and analyzed using TILLvisION 4.0 software (Till Photonics, Gräfelfing, Germany). For puff application, a DMI6000 B fluorescence microscope was used (Leica; Wetzlar, Germany). It contains an external filter wheel (EL6000 Fura2 set; Leica), and a heated perfusion chamber (POC-mini, Tempcontrol 37-2digital, Leica), which kept the temperature at 37°C throughout the measurements. Cells were illuminated with an EL600 light source (Leica) and observed with a 40× HCX PL APO objective lens (Leica). Images were captured using a Leica DFC 360 Fx High Speed Kit Camera, captured and analyzed using LAS AF 6000 3.5.0 software (Leica).

We perfused the neurons continuously with Ringer solution (in mM: 136 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 0.33 NaH_2PO_4, 10 Glucose, 10 HEPES) at a speed of 2 mL/minute, captured images at a rate of 1 frame per 2 seconds, and measured intracellular $[Ca^{2+}]_i$ fluorimetrically as absorbance ratio at 340 nm and 380 nm (F340/380) (510 nm for emission). Baseline ratios were recorded for 60 seconds before puff application of IL-16 (for 2 seconds, 10–12 bar) and 100 seconds before bath application of chemokine (CC motif) ligand 2 (CCL2) or TNFα, respectively. Perfusion with CCL2 or TNFα lasted 180 seconds. All cytokines were applied at a concentration of 100 ng/mL. After a wash-out period, cells were perfused with high potassium (50 mM KCl in Ringer) to check the viability of the neurons. Data are presented as changes in fluorescence ratio (F340/F380) normalized to baseline ratios. The fraction of neurons responding to the stimulus with a >2-fold increase of $[Ca^{2+}]_i$ and the maximum-fold increase was used for statistical comparisons.

Statistics

We used SPSS 19.0 (SPSS Inc, Chicago, IL) for statistical evaluation. Data are presented as means ± SEM or SD as indicated. Calcium imaging results were analyzed by comparing the maximum response with Student t-tests and the fraction of responding cells with chi-square statistics. Behavioral data were analyzed using analysis of variance (ANOVA) for repeated measurements (rmANOVA) for time courses employing the within-subject factor time and between-subject factor genotype. For experiments involving drug treatments, one-way ANOVA or t-tests were used to compare the areas under the curve (AUCs). The latter were calculated according to the linear trapezoidal rule. Counts of immunoreactive neurons, quantitative real-time polymerase chain reaction (QRT-PCR), and Western blot results were analyzed with Student t-tests. *P* was set at .05 for all statistical comparisons. The number of animals used for the experiments and the number of replicate tests are given in the respective figure legends.

Results

Activation of IKKβ/NF-κB After Sciatic Nerve Injury

In the spinal cord, we observed an early increase of IκBα phosphorylation during the first 24 hours after nerve injury (Figs 1A, 2–6h) followed by a decrease of total IκBα (6h) showing its degradation. One to 3 weeks after the nerve injury, there was a late weak increase of the expression of IKKβ (Fig 1A).

In the DRG we found an increase of IKKβ mRNA and protein expression after SNI (Figs 1B and 1C) that was associated with an increase of the DNA binding activity of NF-κB in the DRG (ANOVA F = 4.396; *P* = .011) and transactivation of IκBα expression (Figs 1D and 1E). NF-κB DNA binding activity from SNI-treated $IKK\beta^{fl/fl}$ mice was significantly higher than that of the naïve $IKK\beta^{fl/fl}$ mice (*P* = .032). Such an increase did not occur in mice carrying a specific deletion of IKKβ in primary sensory neurons of the DRG (SNS-$IKK\beta^{-/-}$ mice) (Figs 1D and 1E) (*P* = .143).

Nerve Injury and Nociception in Sensory Neuron-Specific IKKβ Knockout Mice

SNI-evoked nociception was assessed in SNS-$IKK\beta^{-/-}$ mice, which lack IKKβ specifically in primary sensory neurons of the DRG. Naïve SNS-$IKK\beta^{-/-}$ mice showed significantly shorter reaction latencies to noxious heat or mechanical stimuli than $IKK\beta^{fl/fl}$ littermates (Fig 2). However, nociceptive sensitivity after SNI (Figs 2A–C) was reduced in SNS-$IKK\beta^{-/-}$ mice as compared to their $IKK\beta^{fl/fl}$ littermates. The protective effect of IKKβ deletion became apparent 3 days after nerve injury. ANOVA for repeated measurements revealed significant differences in the time courses between genotypes for all stimuli.

