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**Intensity-dependent activation of extracellular signal-regulated protein kinases
(ERK) 5 in sensory neurons contributes to pain hypersensitivity**

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Abbreviations:

AS, antisense; CFA, complete Freund's adjuvant; DRG, dorsal root ganglion; ERK, extracellular signal-regulated protein kinase; IR, immunoreactive; ISHH, *in situ* hybridization histochemistry; JNK/SAPK, c-Jun N-terminal kinase/stress-activated protein kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; MM, mismatch; ODN, oligodeoxynucleotide; PB, phosphate buffer; p-ERK5, phosphorylated-ERK5; S/N, signal/noise; TRP, transient receptor potential.

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Abstract

Alterations in the intracellular signal transduction pathway in primary afferents may contribute to pain hypersensitivity. Recently, we have reported that the phosphorylation of extracellular signal-regulated protein kinase 1/2 (ERK1/2) and p38 mitogen-activated protein kinase (MAPK) occurs in primary afferent neurons in response to noxious stimulation of the peripheral tissue, i.e. activity-dependent activation of ERK1/2 and p38 MAPK in dorsal root ganglion (DRG) neurons. In the present study, we investigated the phosphorylation of ERK5, also known as big MAPK 1, in the DRG by noxious stimulation using immunohistochemistry. Capsaicin injection induced phosphorylated-ERK5 (p-ERK5) in small-to-medium diameter sensory neurons with a peak at 2 min after capsaicin injection. Furthermore, we examined the p-ERK5 labeling in the DRG after noxious heat and cold stimuli and found a stimulus intensity-dependent increase in the number of activated neurons. Most of these p-ERK5-immunoreactive neurons were small- and medium-sized neurons, which coexpressed transient receptor potential ion channel TRPV1 and TRPA1 after noxious heat and cold stimuli, respectively. In contrast, there was no change in ERK5 phosphorylation in the spinal dorsal horn. Intrathecal administration of ERK5 antisense oligodeoxynucleotide reversed heat hyperalgesia, but not mechanical allodynia, produced by capsaicin injection. Taken together, these findings suggest that the *in vivo* activation of the ERK5 signaling pathway in sensory neurons by noxious stimulation may be, at least in part, correlated with functional activity, and further, involved in the development of pain hypersensitivity.

Introduction

The mitogen-activated protein kinase (MAPK) transduces extracellular stimuli into intracellular posttranslational and transcriptional responses (Lewis *et al.*, 1998; Widmann *et al.*, 1999). The MAPK family includes extracellular signal-regulated protein kinase 1/2 (ERK1/2), p38 MAPK, c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK), and ERK5. Activity-dependent activation of ERK1/2 has been reported in the central nervous system, especially in the hippocampus (English and Sweatt, 1996; Durek and Fields, 2001). Several studies have reported the ERK1/2 phosphorylation in spinal dorsal horn neurons after acute noxious stimulation (Ji *et al.*, 1999; Karim *et al.*, 2001). Recently, we have reported that phosphorylation of ERK1/2 in primary afferent neurons occurred in response to natural noxious stimulation of the peripheral tissue, i.e. activity-dependent activation of ERK1/2 in dorsal root ganglion (DRG) neurons (Dai *et al.*, 2002; Mizushima *et al.*, 2005; Seino *et al.*, 2006). ERK1/2 activation in primary afferents has been suggested to be involved in peripheral sensitization in acute pain conditions (Aley *et al.*, 2001; Averill *et al.*, 2001; Dai *et al.*, 2002; Rashid *et al.*, 2004).

ERK5, also known as big MAPK 1, is twice the size of other MAPKs (Nishimoto and Nishida, 2006; Wang and Tournier, 2006). ERK5 is phosphorylated and activated by MAP kinase kinase (MEK) 5 but not by MEK1 or MEK2 (Hayashi and Lee, 2004). MEK5 is specific for ERK5 and does not phosphorylate or activate other MAP kinases (English *et al.*, 1995; Cavanaugh *et al.*, 2001). The amino-terminal half contains the kinase domain, which is similar to that of ERK1/2 and has the Thr-Glu-Tyr activation motif, whereas the carboxy-terminal half is unique. Remarkably, PD98059 and U0126, which were identified as MEK1/2-specific inhibitors, also inhibit the MEK5-ERK5 pathway (Kamakura *et al.*, 1999; Mody *et al.*,

2001). This therefore creates some doubt as to whether ERK5 might regulate some cellular functions originally attributed to ERK1/2. Although the ERK5 pathway involvement in neurotrophin-dependent survival and differentiation of developing peripheral neurons has been characterized in detail (Heerssen and Segal, 2002; Hayashi and Lee, 2004), there has been no study examining ERK5 activation in DRG neurons after noxious stimulation of normal tissue. Here, we report intensity-dependent ERK5 phosphorylation in primary afferents by painful stimulation and its involvement in the development of thermal hyperalgesia.

