

JPET #105569

Prostaglandin E2 receptor EP4 contributes to inflammatory pain hypersensitivity

Chung-Ren Lin, Fumimasa Amaya, Lee Barrett, Haibin Wang, Junji Takada, Tarek A. Samad,  
and Clifford J Woolf

Neural Plasticity Research Group, Massachusetts General Hospital and Harvard Medical School,  
Boston, USA (F.A., L.B., H.W., T.S., C.J.W.); Pfizer Global Research and Development,  
Discovery Biology Research, Aichi, Japan (J.T.); Department of Anesthesiology, Chang Gung  
Memorial Hospital and Chung Gang University, Kaohsiung, Taiwan, 833 (C-RL)

JPET #105569

**Running Title:** EP4 and inflammatory pain

**Address correspondence to:** Dr. Clifford J. Woolf, Neural Plasticity Research Group,  
Department of Anesthesia and Critical Care, Massachusetts General Hospital, 149 13<sup>th</sup> Street,  
Room 4309, Charlestown, MA 02129, USA. Telephone: 617-7243622; Fax: 617-7243632

Email: [cjwoolf@partners.org](mailto:cjwoolf@partners.org)

Number of text pages: 20

Number of tables: 0

Number of figures: 5

Number of references: 38

Number of words in Abstract: 144

Number of words in Introduction: 450

Number of words in Discussion: 1495

**Abbreviations:**

PG, prostaglandin; EP receptor, prostaglandin E2 G-protein-coupled receptor; DRG, dorsal root ganglion; NSAIDs, nonsteroidal anti-inflammatory drugs; COX, cyclooxygenase; CVS, cardiovascular system; CFA, complete Freund's adjuvant; siRNA, short interfering RNA; shRNA, short hairpin RNA; MS shRNA, mismatched shRNA; PKA, protein kinase A; TXA2, thromboxin A2; ANOVA, analysis of variance.

**Recommended section assignment:** Neuropharmacology

JPET #105569

## Abstract

Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) is both an inflammatory mediator released at the site of tissue inflammation and a neuromodulator that alters neuronal excitability and synaptic processing. The effects of PGE<sub>2</sub> are mediated by four G-protein-coupled EP receptors (EP1-EP4). Here we show that the EP4 receptor subtype is expressed by a subset of primary sensory dorsal root ganglion (DRG) neurons and that its levels, but not that of the other EP1-3 subtypes, increase in the DRG after complete Freund' adjuvant –induced peripheral inflammation. Administration of both an EP4 antagonist (AH23848, ((4Z)-7-[(rel-1S,2S,5R)-5-((1,1'-Biphenyl-4-yl)methoxy)-2-(4-morpholinyl)-3-oxocyclopentyl]- 4-heptenoic acid) and EP4 knockdown with intrathecally delivered short hairpin RNA (shRNA) attenuate inflammation-induced thermal and mechanical behavioral hypersensitivity, without changing basal pain sensitivity. AH23848 also reduces the PGE<sub>2</sub> mediated sensitization of capsaicin-evoked currents in DRG neurons *in vitro*. These data suggest that EP4 is a potential target for the pharmacological treatment of inflammatory pain.

JPET #105569

## Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are the most commonly used class of analgesics. Traditional NSAIDs inhibit prostaglandin synthesis through a nonselective blockade both of the constitutively expressed cyclooxygenase, COX-1, and the inducible isomer, COX-2. Their clinical use is hampered, however, by side effects, most notably gastric erosion, ulceration and hemorrhage. Although selective COX-2 inhibitors were designed to prevent the adverse effects mediated by inhibition of COX-1, prolonged use of COX-2–selective inhibitors may, like NSAIDs, confer a risk for cardiovascular events, including myocardial infarct and stroke. The cause of the cardiovascular adverse effects is uncertain but may include an imbalance in prostacyclin and thromboxane levels in the endothelium and blockade of prostanoid actions on renal function. Identification of therapeutic targets downstream of COX may provide opportunity for the development of analgesics that interfere with prostanoid pro-inflammatory and pro-nociceptive actions, but with less cardiovascular risk.

Prostaglandin E2 (PGE2) is the principal pro-inflammatory prostanoid and contributes in particular, to one of the key features of inflammation, pain hypersensitivity. At the site of inflammation PGE2 sensitizes peripheral nociceptors through activation of EP receptors present on the peripheral terminals of these high threshold sensory neurons, reducing threshold and increasing responsiveness, the phenomenon of peripheral sensitization (Omote et al., 2002a). PGE2 is also produced in the spinal cord after tissue injury (Samad et al., 2001), where it contributes to central sensitization (Minami et al., 2001), an increase in excitability of spinal dorsal horn neurons that produces pain hypersensitivity.

JPET #105569

Four PGE<sub>2</sub> G-protein-coupled receptor subtypes (EP1, EP2, EP3, and EP4) that are products of different genes have been identified and these mediate the diverse effects of the prostanoid, based on their differential tissue distribution and coupling to intracellular signal transduction pathways. EP2 and EP4 are coupled to G<sub>s</sub>, some splice variants of EP3 to G<sub>s</sub> and some to G<sub>i</sub>, while EP1 is coupled to G<sub>q</sub>/G<sub>11</sub>. Studies performed either in mutant mice lacking the individual PG receptors (Minami et al., 2001; Murata et al., 1997; Stock et al., 2001) or with EP receptor specific ligands (Minami et al., 1994) have not yet provided a coherent picture of which EP receptor(s) are responsible for inflammatory pain. This is partly due to the fact that PGE<sub>2</sub> facilitates nociception at multiple levels in the neuraxis, and that multiple receptors are involved. EP1, EP2, EP3 and EP4 (Oida et al., 1995) receptors are expressed by dorsal root ganglion neurons, while EP2 receptors are expressed by spinal cord neurons (Kawamura et al., 1997).

We have now investigated the expression and regulation of EP4 in DRG neurons after peripheral inflammation and have explored its involvement in inflammatory pain by either administering an EP4 antagonist or by knockdown of EP4 *in vivo* with intrathecally delivered shRNA.

## **Materials and Methods**

### *Animals*

All experiments were carried out with the approval of the MGH subcommittee on research animal care. Adult male (~200g) Sprague-Dawley rats were used. Peripheral inflammation was induced by intradermal injection of 0.1 ml of complete Freund's adjuvant (5.0 mg/ ml of heat-

JPET #105569

killed *Mycobacterium butyricum* in mineral oil, CFA) into the plantar surface of the left hind paw. The adjuvant was injected into rats anesthetized with 3% isoflurane. Under 2.5% isoflurane anesthesia an intrathecal catheter (32G, Recathco) was implanted into the intrathecal space of the vertebral column with its tip at the lumbar (L3-5) level through a cisternal incision. The catheter was connected to an osmotic minipump (model 2001 delivering 1µL/hr; Alzet). EP4 shRNA and MS shRNA were continuously infused at 200 µg/day for 7 days. The EP4 antagonist AH23848 ((4Z)-7-[(rel-1S,2S,5R)-5-((1,1'-Biphenyl-4-yl)methoxy)-2-(4-morpholinyl)-3-oxocyclopentyl]-4-heptenoic acid hemicalcium salt) (Sigma) (Davis and Sharif, 2000) was injected intraperitoneally and inflammatory nociceptive hypersensitivity measured 30 minutes post-injection.

#### *shRNA*

shRNA was synthesized using the T7 Ribomax express RNA system (Promega). SiRNA sequences were designed with the aid of shRNA Target Designer (Promega). The top strand for the hairpin shRNA1 was GGATCCTAAT ACGACTCACT ATAGGAAGAC TGTGCTCAGT AATTCAAGAG ATTACTGAGC ACAGTCTTCC and the bottom strand AAGGAAGACT GTGCTCAGTA ATCTCTTGAA TTA CTGAGCA CAGTCTTCCT ATAGTGAGTC GTATTAGGAT CC. The top strand for the hairpin shRNA 2 was GGATCCTAAT ACGACTCACT ATAGACTGGA CCACCAACGT AATTCAAGAG ATTACGTTGG TGGTCCAGTC and the bottom strand AAGACTGGAC CACCAACGTA ATCTCTTGAA TTACGTTGGT GGTCCAGTCT ATAGTGAGTC GTATTAGGAT CC. The top strand for the mismatch control shRNA was GGATCCTAAT ACGACTCACT ATAGACTGCT CCAGCAACCT AATTCAAGAG ATTAGGTTGC TGGAGCAGTC and the bottom strand

JPET #105569

AAGACTGCTC CAGCAACCTA ATCTCTTGAA TTAGGTTGCT GGTCCAGTCT  
ATAGTGAGTC GTATTAGGAT CC.