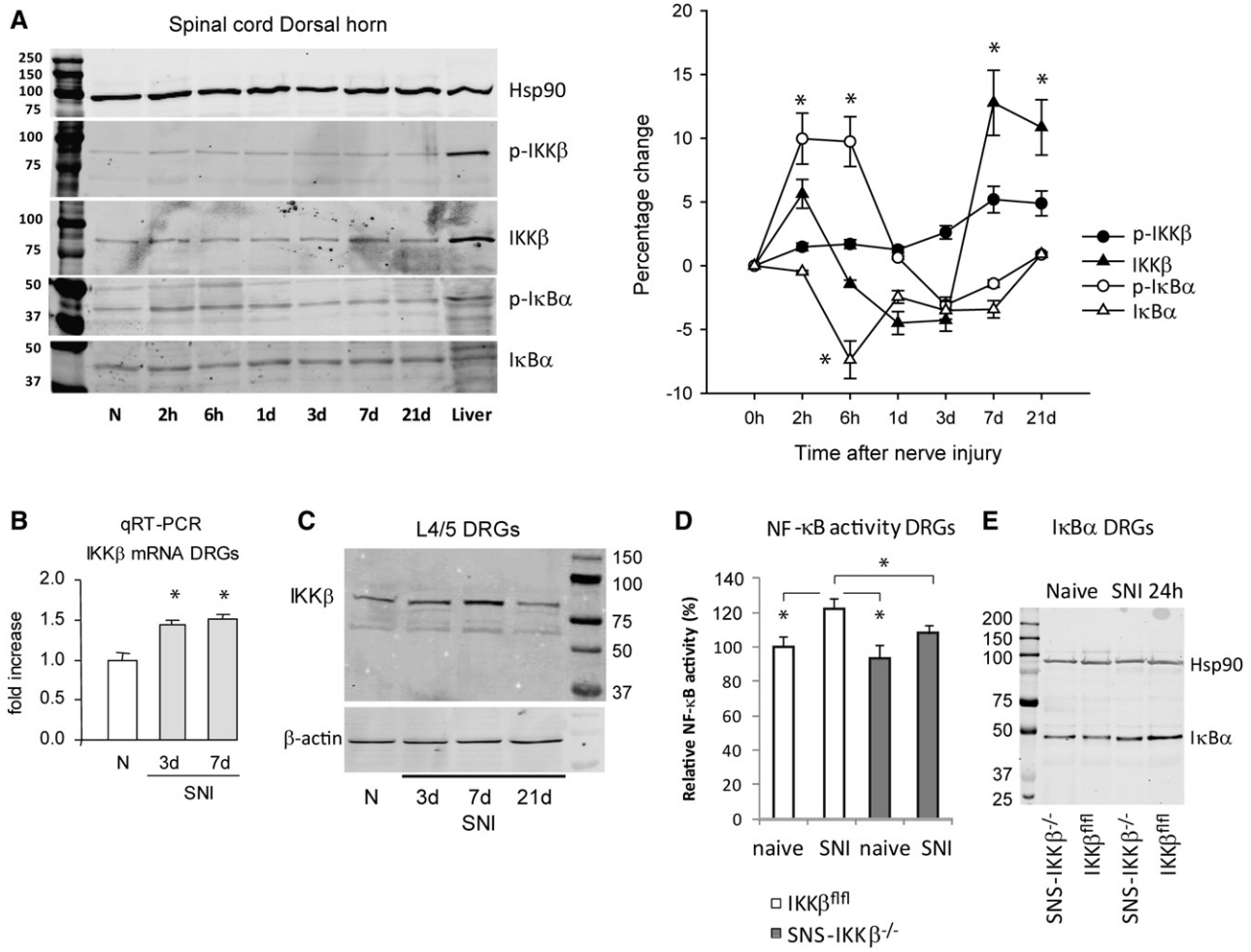

Figure 1. Expression and regulation of IKKβ in the spinal cord and DRG. **(A)** Western blot and semiquantitative analysis of IKKβ and IκBα expression and phosphorylation (p-IKKβ, p-IκBα) in the mouse spinal cord ipsilateral to a peripheral sciatic nerve injury in the SNI model of neuropathic pain. Hsp90 was used as loading control. The semiquantitative analysis represents 3 × 3 mice per time point, $P < .05$). **(B)** Quantitative RT-PCR of IKKβ mRNA in DRG of C57BL/6 mice after SNI and **(C)** Western blot of IKKβ protein expression in DRG after SNI. β-actin was used as loading control. **(D)** NF-κB DNA binding activity in DRG of IKKβ$^{fl/fl}$ mice and SNS-IKKβ$^{-/-}$ mice at baseline and 24 hours after SNI. **(E)** Expression of cytosolic IκBα in DRG at baseline and 24 hours after SNI of IKKβ$^{fl/fl}$ mice and SNS-IKKβ$^{-/-}$ mice. Data are representative results of 3 independent experiments. For the NF-κB activity assays, pooled tissue of each 3 animals was used for duplicate tests. The * indicates statistically significant differences versus baseline and between genotypes with $P < .05$.

For the interaction genotype × time, statistical results were as follows: mechanical F = 7.513, $P < .001$; heat F = 4.326, $P = .009$; cold F = 2.924, $P = .043$. For genotype, respective results were as follows: mechanical F = 4.592, $P = .042$; heat F = 5.254, $P = .030$; cold F = 3.055, $P = .092$. Similar statistical results for genotype were obtained when the AUCs from 3 to 24 days after SNI were compared per one-way ANOVA.

IKKβ Inhibition

We further assessed the role of IKKβ activation in the spinal cord. Whereas systemic treatment with an IKKβ inhibitor reduced mechanical and cold allodynia after SNI at a dose of .75 mg/kg twice daily as compared with vehicle-treated mice (Fig 3A mechanical: ANOVA F = 3.632, $P = .042$; vehicle versus IKKβ inhibitor twice daily $P = .042$; Fig 3B cold: ANOVA F = 10.325, $P = .001$; vehicle versus IKKβ inhibitor twice daily $P < .001$), no such consistent effect was achieved by spinal cord specific inhibition of IKKβ (Fig 4).

Spinal cord-specific inhibition of IKKβ was achieved by continuous intrathecal infusion of the IKKβ inhibitor through a lumbar spinal catheter for 4 weeks starting right after SNI. The efficacy of IKKβ inhibition was confirmed by Western blot analysis of phosphorylated IκBα (Fig 4A), which was reduced in the treatment group. IKKβ inhibition in lumbar spinal cord (Figs 4B–D) was not associated with a significant change of nociceptive behavior after SNI compared with vehicle-treated mice (t-test mechanical T = .596, $P = .561$; thermal T = 2.022, $P = .065$; cold T = 1.918, $P = .077$). A temporal reduction of heat hypersensitivity but increase of cold hypersensitivity occurred around 20 days after SNI in the treatment group, although neither effect reached statistical significance.

Regulation of Cytokines and Chemokines in IKKβ-Deficient DRG

Because the IKKβ-NF-κB pathway is a master regulatory pathway for stress-evoked transcriptional

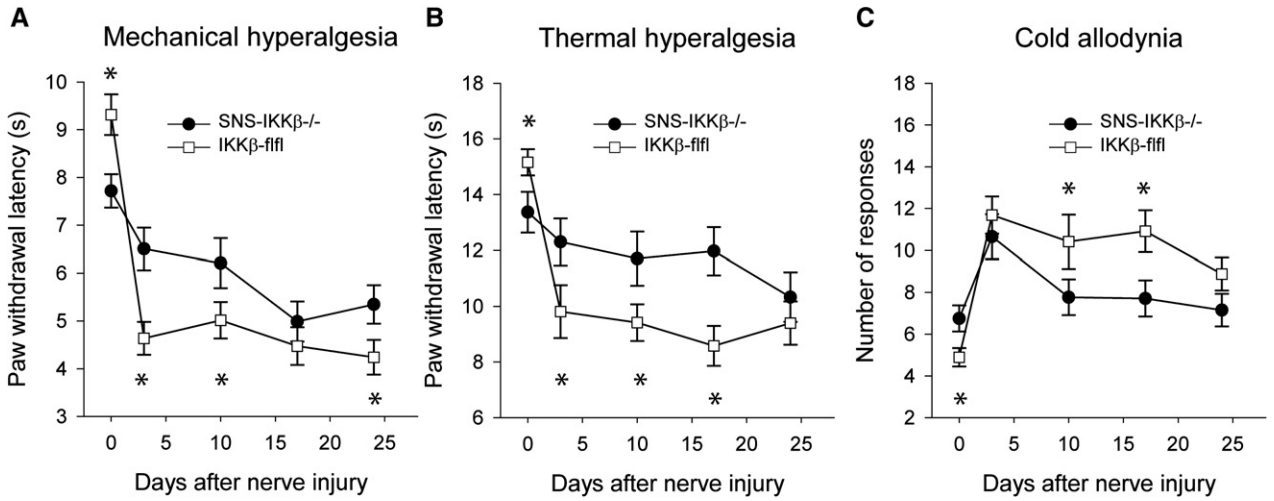

Figure 2. Nociceptive mechanical, thermal, and cold sensitivity in SNS-IKK$\beta^{-/-}$ and control IKK$\beta^{fl/fl}$ mice. **(A–C)** Withdrawal latencies in aesthesiometer and hot plate tests and number of withdrawal reactions in cold plate tests of SNS-IKK$\beta^{-/-}$ and control IKK$\beta^{fl/fl}$ mice before and after injury of the sciatic nerve in the SNI model. Data are means ± SEM of n = 9 to 10 mice in each group. The * indicates significant differences between genotypes ($P < .05$).

regulation of pro-inflammatory genes, we assessed whether the attenuation of nerve injury–evoked nociception in SNS-IKK$\beta^{-/-}$ mice resulted from an attenuated induction of pro-inflammatory factors. We screened the expression of an assortment of cytokines and chemokines in the DRG before and after nerve injury on a proteomic array. The array did not reveal differences in naïve mice (data not shown). However after SNI, upregulation of IL-16, TNFα, and MCP-1/CCL2 was abolished in SNS-IKK$\beta^{-/-}$ mice (Fig 5A). We confirmed the differential regulation of these cytokines by QRT-PCR (Figs 5B–D) and immunofluorescent analysis of DRG (Figs 6A and 6B, representative images; Figs 6C and 6D, quantitative analysis). Quantitative parameters for intensity and area provided unequivocal results. Figs 6C and 6D show the percentage of immunoreactive neurons with intensity above threshold relative to all neurons in the section.

