Supplemental Information

Extracellular Caspase-6 Enhances Spinal Synaptic Transmission and Inflammatory Pain via Microglial TNF-α in Mice

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Supplemental methods

Supplementary tables: 2

Supplementary Figures: 10

Supplemental Methods

Reagents

Bradykinin, capsaicin, carrageenan, complete Freund's Adjuvant (CFA), formalin, lipopolysaccharide (LPS, *Escherichia coli*), LY294002 (PI3K inhibitor), and thiamine monophosphatase (TMB) were from Sigma. We also purchased recombinant full length active caspase-6 protein (rCASP6) from Abcam or Enzo Life Science, recombinant active caspase-3 (rCASP3) from Enzo Life Science, VEID-fmk (caspase-6 inhibitor), and TNF-α from R&D System. SB203580 (p38 inhibitor), U0126 (MEK inhibitor), and SP600125 (JNK inhibitor) were from Calbiochem. CASP6 and TNF-α neutralizing antibodies were obtained from Imgenex and R&D System, respectively. Specific siRNA against CASP6 was obtained from Dharmacon and RVG peptide was synthetized by Invitrogen.

Animals

Knockout mice lacking *Casp6* (*Casp6$^{-/-}$*) and *Tnfr1a/1b* (*Tnfr1/2* DKO) mice, *Cx3cr1*-GFP mice, *Gfap*-GFP mice, and C57BL/6 background WT control mice were purchased from Jackson Laboratories. *Casp6$^{-/-}$ and Tnfr1/2* DKO mice were viable and showed no developmental defects. Neonatal mice were used to prepare primary cultures of microglia and astrocytes. Young mice (4-7 weeks) were used for electrophysiological studies in spinal cord slices to obtain high quality recordings. Of note, DRG neurons and spinal cord circuit are fully developed by postnatal age of three weeks (1). Adult CD1 mice (male, 8-10 weeks) were used for behavioral and pharmacological studies. To produce inflammatory pain, diluted capsaicin (1 µg/µl, 5µl), formalin (5%, 20 µl), carrageenan (1.5 %, 20 µl), or complete Freund's adjuvant (1 mg/ml, 20 µl) was injected into the plantar surface of a hindpaw. For intrathecal injection, spinal cord puncture was made with a 30G needle between the L5 and L6 level to deliver reagents (10 µl) to the cerebral spinal fluid (2). Adult rats (200-220 g, Charles River) were also used for the collection of CSF. For peri-sciatic injection, Casp6 siRNA (2 µg, 6 µl, mixed with RVG peptide

(molar ratio siRNA:RVG =1:10) or control siRNA (scrambled) was injected 48 h prior to the behavioral test.

Behavioral testing

Animals were habituated to the environment for at least 2 days before the testing. All the behaviors were tested blindly. We assessed formalin-evoked spontaneous inflammatory pain by measuring the time (seconds) mice spent on licking or flinching the affected paw every 5 min for 45 min. For testing mechanical sensitivity after plantar or intrathecal injections of different substances, we confined mice in boxes placed on an elevated metal mesh floor and stimulated their hindpaws with a series of von Frey hairs with logarithmically increasing stiffness (0.02-2.56g, Stoelting), presented perpendicularly to the central plantar surface. We determined the 50% paw withdrawal threshold by Dixon's up-down method (3). Thermal sensitivity was tested using Hargreaves radiant heat apparatus (4) (IITC Life Science) and expressed as paw-withdrawal latency (PWL). The radiant heat intensity was adjusted so that basal PWL was between 9 and 12 s, with a cutoff of 20 s to prevent tissue damage.

Spinal cord slice preparation and patch clamp recordings

As we previously reported (5;6), a portion of the lumbar spinal cord (L4-L5) was removed from mice (4-7 weeks old) under urethane anesthesia (1.5 - 2.0 g/kg, i.p.) and kept in pre-oxygenated ice-cold Krebs solution. Transverse slices (400-600 μm) were cut on a vibrating microslicer. The slices were perfused with Kreb's solution (8-10 ml/min) that was saturated with 95% O_2 and 5% CO_2 at 36±1°C for at least 1-3 h prior to experiment. The Kreb's solution contains (in mM): NaCl 117, KCl 3.6, $CaCl_2$ 2.5, $MgCl_2$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 25, and glucose 11. The whole cell patch-clamp recordings were made from lamina IIo neurons in voltage clamp mode. Patch pipettes were fabricated from thin-walled, borosilicate, glass-capillary tubing (1.5 mm o.d., World Precision Instruments). After establishing the whole-cell configuration, neurons were held at the potential of -70 mV to record spontaneous EPSCs (sEPSCs). The miniature EPSCs (mEPSCs) were recorded in the

presence of 1 μM TTX. The resistance of a typical patch pipette is 5-10 MΩ. The internal solution contains (in mM): potassium gluconate 135, KCl 5, $CaCl_2$ 0.5, $MgCl_2$ 2, EGTA 5, HEPES 5, and ATP-Mg 5. Membrane currents were amplified with an Axopatch 200B amplifier (Axon Instruments) in voltage-clamp mode. Signals were filtered at 2 kHz and digitized at 5 kHz. Data were stored with a personal computer using pCLAMP 10 software and analyzed with Mini Analysis (Synaptosoft Inc.).

To measure evoked EPSCs (eEPSCs) in lamina IIo neurons, dorsal root enter zone was stimulated through a concentric bipolar electrode (FHC) with an isolated current stimulator. QX-314 (5 mM) was added to the pipette solution to prevent discharge of action potentials. Test pulses of 0.1 ms with intensity of 3 mA were given at 30 sec intervals and monosynaptic C-fiber responses were recorded. The responses were considered as monosynaptic in origin when the latency remained constant and there was no failure during stimulation at 20 Hz for 1s, or when failures did not occur during repetitive stimulation at 2 Hz for 10s (7). Synaptic strength was quantified by the peak amplitudes of eEPSCs. The mean amplitude of 4-5 EPSCs evoked by test stimuli prior to conditioning stimulation served as control.