The concentration of shRNA was determined by spectrophotometry OD 260.

### *Immunohistochemistry*

Sections were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (PB) for 15 min at 4<sup>0</sup>C. For colocalization of EP4 and NF200 or peripherin, sections were immunostained for EP4 using tyramide signal amplification (Perkins Elmer). Following primary antibody incubation (1:2000 EP4, PerkinElmer Life Sciences) in TNB buffer (0.1 M Tris buffered saline, pH 7.4, containing 1% blocking reagent), overnight at RT, sections were incubated with biotin-conjugated anti-rabbit antiserum (1:100 in TNB buffer, 2 h at RT), streptavidin-HRP (1:100 in TNB buffer, 30 min at RT), and Fluorophore Tyramide (1:50 in amplification diluent, 10 min at RT). After washing, sections were incubated with mouse anti-NF200 (1:1000, Sigma,) or mouse anti-peripherin (1:1000, Sigma) antibody in 0.1 M PBS containing 1% BSA, 0.5% Triton X-100 for 16 h at RT, followed by Rhodamine-conjugated anti-rabbit incubation (1:200 in 0.1 M PBS for 2 h at RT).

### *Image Analysis*

Four EP4 antibody stained sections (minimum separation 90  $\mu$ m) were selected from the L5 DRG from four animals in each group to analyze the proportion of EP4 labeled neuronal profiles and determine size frequency distribution. Signals were analyzed under X400 magnification. Signal intensity and area were calculated using the NIH Image software system. Only neurons

JPET #105569

with visible nuclei were analyzed. Neurons were determined as EP4 positive if their signal intensity was 3-fold higher than background. All counting was performed blinded.

### *In situ hybridization*

A digoxigenin (DIG)-labeled RNA probe was transcribed from a human EP4 fragment cloned into pCRII (Invitrogen) using 10X DIG labeling mixture and SP RNA polymerase (Roche). Signal specificity was confirmed by the absence of signal when sections were hybridized with the sense probe. Tissue preparation, sectioning and fixation proceeded as described for the immunohistochemistry. After washing in 0.1 M PBS, sections were acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine and 0.9% NaCl for 15 min at room temperature. Sections were then incubated with RNA probe in hybridization buffer (Sigma) at 60<sup>0</sup>C for 12 h. After three washes in 50% formamide in 2XSSC at 60<sup>0</sup>C for 15 min, sections were incubated with anti DIG Ab (Roche; 1:1000) diluted in DIG buffer 1 (100 mM Tris-HCl pH 7.5, 150 mM NaCl), 1% blocking reagent and 0.3% Triton X-100 for 12 h at 4<sup>0</sup>C. Signals were visualized by incubating with 4.5 ml/ml of BCIP and 3.5 ml/ml of NBT in DIG buffer 3 (100 mM Tris-HCl pH 7.5, 100 mM NaCl, 50 mM MgCl<sub>2</sub>).

### *Western blots*

DRGs or HEK293T cells transfected with EP4 were homogenized-sonicated in a lysis buffer (200 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40, and 10% glycerol) containing a mixture of proteinase inhibitors (Roche). Protein samples were separated on a SDS-PAGE gradient gel (4-15%; Bio-Rad) and transferred to a polyvinylidene difluoride filter (Immobilon-P; Millipore). The blots were blocked with 6% skim milk (Difco) in TBS-T (Tris-



JPET #105569

buffered saline, 0.15% Tween 20) for 1 hr and incubated with EP4 antibody (1:6000) overnight at 4°C. The blots were incubated with HRP-conjugated anti-rabbit secondary antibody (1:2000; Amersham Biosciences) for 1 hr at room temperature, developed in ECL solution (PerkinElmer Life Sciences) for 1 min, and exposed on x-ray film (Amersham Biosciences) for 1-10 min. For densitometric analysis, blots were scanned using the Model GS-710 imaging densitometer, and results expressed as the ratio of EP4 immunoreactivity to beta-actin immunoreactivity.

#### *Behavioral assessment of thermal and mechanical stimulation*

All behavioral tests were performed blinded to the treatment. The thermal nociceptive threshold was measured using a radiant heat technique (Hargreaves et al., 1988). Latency between the application of a focused light beam and a hind-paw-withdrawal response was measured to the nearest 0.1 sec, with a cut-off time of 20 sec. The punctate mechanical withdrawal threshold was determined using a series of calibrated von Frey filaments (Stoelting), ranging from 0.23 to 59.0 g. Animals were placed in a plastic cage with a metal mesh floor, allowing them to move freely. The animals were habituated to the testing environment and allowed to acclimatize to this environment for at least 20 min before the experiment and the filaments were presented to the midplantar surface. Withdrawal threshold measurements were collected using an up-down method.

#### *Electrophysiology*

Primary adult rat DRG neuron cultures were prepared as described and incubated in Neurobasal medium containing nerve growth factor (Sigma, 100 ng/ml). Whole-cell patch-clamp recordings were performed 2-4 days after dissociation of the DRG. Conventional whole-cell patch clamp

JPET #105569

was used. Briefly, only small DRG neurons (< 30 microns) were recorded. A typical recording pipette resistance was about 2 M $\Omega$ ; once the whole-cell configuration was achieved, the neurons were held at -70 mV and voltage error was not compensated. Signals were recorded by Axon 200A (Molecular Devices, CA) in gap-free mode filtered at 5KHz and sampled at 2 KHz with pClamp 8 (Molecular Devices, CA). External solution (in mM, pH 7.4 with NaOH): 145 NaCl, 5 KCl, 2 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 10 Glucose and 10 Hepes; internal solution (in mM, pH 7.4 with KOH): 15 KCl, 130 K aspartate, 1 MgCl<sub>2</sub>, 0.5 CaCl<sub>2</sub>, 10 EGTA, 10 Hepes and 2 Glucose. Capsaicin was delivered for 30 seconds through a custom-made multibarrel fast exchange drug applicator positioned ~300 microns from the recording neurons, to achieve a final concentration of ~0.2  $\mu$ M. To avoid inadvertent desensitization due to previous application of capsaicin, only one cell was studied from each culture dish. Between two capsaicin applications, cells were treated with either vehicle or PGE2 (10  $\mu$ M, 3 min). In the EP4 antagonist AH23848 study, AH23848 (10  $\mu$ M, Tocris) was pretreated for 30 minutes before recording. Capsaicin receptor desensitization was expressed as the ratio of the 2<sup>nd</sup> peak response to the 1<sup>st</sup> peak.

### *Statistics*

All behavior and Western data are presented as mean  $\pm$  SD. ANOVA followed by Tukey's post hoc test was used for statistical analysis, with  $P < 0.05$  considered significant. The capsaicin response data are presented as mean  $\pm$  SEM, and p values analyzed by one-way ANOVA.

## **Results**

*EP4 receptors are expressed by a subset of dorsal root ganglion neurons*

JPET #105569

The expression of EP4 receptors was analyzed in the lumbar (L5) DRG and spinal cord of naïve rats. EP4 was detected by immunohistochemistry in many cellular profiles in the DRG (Figure 1A). Double label indicated expression of EP4 in both small unmyelinated (peripherin positive) and myelinated (NF200 positive) primary sensory neurons (Fig. 1B), but not in spinal cord neurons (data not shown). EP4 mRNA expression in DRG neurons as detected by *in situ* hybridization had a neuronal expression pattern very similar to that of immunolabeled EP4 (Figure 1C). A Western blot using the same antibody as for the immunohistochemistry revealed a single band of the expected size (Figure 1D). This antibody also recognized recombinant EP4 expressed in HEK cells (data not shown). EP4 mRNA levels analyzed using real time quantitative PCR revealed much higher expression in the DRG than the spinal cord (>12-fold) and both immunohistochemical and Western blot analyses found only low levels of EP4 protein in the dorsal horn (data not shown). The DRG:DH mRNA ratios for EP1, 2 and 3 were 1.2, 2.3 and 1.3 respectively.