TNFα-, CCL2-, and IL-16-Evoked Calcium Influx in Adult DRG Neurons

To assess the potential contribution of the cytokines in nociceptive signaling we analyzed effects of these pro-inflammatory factors on calcium fluxes in primary small- and medium-sized DRG neurons (Fig 7). Perfusion of primary sensory neurons with TNFα evoked an increase of intracellular calcium ($[Ca^{2+}]_i$) in about 70% of small- to medium-sized neurons (Table 1, Fig 7A). The effect was equivalent in neurons of SNS-IKK$\beta^{-/-}$ and IKK$\beta^{fl/fl}$ mice. Perfusion with MCP-1/CCL2 evoked an intracellular calcium rise in 45.7% of IKK$\beta^{fl/fl}$ neurons and in 32.8% of SNS-IKK$\beta^{-/-}$ neurons (Table 1, Fig 7B). The difference was statistically significant, suggesting a differential regulation not only of the chemokine CCL2 but also of its receptor, CCR2. Puff application with IL-16 resulted in an increase of $[Ca^{2+}]_i$ in 5% to 10% of neurons, with no difference between genotypes (Table 1, Fig 7C).

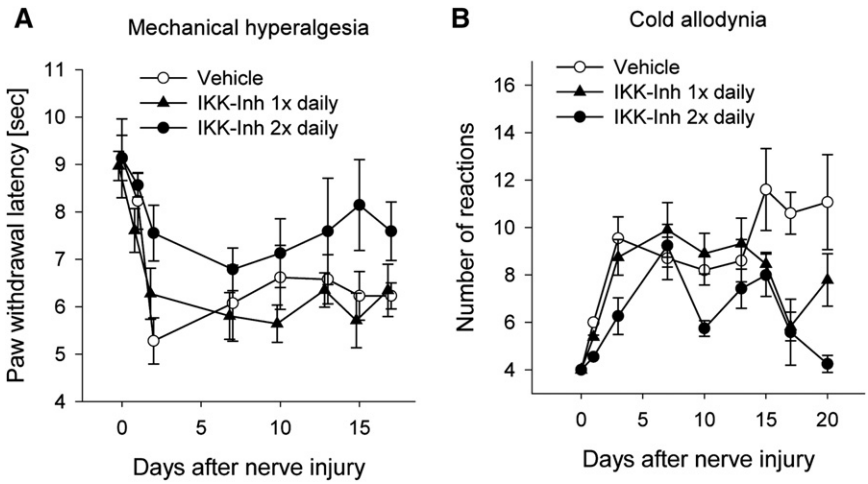

Figure 3. **(A, B)** Effects of IKKβ inhibition on nerve injury–evoked mechanical and cold nociception in the SNI model. Mice were treated with daily intraperitoneal injections (1× or 2× .75 mg/kg daily) of the IKKβ-inhibitor. Data are means ± SEM of 8 mice per group. Antinociceptive effects of the IKKβ-inhibitor twice daily were significant with ANOVA for AUCs, $P < .05$.

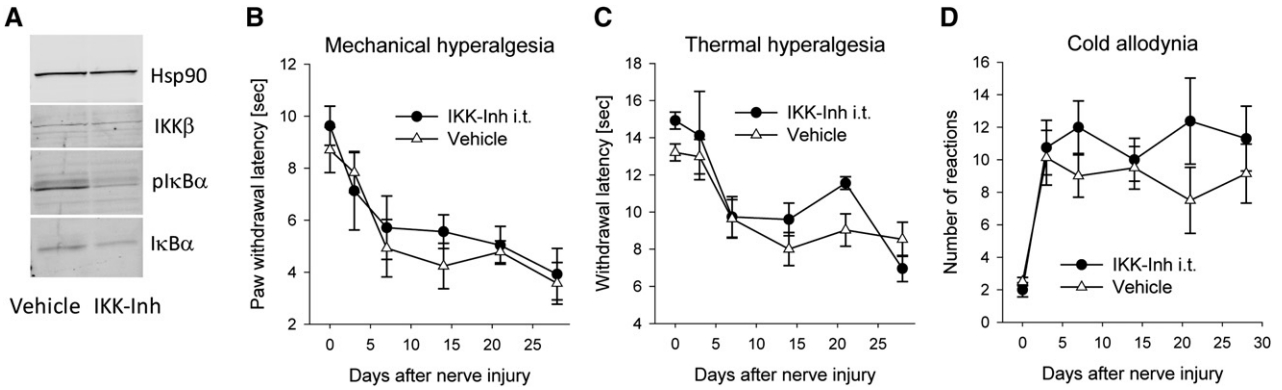

Figure 4. Nerve injury–evoked nociception with spinal cord-specific inhibition of IKKβ. **(A)** Western blot of the ipsilateral spinal cord of mice treated with IKKβ-inhibitor or vehicle at the end of the 4-week treatment period. The reduction of phospho-IκBα shows the efficacy of IKKβ-inhibitor in terms of blocking IKKβ activity. Hsp90 was used as loading control. **(B–D)** Nociceptive mechanical, thermal, and cold sensitivity in the SNI model in mice treated with intrathecally delivered IKKβ inhibitor. The drug was administered as a continuous intrathecal spinal infusion at a constant infusion rate of 7.5 ng/g/hr starting right after SNI surgery. Mechanical sensitivity was assessed by recording the paw withdrawal latency to mechanical stimulation using a Dynamic Plantar Aesthesiometer. Heat pain sensitivity was assessed in the hot plate (52°C) test, and cold pain sensitivity was quantified by measuring the number of withdrawal reactions on a cold plate at 5°C. Data are means ± SEM of n = 7 to 10 mice in each group. AUCs of the nociception versus time curves did not significantly differ between treatment groups.

Discussion

We showed in the present study that mice deficient of IKKβ in primary sensory neurons of the DRG develop attenuated neuropathic pain after sciatic nerve injury, which was associated with a reduced upregulation of TNFα, MCP-1, and IL-16 in the IKKβ-deficient DRG neurons and therefore suggests that the mechanisms involve an attenuated nerve injury–evoked and IKKβ-NF-κB-mediated upregulation of these cytokines. Because IKKβ-NF-κB was activated mainly in the DRG but only weakly in the spinal cord and because a spinally delivered IKKβ-inhibitor did not significantly reduce nociception, it is likely that the major site of the IKKβ-NF-κB-mediated pro-nociceptive adaptation was the injured or neighboring primary sensory neurons and much less so secondary neurons in the spinal cord.

We have shown previously that SNS-Cre mice have no alteration in acute responses to noxious heat and pressure[1] or to noxious chemical stimuli, such as capsaicin and formalin, nor do they differ from wild-type littermates with respect to development of chronic inflammatory or neuropathic pain,[2] showing that the alterations in nociception observed in SNS-IKKβ$^{-/-}$ mice do not arise from expression of cre recombinase in sensory neurons.

The attenuation of the nociceptive behavior after SNI in SNS-IKKβ$^{-/-}$ mice was unexpected because these mice have a higher sensitivity to TRPV1 stimulation at baseline.[6] This baseline effect on TRPV1 is likely mediated by the constitutively active IKKβ[6] and may involve alterations of the axonal transport and clustering of ion channels, whereas the additional activation observed herein of IKKβ-NF-κB in the DRG after nerve injury apparently promotes nociceptive hyperexcitability through transcriptional control of pro-inflammatory factors.