Spinal cord LTP recordings in anesthetized mice

As we previously reported (5;6), mice were anesthetized with urethane (1.5 g/kg, i.p.). The trachea was cannulated to allow mechanical ventilation, when needed. PBS (0.5-1 ml, i.p.) was injected prior to surgery and every 2 h after surgery to maintain electrolyte balance. A laminectomy was performed at vertebrae T13-L1 to expose the lumbar enlargement, and the left sciatic nerve was exposed for bipolar electrical stimulation. The vertebral column was firmly suspended by rostral and caudal clamps on the stereotaxic frame. The exposed spinal cord and the sciatic nerve were covered with paraffin oil. Colorectal temperature was kept constant at 37-38°C by a feedback-controlled heating blanket. Following electrical stimulation of the sciatic nerve, the field potentials were recorded in the ipsilateral L4-5 spinal cord segments with glass microelectrodes, 100-300 μm from the surface of the cord. After recording stable responses following test stimuli (2x C-fiber threshold, 0.5 ms, every 5 min) for > 40 min, conditioning tetanic stimulation (5 times of C-fiber threshold, 100 Hz, 1 s

duration, 4 trains, and 10 s interval) was delivered to the sciatic nerve for inducing LTP of C-fiber-evoked field potentials. For intrathecal drug delivery, a PE5 catheter was inserted at L5-L6 level via lumbar puncture.

Primary cultures of microglia and astrocytes

Microglia and astrocytes cultures were prepared from cerebral cortexes and spinal cords of 2 day-old postnatal mice (8;9). Tissues were transferred to ice-cold Hank's buffer, and the meninges were carefully removed. Tissues were then minced into <1 mm pieces, triturated, filtered through a 100 μm nylon screen, and collected by centrifugation at <3000 × g for 5 min. The cell pellets were dissociated with a pipette and resuspended in a medium containing 10% fetal bovine serum in high-glucose DMEM. After trituration, the cells were filtered through a 10 μm screen, plated into T75 flasks and cultured at 37°C with 5% CO_2/95% air. Medium was replaced every 3–4 days, and confluence was achieved after 3 weeks in culture. The mixed glial cells were shaken for 4 h, and the floating cells (microglial pool) were collected and subcultured at a density of 2.5×10^5 cells/ml. Most of the remaining cells could be astrocytes. After 1 day of plating of potential microglial cells, the medium was changed to discharge all non-adherent cells. This method resulted in >95% purity of microglia, as assessed by IBA-1 and DAPI staining. The same procedure was also used for the isolation of astrocytes except that we collected the cells remaining in the plate and used the low-glucose DMEM medium. Cells were cultured on six-well plates at the density of 2.5×10^5 cells/ml. The cultures were maintained for 10–12 days. Once the astrocyte cells were grown to 95% confluence, 0.15 mM dibutyryl cAMP (Sigma) was added to induce differentiation. For intreathecal injection of microglial cells, the rCASP6-stimulated and non-stimulated microglial cells were washed 3 times with PBS 3 h after the rCASP6 stimulation and intrathecally injected (20 μl) into mice.

Immunohistochemistry

After appropriate survival times, animals were deeply anesthetized with isoflurane and perfused through the ascending aorta with PBS, followed by 4% paraformaldehyde with 1.5% picric acid in 0.16 M phosphate buffer (10). After the perfusion, the L4–L5 spinal cord segments and DRGs were removed and postfixed in the same fixative overnight. Spinal cord sections (30 μm, free-floating) and DRG sections (12 μm) were cut in a cryostat. The sections were first blocked with 2% goat or horse serum for 1 h at room temperature. The sections were then incubated overnight at 4°C with the primary antibodies against CASP6 (rabbit, 1:200 to 1:1000; Cell Signaling) and CGRP (goat, 1:200; Abcam). The sections were then incubated for 1 h at room temperature with cyanine 3 (Cy3)- or FITC-conjugated secondary antibodies (1:400; Jackson ImmunoResearch). For double immunofluorescence, sections were incubated with a mixture of polyclonal and monoclonal primary antibodies, followed by a mixture of Cy3- and FITC- conjugated secondary antibodies or FITC-conjugated IB4 (10 μg/ml, Sigma). In some cases DAPI (Vector laboratories) was used to stain cell nucleus. The stained and mounted sections were examined with a Nikon fluorescence microscope, and images were captured with a CCD Spot camera. Some sections were also examined under a Zeiss 510 inverted confocal microscope.

Primary cultures of DRG neurons

We aseptically removed DRGs from 4-5 week-old mice and digested the tissues with collagenase (1.25 mg/ml, Roche) and dispase-II (2.4 units/ml, Roche) for 90 min, followed by 0.25% trypsin for 8 min at 37 °C. We plated cells on slide chambers coated with poly-D-lysine and laminin or plates coated with poly-D-lysine and grew them in a neurobasal defined medium (with 2% B27 supplement) in the presence of 5 μM AraC, at 37°C, with 5% CO_2/95% air for 24 h before experiments.

ELISA

For *in vitro* experiments, culture medium and cells were collected separately after treatment. For *in vivo* experiments, animals were transcardially perfused with PBS, and

DRG and spinal cord tissues were dissected. Cells or tissues were homogenized in a lysis buffer containing protease and phosphatase inhibitors. Protein concentrations were determined by BCA Protein Assay (Pierce). Mouse ELISA kits (TNF-α, IL-1β, and IL-6) were purchased from R & D Systems. For each assay, 50 μg proteins or 50 μl of culture medium were used according to manufacturer's instructions. The standard curve was included in each experiment.