Materials and Methods

Animals

A total of 88 Male Sprague Dawley rats weighing 220-250 g were used. All animal experimental procedures were approved by the Hyogo College of Medicine Committee on Animal Research and were performed in accordance with the National Institutes of Health guidelines on animal care.

Stimulation

All experimental procedures were done on rats that were deeply anesthetized with sodium pentobarbital (50 mg/kg body weight, i.p.). Ten mM of capsaicin (8-methyl-N-vanillyl-6-noneamide, Sigma, St. Louis, MO) was dissolved in 10% Tween 80 and injected into the plantar surface of the left hind paw (200 μ l). Heat (38-54°C) and cold (20-4°C) stimuli (repeat 6 times of 10 sec of stimulation and 10 sec off interval, total 2 min) were produced by immersion of the rat's hind paw into a circulating water bath (Thermo Minder; Taitec Corp, Tokyo, Japan). The room

temperature and humidity remained stable for all experiments, and the temperature of the water bath was monitored continuously, with a precision of 0.1°C. Rats (n = 4) without stimuli were used as naive controls for immunohistochemistry. Naive control rats were anesthetized and handled in the same manner as rats with stimuli.

Antisense knockdown of ERK5 expression

Antisense oligodeoxynucleotide (AS-ODN; 5'-GAGACTCAATGTCAGCG-3'), mismatch ODN (MM-ODN; 5'-ACTACTACACTAGACTAC-3'), and FITC-labeled ODN directed to ERK5 have been designed and manufactured by BIOGNOSTIK, Germany. The control MM-ODN sequence was similar in length and GC content to the ERK5 AS-ODN sequence, passed a homology search against all sequences in the GenBank database, and was observed not to contain any problematic motifs. These ODN were fully phosphorothioated. The intrathecal delivery of AS-ODN, MM-ODN, and FITC-labeled ODN was performed as previously described (Fukuoka *et al.*, 2001). A laminectomy of the L5 vertebra was performed under adequate anesthesia with sodium pentobarbital. The dura was cut, and a soft tube (Silascon; outer diameter, 0.64 mm; Kaneka Medix, Osaka, Japan) was inserted into the subarachnoid space of the spinal cord at the L4/5 DRG level. To obtain a sustained drug infusion, an ALZET osmotic pump (3 day pump, 1 µl/hr; Durect, Cupertino, CA) was filled with AS-ODN (0.5 nmol/µl) or MM-ODN (0.5 nmol/µl), and the associated catheter was implanted intrathecally 48 hours before capsaicin (15 µg in 5 µl/paw) injection. Saline was used as vehicle control. The concentration of oligonucleotide used in this study is based on experimental protocol of the BIOGNOSTIK Antisense Oligonucleotides Application Notes. No evidence of neurotoxicity such as paralysis, vocalization, or anatomical damage to the spinal cord was noted at this dose.

Although it has frequently been questioned whether ODN can reach the DRG in a sufficient concentration by intrathecal delivery, several reports have demonstrated that intrathecal ODN accumulates in DRG cells (Lai *et al.*, 2002). In fact, consistent with previous reports (Lai *et al.*, 2002), the distribution of FITC-labeled ODN showed the progressive increase in the fluorescence associated with the DRG cell bodies, indicating that the uptake of the ODN occurred in a time-dependent manner and was detectable within 6 hours, and this fluorescence labeling was maintained in neuronal cells 24 hours after the bolus injection (data not shown).

