Inhibitor κ B Kinase β Deficiency in Primary Nociceptive Neurons Increases TRP Channel Sensitivity

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Inhibitor κ B kinase (IKK) regulates the activity of the transcription factor nuclear factor- κ B that normally protects neurons against excitotoxicity. Constitutively active IKK is enriched at axon initial segments and nodes of Ranvier (NR). We used mice with a Cre-loxP-mediated specific deletion of IKK β in sensory neurons of the dorsal root ganglion (*SNS-IKK β ^{-/-}*) to evaluate whether IKK plays a role in sensory neuron excitability and nociception. We observed increased sensitivity to mechanical, cold, noxious heat and chemical stimulation in *SNS-IKK β ^{-/-}* mice, with normal proprioceptive and motor functions as revealed by gait analysis. This was associated with increased calcium influx and increased inward currents in small- and medium-sized primary sensory neurons of *SNS-IKK β ^{-/-}* mice during stimulation with capsaicin or Formalin, specific activators of transient receptor potentials TRPV1 and TRPA1 calcium channels, respectively. *In vitro* stimulation of saphenous nerve preparations of *SNS-IKK β ^{-/-}* mice showed increased neuronal excitability of A- and C-fibers but unchanged A- and C-fiber conduction velocities, normal voltage-gated sodium channel currents, and normal accumulation of ankyrin G and the sodium channels Nav1.6 at NR. The results suggest that IKK β functions as a negative modulator of sensory neuron excitability, mediated at least in part by modulation of TRP channel sensitivity.

Introduction

Nuclear factor- κ B (NF- κ B) is a ubiquitously expressed transcription factor that modulates inducible gene expression crucial for the regulation of inflammatory processes (Li et al., 2002; Karin et al., 2004), immunity (Sha et al., 1995), and cell survival (Foehr et al., 2000). The classical NF- κ B heterodimer p50/p65 is mainly controlled by the inhibitory protein I κ B- α (nuclear factor κ B inhibitor), which inactivates NF- κ B by preventing its nuclear translocation (Ghosh and Karin, 2002). Phosphorylation of I κ B- α at serines 32 and 36, mediated by the activated I κ B kinase complex (IKK), leads to polyubiquitination and proteasomal degradation of I κ B- α , thus allowing nuclear translocation of NF- κ B. IKK consists of the catalytic subunits α and β (DiDonato et al., 1997; Mercurio et al., 1997) and a varying number of regulatory IKK γ subunits (Rothwarf et al., 1998). Its activation is mediated through phosphorylation of the catalytic

subunits (Delhase et al., 1999) by various kinases that are recruited to the IKK complex via IKK γ subunits (Yamaoka et al., 1998). After stimulation by these upstream kinases, IKK itself controls its additional activity by autophosphorylation that, at a certain level, results in inhibition of IKK activity and increased sensitivity to phosphatases (Delhase et al., 1999).

In addition to this canonical IKK-mediated NF- κ B activation pathway, NF- κ B is activated during tyrosine phosphorylation (Carter et al., 1996) of I κ B- α or phosphorylation-independent degradation of I κ B- α via calpain (Han et al., 1999; Scholzke et al., 2003), cathepsins, or other proteases (Schütze et al., 1992). In neurons, NF- κ B dimers are located in the cytoplasm and neurites and also constitutively in the nucleus (Kaltschmidt et al., 1994). The latter may be maintained by spontaneous calcium transients (Lilienbaum and Israël, 2003). Depolarization or stimulation with glutamate leads to a redistribution of NF- κ B from neurites to the nucleus, suggesting that NF- κ B may act as a signal transducer, transmitting transient glutamatergic signals from distant synaptic sites to the nucleus (Kaltschmidt et al., 1994). Supporting this idea, we demonstrated recently a striking enrichment of phosphorylated I κ B- α (phospho-I κ B- α) and activated IKK (phospho-IKK) in the axon initial segment (Schultz et al., 2006) and nodes of Ranvier (NR) (Politi et al., 2008) or along axonal fibers in unmyelinated nerves, providing the first evidence for compartmentalized NF- κ B activation at these sites (Schultz et al., 2006). The enrichment of active IKK at these sites suggested a specific role of IKK for sensory neuronal functions, including

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thermal, mechanical, and nociceptive sensitivity. To address this question, we used mice with a Cre-loxP-mediated specific deletion of IKK β in sensory neurons of the dorsal root ganglion (DRG) (*SNS-IKK β ^{-/-}*) and analyzed the sensitivity toward heat, cold, punctate pressure and noxious chemical stimuli, neuronal excitability, and sensitivity to capsaicin and Formalin, which are activators of transient receptor potential (TRP) calcium channels.

Materials and Methods

Animals. We generated mice deficient of the β subunit of IKK (IKK β) in peripheral primary sensory neurons (*SNS-IKK β ^{-/-}*) via Cre-loxP-mediated recombination by mating mice carrying the IKK β -floxed allele (IKK β ^{fl/fl}) (Pasparakis et al., 2002) with a mouse line expressing Cre recombinase under control of the Nav1.8 promoter (*SNS-Cre*) (Agarwal et al., 2004). 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 (Agarwal et al., 2004, 2007). Genotyping was done as described previously (Pasparakis et al., 2002; Agarwal et al., 2004) and littermates were used for experiments. The mice were housed in climate- and light-controlled quiet rooms. 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.

Reverse transcription-PCR and in situ hybridization. Total RNA was extracted from mouse DRG tissue and reversely transcribed with random primers. The primers 5'-GCAGTCTGTGCACGTCATTT-3' and 5'-TATGTGTGAACGGTGCCTGT-3' were used for reverse transcription (RT)-PCR analysis of IKK β , with β -actin as housekeeping control gene. These primers were also used to synthesize a fragment corresponding to nucleotides 548–995 of IKK β (the adenine of the ATG referred to as nucleotide 1) by PCR. The fragment was cloned into the pCR4-TOPO plasmid vector (Invitrogen) and sequenced. Riboprobes were obtained by *in vitro* transcription and labeling with digoxigenin (Dig-labeling kit; Roche Diagnostics).