Western blot

Protein samples were prepared in the same way as for ELISA analysis, and 20-50 μg of proteins were loaded for each lane and separated by SDS-PAGE gel (4–15%; Bio-Rad). After the transfer, the blots were incubated overnight at 4°C with polyclonal antibody against total CASP6 (1:1000, rabbit; Cell Signaling Technology), aCASP6 (active/cleaved, 1:2000, rabbit; Imgenex), p-p38 (1:500, rabbit; Cell Signaling Technology), pERK (1:500, rabbit; Cell Signaling Technology), and TNF-α (1:500, rabbit; Millipore). For loading control, the blots were probed with beta tubulin (β-TUB) antibody (1:5000, mouse; Millipore). These blots were further incubated with HRP-conjugated secondary antibody, developed in ECL solution (Pierce), and the chemiluminescence was revealed by Bio-Rad ChemiDoc XRS for 1–5 min. Specific bands were evaluated by apparent molecular sizes. The intensity of the selected bands was analyzed using NIH Image J software.

Quantitative real-time RT-PCR

Tissues or cells were rapidly isolated in RNAse free conditions. Total RNAs were extracted using RNeasy Plus Mini kit (Qiagen). Quantity and quality of the eluted RNA samples were verified by NanoDrop spectrophotometer (Thermo Fisher Scientific). Total RNAs (0.5-1 μg) were reverse-transcribed using the SuperScript III reverse transcriptase according to the protocol of the manufacturer (Invitrogen) and our previous study (6). Specific primers including GAPDH control were designed using IDT SciTools Real-Time PCR software (Integrated DNA Technologies). The sequences of these primers are

described in **Supplemental Table 1**. We performed gene-specific mRNA analyses using the MiniOpticon Real-Time PCR system (BioRad). Quantitative PCR amplification reactions contained the same amount of Reverse transcription (RT) product, including 7.5 μL of 2× iQSYBR-green mix (BioRad) and 100-300 nM of forward and reverse primers in a final volume of 15 μL. The thermal cycling conditions comprised 3 min of polymerase activation at 95 °C, 45 cycles of 10 s denaturation at 95 °C, and 30 s annealing and extension at 60 °C, followed by a DNA melting curve for the determination of amplicon specificity.

Single-cell RT-PCR

Single-cell RT-PCR was performed as previously described (12). Single cell (GFP-labeled microglial cell, GFP-labeled astrocyte, or non-labeled neuron) in IIo of spinal cord slices was aspirated into a patch pipette with a tip diameter of 5-10 μm and gently put into a reaction tube containing reverse transcription reagents, and incubated for 1 h at 50°C (superscript III, Invitrogen, Carlsbad, CA, USA). The cDNA product was then used in separate PCR. The sequences of all the "outer" and "inner" primers used for single-cell PCR are described in **Supplemental table 2**. The first round of PCR was performed in 50 μl of PCR buffer containing 0.2 mM dNTPs, 0.2 μM "outer" primers, 5 μl RT product and 0.2 μl platinum Taq DNA polymerase (Invitrogen). The protocol for the first round PCR included a 5-min initial denaturation step at 95 °C followed by 40 cycles of 40 s denaturation at 95 °C, 40 s annealing at 55 °C, 40 s elongation at 72 °C. The reaction was completed with 7 min of final elongation. For the second round of amplification, the reaction buffer (20 μl) contained 0.2 mM dNTPs, 0.2 μM "inner" primers, 5 μl of the first round PCR products, and 0.1 μl platinum Taq DNA polymerase. The reaction procedure for these inner primers was the same as the first round except that (1) the reaction buffer (20 μl) contains 0.2 μM "inner" primers and 5 μl of the first round PCR products and (2) the amplification cycles are 35 for most cDNAs and 45 only for TNF-α cDNAs from microglia and astrocytes. GAPDH was used as a positive control. A negative control was obtained from pipettes that did not harvest any cell contents, but were submerged in the

bath solution. The PCR products were displayed on ethidium bromide-stained 1.5 % agarose gels.

Statistical analyses

All data were expressed as mean ± s.e.m. For electrophysiology in spinal cord slices, those cells showed >5% changes from the baseline levels during drug perfusion were regarded as responding ones. We collected the baseline recordings for 2 min and the recordings in the first 2 min of drug treatment for statistical analysis using paired or unpaired two-tailed student's t-test (11). LTP data were tested using Two-Way ANOVA (12). Behavioral data were analyzed using student's t-test (two groups) or One-Way ANOVA followed by post-hoc Bonferroni test. The criterion for statistical significance was *P*<0.05.

All the animal procedures were approved by the Animal Care Committee of Duke University and Harvard Medical School.

References

1. Fitzgerald,M. 2005. The development of nociceptive circuits. *Nat. Rev. Neurosci.* **6**:507-520.

2. Hylden,J.L., and Wilcox,G.L. 1980. Intrathecal morphine in mice: a new technique. *Eur. J. Pharmacol.* **67**:313-316.

3. Dixon,W.J. 1980. Efficient analysis of experimental observations. *Annu. Rev. Pharmacol. Toxicol.* **20**:441-462.

4. Hargreaves,K., Dubner,R., Brown,F., Flores,C., and Joris,J. 1988. A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain* **32**:77-88.

5. Park,C.K., Xu,Z.Z., Liu,T., Lu,N., Serhan,C.N., and Ji,R.R. 2011. Resolvin d2 is a potent endogenous inhibitor for transient receptor potential subtype v1/a1, inflammatory pain, and spinal cord synaptic plasticity in mice: distinct roles of resolvin d1, d2, and e1. *J. Neurosci.* **31**:18433-18438.

6. Liu,T., Berta,T., Xu,Z.Z., Park,C.K., Zhang,L., Lu,N., Liu,Q., Liu,Y., Gao,Y.J., Liu,Y.C. et al 2012. TLR3 deficiency impairs spinal cord synaptic transmission, central sensitization, and pruritus in mice. *J Clin. Invest* **122**:2195-2207.

7. Luo,C., Kumamoto,E., Furue,H., Chen,J., and Yoshimura,M. 2002. Nociceptin inhibits excitatory but not inhibitory transmission to substantia gelatinosa neurones of adult rat spinal cord. *Neuroscience* **109**:349-358.