Based on the well-known key regulatory role of the IKK-NF-κB pathway for pro-inflammatory molecules, we hypothesized that a differential upregulation of pro-inflammatory factors might explain the observed antinociceptive effect of IKKβ-deletion in sensory neurons after nerve injury. In a cytokine screen we found a reduced upregulation of TNFα, MCP-1, and IL-16, suggesting that a differential regulation of these cytokines may explain in part the phenotype of SNS-IKKβ$^{-/-}$ mice. However, NF-κB controls the transcription of more than 200 different genes so that further differences in gene regulation might have contributed to the observed attenuated SNI-evoked nociception in SNS-IKKβ$^{-/-}$ mice. In addition, the upregulation of TNFα, MCP-1, and IL-16 may be further enhanced by other transcription factors, which are activated in the DRG upon axonal injury of the sciatic nerve, such as WW domain-containing oxidoreductase, c-Jun, and CREB.[29]

The axonal injury may also trigger direct IKKβ-mediated phosphorylation of proteins independent of NF-κB such as SNAP-23,[48] FOXO3a,[19] p53, and TSC1 (reviewed in Chariot[7]). Particularly, IKKβ-mediated phosphorylation, and thereby suppression of TSC1,[28] may increase endoplasmic reticulum stress and hyperexcitability[4,11] and may promote neuronal death after axonal injury. A potentially harmful outcome resulting from IKKβ activation in the brain has also been observed following inflammation or hypoxic insults,[18,40,57] suggesting that a pro-inflammatory drive that occurs with stressful stimuli may override the constitutive protective functions of NF-κB in neurons.[5,12,15,23,26]

The beneficial antinociceptive effects of DRG-specific deletion of IKKβ were mimicked by systemic pharmacological inhibition of IKKβ but not by spinal cord-specific inhibition of IKKβ, and NF-κB was mainly activated in the DRG but much less so in the spinal cord. Because we could demonstrate efficacy of spinal cord-specific inhibition of IKKβ in terms of IκBα phosphorylation, it is unlikely that the intrathecal treatment was just ineffective in terms of blocking IKKβ activity, but rather that IKKβ in the spinal cord did not considerably contribute to the nerve injury–evoked IKKβ-mediated pro-nociceptive adaptation. This was also suggested by the

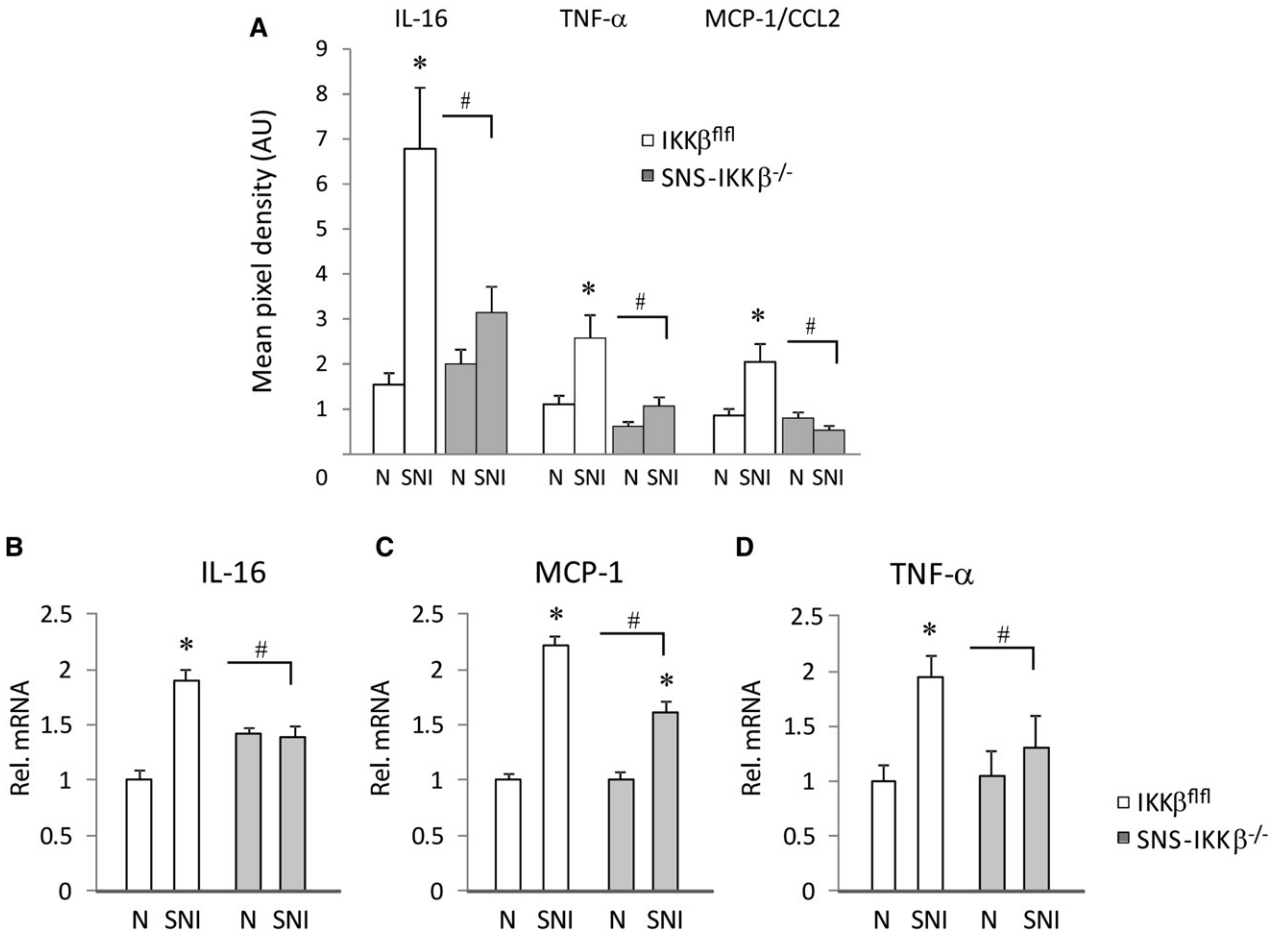

Figure 5. **(A)** IL-16, MCP-1/CCL2, and TNFα protein expression assessed with a cytokine/chemokine protein array in the ipsilateral L4/5 DRG of IKKβ$^{fl/fl}$ mice and SNS-IKKβ$^{-/-}$ mice in naïve mice (N) and 7 days after SNI. Results are means of triplicate arrays employing each pooled DRG tissue of 3 animals. Two-fold increases after SNI and >2-fold differences between genotypes were considered relevant. No differences were observed at baseline. After SNI, IL-16, MCP-1/CCL2, and TNFα differed between genotypes, $P < .05$. **(B–D)** QRT-PCR of IL-16, MCP-1/CCL2, and TNFα in the ipsilateral L4/5 DRG of IKKβ$^{fl/fl}$ mice and SNS-IKKβ$^{-/-}$ mice at baseline and 7 days after SNI. Data show results of 3 × 3 mice per genotype and time point. The * indicates significant differences versus the respective naïve mice and the # indicates the differences between the SNI-treated groups; $P < .05$.

unchanged amount of IKKβ protein, total and phosphorylated, in the spinal cord after nerve injury and the only weak and transient increase of phosphorylated IκBα at this site. We showed recently that NF-κB activation in the spinal cord after nociceptive stimulation strongly depended on the IKK-related kinase IKKε,[33] which was abundantly expressed in neurons and glia[33] and might adopt some of the stimulus-dependent NF-κB activation at this site and may also be involved in the glia activation in the spinal cord after nerve injury.