Fresh frozen DRGs were cut at 14 μ m, fixed for 10 min in 4% paraformaldehyde in 0.1 M PBS, and acetylated. Sections were prehybridized for 2 h at room temperature and hybridized at 70°C for 16 h with 200 ng/ml sense and antisense probes in the prehybridization mix (50% formamide, 5 $\times$ SSC, 5 $\times$ Denhardt's solution, 500 μ g/ml herring sperm DNA, and 250 μ g/ml yeast tRNA) (Tegeder et al., 2006), washed in 0.2% SSC at 60°C and incubated with anti-Dig-alkaline phosphatase (AP) (1:1000; Roche Diagnostics) in 0.12 M maleic acid buffer with 0.15 M NaCl, pH 7.5, and 1% Blocking Reagent (Roche Diagnostics), washed in TBS, equilibrated in alkaline buffer (0.1 M Tris-HCl, 0.1 M NaCl, 0.05 M MgCl₂, pH 9.5, and 2 mM levamisole), and developed with BM Purple AP substrate (Roche Diagnostics). Slides were embedded in glycerol/gelatin or were further processed for post-*in situ* immunofluorescence studies. Images were ob-

Figure 1. Tissue-specific deletion of IKK β in neurons of the dorsal root ganglia. *a*, *In situ* hybridization of IKK β in naive *SNS-IKK β ^{-/-}* and control *IKK β ^{fl/fl}* mice. *b*, RT-PCR analysis of IKK β mRNA expression in DRG and spinal cord (SC) tissue of naive *SNS-IKK β ^{-/-}* and control *IKK β ^{fl/fl}* mice. β -Actin was used as housekeeping control gene. *c*, IKK β protein expression in cytosolic extracts of dorsal root ganglia and spinal cord and in cytosolic and membrane fractions of the frontal cortex of naive *SNS-IKK β ^{-/-}* and control *IKK β ^{fl/fl}* mice. β -Actin was used as loading control. *d*, Nuclear NF- κ B DNA binding activity in DRGs of naive *SNS-IKK β ^{-/-}* and control *IKK β ^{fl/fl}* mice. Data are representative results of three independent experiments. For Western blots and NF- κ B activity assay, pooled tissue of three animals was used.

tained using an Eclipse E600 fluorescence microscope equipped with a Kappa DX 20 H camera and Kappa ImageBase software.

Quantitative real-time PCR of TRPV1 was performed on a TaqMan (Applied Biosystems) using the Sybrgreen detection system with primer sets designed on Primer Express. Specific PCR product amplification was confirmed with gel electrophoresis. Transcript regulation was determined using the relative standard curve method per the instructions of the manufacturer (Applied Biosystems).

Western blot analysis. Tissue samples were homogenized in Phospho-Safe Buffer (Sigma) and protease inhibitor mixture (Complete; Roche

Diagnostics). Proteins were separated by SDS-PAGE (30 μ g/lane), transferred onto nitrocellulose membranes (GE Healthcare) by wet blotting, and incubated with primary antibodies overnight at 4°C. We used β -actin or heat shock protein (HSP90) as loading controls. After incubation with the secondary antibody conjugated with IRDye 680 or 800 (1:10,000; LI-COR Biosciences), 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. Primary antibodies were for IKK β (catalog #2684; Cell Signaling Technology) and TRPV1 [RA10110, rabbit N-terminal (Neuromics); Sc-12498, goat C-terminal (Santa Cruz Biotechnology)].

NF- κ B activity. Nuclear extracts were prepared from DRGs of naive mice using a Nuclear-Cytosol Fractionation kit (PromoKine). To assess NF- κ B activity, we analyzed nuclear extracts with the NF- κ B Transcription factor ELISA kit from ActifMotif that allows for the detection and quantification of transcription factor activation by a combination of NF- κ B-specific oligonucleotide binding and subsequent detection of the p65 subunit with a specific antibody. The assay was performed as recommended by the manufacturer.

Behavioral experiments. All tests were performed by an investigator blinded of the mouse genotype. After habituation, we determined the latency for paw withdrawal using a Dynamic Plantar Aesthesiometer (Ugo Basile) to assess the sensitivity to mechanical stimulation. The steel rod was pushed against the paw with ascending force (0–5 g over a 10 s period, 0.2 g/s) and then maintained at 5 g until the paw was withdrawn. The paw-withdrawal latency was the mean of three consecutive trials with at least 30 s intervals. To assess the sensitivity to cold, we recorded the number of flinches, paw lickings, and withdrawals on a cold plate at 5°C during a period of 90 s starting immediately after placing the mouse onto the plate (AHP-1200CPHC; Teca). We assessed the sensitivity to painful heat stimuli by means of a hotplate at 52°C and by means of the tail-flick test with a radiant heat source. The mean paw-withdrawal or tail-flick latency of each three tests with at least 1 h intervals was used for statistical analysis. We used the Formalin test and the capsaicin test to assess chemically evoked acute A-fiber-mediated nociception in the first phase and C-fiber sensitization in the second phase of the test. We injected 20 μ l of 5% Formalin or 0.1% capsaicin into a hindpaw and monitored the licking time for 45 min in 5 min intervals starting directly after injection of the irritant into the paw. The time the mouse spent licking the paw during the first and second phases of the test was used for statistical comparisons. We performed rotarod tests and a gait analysis to assess sensorimotor functions. Gait analysis was performed on footprints obtained by painting the hindpaws of mice with nontoxic blue and the forepaws with red dye and having them walk on paper along a 50-cm-long, 8-cm-wide runway, with 6-cm-high walls on either side. Seven consecutive steps were used to determine several gait parameters, including stride length, stride width, linearity, and uniformity of step alternation (Patel and Hillard, 2001).

Nerve conduction velocity and activation thresholds. To evaluate the potential role of IKK β for nerve conduction velocity and sensory neuron excitability, we used extracellular recordings of action potentials in single fibers isolated from the saphenous nerve of SNS-IKK $\beta^{-/-}$ mice and control IKK $\beta^{+/+}$ littermates (Agarwal et al., 2007). Briefly, the saphenous nerve was dissected; the proximal end was mounted into an organ bath chamber and superfused with an oxygen-saturated modified synthetic interstitial fluid solution containing 108 mM NaCl, 3.48 mM KCl, 3.5 mM MgSO₄, 26 mM NaHCO₃, 1.7 mM NaH₂PO₄, 2.0 mM CaCl₂, 9.6 mM sodium gluconate, 5.5 mM glucose, and 7.6 mM sucrose at a temperature of 31 $\pm$ 1°C and pH 7.4 $\pm$ 0.05. The distal end of the saphenous nerve was pulled into a separate chamber, and fine filaments were placed on a gold wire recording electrode. Action potentials in single sensory neurons were recorded extracellularly, amplified (5000-fold), filtered (low pass at 1 kHz, high pass at 100 Hz), visualized on an oscilloscope, and stored on a personal computer-type computer. For data storage and offline analysis, the Spike/Spidi software package was used (Forster and Handwerker, 1990). The nerve trunk was stimulated with square-wave pulses of increasing magnitude (up to 100 V) and the activation threshold and conduction velocity of the nerve fibers were computed. Fibers conducting