8. Gao,Y.J., Zhang,L., and Ji,R.R. 2010. Spinal injection of TNF-alpha-activated astrocytes produces persistent pain symptom mechanical allodynia by releasing monocyte chemoattractant protein-1. *Glia* **58**:1871-1880.

9. Xu,Z.Z., Berta,T., and Ji,R.R. 2013. Resolvin E1 inhibits neuropathic pain and spinal cord microglial activation following peripheral nerve injury. *J Neuroimmune Pharmacol.* **8**:37-41.

10. Zhuang,Z.Y., Gerner,P., Woolf,C.J., and Ji,R.R. 2005. ERK is sequentially activated in neurons, microglia, and astrocytes by spinal nerve ligation and contributes to mechanical allodynia in this neuropathic pain model. *Pain* **114**:149-159.

11. Kawasaki,Y., Zhang,L., Cheng,J.K., and Ji,R.R. 2008. Cytokine mechanisms of central sensitization: distinct and overlapping role of interleukin-1beta, interleukin-6, and tumor necrosis factor-alpha in regulating synaptic and neuronal activity in the superficial spinal cord. *J. Neurosci* **28**:5189-5194.

12. Park,C.K., Lu,N., Xu,Z.Z., Liu,T., Serhan,C.N., and Ji,R.R. 2011. Resolving TRPV1- and TNF-a-mediated spinal cord synaptic plasticity and inflammatory pain with neuroprotectin D1. *J Neurosci* **31**:15072-15085.
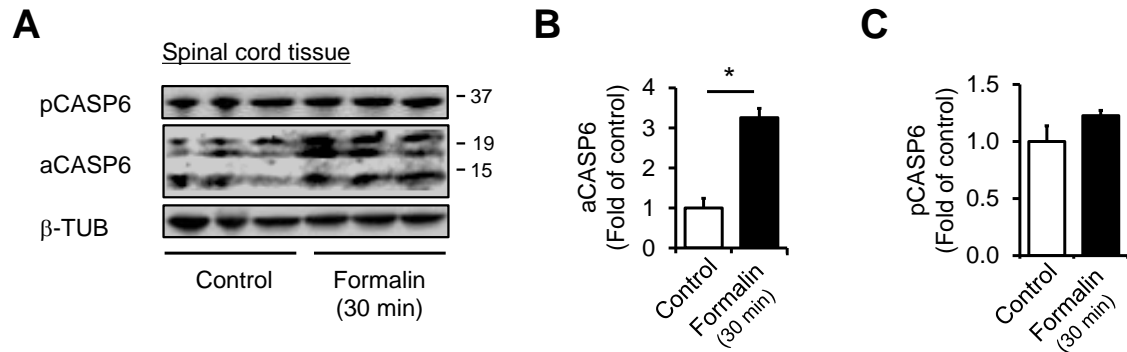
Supplemental table 1. Sequences of the primers for the quantitative real-time RT-PCR

Target gene	Forward primers	Reverse primers	Genbank No.
GAPDH	AGG TCG GTG TGA ACG GAT TTG	GGG GTC GTT GAT GGC AAC A	XM_001479371
ATF3	GGT CGC ACT GAC TTC TGA GG	CTC TGG CCG TTC TCT GGA	NM_007498
CASP1	TCT GTA TTC ACG CCC TGT TG	GAT AAA TTG CTT CCT CTT TGC CC	NM_009807
CASP3	GAC TGA TGA GGA GAT GGC TTG	TGC AAA GGG ACT GGA TGA AC	NM_009810
CASP6	TCA GGG CTA GGA CAC CG	TTG AAG ATG AGG GCA ACT CC	NM_009811

Supplemental table 2. Sequences of the outer and inner primers for single-cell PCR

| Target gene (Product length)[a]	Outer primers	Inner primers	Genbank No.
TNF-α (393 bp, 207 bp)	GTCTCAGAATGAGGCTGGATAAG GAGCAGAGGTTCAGTGATGTAG	TAAGATCTCAGGCCTTCCTACC CTGAAGACAGCTTCCCACAC	NM_013693
IBA-1 (381 bp, 218 bp)	GCAGACTTCATCCTCTCTCTTC GACGGCAGATCCTCATCATT	CCTCAGCTGCCTGTCTTAAC AATCCCTGCTTTGGCTCAT	NM_019467
GFAP (371 bp, 218 bp)	CAGAGCGAGCCTATGCTAAA CGTCCAGAGGGAACTAACTAAC	GCCTATGCTAAAGGTTAGGTTGTA AGCACTGAAGTGAAGCAATAGA	NM_001131020
GAPDH (367 bp, 313 bp)	AGCCTCGTCCCGTAGACAAAA TTTTGGCTCCACCCCTTCA	TGAAGGTCGGTGTGAACGAATT GCTTTCTCCATGGTGGTGAAGA	XM_001473623

[a] (n, n) indicates product size obtained from outer and inner primers, respectively.

A

Spinal cord tissue

pCASP6 — 37

aCASP6 — 19
— 15

β-TUB

Control   Formalin
(30 min)

B

aCASP6
(Fold of control)

*

Control   Formalin
(30 min)

C

pCASP6
(Fold of control)

Control   Formalin
(30 min)

Supplemental Figure 1. **(A)** Western blotting analysis showing the bands of active CASP6 (aCASP6, ≈20 and 10 kDa) and proCASP6 (pCASP6, ≈ 35 kDa) in the ipsilateral and contralateral (control) side of the spinal cord dorsal horn 30 min after the formalin injection. **(B, C)** Intensity of aCASP6 (B) and pCASP (C) bands. The data are shown as fold of control. Note that formalin only increases aCASP6. *$P<0.05$, n=3 mice.