#### *Increased Expression of EP4 receptors in the DRG after peripheral inflammation*

After induction of a localized area of peripheral inflammation in the hindpaw by an intraplantar injection of CFA, EP4 protein levels increased significantly in the L5 DRG ipsilateral to the inflamed hindpaw but not in the contralateral non-inflamed hindpaw (Figure 2 A). The increase in EP4 immunoreactivity was present from day 2 through day 7 (the longest time measured). In the L5 DRG ipsilateral to a CFA-treated hindpaw (2 days)  $57 \pm 9.8$  % of peripherin positive neuronal profiles were found to be EP4 positive compared with  $39 \pm 6$  % in the contralateral DRG ( $P < 0.05$ , ipsilateral vs contralateral) (Fig. 2B). The proportion of NF200 positive cells that are EP4 positive did not differ, however, between DRGs ipsilateral and contralateral to the

JPET #105569

inflamed hindpaw (data not shown). Real time RT-PCR analysis of naïve and inflamed L5 DRGs confirmed an increase in EP4 mRNA expression in the DRG, but showed that mRNA for the other EP receptors, EP1, EP2, and EP3 did not change in the DRG (Fig. 2C). In the spinal cord no change in EP1, EP2, EP3 or EP4 mRNA levels occurred after the CFA-induced inflammation (Fig. 2C).

*The EP4 antagonist AH23848 attenuates CFA-induced pain hypersensitivity*

The inflammation produced by intraplantar CFA results in pronounced thermal and mechanical hypersensitivity with a reduction in the thermal (radiant heat) withdrawal latency (Fig. 3A) and in the von Frey mechanical threshold for eliciting a withdrawal response (Fig. 3B). The ipsilateral thermal withdrawal latency was reduced from  $17.4 \pm 1.8$ s to  $10.9 \pm 0.6$ s the mechanical threshold from  $20.8 \pm 2.5$ g to  $8.1 \pm 1.3$ g (n=7 or more). Intraperitoneal injection of the EP4 antagonist AH23848 (Davis and Sharif, 2000) resulted in a dose-dependent reduction in the inflammatory nociceptive hypersensitivity measured 30 minutes post-injection (Figure 3). AH23848 produced no change in thermal latency or mechanical withdrawal thresholds on the contralateral non-inflamed paw (data not shown).

*AH23848 attenuates PGE2 sensitization of capsaicin currents in DRG neurons*

To test if PGE2 has direct actions via EP4 in DRG neurons we used the *in vitro* PGE2 sensitization of capsaicin currents as a model system. Capsaicin is a ligand for the TRPV1 receptor. Repeated exposure of TRPV1 to capsaicin results in a desensitization of the ion channel that is reduced by exposure of the cells to PGE2 (Bhave et al., 2002). More than 90% of TRPV1 positive cells observed in DRG cultures were found to express EP4 (Fig. 4A). This

JPET #105569

indicates that EP4 is expressed by heat sensitive nociceptors.

In order to study the desensitization of capsaicin-activated current in DRG neurons we applied a series of brief (30 sec) pulses of 0.2  $\mu$ M capsaicin at 3-min intervals and measured current using whole cell patch-clamp recordings. Capsaicin-responsive DRG neurons showed a pronounced desensitization on repeated exposure. The mean current amplitude on the second application was  $50 \pm 5\%$  (n=11) of that found with first application (Figure 4B and E). The extent of the desensitization was significantly decreased when the cells were pretreated for 3 min with 10  $\mu$ M PGE2. In this situation, the current amplitude on the second application of capsaicin was  $74 \pm 10\%$  (n=9) of the first application (Figure 4C and E). The EP4 antagonist AH23848 (1  $\mu$ M) blocked PGE2's reduction of the desensitization ( $49 \pm 6\%$ , n=11) without producing an effect by itself (8 cells) (Figure 4D and E).

#### *Intrathecal shRNA infusion knocks down EP4 and reduces CFA pain hypersensitivity*

In preliminary experiments we injected dig-labeled EP4 shRNA intrathecally and found extensive label in the lumbar DRG but only limited penetration into the spinal cord (data not shown). To assess potential neurotoxicity, increasing doses of the EP4 shRNA were continuously delivered intrathecally to naïve rats (n = 3 per dose) via an indwelling cannula with its tip positioned at the levels of L4/L5 and attached to an osmotic minipump. Doses up to 8.3  $\mu$ g/hour did not elicit any signs of hind limb paralysis, sensory loss, vocalization or anatomical damage to the spinal cord and were used in all subsequent experiments. This dose of two independent EP4 shRNAs resulted in a down regulation of EP4 mRNA (Fig. 5A) and protein levels (Fig. 5B) in the L5 DRG (69% reduction). The MS shRNA and vehicle controls had no

JPET #105569

effect (Fig. 5). The shRNA targeted against EP4 had no effect on the levels of the mu opiate receptor (MOR) another GPCR (Fig. 5B). EP4 shRNAs delivered intrathecally, but not MS shRNA or vehicle, produced within 12 hrs a significant reduction ( $P < 0.05$ ) in the CFA-induced thermal and mechanical hyper responsiveness (Figure 5C,D). In order to address the issue of the specificity of EP4 shRNA, we quantified by real time RT-PCR levels of EP1, EP2, EP3 and EP4 mRNA following EP4 shRNA treatment. Only EP4 mRNA levels showed a significant decrease ( $p < 0.05$ ) (Fig. 5E). Similar behavioral effects were produced by the EP4 antagonist and shRNA. The shRNA was administered intrathecally to target lumbar spinal cord and DRG cells that produce the EP4 receptor, to reduce the synthesis of the protein, while the antagonist was administered systemically to block activity of receptors distributed in both the peripheral and central terminals of sensory neurons.

## Discussion

PGE2 is a major mediator of peripheral inflammation. This prostanoid when produced at the site of tissue injury and inflammation after induction of COX-2 and prostaglandin synthases, acts on capillaries to produce vasodilatation and edema, and on macrophages to stimulate release of cytokines (McCoy et al., 2002). Inhibition of PGE2 synthesis produces therefore, an anti-inflammatory effect. PGE2 also has, however, a direct action on the peripheral terminals of nociceptor sensory neurons, reducing their threshold to peripheral stimuli by promoting the phosphorylation of TRPV1 and other TRP channels, and increasing terminal membrane excitability by phosphorylating voltage-gate sodium channels (Lopshire and Nicol, 1998; Smith et al., 2000). Treatment with a monoclonal anti-PGE2 antibody reverses carrageenan-induced

JPET #105569

hyperalgesia to the same extent as NSAIDs, indicating that PGE2 plays a key role in inflammatory pain (Zhang et al., 1997). Peripheral sensitization contributes to pain hypersensitivity within the confines of inflamed tissue, the zone of primary hyperalgesia and inhibiting PGE2 synthesis or action produces an analgesic action by reducing peripheral sensitization.

PGE2 also has a central action causing allodynia and hyperalgesia when applied directly to the spinal cord (Malmberg and Yaksh, 1995). The central action of PGE2 includes a presynaptic action, increasing transmitter release from the central terminals of nociceptors (Vasko, 1995), and postsynaptic actions, including a blockade of glycinergic inhibition (Harvey et al., 2004). The low constitutive levels of COX-1 and COX-2 in the spinal cord generate a short latency, activity-dependent release of PGE2 and peripheral inflammation leads after several hours to a large induction of COX-2 in the spinal cord that contributes to inflammation-induced central sensitization and pain (Samad et al., 2001).

PGE2 exerts its cellular effects through four different G protein-coupled EP receptors.

Activation of the EP1 receptor leads to calcium influx, stimulation of EP2, some isoforms of EP3, and EP4 receptors all increase intracellular cAMP through activation of adenylate cyclase, while activation of other EP3 receptor splice variants inhibit adenylyl cyclase (Funk, 2001; Negishi et al., 1995). We find that EP4 receptors are, as previously described (Oida et al., 1995), expressed by a subset of dorsal root ganglion but not by spinal cord neurons. The actions of PGE2 on peripheral sensitization could include an EP4 component, but any central action of PGE2 mediated by EP4 could act only presynaptically.