Immunohistochemistry

After appropriate survival times (the survival time after stimulation in all experiments was 2 min, except in the time course study), rats were perfused transcardially with 1% paraformaldehyde in 0.1 M phosphate-buffer (PB) (pH 7.4), followed by 4% paraformaldehyde in 0.1 M PB (n = 4/group). After the perfusion, the L4/5 DRGs and L5 spinal cord segments were dissected out and post-fixed in the same fixative for 12 hours, then replaced with 20% sucrose overnight. DRG sections (10 μ m) and transverse spinal sections (free-floating, 25 μ m) were cut in a cryostat and processed for phosphorylated-ERK5 (p-ERK5) immunohistochemistry according to the procedure used in our previous study (Noguchi *et al.*, 1995). The polyclonal primary antibody for p-ERK5 (1:400; Affinity Bioreagents, Golden, CO) at 1:400 was used for DAB staining. For double immunofluorescent staining, the tyramide signal amplification (TSA, NEN, Boston, MA) fluorescence procedures (Michael *et al.*, 1997) were used for p-ERK5 (1:10000) staining. Then, the p-ERK5 antibody was combined with monoclonal anti-neurofilament 200 (NF200, 1:400; Sigma) or rabbit polyclonal TRPV1 antibody (1:400, Oncogene, San Diego, CA).

When two primary antisera raised in rabbit were combined, non-specific double labeling was not observed. The lack of cross-reactivity is thought to be attributable to the fact that the TSA procedure allows the first series primary antibody to be used at a dilution that is too high to be detected by the second reagent set (Michael *et al.*, 1997). Our data support this explanation. In control single labeling using indirectly labeled immunofluorescence, we were unable to visualize the p-ERK5 antiserum at the dilutions used for the TSA procedure.

The number of p-ERK5-immunoreactive (IR) neurons per section was counted in the left DRG. In each rat, 8-12 sections of the L4/5 DRG were selected randomly and 2000-3000 profiles were counted. The total number and the number of labeled neurons per section were counted to determine changes in p-ERK5 expression in the L4/5 DRG after various types of stimulation. The proportion of p-ERK5-expressing DRG neurons was determined by counting the neuronal profiles that show distinctive p-ERK5 labeling compared to background labeling in DRG sections. The total number of DRG neurons was obtained by the background staining of neurons and the Nomarski DIC (differential interference contrast) image. A number of labeled neurons per total neurons was obtained for each animal across the different tissue sections, then the mean \pm SD across animals was determined. Since a stereological approach was not used in this study, quantification of the data may represent a biased estimate of the actual number of neurons. An assistant, who was unaware of the treatment group of the tissue sections, performed all counting. A statistical significance of differences was analyzed using pair-wise comparisons (*t* test) or one-way ANOVA, followed by individual *post-hoc* comparisons (Fisher exact test). A difference was accepted as significant if $p < 0.05$.

Double labeling for TRPA1 mRNA with in situ hybridization histochemistry and p-ERK5-IR with immunohistochemistry

For the double labeling for TRPA1 mRNA and the p-ERK5 protein, the left L4/5 DRGs were sectioned (10 μ m thick) with a cryostat, thaw-mounted onto Vectabond- (Vector Laboratories, Burlingame, CA) coated slides and stored at -80°C until ready for use. In this procedure, the p-ERK5 was labeled immunohistochemically using the avidin-biotin-peroxidase complex (ABC) method followed by the detection of TRPA1 mRNA using *in situ* hybridization histochemistry (ISHH) with a radioisotope-labeled probe according to the procedure of our previous study (Tsuzuki *et al.*, 2001).

The sections were first immunostained using the ABC method. The polyclonal primary antibody for p-ERK5 were diluted 1:200 with 0.1 M Tris-HCl-buffered saline (TBS; pH 7.4) containing 2% NGS, 1 mM DTT, and 200 U RNase inhibitor, in which sections were incubated for 12-17 h at 4°C. After rinsing with 0.1 M TBS, the sections were incubated in biotinylated goat anti-rabbit IgG (Vector Laboratories) diluted 1:200 in TBS containing 2% NGS, 1 mM DTT, and 200 U RNase inhibitor for 1 h at 37°C. After rinsing with TBS, the sections were incubated with ABC reagent (Vector Laboratories) in TBS for 30 min at room temperature. After rinsing in 0.1 M TBS, the sections were reacted in 0.05% diaminobenzidine tetrahydrochloride (Wako, Tokyo, Japan) and 0.01% hydrogen peroxidase. Sections were then washed in TBS. After immunohistochemistry, these sections were immediately processed for ISHH. The procedure for ISHH was basically the same as that used in previous studies (Yamanaka *et al.*, 1999). Briefly, the rat TRPA1 cRNA probe corresponding to nucleotides 302-788 was prepared. The sections were treated with 10 μ g/ml proteinase K in 50 mM Tris-HCl and 5 mM EDTA for 3 min and acetylated with 0.25%