A Primary mechanical allodynia

Paw withdrawal frequency (%)

WT
$Casp6^{-/-}$

BL | 0.5 h | 1 h | 2 h | 4 h

Time after capsaicin injection

B Secondary mechanical allodynia

Paw withdrawal threshold (%)

BL | 0.5 h | 1 h | 2 h | 4 h

Time after capsaicin injection
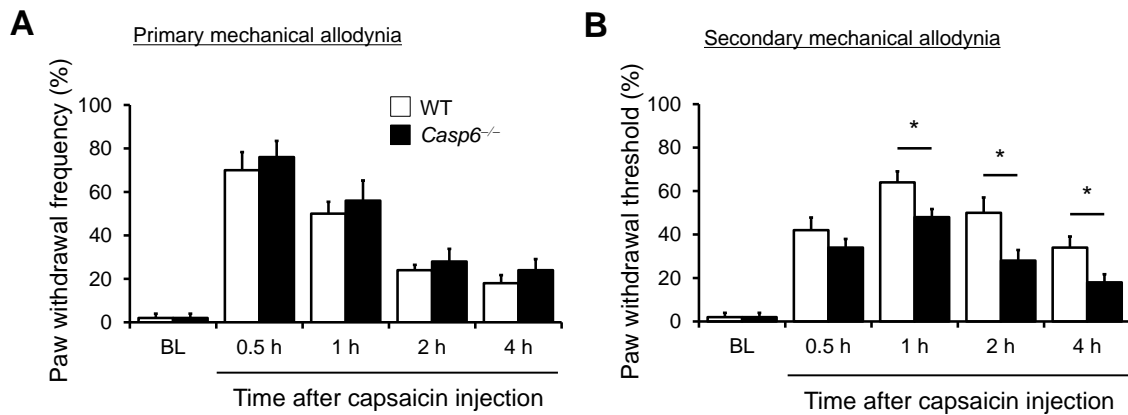
Supplemental Figure 2. Primary **(A)** and secondary **(B)** mechanical allodynia in WT and $Casp6^{-/-}$ mice following intraplantar injection of capsaicin (5 μg, 5 μl). Primary and secondary mechanical allodynia were measured, in response to a 0.16g Von Frey hair, at the injection site (center of paw, primary) and the base of the third toes (0.5 cm away from the injection site, secondary), respectively. Note only the secondary mechanical allodynia was reduced in $Casp6^{-/-}$ mice. *$P<0.05$, compared to corresponding WT control, n=5 mice.
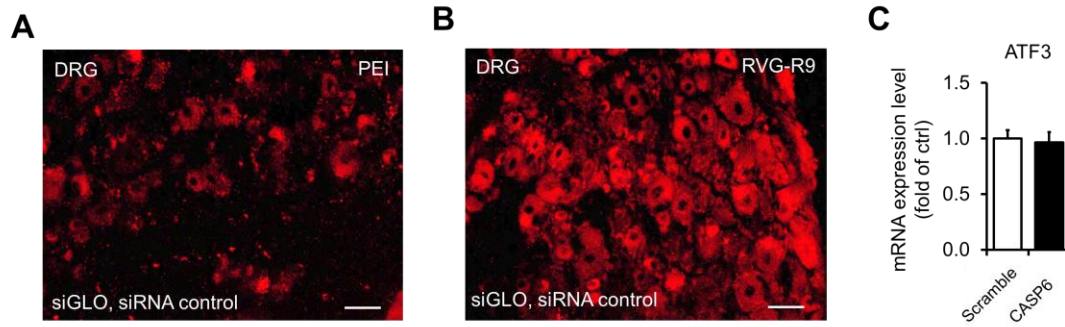
Supplemental Figure 3. Peri-sciatic delivery of Cy3-labeled siRNA results in uptake of siRNA by DRG neurons without causing axonal damage. **(A,B)** DRG sections showing neuronal uptake of fluorescence-labeled siRNA 2 days after peri-sciatic injection of labeled control siRNA (siGLO, 2 μg, 6 μl, mixed with PEI (A) or RVG peptide (B), molar ratio RVG:siRNA=1:10). Scales, 20 μm. Note that more DRG neurons up-take siRNA when siRNA was mixed with RVG-R9 peptide than mixed with PEI (Polyethylenimine). **(C)** RT-PCR in DRG tissues shows that peri-sciatic injection of CASP6 siRNA does not affect the expression of ATF3, a marker for axonal degeneration. $P>0.05$, compared to control siRNA (scramble siRNA), n=6 mice.
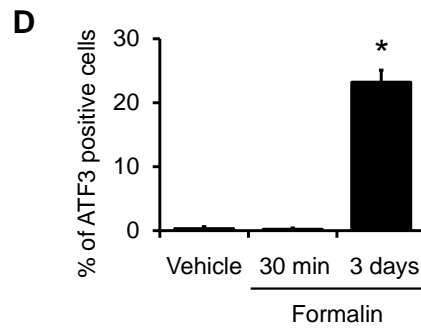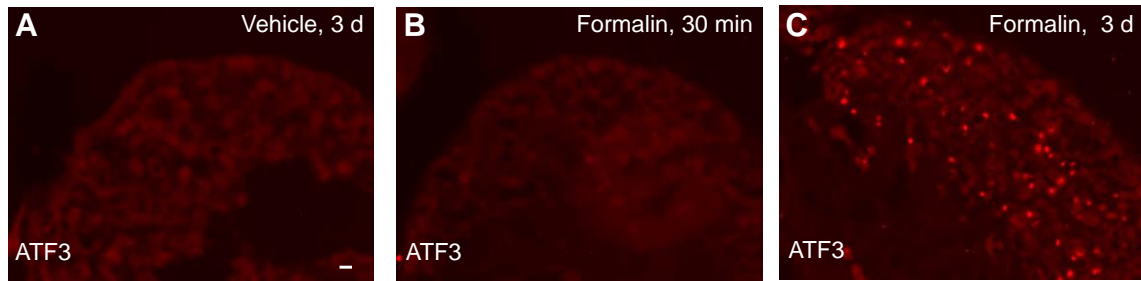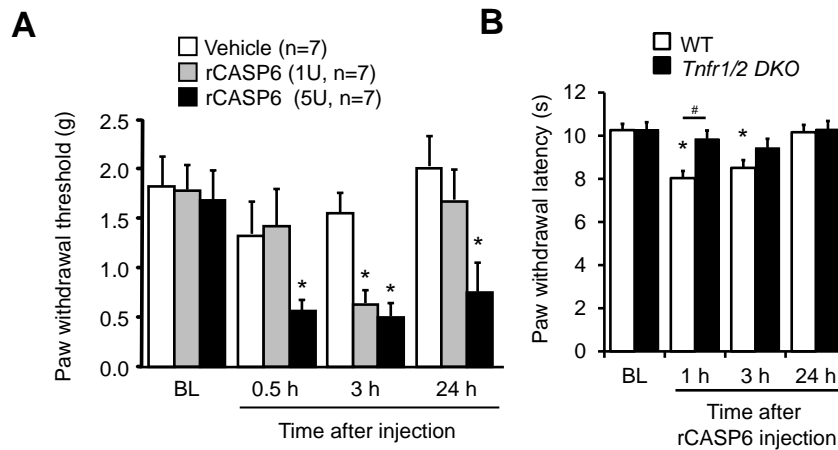