We infer that the DRG are the primary site of the IKKβ-mediated pro-nociceptive adaptations after nerve injury and likely depend on gene regulation. The time course of the nociceptive behavior after nerve injury and the reduced upregulation of TNFα, MCP-1/CCL2, and IL-16 in the DRG of SNS-IKKβ$^{-/-}$ mice were compatible with this idea. For TNFα and MCP-1/CCL2, pronociceptive effects have been extensively studied previously (see Liu et al,[31] Opree and Kress,[36] Parada et al,[37] Shafers et al,[42] and Xu et al[56] for TNFα and see de jong et al,[9] Jung et al,[20] Thacker et al,[52] and White et al[55] for CCL2). The release of TNFα and MCP-1 increases neurogenic inflammation and neuronal excitability by autocrine stimulation of the respective receptors, which are expressed on subsets of primary sensory neurons in the DRG, particularly in models of ongoing nociception,[3,8,22,55] and anti-TNFα-directed treatments strongly reduce neuropathic pain in animal models.[45,46] In agreement, we found that about two-thirds of small- to medium-sized primary DRG neurons showed an increase of intracellular calcium upon stimulation with TNFα.

For MCP-1, there is also strong evidence that it is released from neurons and contributes to the glia activation and development of neuropathic pain after nerve injury[47,49,55] that is promoted by upregulation of its receptor, CCR2.[55] The calcium response to MCP-1 was reduced in primary DRG neurons of SNS-IKKβ$^{-/-}$ mice as compared to the IKKβ$^{fl/fl}$ controls, suggesting that IKKβ-NF-κB stimulated not only the production of MCP-1 but also expression of its receptor, CCR2, which is a target gene of NF-κB but is also controlled by nuclear factor of activated T-cells in the DRG.[21]

The functions of IL-16 in the peripheral nervous system are so far elusive. In a mouse model of autoimmune

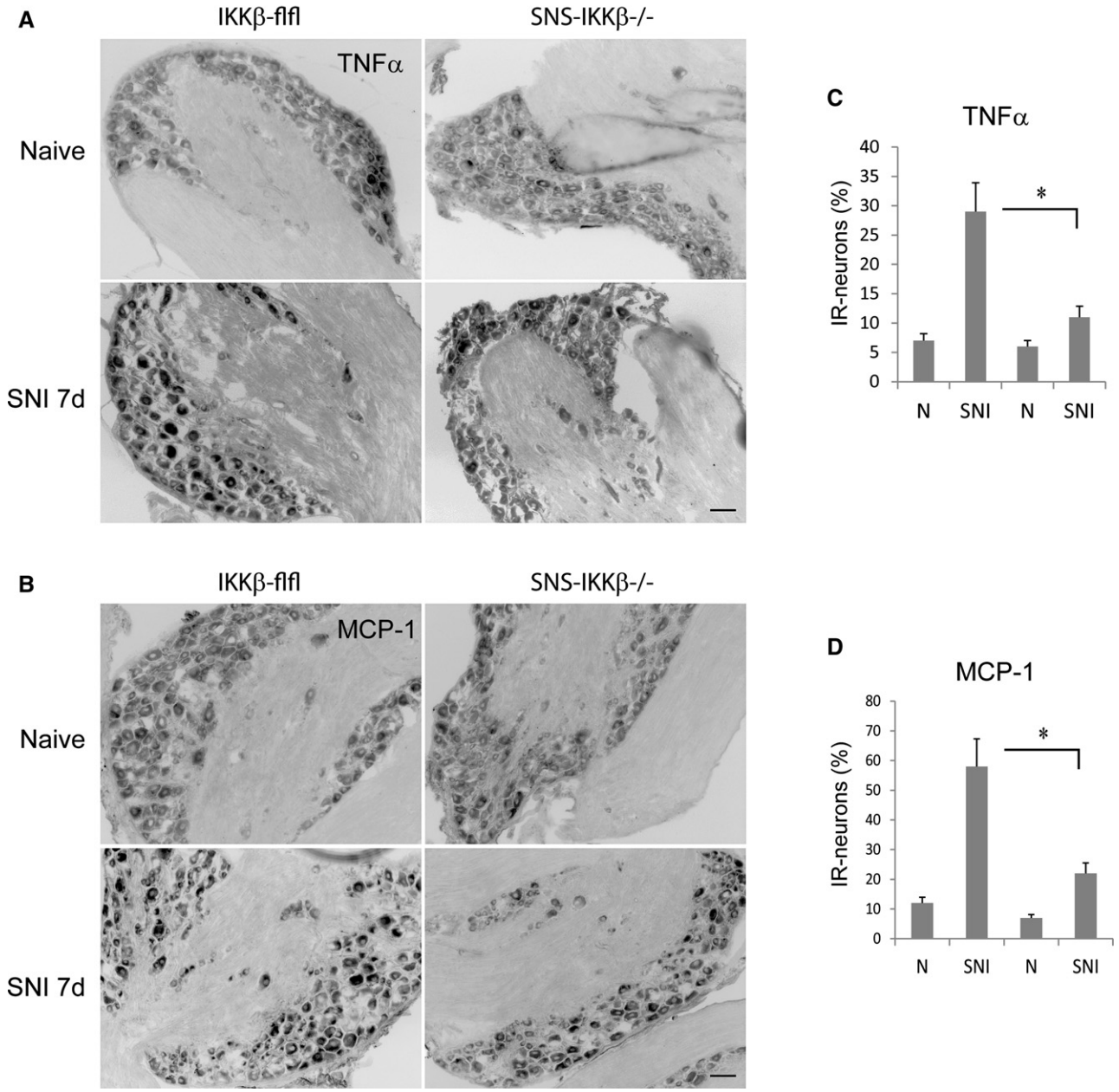

Figure 6. Immunofluorescent analysis of **(A, C)** TNFα and **(B, D)** MCP-1/CCL2 expression in L4/5 DRG in IKKβ$^{fl/fl}$ mice and SNS-IKKβ$^{-/-}$ mice in the naïve (N) and 7 days after SNI. For quantification **(C, D)** the number of immunoreactive neurons was counted. Results are the mean counts of 3 DRG sections per mouse from each 3 mice per group. Scale bar = 50 μm.

Table 1. Calcium Imaging in Primary Small- to Medium-Sized Neurons of the Dorsal Root Ganglia

	Genotype	Number of Neurons With $[Ca^{2+}]_i$ Increase*	Number of Neurons With No Change of $[Ca^{2+}]_i$	Percentage of Sensitive Neurons
TNFα	IKKβ$^{fl/fl}$	51	19	72.9
TNFα	SNS-IKKβ$^{-/-}$	43	18	70.5
MCP-1/CCL-2	IKKβ$^{fl/fl}$	58	69	45.7
MCP-1/CCL-2	SNS-IKKβ$^{-/-}$	40	82	32.8*
IL-16	IKKβ$^{fl/fl}$	8	130	5.8
IL-16	SNS-IKKβ$^{-/-}$	16	151	9.6

NOTE. The neurons showing a >2-fold rise of intracellular calcium ($[Ca^{2+}]i$) upon perfusion with TNFα or MCP-1/CCL2 or a >1.5-fold increase upon puff application of IL-16 were considered to be sensitive to the respective stimulus. The percentages of sensitive and insensitive neurons were compared using chi-square statistics.
*Indicates a statistically significant difference between genotypes with $P < .05$.