Figure 2. Nociceptive thermal, mechanical, and cold sensitivity in naive SNS-IKK $\beta^{-/-}$ and control IKK $\beta^{+/+}$ mice. **a**, Mechanical sensitivity was assessed by recording the paw-withdrawal latency to punctate mechanical stimulation using a Dynamic Plantar Aesthesiometer. Heat pain sensitivity was assessed in the tail-flick and hotplate (52°C) tests by recording the tail or paw-withdrawal latency. Cold pain sensitivity was quantified by counting the number of flinches and paw-withdrawals during a 90 s exposure to a cold plate at 5°C. **b**, Chemical nociceptive sensitivity was assessed by recording the time course of paw-licking behavior after injection of Formalin into a hindpaw and summary of the total licking time in the first (1–10 min) and second (11–45 min) phases of the Formalin test. **c**, Capsaicin-evoked nociception was analyzed in analogy to the Formalin test by recording the time course of paw-licking behavior after injection of 0.1% capsaicin into a hindpaw. Data are means $\pm$ SEM of $n = 9$ –12 in each group; * $p < 0.05$.

with conduction velocity < 2 m/s were considered as unmyelinated C-fibers.

Primary neuron cultures. Primary neuron-enriched cultures of DRG neurons were prepared by dissecting dorsal root ganglia of adult mice into HBSS (Dulbecco's) and 10 mM HEPES, followed by digestion with 5 mg/ml collagenase A and 1 mg/ml dispase II (Roche Diagnostics) before treatment with 0.25% trypsin (Invitrogen). Triturated cells were centrifuged through a 10% bovine serum albumin (BSA) solution before plating and cultivating them on poly-L-lysine- and laminin-coated coverslips in serum-free Neurobasal medium (Invitrogen) containing 2% (v/v) B27 supplement (Invitrogen), 50 μ g/ml penicillin–streptomycin (Sigma), 10 μ M AraC (Sigma), 100 ng/ml NGF (Invitrogen), and 200 mM L-glutamine (Invitrogen) at 5% CO₂. Calcium imaging experiments and recordings of capsaicin-activated and voltage-gated sodium currents were performed 24–36 h after plating.

Calcium imaging. Cultured adult DRG neurons were loaded for 1 h with 5 μ M fura-2 (Invitrogen) in Neurobasal medium with 0.02% Pluronic (w/v) and then rinsed for 30 min in Ringer's solution composed of the following (in mM): 145 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 10 glucose, and 10 HEPES. Coverslips 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 40 $\times$ Achromplan water-immersion objective lens (Carl Zeiss). We perfused the neurons continuously with Ringer's solution at 2 ml/min and measured intracellular $[Ca^{2+}]_i$ fluorimetrically as absorbance ratio at 340 and 380 nm (F_{340}/F_{380}) (510 nm for emission). Images were captured at a rate of 1 frame per second using a digital CCD camera (Photometrics; Roper Scientific), captured, and analyzed using TILLvisION 4.0 software (TILL Photonics). Baseline ratios were recorded for 100 s before bath application of 0.1 or 1 μ M capsaicin, 100 μ M ATP or 100 μ M UTP, or 0.01% Formalin for 50 s. After a wash-out period, cells were perfused with high potassium (50 mM KCl in Ringer's solution) to check the viability of the neurons. Data are presented as changes in fluorescence ratio (F_{340}/F_{380}) normalized to baseline ratios. The fraction of neurons responding to the stimulus with a >1.5-fold increase of $[Ca^{2+}]_i$ and the maximum fold increase was used for statistical comparisons.

Patch-clamp recording of capsaicin-activated currents and voltage-gated Na^+ currents. Using the whole-cell voltage-clamp configuration of the patch-clamp technique, capsaicin-activated currents were recorded from isolated neurons as described previously (Obreja et al., 2002). External solution contained the following (in mM): 150 NaCl, 5 KCl, 0.1 $CaCl_2$, and 1 $MgCl_2$ (all from Sigma-Aldrich), and 10 glucose and 10 HEPES (both from Merck), at pH 7.3 adjusted with NaOH (Merck). Patch-clamp pipettes from borosilicate glass (Science Products), filled with internal solution [in mM: 138 KCl, 2 $MgCl_2$, 2 Na_2 -ATP, 0.5 $CaCl_2$, 0.2 Li-GTP, and 5 EGTA (all from Sigma-Aldrich) and 10 HEPES (Merck), at pH 7.3 adjusted with KOH (Merck)], had a resistance of 4–5 M Ω . Currents were filtered at 2.9 kHz, sampled at 3 kHz, and recorded using an EPC 10 (HEKA) and the Pulse version 8.74 software (HEKA). Capsaicin-activated inward currents (I_{Caps}) were evoked at -80 mV holding potential. A seven-barrel system with common outlet was used for fast application of increasing capsaicin concentrations of 10 nM to 10 μ M (Dittert et al., 1998). Capsaicin was purchased from Sigma-Aldrich.

Using the voltage-clamp configuration, voltage-gated sodium currents were recorded in small-diameter DRG neurons at room temperature using an EPC-10 amplifier and Pulse software program version 8.78 (HEKA). Patch-clamp pipettes from borosilicate glass (Science Products) were fabricated using a P-97 puller (Sutter Instruments) and fire-polished to have resistances of 2.5–3.7 M Ω after filling with the pipette solution containing the following (in mM): 138 cesium methane sulfonate, 10 NaCl, 0.1 $CaCl_2$, 2 $MgCl_2$, 1 EGTA, 2 Na_2 -ATP, and 10 HEPES, adjusted to 7.3 with CsOH. The standard bath solution contained the following (in mM): 125 NaCl, 5 cesium methane sulfonate, 1 $MgCl_2$, 2 $CaCl_2$, 0.5 $CdCl_2$, 20 tetraethylammonium (TEA)-Cl, 10 D-glucose, and 10 HEPES, adjusted to pH 7.3 with NaOH. TEA-Cl was added to block voltage-activated K^+ currents, and Cd^{2+} was included to block voltage-activated Ca^{2+} currents. Voltage errors were minimized by using 80% series resistance compensation. Leakage currents were digitally subtracted by the P/5 procedure, based on

naive

- (1) Stride length
- (2) Stride width
- (3) Intrastep distance
- (4) Coordination

Figure 3. Motor functions in naive *SNS-IKK $\beta^{-/-}$* and control *IKK $\beta^{fl/fl}$* mice. Gait analysis was performed after painting forepaws with red and hindpaws with blue dye. Stride length, width, intrastep distance, and coordination were analyzed as indicated. Data are means $\pm$ SEM of $n = 12$ in each group.

resistance estimates from five hyperpolarizing pulses applied before the prepulse potential. Membrane currents were filtered at 2.9 Hz and digitally sampled at 20 kHz. Cell capacitance was used as a measure of cell size and was estimated by the electronic cancellation circuitry on the amplifier.