Supplemental Figure 4. Intraplantar injection of formalin induces ATF3 expression in DRG neurons after 3 days. **(A-C)** ATF3 immunostaining in DRG sections of vehicle (A) and formalin treated animals for 30 min (B) and 3 d (C). Scale, 20 μm. **(D)** Percentage of ATF3-positive neurons in DRGs. *$P<0.05$, n=4 mice.
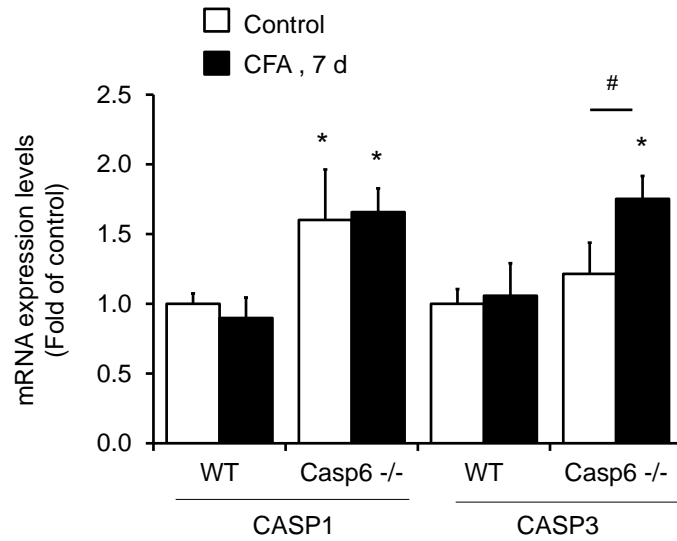
Supplemental Figure 5. *Casp6$^{-/-}$* mice show compensation of CASP1 and CASP3 in the spinal cord, as revealed by quantitative real-time PCR. Note that CFA inflammation only increases CASP3 in KO mice.   *P<0.05, compared to corresponding WT control mice; [#] P<0.05, compared to non-inflamed KO mice, n=4 mice.
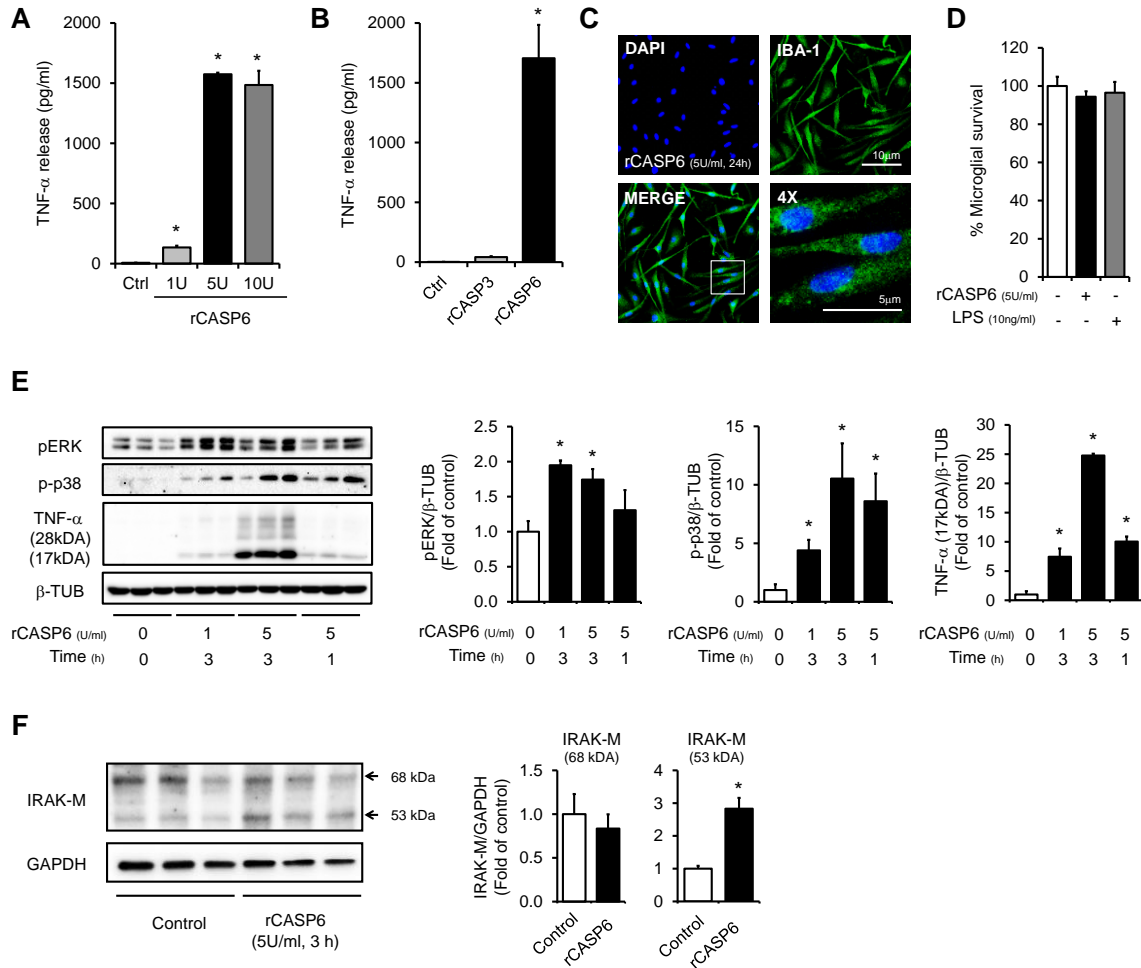
Supplemental Figure 6. Recombinant caspase-6 (rCASP6) induces TNF-α release and expression, activation of MAP kinases, and cleavage of IRAK-M without causing cell death in primary cultures of microglia. **(A)** rCASP6 (1, 5, and 10 U/ml, 3 h) evokes dose-dependent TNF-α release in microglial cultures, as revealed by ELISA analysis. *P<0.05, compared to control, n=3 cultures. **(B)** rCASP6 but not rCASP3 (5 U/ml, 3 h) induces TNF-α release in microglial cultures. *P<0.05, compared to control, n=4 cultures. **(C)** Double staining IBA-1 and DAPI in microglial cultures. Note that all cells (DAPI+) in the culture are microglia (IBA-1+). **(D)** Cell survival rate, as revealed by MTT cell proliferation assay, after rCASP6 treatment (5 U/ml, 3 h). *P>0.05, n=4 cultures. Note that extracellular rCASP6 does not change microglial survival rate. **(E)** rCASP6 induces ERK and p38 activation (phosphorylation) and TNF-α activation in microglia, as shown by western blotting. Right, intensity of pERK, p-p38, and TNF-α (active form, 17 kDa) bands. *P<0.05, compared to control, n=3 cultures. **(F)** rCASP6 induces cleavage of IRAK-M in microglia, as shown by western blotting. Right, intensity of pre-IRAK-M (68 kDa) and cleaved IRAK-M (53 kDa) bands. *P<0.05, compared to control, n=3 cultures.