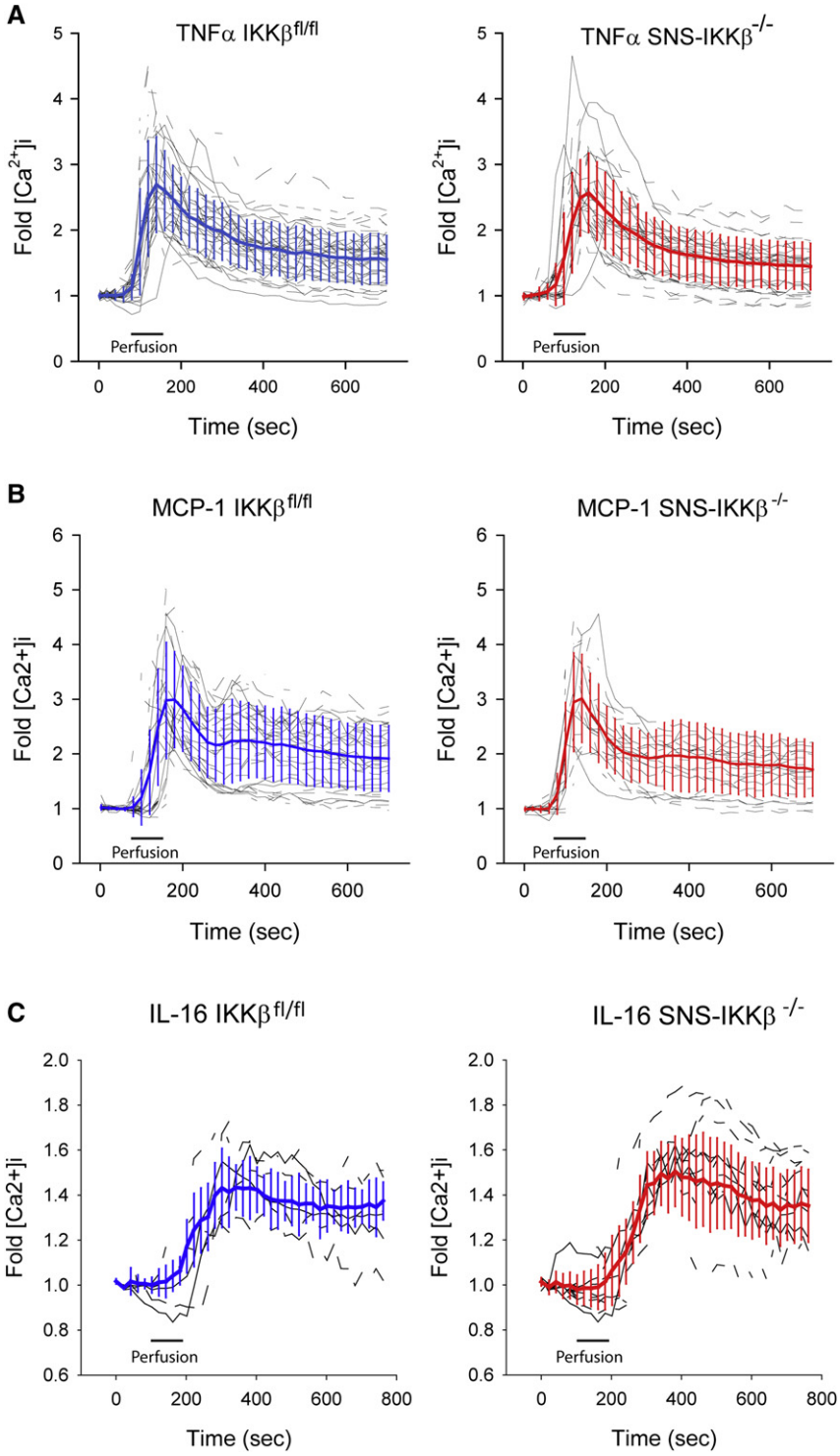

Figure 7. Calcium imaging in primary DRG neuron cultures of adult SNS-IKK$\beta^{-/-}$ and control IKK$\beta^{fl/fl}$ mice stimulated by perfusion with **(A)** 100 ng/mL TNFα, **(B)** 100 ng/mL MCP-1/CCL2, and **(C)** puff application of 100 ng/mL IL-16. $[Ca^{2+}]_i$ was measured fluorometrically in neurons loaded with fura-2 as absorbance ratio at 340 to 380 nm (ΔF 340/380). The figures show the fold changes of ΔF as compared to the baseline of individual responsive neurons and the mean ΔF $\pm$ SD as in blue and red. For MCP-1/CCL2, the number of neurons with increase of ΔF was significantly reduced in SNS-IKK$\beta^{-/-}$ cultures (Table 1). We analyzed n = 70 to 150 neurons for each stimulus of 4 to 6 mice in each group. Statistical results and number of neurons are shown in Table 1.

neuritis, IL-16 was detected in sciatic nerves, spinal roots, and spinal cord.[58] In neurons of the cerebellum and hippocampus, a neuron-specific IL-16 protein was described and referred to as NIL-16,[27] which regulates neuronal excitability through a modulation of potassium channel currents[14] and of voltage-gated calcium channels.[53] NIL-16 is processed by proteases, resulting in the release of IL-16, which may then act at its receptor, CD4, which is expressed mainly by immune cells but also by some neurons.[13,35] In line with this reasoning we observed that a small proportion of DRG neurons showed a calcium influx upon IL-16 stimulation, suggesting that CD4

positive DRG neurons may be activated by this cytokine. It is unclear though whether activation of this proportion of neurons contributes to the development of chronic pain after nerve injury.

In terms of pharmacological therapeutic advances that aim at targeting IKKβ for the inhibition of neuropathic pain,[34,50] our results suggest that potential drug candidates would profit from low brain penetration rates to act only in the periphery and avoid interference with constitutive functions of IKKβ in the central nervous system. Although constitutive IKKβ-NF-κB functions at axon initial segments are not completely understood, it is likely that NF-κB translates electrical activity into gene expression by shuttling between axons and nucleus.[32,54] IKKβ inhibitors not passing the blood-brain barrier would therefore avoid some toxicity without loss of antinociceptive functions mediated in part by attenuation of cytokine upregulation in DRG neurons after axonal injury.