JPET #105569

Dissecting out the specific contributions of the different EP receptors to inflammatory pain and indeed other functions has proved relatively difficult. Specific antagonists for each receptor are not available and in consequence knockout mice have been extensively used to study the function of each receptor. IP and EP3, but not EP1, EP2 or EP4 receptors contribute to abdominal writhing in response to an intraperitoneal pro-inflammatory irritant (Ueno et al., 2001), but another study found a contribution of EP1 in a similar model (Stock et al., 2001). Intrathecal PGE2 induces allodynia in wildtype and EP3-deficient mice, but not in EP1-deficient mice (Minami et al., 2001). EP2 deficient mice also show a lack of intrathecal PGE2-evoked hyperalgesia (Reinold et al., 2005). Inoculation of mice with herpes simplex virus type-1 elicits both acute herpetic pain and a delayed “postherpetic” pain and deficiency of EP3, but not EP1, IP or TP, prostanoid receptors diminishes both the acute herpetic pain-like behavior and the delayed “postherpetic” pain (Takasaki et al., 2005). Local PGE2 induced thermal hyperalgesia is reduced in EP1 deficient mice (Moriyama et al., 2005), while EP4 deficient mice display a marked reduction in the development of joint inflammation in a model of rheumatoid arthritis (McCoy et al., 2002). It is difficult to get a clear view of the specific role of the EP receptors from these studies. Interpreting phenotypic differences in null mutations is problematic due to variations in genetic background, developmental effects, genetic compensation, and lack of regional or cell specificity.

Intrathecal injection of the EP1 specific antagonists SC-51089 and SC-51234A result in a suppression of the second phase of formalin test (Malmberg et al., 1994). Another EP1 specific antagonist ONO-8711 decreases incision-induced mechanical hyperalgesia (Omote et al.,



JPET #105569

2002b), chronic constriction injury induced hyperalgesia (Kawahara et al., 2001) and inflammatory pain (Nakayama et al., 2002). AH23848 is reported to be a selective if weak antagonist for EP4 receptors (Coleman et al., 1994). Binding studies utilizing HEK-293 cells expressing cloned EP2 and EP4 receptors produced  $K_i$  values  $> 100\ 000$  nM and 8010 nM, respectively for AH23848 (Boie et al., 1997) and AH23848B produces a  $>90\%$  inhibition of PGE<sub>2</sub> stimulated cAMP formation in human lens epithelial cells (Mukhopadhyay et al., 1999). Paradoxically, ON-AE1-329 a selective EP4 receptor agonist ( $K_i=9.7$  nM for EP4 with selectivity at least 100- fold 100 over EP1, EP2 and EP3) inhibits hyperalgesia and swelling in CFA-induced arthritis (Omote et al., 2002a).

One mechanism with a major role in inflammatory heat pain sensitivity is sensitization of TRPV1 receptors. Both EP<sub>2</sub>, and EP<sub>4</sub> by virtue of their coupling with G<sub>s</sub> to activate adenylate cyclase, increase intracellular adenosine 3',5'-cyclic monophosphate levels which in turn activates protein kinase A (PKA). Activation of PKA by PGE<sub>2</sub> in DRG neurons enhances both capsaicin and heat-mediated responses (Lopshire and Nicol, 1998; Smith et al., 2000), an effect mimicked by membrane-permeant cAMP analogues (Southall and Vasko, 2001). A null mutation of the type I PKA regulatory subunit eliminates PGE<sub>2</sub> induced heat pain hypersensitivity (Malmberg et al., 1997). Our study demonstrates that the EP4 specific antagonist AH23848 attenuates PGE<sub>2</sub> sensitization of capsaicin currents in DRG culture and CFA induced hypersensitivity *in vivo*. In HEK293 cells and DRG neurons PGE<sub>2</sub> increases TRPV1 activity via EP1 receptors in a PKC dependent manner and through PKA via EP4 (Moriyama et al., 2005). These results suggest that both EP4 and EP1 receptors may contribute to the acute PGE<sub>2</sub>-induced peripheral sensitization of sensory neurons. EP4, because of its increased levels after peripheral

JPET #105569

inflammation, may play a more substantial role in peripheral chronic inflammatory pain. In DRG cultures pretreated with antisense oligonucleotides directed against EP3C and EP4 receptor subtype mRNA, the PGE<sub>2</sub>-augmented release of substance P and CGRP is abolished (Southall and Vasko, 2001) suggesting that these receptors mediate neurogenic inflammation.

RNAi induces sequence-specific gene silencing by production of double stranded dsRNA, a phenomenon first discovered in *Caenorhabditis elegans* (Fire et al., 1998). Long dsRNA is processed by the nuclease 'dicer' into 21–23 duplexes, called short interfering RNA (siRNA). siRNAs associate with a RISC nuclease complex to degrade mRNA in a sequence-specific manner. A similar process occurs in mammals (Billy et al., 2001). In cultured mammalian cell lines however, uninterrupted RNA duplexes longer than 30 bp trigger non-specific deleterious cellular responses as the result of the activation of the dsRNA-dependent protein kinase PKR and RnaseL (Stark et al., 1998). This can be avoided by direct administration of 21 nucleotide siRNAs to elicit only a sequence-specific RNAi-mediated inhibition of gene expression (Elbashir et al., 2001). Short hairpin RNA (shRNA) produced *in vitro* or expressed *in vivo* silences genes as effectively as short dsRNAs (Yu et al., 2002). The shRNA system is more effective than the tandem system and shRNAs are more potent inducers of RNAi than siRNA (Siolas et al., 2005). We show here that intrathecal delivery of shRNA suppresses EP4 expression in DRG neurons. Why shRNA penetrates the DRG more successfully than the spinal cord is not clear, although it may reflect in part the relative position of the tip of the intrathecal cannula targeted more caudally than the lumbar enlargement. Nevertheless, considering its effectiveness *in vivo* and relative lack of toxicity even at a high concentrations, shRNA can be used to validate potential novel targets in sensory neurons where small molecule antagonists or inhibitors are not available.

JPET #105569

In conclusion, our data indicate that the EP4 receptor is expressed by primary sensory neurons and that EP4 levels increase in the DRG after peripheral inflammation. Both administration of an EP4 antagonist and EP4 knockdown with shRNA attenuate inflammatory pain hypersensitivity. EP<sub>4</sub> receptor specific antagonists or EP4 shRNA may be, therefore, useful drugs for treating inflammatory pain including conditions like rheumatoid and osteoarthritis. EP4 antagonists as analgesics may have an advantage over other approaches that target prostaglandins such as NSAIDs, COX-2 inhibitors, prostaglandin synthase inhibitors. This will be determined in part by the specific role of EP4 in other PGE<sub>2</sub> mediated functions such as renal homeostasis, endothelial and platelet function, control of blood pressure and GI mucosal function, which remain to be systematically investigated. The overall safety profile of selective EP4 antagonists should be nevertheless different from COX-2 inhibitors and NSAIDs that non-selectively inhibit all prostaglandin synthesis. In particular, EP4 antagonists would not be expected to block PGE<sub>2</sub>/PGI<sub>2</sub> biosynthesis or affect the PGI<sub>2</sub>/TXA<sub>2</sub> balance that may be an underlying cause of renal or cardiovascular side effects associated with COX-2 inhibitors. In the late phase of inflammation COX-2 may actually facilitate the production of anti-inflammatory PGs such as PGA<sub>1</sub> that play roles in the resolution of inflammation by suppressing NF-κB activation and thereby COX-2 gene expression (Mandal et al., 2005). Unlike COX-2 inhibitors, therapies targeting the EP4 receptor may selectively reduce pain without prolonging inflammation.

JPET #105569

## References:

- Bhave G, Zhu W, Wang H, Brasier DJ, Oxford GS and Gereau RWt (2002) cAMP-dependent protein kinase regulates desensitization of the capsaicin receptor (VR1) by direct phosphorylation. *Neuron* **35**:721-773.
- Billy E, Brondani V, Zhang H, Muller U and Filipowicz W (2001) Specific interference with gene expression induced by long, double-stranded RNA in mouse embryonal teratocarcinoma cell lines. *Proc Natl Acad Sci U S A* **98**:14428-14433.
- Boie Y, Stocco R, Sawyer N, Slipetz DM, Ungrin MD, Neuschafer-Rube F, Puschel GP, Metters KM and Abramovitz M (1997) Molecular cloning and characterization of the four rat prostaglandin E2 prostanoid receptor subtypes. *Eur J Pharmacol* **340**:227-241.
- Coleman RA, Grix SP, Head SA, Louttit JB, Mallett A and Sheldrick RL (1994) A novel inhibitory prostanoid receptor in piglet saphenous vein. *Prostaglandins* **47**:151-168.
- Davis TL and Sharif NA (2000) Pharmacological characterization of [(3)H]-prostaglandin E(2) binding to the cloned human EP(4) prostanoid receptor. *Br J Pharmacol* **130**:1919-1926.
- Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K and Tuschl T (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411**:494-498.
- Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE and Mello CC (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **391**:806-811.
- Funk CD (2001) Prostaglandins and leukotrienes: advances in eicosanoid biology. *Science* **294**:1871-1875.