Supplemental Figure 7. Intrathecal injection of rCASP6 induces mechanical and thermal hypersensitivity. **(A)** Intrathecal rCASP6 (1 and 5 U) elicits dose-dependent mechanical allodynia. $*P<0.05$, vs. vehicle (PBS), n=7 mice. **(B)** rCASP6 (i.t., 5 U) also induces heat hyperalgesia, which is impaired in *Tnfr1/2* double knockout (*Tnfr1/2* DKO) mice. $*P<0.05$, vs. WT control, n=7 mice.
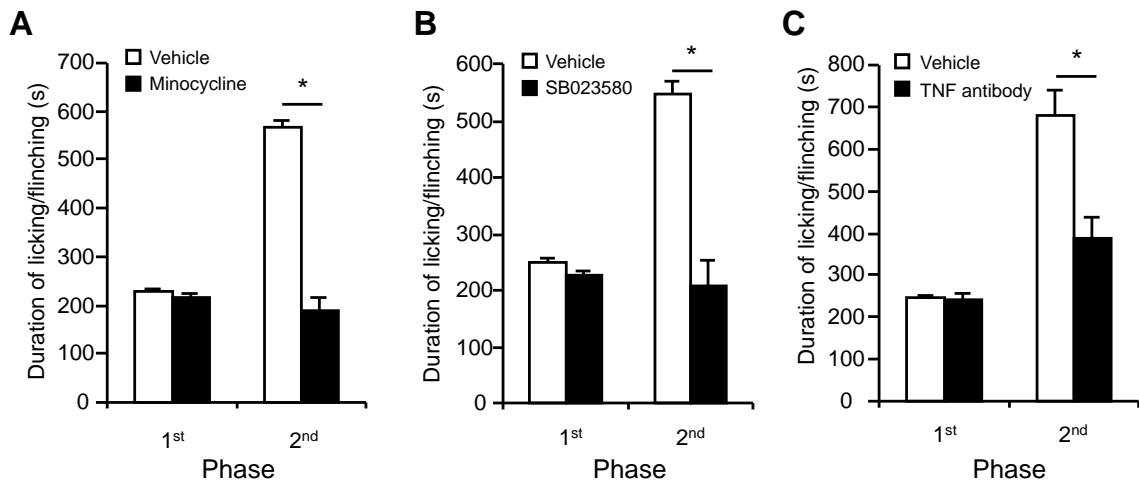
Supplemental Figure 8 (A-C) Spinal cord microglial activation contributes to formalin-induced acute inflammatory pain in the 2nd phase. Intraplantar injection of formalin (5%) induces spontaneous pain, characterized by the 1st phase (0-10 min) and the 2nd phase (10-45 min) responses. Intrathecal injection of the microglial inhibitor minocycline (50 μg, A), p38 MAPK inhibitor SB203580 (10 μg, B), or TNF-α neutralizing antibody (1 μg, C), significantly reduces the formalin-induced 2nd phase pain. *$P<0.05$, compared to vehicle (PBS in A and B and control serum in C). n=5-7 mice.
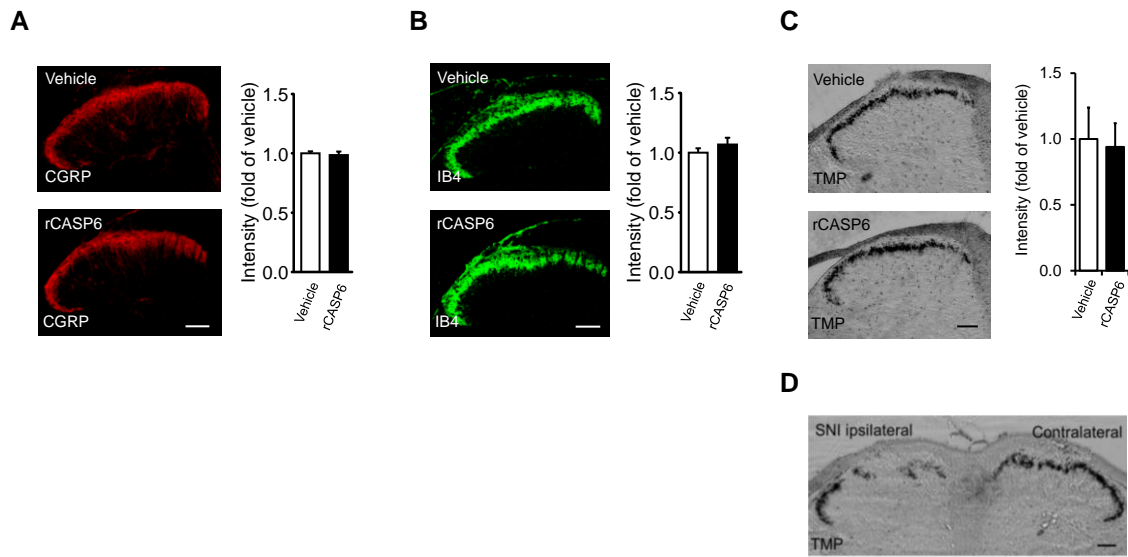
Supplemental Figure 9. Intrathecal injection of rCASP6 (5 U) does not cause axonal degeneration in the spinal cord. **(A-C)** Staining of CGRP (A), IB4 (B), and thiamine monophosphatase (TMP) (C) in the spinal cord 24 h after vehicle (saline) and rCASP6 treatment. Graphs show the density of staining in the dorsal horn. $P>0.05$, rCASP6 vs. vehicle control, n=4-5 