Voltage-gated Na^+ currents were evoked by 40 ms depolarizing steps to potentials ranging from -80 to $+60$ mV in 10 mV increments. To separate and quantify tetrodotoxin (TTX)-sensitive (TTX-S) and TTX-resistant (TTX-R) current components, a prepulse inactivation protocol was used. Prepulse inactivation takes advantage of the differences in the inactivation properties of the TTX-S and TTX-R currents. When in the voltage-clamp protocol a prepulse of 500 ms to -120 mV preceded the step

Figure 4. *a, b*, Electrophysiological recordings from A- and C-fibers of the saphenous nerve in an *in vitro* nerve preparation derived from *SNS-IKKβ*^{−/−} and control *IKKβ*^{fl/fl} mice ($n = 11$ in each group). Fibers were identified by conduction velocities. The number of fibers responding at a threshold range or conducting at a certain range of velocities were recorded and compared by χ^2 statistics. The percentage of A- and C-fibers responding at 0.1–5 V was significantly higher in *SNS-IKKβ*^{−/−} (84.6 and 73.0%) than in control *IKKβ*^{fl/fl} mice (63.1 and 33.3%), indicating enhanced sensitivity ($p < 0.01$, χ^2 test). Fibers ($n = 65$ –87, as indicated) of 10 mice per group were analyzed. $**p < 0.01$, $***p < 0.001$.

to the test potential, both TTX-S and TTX-R currents were apparent. When a prepulse of 500 ms to -40 mV preceded the test potential, only the slow TTX-R currents were activated, because TTX-S currents were inactivated by the pre-potential. TTX-S currents were obtained by digitally subtracting currents obtained with the -40 mV prepulse from currents recorded with the hyperpolarized prepulse (-120 mV).

The peak amplitudes of total TTX-R and TTX-S sodium currents were normalized to the cell capacitance. The sodium currents were converted to sodium conductance (g_{Na}) by dividing the peak evoked current (I_{Na}) by the driving force of the current, such that $g = I/(V_m - V_{rev})$. g_{Na} is the sodium conductance, I_{Na} is the sodium current, V_m is the potential at which the current was evoked, and V_{rev} is the reversal potential. The sodium conductance was plotted as a function of the test potentials to generate the conductance–voltage relationship.

Immunofluorescence. We perfused terminally anesthetized mice transcardially with 0.9% saline followed by 4% paraformaldehyde in 0.1 M PBS, pH 7.4. The L4 and L5 spinal cord segments, DRGs, and sciatic nerve were dissected and postfixed for 2 h and then transferred into 20% sucrose in PBS for overnight cryoprotection at 4°C. We embedded the tissue in Tissue-Tek O.C.T. Compound (Science Services) and cut transverse sections (10 μ m for DRGs, sciatic nerve, and 14 μ m spinal cord) using a cryotome. Sections were permeabilized for 5 min in PBST (0.1% Triton X-100 in 0.1 M PBS), blocked for 1 h with 10% normal goat serum and 3% BSA in PBST or with 1% blocking reagent containing casein (Roche Diagnostics) in PBST, and incubated overnight at 4°C with primary antibodies dissolved in 3% BSA or 1% blocking reagent in PBST. Polyclonal antibodies directed against phospho-Ser32-I κ B- α and phospho-Ser181-IKK α/β (phospho-IKK, corresponding to the activated IKK) were purchased from Cell Signaling Technology and Santa Cruz Biotechnology, respectively. The polyclonal ankyrin G and Nav1.6 antibodies were purchased from Sigma-Aldrich and Santa Cruz Bio-

technology, respectively. Polyclonal antibodies directed against TRPV1 were from Neuromics. After washes in PBS, we incubated the sections for 2 h at room temperature with species-specific secondary antibodies conjugated with Alexa dyes (Invitrogen). To reduce lipofuscin-like autofluorescence, slides were briefly immersed in 0.1% Sudan black B (in 70% ethanol) (Schnell et al., 1999), rinsed in PBS, and coverslipped in antifade medium. Conventional microscopic images were acquired using a Kappa DX 20 H camera attached to an Eclipse E600 microscope (Olympus). For confocal microscopy, a laser scanning microscope (LSM 510; Carl Zeiss) was used.

Statistics. We used SPSS 15.0 for statistical evaluation. Data are presented as means $\pm$ SEM or SD as indicated. Calcium imaging results were analyzed by comparing the maximum response and area under the curve (AUC) with Student's t tests and the fraction of responding cells with χ^2 statistics. Data of electrophysiology experiments (thresholds and conduction velocity) were submitted to χ^2 analysis. Patch-clamp data were analyzed using ANOVA and Mann–Whitney test. Counts of immunoreactive neurons, quantitative RT-PCR, and Western blot results were analyzed with Student's t tests. p was set at 0.05 for all statistical comparisons.

Results

Selective deletion of IKK β in sensory neurons

In situ mRNA hybridization using IKK β -specific riboprobes revealed the Cre-loxP-mediated IKK β deletion in the DRG showing a significant loss of IKK β in DRG neurons with a diameter <30 μ m but not in neurons with a cell diameter ≥ 30 μ m, as expected from the profile of *SNS-Cre* mice (Agarwal et al., 2004) (Fig. 1). The spared large DRG neurons represent proprioceptive neurons that account for $\sim 10\%$ of all DRG neurons (Agarwal et al., 2004). RT-PCR analysis showed a reduction of the IKK β transcript in DRG tissue but not a complete loss (Fig. 1*b*). A similar result was obtained in Western blots from DRG tissue (Fig. 1*c*). As expected, IKK β protein in the spinal cord and brain were not altered in *SNS-IKKβ*^{−/−} mice compared with *IKKβ*^{fl/fl} controls. The expected size of IKK β is 87 kDa in Western blots. The nuclear DNA binding activity of NF- κ B did not differ between genotypes (Fig. 1*d*), showing that IKK β deletion did not affect the constitutive neuronal NF- κ B activity in the DRGs.