JPET #105569

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.

Harvey RJ, Depner UB, Wassle H, Ahmadi S, Heindl C, Reinold H, Smart TG, Harvey K, Schutz B, Abo-Salem OM, Zimmer A, Poisbeau P, Welzl H, Wolfer DP, Betz H, Zeilhofer HU and Muller U (2004) GlyR alpha3: an essential target for spinal PGE2-mediated inflammatory pain sensitization. *Science* **304**:884-887.

Kawahara H, Sakamoto A, Takeda S, Onodera H, Imaki J, and Ogawa R (2001) A prostaglandin E2 receptor subtype EP1 receptor antagonist (ONO-8711) reduces hyperalgesia, allodynia, and c-fos gene expression in rats with chronic nerve constriction. *Anesth Analg* **93**:1012-1017.

Kawamura T, Yamauchi T, Koyama M, Maruyama T, Akira T and Nakamura N (1997) Expression of prostaglandin EP2 receptor mRNA in the rat spinal cord. *Life Sci* **61**:2111-2116.

Lopshire JC and Nicol GD (1998) The cAMP transduction cascade mediates the prostaglandin E2 enhancement of the capsaicin-elicited current in rat sensory neurons: whole-cell and single-channel studies. *J Neurosci* **18**:6081-6092.

Malmberg AB, Brandon EP, Idzerda RL, Liu H, McKnight GS and Basbaum AI (1997) Diminished inflammation and nociceptive pain with preservation of neuropathic pain in mice with a targeted mutation of the type I regulatory subunit of cAMP-dependent protein kinase. *J Neurosci* **17**:7462-7470.

Malmberg AB, Rafferty MF and Yaksh TL (1994) Antinociceptive effect of spinally delivered prostaglandin E receptor antagonists in the formalin test on the rat. *Neurosci Lett* **173**:193-196.

JPET #105569

Mandal AK, Zhang Z, Kim SJ, Tsai PC and Mukherjee AB (2005) Yin-yang: balancing act of prostaglandins with opposing functions to regulate inflammation. *J Immunol* **175**:6271-6273.

McCoy JM, Wicks JR and Audoly LP (2002) The role of prostaglandin E2 receptors in the pathogenesis of rheumatoid arthritis. *J Clin Invest* **110**:651-658.

Minami T, Nakano H, Kobayashi T, Sugimoto Y, Ushikubi F, Ichikawa A, Narumiya S and Ito S (2001) Characterization of EP receptor subtypes responsible for prostaglandin E2-induced pain responses by use of EP1 and EP3 receptor knockout mice. *Br J Pharmacol* **133**:438-444.

Minami T, Nishihara I, Uda R, Ito S, Hyodo M and Hayaishi O (1994) Characterization of EP-receptor subtypes involved in allodynia and hyperalgesia induced by intrathecal administration of prostaglandin E2 to mice. *Br J Pharmacol* **112**:735-740.

Moriyama T, Higashi T, Togashi K, Iida T, Segi E, Sugimoto Y, Tominaga T, Narumiya S and Tominaga M (2005) Sensitization of TRPV1 by EP1 and IP reveals peripheral nociceptive mechanism of prostaglandins. *Mol Pain* **1**:3.

Mukhopadhyay P, Bhattacharjee P, Andom T, Geoghegan TE, Andley UP and Paterson CA (1999) Expression of prostaglandin receptors EP4 and FP in human lens epithelial cells. *Invest Ophthalmol Vis Sci* **40**:105-112.

Murata T, Ushikubi F, Matsuoka T, Hirata M, Yamasaki A, Sugimoto Y, Ichikawa A, Aze Y, Tanaka T, Yoshida N, Ueno A, Oh-ishi S and Narumiya S (1997) Altered pain perception and inflammatory response in mice lacking prostacyclin receptor. *Nature* **388**:678-682.

Nakayama Y, Omote K and Namiki A (2002) Role of prostaglandin receptor EP1 in the spinal dorsal horn in carrageenan-induced inflammatory pain. *Anesthesiology* **97**:1254-1262.

JPET #105569

- Oida H, Namba T, Sugimoto Y, Ushikubi F, Ohishi H, Ichikawa A and Narumiya S (1995) In situ hybridization studies of prostacyclin receptor mRNA expression in various mouse organs. *Br J Pharmacol* **116**:2828-2837.
- Omote K, Kawamata T, Nakayama Y, Yamamoto H, Kawamata M and Namiki A (2002a) Effects of a novel selective agonist for prostaglandin receptor subtype EP4 on hyperalgesia and inflammation in monoarthritic model. *Anesthesiology* **97**:170-176.
- Omote K, Yamamoto H, Kawamata T, Nakayama Y and Namiki A (2002b) The effects of intrathecal administration of an antagonist for prostaglandin E receptor subtype EP(1) on mechanical and thermal hyperalgesia in a rat model of postoperative pain. *Anesth Analg* **95**:1708-1712.
- Reinold H, Ahmadi S, Depner UB, Layh B, Heindl C, Hamza M, Pahl A, Brune K, Narumiya S, Muller U and Zeilhofer HU (2005) Spinal inflammatory hyperalgesia is mediated by prostaglandin E receptors of the EP2 subtype. *J Clin Invest* **115**:673-679.
- Samad TA, Moore KA, Sapirstein A, Billet S, Allchorne A, Poole S, Bonventre JV and Woolf CJ (2001) Interleukin-1beta-mediated induction of Cox-2 in the CNS contributes to inflammatory pain hypersensitivity. *Nature* **410**:471-475.
- Siolas D, Lerner C, Burchard J, Ge W, Linsley PS, Paddison PJ, Hannon GJ and Cleary MA (2005) Synthetic shRNAs as potent RNAi triggers. *Nat Biotechnol* **23**:227-231.
- Smith JA, Davis CL and Burgess GM (2000) Prostaglandin E2-induced sensitization of bradykinin-evoked responses in rat dorsal root ganglion neurons is mediated by cAMP-dependent protein kinase A. *Eur J Neurosci* **12**:3250-3258.

JPET #105569

- Southall MD and Vasko MR (2001) Prostaglandin receptor subtypes, EP3C and EP4, mediate the prostaglandin E2-induced cAMP production and sensitization of sensory neurons. *J Biol Chem* **276**:16083-16091.
- Stark GR, Kerr IM, Williams BR, Silverman RH and Schreiber RD (1998) How cells respond to interferons. *Annu Rev Biochem* **67**:227-264.
- Stock JL, Shinjo K, Burkhardt J, Roach M, Taniguchi K, Ishikawa T, Kim HS, Flannery PJ, Coffman TM, McNeish JD and Audoly LP (2001) The prostaglandin E2 EP1 receptor mediates pain perception and regulates blood pressure. *J Clin Invest* **10**:325-331.
- Takasaki I, Nojima H, Shiraki K, Sugimoto Y, Ichikawa A, Ushikubi F, Narumiya S and Kuraishi Y (2005) Involvement of cyclooxygenase-2 and EP3 prostaglandin receptor in acute herpetic but not postherpetic pain in mice. *Neuropharmacology* **49**:283-292.
- Ueno A, Matsumoto H, Naraba H, Ikeda Y, Ushikubi F, Matsuoka T, Narumiya S, Sugimoto Y, Ichikawa A and Oh-ishi S (2001) Major roles of prostanoid receptors IP and EP(3) in endotoxin-induced enhancement of pain perception. *Biochem Pharmacol* **62**:157-160.
- Vasko MR (1995) Prostaglandin-induced neuropeptide release from spinal cord. *Prog Brain Res* **104**:367-380.
- Yu JY, DeRuiter SL and Turner DL (2002) RNA interference by expression of short-interfering RNAs and hairpin RNAs in mammalian cells. *Proc Natl Acad Sci U S A* **99**:6047-6052.
- Zhang Y, Shaffer A, Portanova J, Seibert K and Isakson PC (1997) Inhibition of cyclooxygenase-2 rapidly reverses inflammatory hyperalgesia and prostaglandin E2 production. *J Pharmacol Exp Ther* **283**:1069-1075.



JPET #105569

**Footnotes:**

We thank Pfizer and the NIH for financial support.