mice. Note that spinal rCASP6 does not cause the degeneration of CGRP[+] peptidergic axons (A) and IB4^{+} (B) and TMP[+] (C) non-peptidergic axons in the dorsal horn. **(D)** As a positive control, spared nerve injury (SNI, 3 d) causes marked axonal degeneration in the dorsal horn, as shown by TMP staining. Scales, 100 μm.
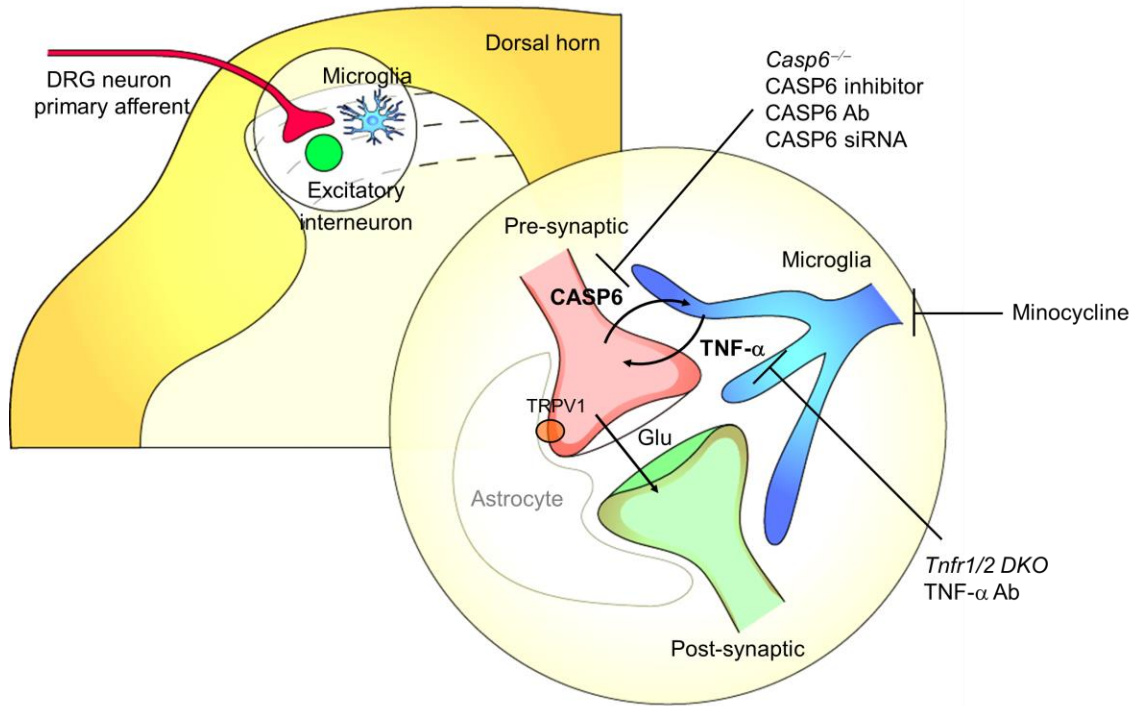
Supplemental Figure 10. Schematic of axonal-microglial interactions in the spinal cord dorsal horn. CASP6 is uniquely localized in spinal cord axonal terminals of C-fibers from dorsal root ganglion (DRG). CASP6-expressing axonal terminals form synapses to lamina IIo excitatory neurons, which in turn synapse to lamina I projection neurons. These CASP6-expressing axonal terminals also have close contacts with microglial cell bodies and processes. Intense and persistent activation of C-fibers results in CASP6 release from axonal terminals, which acts on microglia to trigger TNF-α release. TNF-α binds TNF receptors (TNFR) on presynaptic sites to elicit glutamate release via TRPV1 activation and Ca^{2+} influx, leading to enhanced synaptic transmission (EPSC frequency increase in post-synaptic neurons) and pain hypersensitivity. In this study, CASP6 was targeted by *Casp6* gene deletion and also by CASP6 inhibitor, neutralizing antibody (Ab), and siRNA. Microglia were inhibited by minocycline. TNF-α was targeted by neutralization and *Tnfr* deletion.