acetic anhydride in 0.1 M triethanolamine, then the ^{35}S -labeled RNA probe (5×10^6 cpm/ml) was placed on these sections overnight at 55°C . Hybridized sections were rinsed in 5 X SSC, 5 mM DTT for 30 min at 65°C , washed in high-stringency buffer for 30 min at 65°C , and treated with 2 $\mu\text{g/ml}$ RNase A for 30 min at 37°C . Sections were rinsed, dehydrated in an ascending ethanol series, and air dried. For autoradiography, the sections were coated with NTB-3 emulsion (Eastman Kodak, Rochester, NY), diluted 6:4 with distilled water at 45°C , and exposed for 2 weeks in light-tight boxes at 4°C . After development in D19 (Eastman Kodak) and fixation in 24% sodium thiosulfate, the sections were rinsed in distilled water, stained with hematoxylin-eosin, dehydrated in a graded ethanol series, cleared in xylene, and coverslipped.

Western blotting

Tissue samples from the L4/5 DRGs were lysed by homogenizing in 200 μl of lysis buffer containing 20 mM Tris-HCl, PH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Igepal CA-630, 2 mM Na_3VO_4 , 0.5 mM DTT, 1 mM PMSF, 1 $\mu\text{g/ml}$ pepstatin, 5 $\mu\text{g/ml}$ leupeptin, 9 $\mu\text{g/ml}$ aprotinin and 10% glycerol. Lysates were centrifuged at 14400 g for 60 min and the concentration of protein in each sample (supernatant) was determined using the Bio-Rad dye-binding. Samples with equal amounts of protein were then separated by 10-20% polyacrylamide gel electrophoresis, and the resolved proteins were electrotransferred to Hybond-P Nitrocellulose (Amersham Biosciences, Little Chalfont, UK). Membranes were incubated with 5% nonfat milk in Tris buffer containing Tween 20 (TBST, 10 mM Tris-HCl, PH 8.0, 150 mM NaCl and 0.2% Tween 20) for at least 10 min at room temperature and incubated with the polyclonal primary antibody for total ERK5 (1:1000; GeneTex, San Antonio, TX) and total ERK1/2

(1:1000; Cell Signaling Technology, Beverly, MA) and the monoclonal primary antibody for β -actin (1:1000; Sigma) at 4°C overnight. Membranes were then washed twice with TBST and probed with goat anti-rabbit IgG conjugated with horseradish peroxidase (Vector Laboratories) at room temperature for 2 h. Membranes were finally washed several times with TBST to remove unbound secondary antibodies and visualized using enhanced chemiluminescence (Boehringer Mannheim, Indianapolis, IN). Each experiment was repeated at least twice and in all cases the same results were obtained. The density of specific bands was measured with a computer-assisted imaging analysis system (ATTO Densitograph version 4.02, Tokyo, Japan) and normalized against a loading control (β -actin). The protein level was expressed as a percentage of the protein level in the naive control.

Data are expressed as mean \pm SD. Differences in changes of values over time of each group were tested using one-way ANOVA, followed by individual *post-hoc* comparisons (Fisher exact test). One-way ANOVA, followed by individual *post-hoc* comparisons (Fisher exact test) or pairwise comparisons (*t* test) were used to assess differences of values between the intrathecal groups. A difference was accepted as significant if $p < 0.05$.

Behavioral studies

All tests were performed on male 220-250 g rats ($n = 8/\text{group}$). Heat hyperalgesia to the capsaicin injection site was measured using the Hargreaves method, before and 2, 5, 10, 30, 60 min after injection. The heat stimulus was terminated with a withdrawal response, or at 20 sec to avoid skin damage. Three latencies were recorded and averaged for the ipsilateral hind paw in each test

session. Mechanical allodynia was assessed with a 'dynamic plantar aesthesiometer' (Ugo Basile, Comerio, Italy), which is an automated von Frey type system (Lever *et al.*, 2003). To measure rat hind paw mechanical thresholds, animals were placed in plastic cages with a wire mesh floor and allowed to acclimate for 15 min before each test session. A paw-flick response was elicited by applying an increasing force (measured in grams) using a plastic filament (0.5 mm diameter) focused on the plantar surface of the ipsilateral hind paw. The force applied was initially below detection threshold and then increased from 1 to 50 g in 1 g steps over 20 s, then held at 50 g for a further 10 s. The rate of force increase was 2.5 g/s. The force applied to elicit a reflex removal of the ipsilateral hind paw was monitored. This was defined as the mean of three measurements at 1 min intervals. The variability between trials was approximately 2 g. An assistant, who was unaware of the treatment group, performed all the behavioral experiments.