JPET #105569

### Legends for Figures:

Fig. 1 EP4 expression in the DRG of naïve rats. A, EP4 immunostaining in the L5 DRG. B, Double immunofluorescence for EP4 (green) and peripherin (red) a marker of DRG neurons with unmyelinated axons (left); and NF-200 a marker of cells with myelinated axons (right). C, In situ hybridization of EP4 mRNA in the DRG of naïve rats using sense (left) and antisense probes (right). D, Western blot analysis reveals EP4 expression in L5 DRG of naïve rats.

Fig. 2 Peripheral inflammation upregulates EP4 expression in the DRG. A, Western blots showing an increase in EP4 protein in the L5 DRG after CFA-induced peripheral inflammation, beta actin acts as loading control. EP4 levels (normalized with density of beta-actin bands) increases in the DRG ipsilateral (I) to the inflammation after inflammation compared to naïves (N) or contralateral (C)DRGs. ( $p < 0.05$ ,  $n = 3$ ). B, Double immunofluorescence in a naïve L5 DRG (left) or ipsilateral to CFA inflammation (3 days) (right) for EP4 (green) and peripherin (red). C. Real-time PCR analysis showing an increase in EP4 but not EP1-3 mRNA in the DRG after CFA-induced inflammation. No significant increase was found for any EP receptor in the dorsal horn.

Fig. 3 The effect of the EP4 antagonist AH23848 on the radiant heat withdrawal latency (A) and mechanical threshold (B) in rats 24 hr after CFA injection. Intraperitoneal injection of AH23848 at (0.1-10 mg/kg) prolonged the thermal latency and reduced mechanical thresholds at 30 min (1 and 10mg/kg) and 60 min (10 mg/kg) \* $p < 0.05$  ( $n = 7$  or more).

JPET #105569

Fig. 4 The EP4 antagonist AH23848 attenuates PGE2-mediated sensitization of capsaicin currents in cultured adult DRG neurons. A, Colocalization of EP4 (green) and TRPV1 (red) in cultured rat DRG cells. B, Representative traces showing that repeated capsaicin applications (0.2  $\mu$ M, 0.5 min.) induce receptor desensitization and that PGE2 (10  $\mu$ M, 3 min) treatment prevents this (C). D, The PGE2-mediated sensitization was attenuated by AH23848 (10  $\mu$ M, 30 minutes pre-incubation), while AH23848 by itself did not alter capsaicin receptor desensitization (E). Capsaicin receptor desensitization (E) expressed as ratio of the 2<sup>nd</sup> peak response to the 1<sup>st</sup> peak. Numbers of samples are indicated in the parenthesis. p values analyzed by one-way ANOVA.

Fig. 5 EP4 knockdown with intrathecal shRNA. A, Reduction in EP4 mRNA expression in L5 DRG by in situ hybridization. B, Western blot analysis indicates decreased EP4 protein levels in DRGs from rats that received EP4 shRNA infusion compared to those from naïve and rats that received mismatched shRNA infusion. EP4 knockdown after 7 days of treatment with shRNA reduces thermal (C) and mechanical (D) sensitivity in the CFA induced inflamed hindpaw. For all groups, n=5. \*p<0.05. E. The knockdown produced by the EP4 shRNA1 did not alter EP1, EP2 or EP3 mRNA levels in the DRG, only EP4.

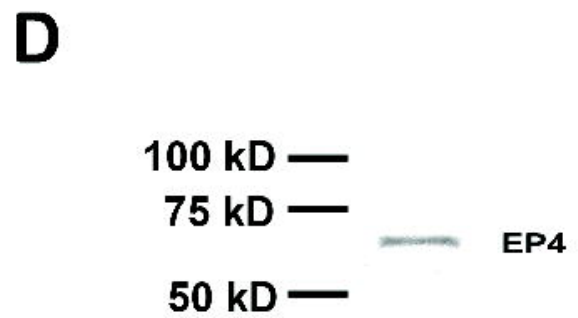
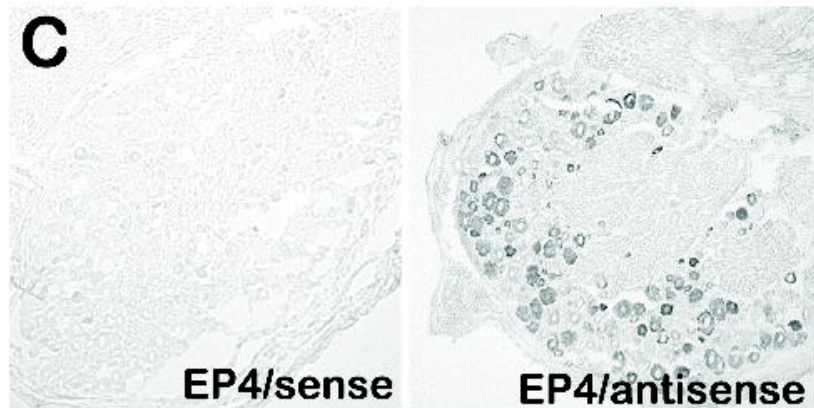
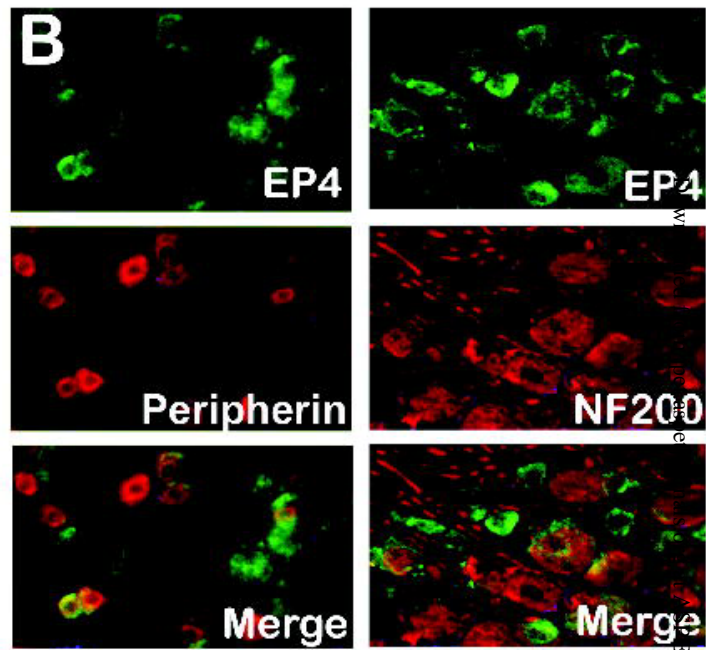
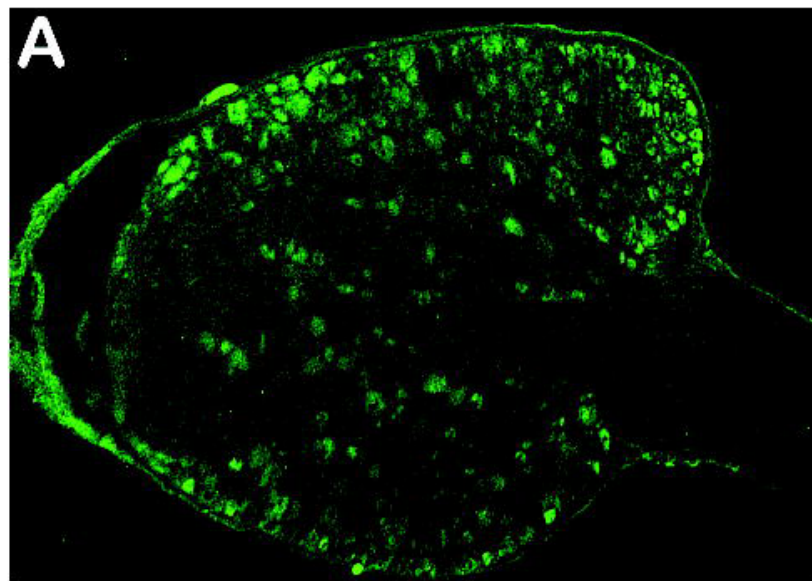