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References

1. Agarwal N, Offermanns S, Kuner R: Conditional gene deletion in primary nociceptive neurons of trigeminal ganglia and dorsal root ganglia. Genesis 38:122-129, 2004

2. Agarwal N, Pacher P, Tegeder I, Amaya F, Constantin CE, Brenner GJ, Rubino T, Michalski CW, Marsicano G, Monory K, Mackie K, Marian C, Batkai S, Parolaro D, Fischer MJ, Reeh P, Kunos G, Kress M, Lutz B, Woolf CJ, Kuner R: Cannabinoids mediate analgesia largely via peripheral type 1 cannabinoid receptors in nociceptors. Nat Neurosci 10:870-879, 2007

3. Andratsch M, Mair N, Constantin CE, Scherbakov N, Benetti C, Quarta S, Vogl C, Sailer CA, Uceyler N, Brockhaus J, Martini R, Sommer C, Zeilhofer HU, Muller W, Kuner R, Davis JB, Rose-John S, Kress M: A key role for gp130 expressed on peripheral sensory nerves in pathological pain. J Neurosci 29:13473-13483, 2009

4. Bateup HS, Takasaki KT, Saulnier JL, Denefrio CL, Sabatini BL: Loss of Tsc1 in vivo impairs hippocampal mGluR-LTD and increases excitatory synaptic function. J Neurosci 31:8862-8869, 2011

5. Bhakar AL, Tannis LL, Zeindler C, Russo MP, Jobin C, Park DS, MacPherson S, Barker PA: Constitutive nuclear factor-kappa B activity is required for central neuron survival. J Neurosci 22:8466-8475, 2002

6. Bockhart V, Constantin CE, Haussler A, Wijnvoord N, Kanngiesser M, Myrczek T, Pickert G, Popp L, Sobotzik JM, Pasparakis M, Kuner R, Geisslinger G, Schultz C, Kress M, Tegeder I: Inhibitor kappaB Kinase beta deficiency in primary nociceptive neurons increases TRP channel sensitivity. J Neurosci 29:12919-12929, 2009

7. Chariot A: The NF-kappaB-independent functions of IKK subunits in immunity and cancer. Trends Cell Biol 19: 404-413, 2009

8. Constantin CE, Mair N, Sailer CA, Andratsch M, Xu ZZ, Blumer MJ, Scherbakov N, Davis JB, Bluethmann H, Ji RR, Kress M: Endogenous tumor necrosis factor alpha (TNFalpha) requires TNF receptor type 2 to generate heat hyperalgesia in a mouse cancer model. J Neurosci 28:5072-5081, 2008

9. de Jong EK, Dijkstra IM, Hensens M, Brouwer N, van Amerongen M, Liem RS, Boddeke HW, Biber K: Vesicle-mediated transport and release of CCL21 in endangered neurons: A possible explanation for microglia activation remote from a primary lesion. J Neurosci 25:7548-7557, 2005

10. Decosterd I, Woolf CJ: Spared nerve injury: An animal model of persistent peripheral neuropathic pain. Pain 87: 149-158, 2000

11. Di Nardo A, Kramvis I, Cho N, Sadowski A, Meikle L, Kwiatkowski DJ, Sahin M: Tuberous sclerosis complex activity is required to control neuronal stress responses in an mTOR-dependent manner. J Neurosci 29:5926-5937, 2009

12. Digicaylioglu M, Lipton SA: Erythropoietin-mediated neuroprotection involves cross-talk between Jak2 and NF-kappaB signalling cascades. Nature 412:641-647, 2001

13. Fenster CP, Chisnell HK, Fry CR, Fenster SD: The role of CD4-dependent signaling in interleukin-16 induced c-Fos expression and facilitation of neurite outgrowth in cerebellar granule neurons. Neurosci Lett 485:212-216, 2010

14. Fenster CP, Fenster SD, Leahy HP, Kurschner C, Blundon JA: Modulation of Kv4.2 K+ currents by neuronal interleukin-16, a PDZ domain-containing protein expressed in the hippocampus and cerebellum. Brain Res 1162:19-31, 2007

15. Fernyhough P, Smith DR, Schapansky J, Van Der Ploeg R, Gardiner NJ, Tweed CW, Kontos A, Freeman L, Purves-Tyson TD, Glazner GW: Activation of nuclear factor-kappaB via endogenous tumor necrosis factor alpha regulates survival of axotomized adult sensory neurons. J Neurosci 25: 1682-1690, 2005

16. Foehr ED, Lin X, O'Mahony A, Geleziunas R, Bradshaw RA, Greene WC: NF-kappa B signaling promotes both cell survival and neurite process formation in nerve growth factor-stimulated PC12 cells. J Neurosci 20: 7556-7563, 2000

17. Ghosh S, Karin M: Missing pieces in the NF-kappaB puzzle. Cell 109(Suppl):S81-S96, 2002

18. Herrmann O, Baumann B, de Lorenzi R, Muhammad S, Zhang W, Kleesiek J, Malfertheiner M, Kohrmann M, Potrovita I, Maegele I, Beyer C, Burke JR, Hasan MT, Bujard H, Wirth T, Pasparakis M, Schwaninger M: IKK mediates ischemia-induced neuronal death. Nat Med 11: 1322-1329, 2005

19. Hu MC, Lee DF, Xia W, Golfman LS, Ou-Yang F, Yang JY, Zou Y, Bao S, Hanada N, Saso H, Kobayashi R, Hung MC: IkappaB kinase promotes tumorigenesis through inhibition of forkhead FOXO3a. Cell 117:225-237, 2004

20. Jung H, Bhangoo S, Banisadr G, Freitag C, Ren D, White FA, Miller RJ: Visualization of chemokine receptor activation in transgenic mice reveals peripheral activation of

CCR2 receptors in states of neuropathic pain. J Neurosci 29: 8051-8062, 2009

21. Jung H, Miller RJ: Activation of the nuclear factor of activated T-cells (NFAT) mediates upregulation of CCR2 chemokine receptors in dorsal root ganglion (DRG) neurons: A possible mechanism for activity-dependent transcription in DRG neurons in association with neuropathic pain. Mol Cell Neurosci 37:170-177, 2008

22. Jung H, Toth PT, White FA, Miller RJ: Monocyte chemoattractant protein-1 functions as a neuromodulator in dorsal root ganglia neurons. J Neurochem 104:254-263, 2008

23. Kaltschmidt B, Uherek M, Wellmann H, Volk B, Kaltschmidt C: Inhibition of NF-kappaB potentiates amyloid beta-mediated neuronal apoptosis. Proc Natl Acad Sci U S A 96:9409-9414, 1999

24. Kaltschmidt C, Kaltschmidt B, Neumann H, Wekerle H, Baeuerle PA: Constitutive NF-kappa B activity in neurons. Mol Cell Biol 14:3981-3992, 1994

25. Karin M, Yamamoto Y, Wang QM: The IKK NF-kappa B system: A treasure trove for drug development. Nat Rev Drug Discov 3:17-26, 2004

26. Kassed CA, Butler TL, Patton GW, Demesquita DD, Navidomskis MT, Memet S, Israel A, Pennypacker KR: Injury-induced NF-kappaB activation in the hippocampus: Implications for neuronal survival. Faseb J 18:723-724, 2004

27. Kurschner C, Yuzaki M: Neuronal interleukin-16 (NIL-16): A dual function PDZ domain protein. J Neurosci 19: 7770-7780, 1999

28. Lee DF, Kuo HP, Chen CT, Hsu JM, Chou CK, Wei Y, Sun HL, Li LY, Ping B, Huang WC, He X, Hung JY, Lai CC, Ding Q, Su JL, Yang JY, Sahin AA, Hortobagyi GN, Tsai FJ, Tsai CH, Hung MC: IKK beta suppression of TSC1 links inflammation and tumor angiogenesis via the mTOR pathway. Cell 130: 440-455, 2007

29. Li MY, Lai FJ, Hsu LJ, Lo CP, Cheng CL, Lin SR, Lee MH, Chang JY, Subhan D, Tsai MS, Sze CI, Pugazhenthi S, Chang NS, Chen ST: Dramatic co-activation of WWOX/WOX1 with CREB and NF-kappaB in delayed loss of small dorsal root ganglion neurons upon sciatic nerve transection in rats. PLoS ONE 4:e7820, 2009