Nociception in sensory neuron-specific IKK β knock-out mice

Compared with *IKKβ*^{fl/fl} littermates, *SNS-IKKβ*^{−/−} mice had significantly reduced reaction latencies to noxious heat applied by hotplate and a radiant heat source (tail flick) and to mechanical stimuli applied via a dynamic aesthesiometer, showing that acute thermal, mechanical, and nociceptive sensitivity was enhanced in *SNS-IKKβ*^{−/−} mice (Fig. 2*a*) ($p = 0.02$, 0.04 , and 0.02 , respectively). Differences for noxious cold reactivity did not reach statistical significance (Fig. 2*a*) ($p = 0.07$). Immediate nociceptive responses elicited by intraplantar injections of the irritant Formalin were significantly increased in *SNS-IKKβ*^{−/−} mice compared with *IKKβ*^{fl/fl} mice in the first 5 min of the Formalin test (Fig. 2*b*) ($p = 0.023$ for the first 5 min interval, $p = 0.089$ for the second phase), indicative of enhanced chemogenic A δ -fiber-mediated no-

ciception. Nociceptive responses evoked by capsaicin injection into the hindpaw were enhanced in *SNS-IKK β ^{-/-}* mice compared with *IKK β ^{fl/fl}* mice, with a significant difference for the second phase of the test (Fig. 2c) ($p = 0.086$ for the first phase, $p = 0.006$ for the second phase). In contrast, proprioceptive and motor performances on the rotarod and catwalk were unaffected in *SNS-IKK β ^{-/-}* mice (Fig. 3). The running time in the rotarod test was 75.2 ± 6.5 s (mean $\pm$ SEM) in *SNS-IKK β ^{-/-}* mice and 78.3 ± 5.6 s in *IKK β ^{fl/fl}* mice ($p = 0.47$).

We have shown previously that *SNS-Cre* mice have no alteration in acute responses to noxious heat and pressure (Agarwal et al., 2004) 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 pain or neuropathic pain (Agarwal et al., 2007), showing that the alterations in nociception observed in *SNS-IKK β ^{-/-}* do not arise from expression of Cre recombinase in the sensory neurons.

Electrical excitability of afferent axons in sensory neuron-specific IKK β knock-out mice

The increased sensitivity to acute noxious stimuli observed in the *SNS-IKK β ^{-/-}* mice could result from the deficiency of the constitutively phosphorylated IKK β at sites of its enrichments in sensory nerves and a modulation of the excitability. To address this question, we performed electrophysiological recordings on peripheral A- and C-fiber sensory neurons identified on the basis of stimulation and conduction properties in saphenous nerve preparations isolated from *SNS-IKK β ^{-/-}* mice and *IKK β ^{fl/fl}* mice. Electrical thresholds of myelinated A-fibers ranged from 0.1 to 53 V. The proportion of A-fibers with low activation thresholds (0.1–5 V) was significantly higher in *SNS-IKK β ^{-/-}* compared with *IKK β ^{fl/fl}* mice (83.6 vs 63.1%; $p < 0.01$, χ^2 test) (Fig. 4a). Unmyelinated C-fibers had a significantly lower median electrical activation threshold in *SNS-IKK β ^{-/-}* mice than in the *IKK β ^{fl/fl}* mice (1.15 vs 8 V; $p < 0.001$, Mann–Whitney test) (Fig. 4a). Furthermore, in *SNS-IKK β ^{-/-}* mice, a significantly higher proportion of C-fibers had very low activation thresholds of 0.1–5 V compared with control *IKK β ^{fl/fl}* mice (73 vs 33.3%; $p < 0.001$, χ^2 test) (Fig. 4a). Together, these data suggest that the presence of IKK β negatively modulates the excitability of A- and C-fibers. Conduction velocities of A- and C-fibers were not significantly altered in IKK β -deficient C- and A-fibers (Fig. 4b).

Increased capsaicin and Formalin evoked calcium influx in adult DRG neurons

To evaluate potential mechanisms for enhanced excitability of IKK β -deficient sensory nerves, we assessed calcium fluxes in primary DRG neurons of adult naive mice during stimulation with

Figure 5. Calcium imaging in freshly dissociated primary DRG neuron cultures from adult *SNS-IKK β ^{-/-}* and control *IKK β ^{fl/fl}* mice stimulated with 100 μ M UTP and 100 μ M ATP (**a**), 0.1 and 1 μ M capsaicin (**b**), and 0.01% Formalin (**c**). KCl at 50 mM (K^+) was used to check the viability of the neurons at the end of the experiment. $[Ca^{2+}]_i$ was measured fluorometrically in neurons loaded with fura-2 as absorbance ratio at 340 to 380 nm ($\Delta F_{340}/F_{380}$). The fold change (mean $\pm$ SEM) of ΔF after stimulation compared with the baseline ΔF demonstrates the stronger increase of $[Ca^{2+}]_i$ during capsaicin stimulation in *SNS-IKK β ^{-/-}* mice than in control *IKK β ^{fl/fl}* mice. The fold increased of ΔF during UTP or ATP stimulation did not differ between genotypes. For Formalin, the peak increase did not differ between groups. However, calcium influx lasted longer as revealed by comparing AUCs. Data are means $\pm$ SEM. We analyzed $n = 74$ –89 neurons of 4–6 mice in each group. Statistical results and number of neurons analyzed are shown in Tables 1–3. **d**, Dose–response curves of capsaicin-evoked currents in DRG neurons from *SNS-IKK β ^{-/-}* and *IKK β ^{fl/fl}* mice. Currents were normalized to the maximal response elicited by capsaicin; the lines are fits to the Hill equation. Neurons ($n = 9$) of four mice per genotype were analyzed. The EC_{50} differed significantly between genotypes with $p < 0.05$.

100 μ M ATP, 100 μ M UTP, 0.1 and 1 μ M capsaicin, 0.01% Formalin, and 50 mM K^+ . ATP evokes a calcium influx in a high proportion of small- and medium-sized DRG neurons acting on P2X and P2Y receptors, and UTP is a selective agonist for P2Y₂/P2Y₄ receptors (Ueno et al., 1999). Capsaicin elicits $[Ca^{2+}]_i$ increases in dissociated DRG neurons by acting specifically on transient receptor potential channel family V, member 1 (TRPV1), the capsaicin receptor also known as vanilloid 1 receptor (Ueno et al., 1999; Petruska et al., 2000). Formalin stimulates transient receptor potential channel family A, member 1 (TRPA1) calcium channels that are also activated by noxious cold (Bautista et al., 2005). During stimulation with ATP and UTP, we observed similar raises of $[Ca^{2+}]_i$ in *SNS-IKK β ^{-/-}* and *IKK β ^{fl/fl}* DRG neurons (Fig. 5a, Tables 1, 2). There was also no significant difference in the fraction of neurons responding to either ATP or UTP (Tables 1, 2). However, small- and medium-sized DRG neurons of *SNS-IKK β ^{-/-}* mice showed a stronger increase in $[Ca^{2+}]_i$ than respective neurons of *IKK β ^{fl/fl}* mice after stimulation with capsaicin as revealed by comparing the maximum fold increase ($p < 0.001$ for 0.1 μ M and $p = 0.002$ for 1 μ M) (Fig. 5b).