Figure 1

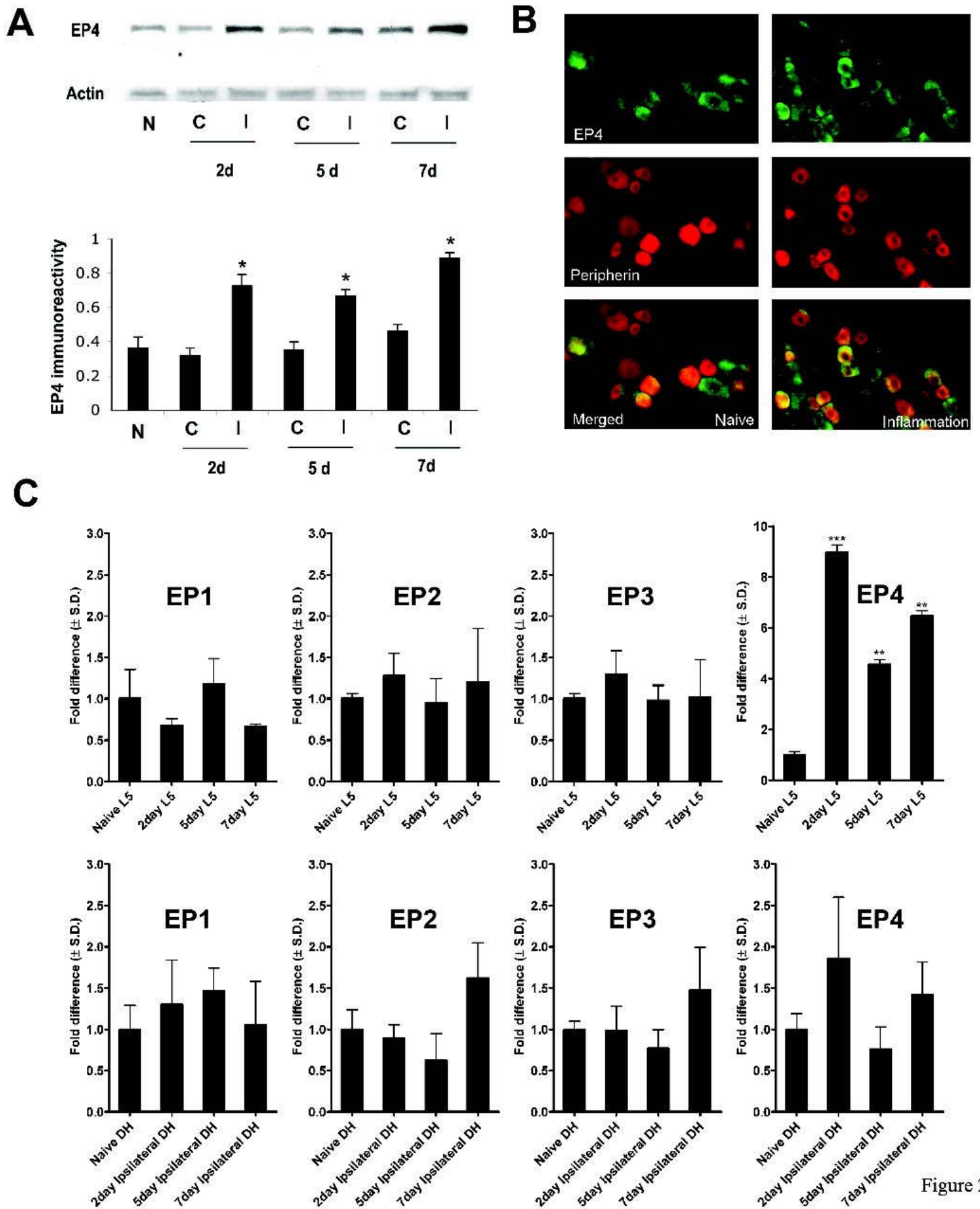


Figure 2

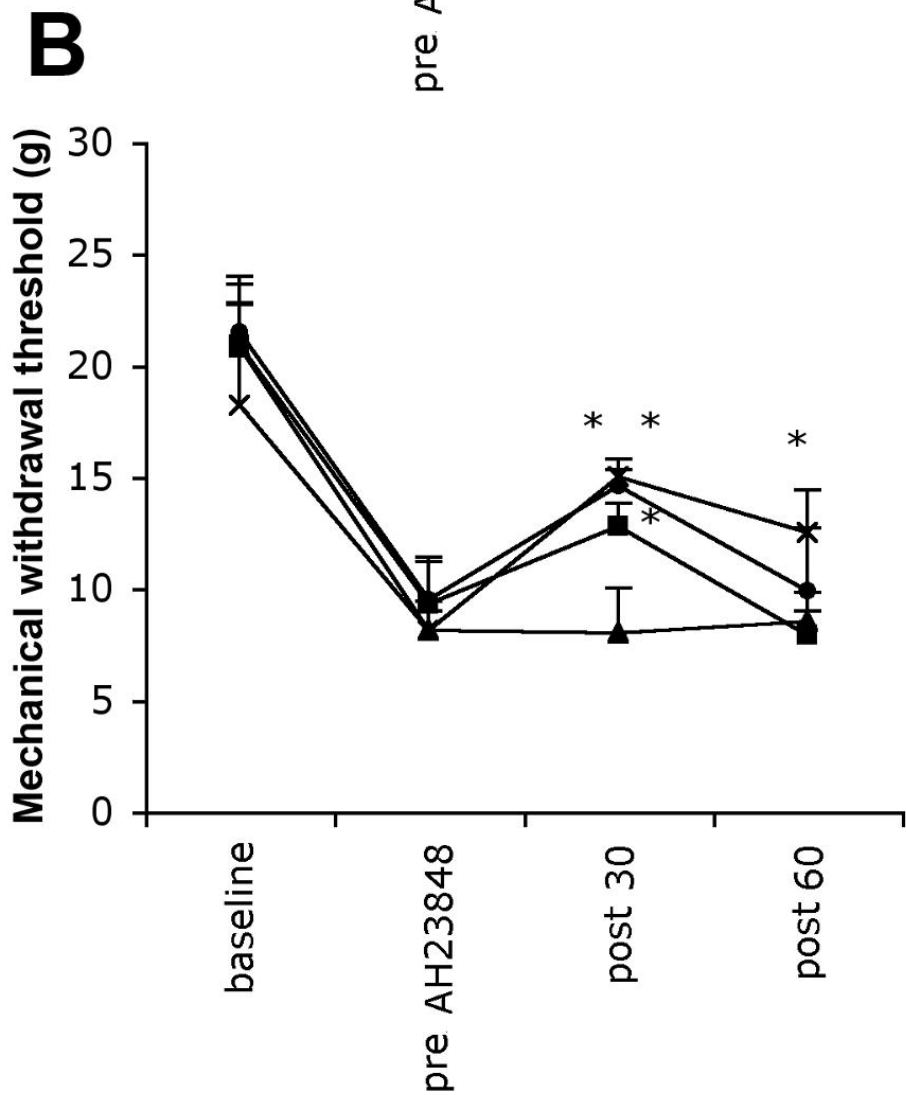
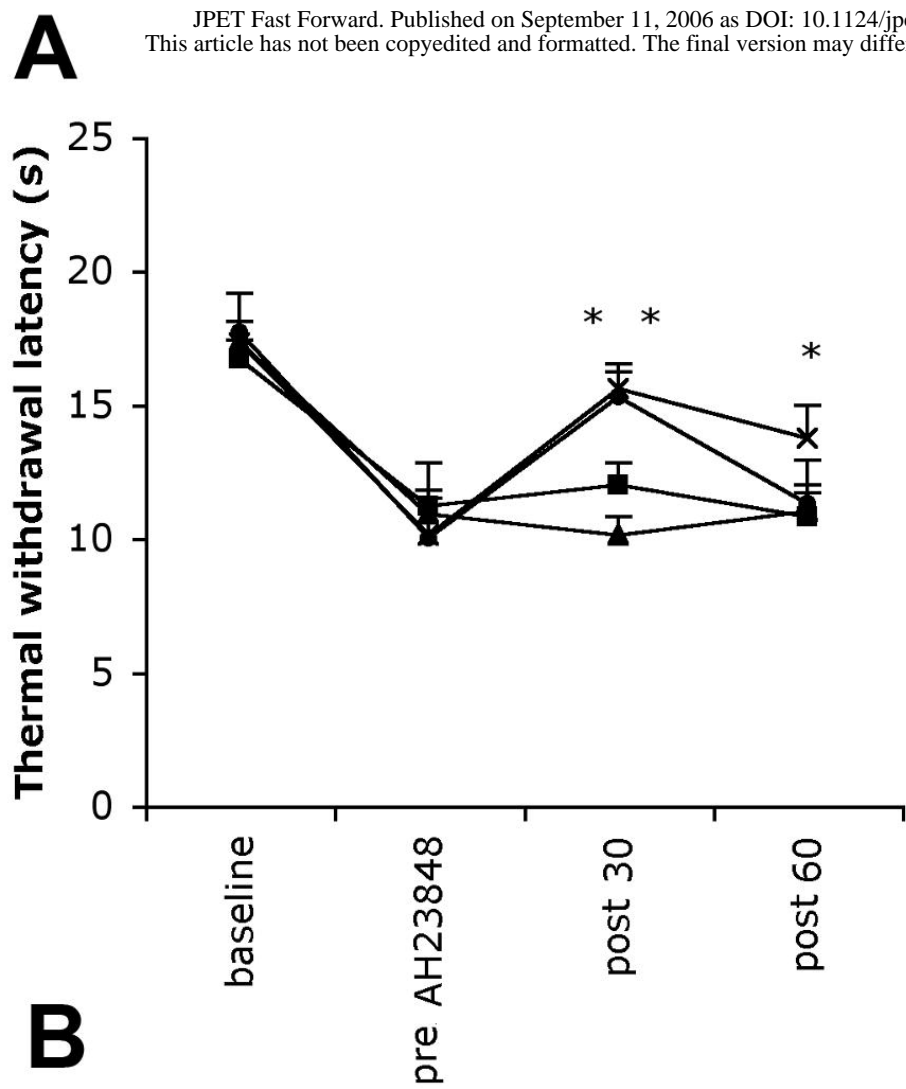