30. Li X, Massa PE, Hanidu A, Peet GW, Aro P, Savitt A, Mische S, Li J, Marcu KB: IKKalpha, IKKbeta, and NEMO/IKKgamma are each required for the NF-kappa B-mediated inflammatory response program. J Biol Chem 277:45129-45140, 2002

31. Liu B, Li H, Brull SJ, Zhang JM: Increased sensitivity of sensory neurons to tumor necrosis factor alpha in rats with chronic compression of the lumbar ganglia. J Neurophysiol 88:1393-1399, 2002

32. Mikenberg I, Widera D, Kaus A, Kaltschmidt B, Kaltschmidt C: Transcription factor NF-kappaB is transported to the nucleus via cytoplasmic dynein/dynactin motor complex in hippocampal neurons. PLoS ONE 2: e589, 2007

33. Moser CV, Kynast K, Baatz K, Russe OQ, Ferreiros N, Costiuk H, Lu R, Schmidtko A, Tegeder I, Geisslinger G, Niederberger E: The protein kinase IKK{varepsilon} is a potential target for the treatment of inflammatory hyperalgesia. J Immunol 187:2617-2625, 2011

34. Niederberger E, Geisslinger G: The IKK-NF-{kappa}B pathway: A source for novel molecular drug targets in pain therapy? Faseb J 22:3432-3442, 2008

35. Omri B, Crisanti P, Alliot F, Marty MC, Rutin J, Levallois C, Privat A, Pessac B: CD4 expression in neurons of the central nervous system. Int Immunol 6:377-385, 1994

36. Opree A, Kress M: Involvement of the proinflammatory cytokines tumor necrosis factor-alpha, IL-1 beta, and IL-6 but not IL-8 in the development of heat hyperalgesia: Effects on heat-evoked calcitonin gene-related peptide release from rat skin. J Neurosci 20:6289-6293, 2000

37. Parada CA, Yeh JJ, Joseph EK, Levine JD: Tumor necrosis factor receptor type-1 in sensory neurons contributes to induction of chronic enhancement of inflammatory hyperalgesia in rat. Eur J Neurosci 17:1847-1852, 2003

38. Pasparakis M, Courtois G, Hafner M, Schmidt-Supprian M, Nenci A, Toksoy A, Krampert M, Goebeler M, Gillitzer R, Israel A, Krieg T, Rajewsky K, Haase I: TNF-mediated inflammatory skin disease in mice with epidermis-specific deletion of IKK2. Nature 417: 861-866, 2002

39. Politi C, Del Turco D, Sie JM, Golinski PA, Tegeder I, Deller T, Schultz C: Accumulation of phosphorylated I kappaB alpha and activated IKK in nodes of Ranvier. Neuropathol Appl Neurobiol 34:357-365, 2008

40. Sarnico I, Boroni F, Benarese M, Alghisi M, Valerio A, Battistin L, Spano P, Pizzi M: Targeting IKK2 by pharmacological inhibitor AS602868 prevents excitotoxic injury to neurons and oligodendrocytes. J Neural Transm 115:693-701, 2008

41. Sarnico I, Lanzillotta A, Boroni F, Benarese M, Alghisi M, Schwaninger M, Inta I, Battistin L, Spano P, Pizzi M: NF-kappaB p50/RelA and c-Rel-containing dimers: Opposite regulators of neuron vulnerability to ischaemia. J Neurochem 108: 475-485, 2009

42. Schafers M, Svensson CI, Sommer C, Sorkin LS: Tumor necrosis factor-alpha induces mechanical allodynia after spinal nerve ligation by activation of p38 MAPK in primary sensory neurons. J Neurosci 23:2517-2521, 2003

43. Schneider A, Martin-Villalba A, Weih F, Vogel J, Wirth T, Schwaninger M: NF-kappaB is activated and promotes cell death in focal cerebral ischemia. Nat Med 5: 554-559, 1999

44. Sha WC, Liou HC, Tuomanen EI, Baltimore D: Targeted disruption of the p50 subunit of NF-kappa B leads to multifocal defects in immune responses. Cell 80:321-330, 1995

45. Sommer C, Lindenlaub T, Teuteberg P, Schafers M, Hartung T, Toyka KV: Anti-TNF-neutralizing antibodies reduce pain-related behavior in two different mouse models of painful mononeuropathy. Brain Res 913:86-89, 2001

46. Sommer C, Schafers M, Marziniak M, Toyka KV: Etanercept reduces hyperalgesia in experimental painful neuropathy. J Peripher Nerv Syst 6:67-72, 2001

47. Sun JH, Yang B, Donnelly DF, Ma C, LaMotte RH: MCP-1 enhances excitability of nociceptive neurons in chronically compressed dorsal root ganglia. J Neurophysiol 96: 2189-2199, 2006

48. Suzuki K, Verma IM: Phosphorylation of SNAP-23 by IkappaB kinase 2 regulates mast cell degranulation. Cell 134:485-495, 2008

49. Tanaka T, Minami M, Nakagawa T, Satoh M: Enhanced production of monocyte chemoattractant protein-1 in the dorsal root ganglia in a rat model of neuropathic pain:

Possible involvement in the development of neuropathic pain. Neurosci Res 48:463-469, 2004

50. Tegeder I, Niederberger E, Schmidt R, Kunz S, Guhring H, Ritzeler O, Michaelis M, Geisslinger G: Specific Inhibition of IkappaB kinase reduces hyperalgesia in inflammatory and neuropathic pain models in rats. J Neurosci 24:1637-1645, 2004

51. Tegeder I, Pfeilschifter J, Geisslinger G: Cyclooxygenase-independent actions of cyclooxygenase inhibitors. Faseb J 15:2057-2072, 2001

52. Thacker MA, Clark AK, Bishop T, Grist J, Yip PK, Moon LD, Thompson SW, Marchand F, McMahon SB: CCL2 is a key mediator of microglia activation in neuropathic pain states. Eur J Pain 13:263-272, 2009

53. Weick JP, Groth RD, Isaksen AL, Mermelstein PG: Interactions with PDZ proteins are required for L-type calcium channels to activate cAMP response element-binding protein-dependent gene expression. J Neurosci 23:3446-3456, 2003

54. Wellmann H, Kaltschmidt B, Kaltschmidt C: Retrograde transport of transcription factor NF-kappa B in living neurons. J Biol Chem 276:11821-11829, 2001

55. White FA, Sun J, Waters SM, Ma C, Ren D, Ripsch M, Steflik J, Cortright DN, Lamotte RH, Miller RJ: Excitatory monocyte chemoattractant protein-1 signaling is up-regulated in sensory neurons after chronic compression of the dorsal root ganglion. Proc Natl Acad Sci U S A 102:14092-14097, 2005

56. Xu JT, Xin WJ, Zang Y, Wu CY, Liu XG: The role of tumor necrosis factor-alpha in the neuropathic pain induced by Lumbar 5 ventral root transection in rat. Pain 123:306-321, 2006

57. Zhang F, Qian L, Flood PM, Shi JS, Hong JS, Gao HM: Inhibition of IkappaB kinase-beta protects dopamine neurons against lipopolysaccharide-induced neurotoxicity. J Pharmacol Exp Ther 333:822-833, 2010

58. Zhang ZY, Zhang Z, Fauser U, Schluesener HJ: Expression of interleukin-16 in sciatic nerves, spinal roots and spinal cords of experimental autoimmune neuritis rats. Brain Pathol 19:205-213, 2009