Table 1. Fold increase of $[Ca^{2+}]_i$ (means $\pm$ SEM)

	UTP 100 μ M		ATP 100 μ M		K ⁺ 50 mM		Capsaicin 0.1 μ M		Capsaicin 1 μ M		K ⁺ 50 mM	
	IKK $\beta^{fl/m}$	SNS-IKK $\beta^{-/-}$	IKK $\beta^{fl/m}$	SNS-IKK $\beta^{-/-}$	IKK $\beta^{fl/m}$	SNS-IKK $\beta^{-/-}$	IKK $\beta^{fl/m}$	SNS-IKK $\beta^{-/-}$	IKK $\beta^{fl/m}$	SNS-IKK $\beta^{-/-}$	IKK $\beta^{fl/m}$	SNS-IKK $\beta^{-/-}$
Large ($n = 27$)	1.57 $\pm$ 0.31	1.16 $\pm$ 0.06	1.65 $\pm$ 0.28	1.21 $\pm$ 0.09	2.61 $\pm$ 0.31	2.29 $\pm$ 0.17	1.07 $\pm$ 0.02	1.10 $\pm$ 0.04	1.16 $\pm$ 0.02	1.22 $\pm$ 0.25	2.27 $\pm$ 0.19	1.99 $\pm$ 0.11
Medium ($n = 25$)	1.88 $\pm$ 0.28	1.51 $\pm$ 0.15	1.57 $\pm$ 0.18	1.97 $\pm$ 0.39	2.39 $\pm$ 0.20	2.02 $\pm$ 0.17	1.27 $\pm$ 0.10	1.69 $\pm$ 0.17*	1.35 $\pm$ 0.08	2.50 $\pm$ 0.41*	1.82 $\pm$ 0.14	2.13 $\pm$ 0.17
Small ($n = 22$)	1.43 $\pm$ 0.14	1.68 $\pm$ 0.25	2.23 $\pm$ 0.29	1.68 $\pm$ 0.20	2.39 $\pm$ 0.22	2.01 $\pm$ 0.19	1.21 $\pm$ 0.06	2.77 $\pm$ 0.36*	1.60 $\pm$ 0.23	4.97 $\pm$ 1.47*	2.18 $\pm$ 0.38	2.30 $\pm$ 0.19

* $p < 0.05$.**Table 2.** Percentage of cells responding with > 1.2 -fold increase of $[Ca^{2+}]_i$

	UTP 100 μ M		ATP 100 μ M		K ⁺ 50 mM		Capsaicin 0.1 μ M		Capsaicin 1 μ M		K ⁺ 50 mM	
	IKK $\beta^{fl/m}$	SNS-IKK $\beta^{-/-}$	IKK $\beta^{fl/m}$	SNS-IKK $\beta^{-/-}$	IKK $\beta^{fl/m}$	SNS-IKK $\beta^{-/-}$	IKK $\beta^{fl/m}$	SNS-IKK $\beta^{-/-}$	IKK $\beta^{fl/m}$	SNS-IKK $\beta^{-/-}$	IKK $\beta^{fl/m}$	SNS-IKK $\beta^{-/-}$
Large ($n = 27$)	27.78	20.00	46.67	30.00	90.00	95.00	11.11	7.41	9.21	29.63*	86.96	81.48
Medium ($n = 25$)	55.56	54.17	61.11	62.5	88.89	83.33	16.67	56.00*	33.33	60.00*	72.22	53.85
Small ($n = 22$)	40.91	51.86	77.27	65.59	95.46	94.80	36.84	81.82*	63.16	95.46*	73.68	83.33

* $p < 0.05$.**Table 3.** Fold increase of $[Ca^{2+}]_i$ (means $\pm$ SEM) and percentage of cells responding with at least twofold increase of $[Ca^{2+}]_i$

Formalin 0.01% Medium + small ($n = 83$ and 89)	Fold increase		Percentage responding	
	IKK $\beta^{fl/m}$	SNS-IKK $\beta^{-/-}$	IKK $\beta^{fl/m}$	SNS-IKK $\beta^{-/-}$
Peak	4.49 $\pm$ 0.20	4.57 $\pm$ 0.18	58.8	66.4 ($p = 0.101$)
AUC	508.0 $\pm$ 16.4	532.8 $\pm$ 16.0*		

* $p < 0.05$.

In addition, the relative number of neurons responding to 0.1 or 1 μ M capsaicin (> 1.5 -fold increase of $[Ca^{2+}]_i$) was higher in SNS-IKK $\beta^{-/-}$ mice (χ^2 analysis, $p < 0.001$ for small-sized cells, $p < 0.01$ for medium-sized cells) (Table 1). After stimulation with 0.01% Formalin, DRG neurons of SNS-IKK $\beta^{-/-}$ mice and IKK $\beta^{fl/m}$ mice reached similar peak increases of $[Ca^{2+}]_i$ ($p = 0.782$), and the fraction of neurons responding to 0.01% Formalin was slightly but not significantly increased in SNS-IKK $\beta^{-/-}$ mice (χ^2 analysis, $p = 0.101$) (Fig. 5c, Table 3). However, the Formalin-evoked calcium influx lasted longer in neurons of SNS-IKK $\beta^{-/-}$ mice as revealed by comparing AUCs ($p = 0.015$) (Table 3).

Because capsaicin produced a stronger increase of $[Ca^{2+}]_i$ in DRG neurons of SNS-IKK $\beta^{-/-}$ mice, we asked whether this was associated with altered ion currents of transient receptor potential channels and found increased capsaicin-activated TRPV1 currents in sensory neuron-specific IKK β -deficient mice. In DRG neurons from SNS-IKK $\beta^{-/-}$ mice, the dose-response curve to capsaicin was significantly shifted to the left compared with IKK $\beta^{fl/m}$ littermates (Fig. 5d). Capsaicin concentrations that induced half-maximal activation of the current (EC_{50}) were 0.55 μ M in SNS-IKK $\beta^{-/-}$ mice and 1 μ M for IKK $\beta^{fl/m}$ littermates, respectively ($p < 0.05$). Together, this suggests that the absence of IKK β makes sensory neurons more sensitive to the stimulation of capsaicin- and Formalin-sensitive TRP channels (Fig. 5d).