Figure 3

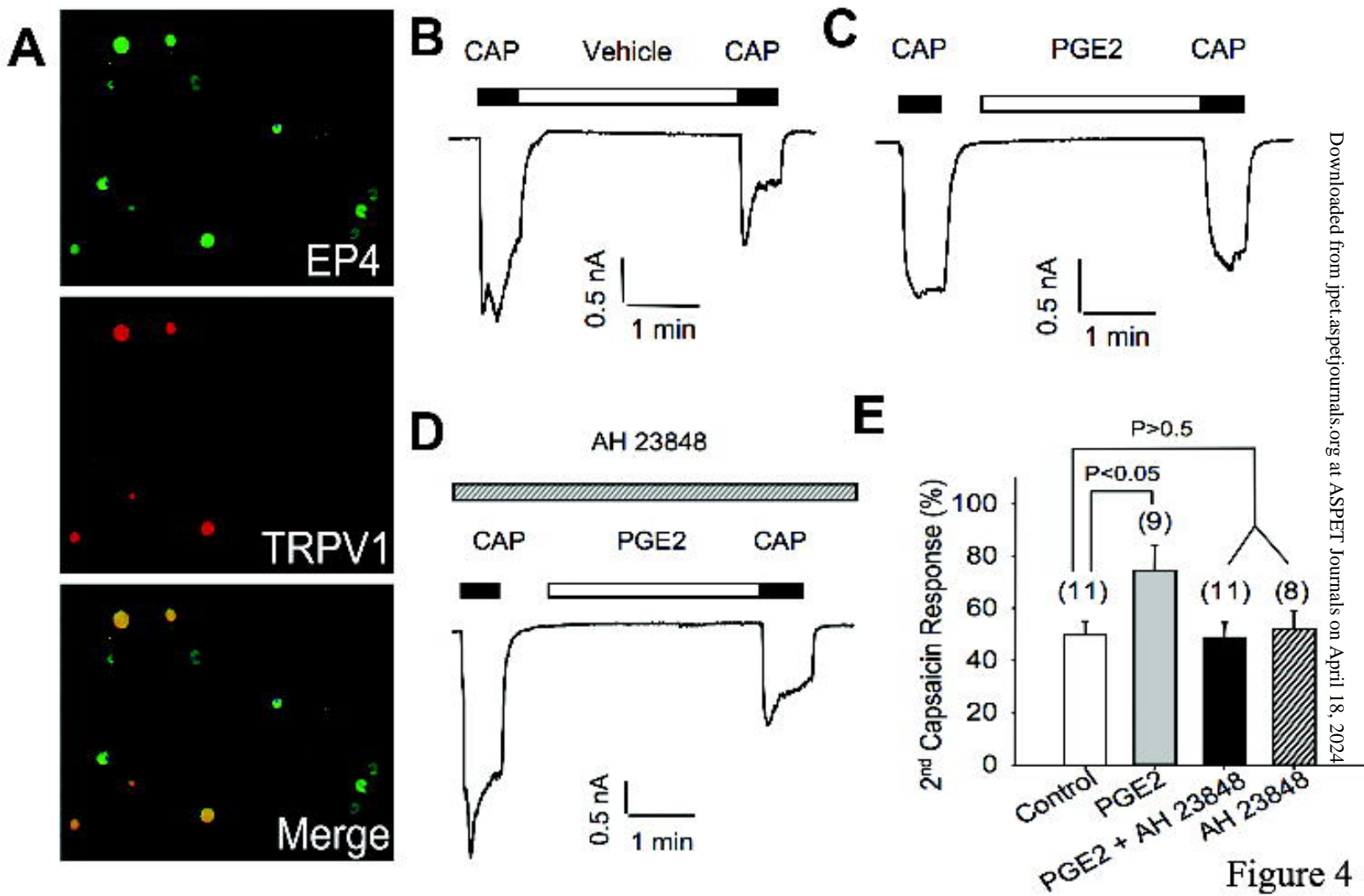


Figure 4

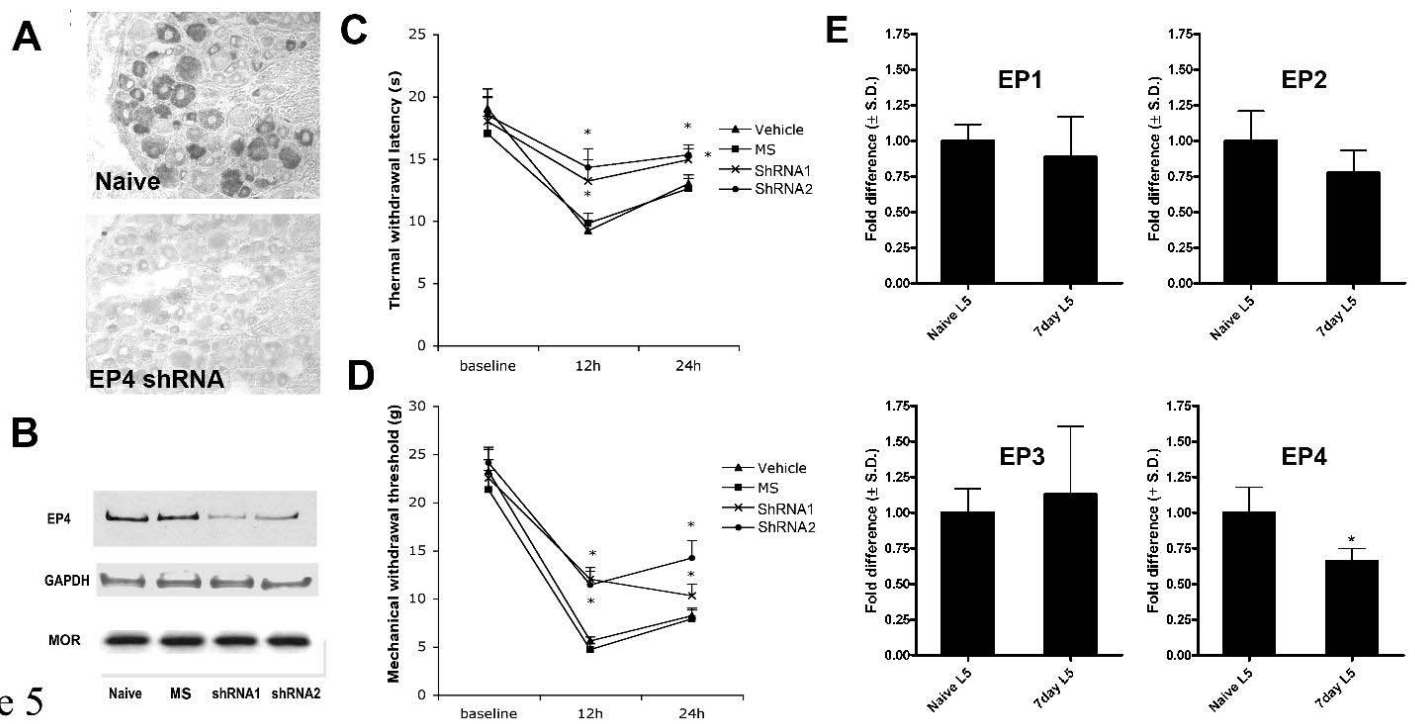


Figure 5