Data are expressed as mean \pm SEM. Differences in changes of values over time of each group were tested using one-way ANOVA, followed by individual *post-hoc* comparisons (Fisher exact test). Differences in values over time between the treatment groups were tested using two-way ANOVA. Pair-wise comparisons (*t* test) were used to assess differences of values between the treatment groups. A difference was accepted as significant if $p < 0.05$.

Results

p-ERK5-IR cells in the primary afferent neurons after capsaicin injection

To clarify the *in vivo* activation of ERK5 in DRG neurons, we stimulated the receptive fields in a variety of ways, and used an antibody that recognizes p-ERK5.

We found that $17.8 \pm 2.2\%$ of the neurons were p-ERK5-IR in the naive DRG ($n = 4$) (Fig. 1A). Capsaicin injection into the plantar surface of the hind paw induced a number of DRG neurons labeled for p-ERK5 (Fig. 1B). The increase in p-ERK5 was seen mainly in small- to medium-diameter neurons, and thus presumed to be nociceptive neurons (arrows). We examined the time course of phosphorylation of ERK5 in DRG neurons after capsaicin injection and found that this response was very quick: the peak was 2 min after stimulation ($35.8 \pm 5.5\%$) and the number of labeled cells rapidly declined for 10 min ($19.4 \pm 2.2\%$) (Fig. 1C-E). Furthermore, we found that noxious stimuli did not induce an increase in p-ERK5 expression 1 and 3 days after stimulation (data not shown).

To investigate whether an increase of p-ERK5 occurs after capsaicin injection in a subpopulation of DRG neurons with myelinated fibers, we examined the immunohistochemical colocalization of p-ERK5 and NF200, a marker for myelinated A-fibers. The results of the colocalization study with p-ERK5 and NF200 in DRG neurons 2 min after stimulation are shown in Fig. 1F-H. p-ERK5- and NF200-IR neurons were clearly distinguishable, indicating that p-ERK5 is predominantly expressed in neurons with unmyelinated axons, the C-fibers.

p-ERK5-IR cells in the primary afferent neurons after heat stimuli

Next, we examined the p-ERK5 labeling after natural stimulation. We examined the relationship between heat stimulation at different temperatures and the induction of p-ERK5 in DRG neurons. We applied the thermal stimuli by immersion of the hind paw into warm to hot water ($38-54^{\circ}\text{C}$), and examined the p-ERK5 labeling 2 min after heat stimulation. We found a small number of cells labeled for p-ERK5 after thermal stimulation at 38°C (Fig. 2B), as well as those of naive control rats (Fig. 2A).

In contrast, noxious heat stimulation at higher temperature induced p-ERK5 in more neurons ($20.4 \pm 1.9\%$ at 42°C , $25.1 \pm 1.4\%$ at 46°C , $29.1 \pm 4.3\%$ at 50°C and $34.4 \pm 5.3\%$ at 54°C ; $n = 4$) (Fig. 2C-E). The increase in p-ERK5 labeling was found mainly in small-to-medium diameter neurons (arrows).

TRPV1, one of the transducer proteins, can generate depolarizing currents in response to noxious thermal stimuli (Caterina *et al.*, 2000). To investigate whether ERK5 activation in DRG neurons by heat stimulation is mediated through TRPV1, which is a heat-gated ion channel, we performed double staining for p-ERK5 and TRPV1 (Fig. 2F-H). We found that a number of p-ERK5-IR neurons (Fig. 2F, green) also expressed TRPV1 (Fig. 2G, red).

p-ERK5-IR cells in the primary afferent neurons after cold stimuli

We next examined the p-ERK5 labeling 2 min after cold stimulation. There were a small number of p-ERK5-positive neurons in the DRG 2 min after thermal stimulation at 20°C (Fig. 3B), as well as in control non-treated rats (Fig. 3A). However, we found that noxious cold stimulation induced a number of DRG neurons labeled for p-ERK5 ($20.4 \pm 2.4\%$ at 16°C , $24.2 \pm 2.1\%$ at 12°C , $26.3 \pm 3.1\%$ at 8°C and $30.5 \pm 3.7\%$ at 4°C ; $n = 4$) (Fig. 3C-E). Most of p-ERK5-labeled neurons were small or medium in size (arrows).