Expression of TRPV1 in IKK β -deficient DRGs

Because of the observed predominant modulation of capsaicin sensitivity in DRG neurons of SNS-IKK $\beta^{-/-}$ mice, we analyzed the expression of TRPV1 in DRGs. The quantitative RT-PCR revealed slightly increased TRPV1 mRNA expression ($p = 0.07$) (Fig. 6a). Western blots from DRGs, sciatic nerve, and spinal cord dorsal horn showed a slight increase of monomeric TRPV1 protein at the expected sizes for glycosylated and nonglycosylated TRPV1 monomers with N- and C-terminal antibodies (Goswami et al., 2004) in SNS-IKK $\beta^{-/-}$ mice (Fig. 6b,c). The differences

were statistically significant in the sciatic nerve and in the membrane fraction of the dorsal horn, suggesting that TRPV1 channels are enriched at peripheral and central terminals in SNS-IKK $\beta^{-/-}$ mice. The number of DRG neurons with TRPV1 immunoreactivity was not increased in SNS-IKK $\beta^{-/-}$ mice compared with control IKK $\beta^{fl/m}$ mice (Fig. 6d). However, the number of intensely labeled small DRG neurons was enhanced (SNS-IKK $\beta^{-/-}$, 10.4 ± 3.8 ; IKK $\beta^{fl/m}$, 4.3 ± 1.4 ; $p = 0.002$). These neurons were negative for isolectin B4, which is a marker for small nonmyelinated glutamatergic DRG neurons (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). Because of these results, we compared the behavioral response of SNS-IKK $\beta^{-/-}$ mice with TRPV1 $^{-/-}$ mice. These mice show strongly reduced heat- and capsaicin-evoked nociceptive responses (Caterina et al., 2000), and we found a reduction of the Formalin-evoked licking response (supplemental Fig. 2, available at www.jneurosci.org as supplemental material) in the early second phase (phase 2a) of the Formalin assay ($p = 0.045$). The different time courses in the Formalin assay of SNS-IKK $\beta^{-/-}$ mice (Fig. 2b) and TRPV1 $^{-/-}$ mice (supplemental Fig. 2, available at www.jneurosci.org as supplemental material) suggest that the enhanced nociceptive response in the first phase of SNS-IKK $\beta^{-/-}$ mice is rather mediated through TRPA1 than TRPV1.

Normal conduction velocity, sodium currents, and clustering of sodium channels at nodes of Ranvier

On the basis of the previously observed clustering of phospho-IKK at nodes of Ranvier, we determined conduction velocities of A- and C-fibers and found them unaffected in IKK β -deficient C- and A-fibers (Fig. 4b). We therefore analyzed the clustering of the voltage-dependent sodium channel Nav1.6 at NR (Jenkins and Bennett, 2001; Shirahata et al., 2006) using double immunofluorescence for Nav1.6 and ankyrin G, a membrane adapter protein enriched in the NR (Dzhashishvili et al., 2007). In IKK $\beta^{fl/m}$ mice, nodal accumulation of activated phospho-IKK showed a membrane-associated distribution in the nodal and paranodal domain as shown previously (Schultz et al., 2006; Politi et al., 2008) and was localized in close proximity to Nav1.6 and ankyrin G (data not shown). Ankyrin G and Nav1.6 displayed the previously established membrane-associated distribution confined to the nodal domains. We observed no alteration of ankyrin G or Nav1.6 clustering in SNS-IKK $\beta^{-/-}$ mice compared with IKK $\beta^{fl/m}$ mice (Fig. 7), suggesting that the gross molecular structure of NRs was not affected by IKK β deficiency.

In addition, we did not observe changes of TTX-S and TTX-R sodium currents in DRG neurons of SNS-IKK $\beta^{-/-}$ mice com-

pared with *IKK β ^{fl/fl}* mice at any of the tested potentials ($p > 0.05$, ANOVA) (Fig. 8). Recordings were obtained from small DRG neurons of similar sizes (17.02 ± 2.86 and 16.56 ± 1.06 pF). The conductance–voltage relationship was best fitted with the Boltzmann function. The normalized Na^+ conductance showed a sigmoidal relationship to the membrane potentials. TTX-S Na^+ channels had similar half-maximal activation potentials (V_{half}) in *SNS-IKK β ^{-/-}* mice and *IKK β ^{fl/fl}* littermates ($V_{\text{half}} = -32.05$ mV in *SNS-IKK β ^{-/-}* mice vs -31 mV in *IKK β ^{fl/fl}*). TTX-R currents showed a depolarized voltage dependency of activation compared with that of the TTX-S currents, but no significant difference was observed between *SNS-IKK β ^{-/-}* mice and *IKK β ^{fl/fl}* littermates ($V_{\text{half}} = -12.0 \pm 3.36$ mV in *SNS-IKK β ^{-/-}* mice vs -8.07 ± 1.55 mV in *IKK β ^{fl/fl}*).

Discussion

The present study assessed the functions of IKK β in peripheral sensory neurons using mice with a specific Cre–loxP-mediated deletion of IKK β in neurons of the dorsal root ganglia. The *SNS-IKK β ^{-/-}* mice were more sensitive to acute thermal, mechanical, and chemical noxious stimulation, and their A- and C-fibers showed lower activation thresholds in nerve preparations. In IKK β -deficient DRG neurons, capsaicin and Formalin elicited stronger calcium influx and calcium currents compared with control neurons. The results suggest that IKK β negatively regulates the excitability in nociceptive, thermosensitive, and mechanosensitive neurons under naive conditions, i.e., without previous sensitization, by negative modulation of the transient receptor potential channels TRPV1 and weakly TRPA1, which play crucial roles in heat, cold, and chemical pain sensitivity (Kress and Zeilhofer, 1999; Zimmermann et al., 2005). In support, IKK β deficiency particularly increased acute heat- and capsaicin-evoked pain sensitivity and calcium transients, whereas responses to purines were unaffected.

We have shown previously that constitutively active phospho-IKK β clusters beneath the cytoplasmic axonal membrane, partially within lipid rafts (Schultz et al., 2006), suggesting close proximity to receptors and ion channels. It may be hypothesized that a TRP channel-mediated calcium influx stimulates IKK β phosphorylation, and this in turn may act on its calcium currents by direct phosphorylation of channel subunits, by modulation of the release of phosphatidylinositol-4, 5-bisphosphate (PIP₂) phosphoinositides, or by NF- κ B-mediated feedback. The deficiency of IKK β in sensory neurons of *SNS-IKK β ^{-/-}* mice may disturb the balance of these membrane events and explain the observed lower activation threshold of A δ - and C-fibers and the faster response toward mechanical and heat stimulation and enhanced nociceptive responses after capsaicin or Formalin injection. Various kinases, including protein kinase C and A, p38 mitogen-activated protein kinase, and cyclin-dependent kinase

Figure 6. *a*, Quantitative RT-PCR analysis of TRPV1 mRNA in DRGs of *SNS-IKK β ^{-/-}* and control *IKK β ^{fl/fl}* mice ($n = 3$ mice per group). *b*, *c*, Western blot analyses of monomeric TRPV1 protein in *SNS-IKK β ^{-/-}* and control *IKK β ^{fl/fl}* mice in cytosolic protein fractions of DRGs and sciatic nerve (ScN) or cytosolic and membrane fractions of the dorsal horn (DH). The sites represent neuronal somata, peripheral, and central nerve terminals. *c* shows the statistical results of semiquantitative Western blot analyses. Two samples of pooled tissue of each of three mice per group were analyzed on triplicate blots. $*p < 0.05$. *d*, Immunofluorescent studies of TRPV1 expression in DRGs of *SNS-IKK β ^{-/-}* and control *IKK β ^{fl/fl}* mice ($n = 4$ mice per group, 37 and 47 DRG sections, respectively). The number of TRPV1-immunoreactive neurons did not differ between genotypes.

Cdk5, were reported to increase TRPV1 channel activity by phosphorylation of individual serine or threonine residues (Ji et al., 2002; Bhawe et al., 2003; Distler et al., 2003; Carlton et al., 2004; Zhang et al., 2005; Siemens et al., 2006; Salazar et al., 2008), i.e., exerting opposite effects on the channel than the IKK β -mediated negative control observed here, suggesting that IKK β either affects residues other than those known to increase TRPV1 activity or a mechanism not exerted by direct TRPV1 phosphorylation. The moderate increase of TRPV1 protein in IKK β -deficient neurons, particularly at their peripheral and central terminals, suggests that IKK may also regulate the expression of TRPV1. Previously, phosphoinositides were shown to modulate TRP channel activity (Prescott and Julius, 2003), particularly those that display PIP₂ binding sites (Prescott and Julius, 2003; Qin, 2007). For TRPV1, this is mediated by a membrane protein, Pirt, which is expressed in most nociceptive DRG neurons (Kim et al., 2008a). Although TRPA1 has no PIP₂ binding sites, it is also modulated by PIP₂ phospholipids (Kim et al., 2008b). It is conceivable that IKK β deficiency alters the PIP₂ release and thereby modulates indirectly TRP channels. In addition, inositoltrisphosphate receptors cluster at nodes of Ranvier in the sciatic nerve, in a distribution similar to Nav1.6 sodium channels and active phospho-IKK β . Phosphatidylinositol 3 kinase inhibitors reduce TRPV1-mediated nociception (Zhuang et al., 2004). An indirect PIP₂-mediated effect would explain the additional sensitization of the TRPA1 channels (Karashima et al., 2008; Kim et al., 2008b) as indicated by an enhanced early nociceptive response to Formalin *in vivo* and increased calcium influx in primary neurons of

Figure 7. Immunofluorescent studies showing the subcellular distribution of Nav1.6 (red) and ankyrin G (green) in the nodal domain of nodes of Ranvier in the dorsal root ganglion (**a**) and sciatic nerve (**b**) of *SNS-IKK β ^{−/−}* and control *IKK β ^{fl/fl}* mice. Nav1.6 and ankyrin G both display a membrane-associated distribution restricted to the nodal domain. No gross structural difference was observed between genotypes.

Figure 8. Current–voltage and conductance–voltage relations for TTX-S and TTX-R sodium currents in DRG neurons of *SNS-IKK β ^{−/−}* and control *IKK β ^{fl/fl}* mice ($n = 4–9$ neurons of 3 mice per group). The conductance values were normalized to the maximum conductance, and the data points were fitted to the Boltzmann function.

SNS-IKK β ^{−/−} mice during Formalin stimulation. Compared with previous results of *TRPA1^{−/−}* mice, which showed a strong reduction of the nociceptive response in both phases of the Formalin test (McNamara et al., 2007), the enhanced nociception in

SNS-IKK β ^{−/−} mice observed here was restricted to acute immediate responses that do not involve a sensitization process in nociceptive neurons. The somewhat not significantly reduced response during the second phase of the Formalin test in *SNS-IKK β ^{−/−}* mice suggests that IKK β -mediated sensitizing effects on TRPA1 channels are rapidly offset by adaptive changes that occur during ongoing stimulation of nociceptors.

Interestingly, mice genetically deficient of the NF- κ B p50 subunit behaved differently in nociceptive tests than the *SNS-IKK β ^{−/−}* mice. The latency to heat and mechanical stimulation was increased, and the second phase of the Formalin assay was reduced (Niederberger et al., 2007). The difference may be attributable to the difference between a general versus sensory neuron-specific knockout. Alternatively, IKK β in sensory neurons may phosphorylate and activate NF- κ B-independent targets or may involve NF- κ B p50 subunit-independent mechanisms such as I κ B- α independent serine-(536) phosphorylation and nuclear translocation of phospho-p65 (Sasaki et al., 2005). The constitutive NF- κ B activity in DRG neurons was not altered in naive *SNS-IKK β ^{−/−}* mice, also suggesting that the IKK β -mediated effects observed here were independent of this baseline NF- κ B activity. IKK β targets other than I κ B undergo IKK-mediated

phosphorylation and subsequent ubiquitination or stabilization such as Bcl10 (Wegener et al., 2006) and p53 family members p73 (Furuya et al., 2007) and TAp63 (MacPartlin et al., 2008) as well as the deubiquitination enzyme CYLD (Reiley et al., 2005). At present, the exact mechanisms that link IKK β to TRPV1 and weakly to TRPA1 remain unknown. However, our results clearly point to a negative regulatory role of IKK β on TRP channel activity, thereby reducing acute nociceptive responses, neuronal excitability, and calcium currents. These physiological functions of IKK in peripheral neurons may be relevant for potential side effects of IKK inhibitors that are being developed, e.g., as anti-inflammatory and anti-cancer drugs.

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