TRPA1 is activated at approximately 17°C , a temperature that is reported as painfully cold by humans (Story *et al.*, 2003). To ascertain whether an increase of p-ERK5 after noxious cold stimulation occurs in a subpopulation of DRG neurons labeled for TRPA1, we examined double labeling for TRPA1 mRNA with ISHH and p-ERK5-IR with immunohistochemistry (Fig. 3F-G). The majority of p-ERK5-labeled neurons 2 min after thermal stimulation at 4°C also expressed TRPA1 mRNA,

indicating the ERK5 activation of neurons with TRPA1 after noxious cold stimulation (Fig. 3G).

No change of p-ERK5 protein in the spinal dorsal horn

Recent studies have shown that acute noxious stimuli, such as formalin or capsaicin, induce ERK1/2 and p38 MAPK phosphorylation in the spinal dorsal horn (Ji *et al.*, 1999; Karim *et al.*, 2001; Svensson *et al.*, 2003). Therefore, we examined p-ERK5 immunoreactivity in the spinal cord (Fig. 4). We found that noxious stimuli did not induce an increase in p-ERK5 expression in the dorsal horn.

Effect of ERK5 AS-ODN on pain behaviors after capsaicin injection

To examine the functional consequences of ERK5 activation, we investigated whether inhibition of ERK5 activation modifies capsaicin-induced heat hyperalgesia and mechanical allodynia. Rats were intrathecally treated with either an AS-ODN targeting ERK5 or a MM-ODN beginning 48 hours before capsaicin injection. Intrathecal administration of AS-ODN or MM-ODN into naive animals produced no significant changes in basal pain sensitivity (Fig. 5A-B). The capsaicin injection induced a rapid heat hyperalgesia at the site of injection that mostly recovered by 1 h after injection (Fig. 5A). Pretreatment with ERK5 AS-ODN dose-dependently prevented heat hyperalgesia at 2, 5 and 10 min after capsaicin injection (Fig. 5A). The time courses of withdrawal latency with 0.5 and 0.05 nmol/ μ l of AS-ODN-treated rats were significantly different from those of vehicle control and MM-ODN-treated rats ($p < 0.001$: two-way repeated ANOVA). In contrast, there was no effect of ERK5 AS-ODN on mechanical allodynia throughout the period we studied (Fig. 5B).

We then confirmed that the level of ERK5 protein in the ipsilateral DRG of the

AS-ODN-treated rats was significantly lower than that in the MM-ODN-treated rats, whereas there was no difference in ERK1/2 expression (Fig. 5C-D).

Discussion

Action potentials that are transmitted from the periphery and associated Ca^{2+} transients could activate the specific intracellular signaling pathway and regulate gene expression in DRG neurons (Fields *et al.*, 1997; Fields, 1998). We have recently reported that phosphorylation of ERK1/2 in DRG neurons occurred in response to noxious stimulation (Dai *et al.*, 2002; Mizushima *et al.*, 2005; Seino *et al.*, 2006). The present study demonstrated that capsaicin injection induced an increase in the phosphorylation of ERK5 in small-to-medium diameter neurons with a peak at 2 min after capsaicin injection. Furthermore, the noxious heat and cold stimulation of the receptive field with different intensities resulted in changes in the number of p-ERK5-labeled neurons. Therefore, these findings suggest that after noxious stimulation, action potentials that are transmitted from the periphery and associated calcium transients could activate the specific intracellular signaling pathway, such as ERK5 and ERK1/2 (Fields *et al.*, 1997; Fields, 1998).

Temperatures over about 43°C evoke not only a thermal sensation, but also a feeling of pain, whereas the threshold for painful (noxious) cold is about 15°C (LaMotte and Campbell, 1978). In the present study, innocuous heat and cold stimulation, such as the 42°C and 16°C stimulation, induced p-ERK5 labeling in a small number of DRG neurons. Furthermore, we found that noxious heat and cold stimulation of the receptive field with different intensities resulted in corresponding changes in the number of p-ERK5-labeled neurons, i.e. it was stimulus intensity

dependent. Therefore, our results suggest that the examination of p-ERK5, as well as ERK1/2, is very useful as an indicator of the activated DRG neurons after noxious, but not innocuous, heat and cold stimulation *in vivo*. However, the reasons why noxious stimulation did not activate ERK5 in the spinal dorsal horn are not clearly understood at this point, although a number of studies have demonstrated the activation of ERK1/2 in dorsal horn neurons after acute noxious stimulation (Ji *et al.*, 1999; Karim *et al.*, 2001).

The present study also demonstrated that most of the increased p-ERK5-IR DRG neurons were small- and medium-sized neurons, which coexpressed TRPV1 and TRPA1 after noxious heat and cold stimulation, respectively. TRPV1, one of the transducer proteins, can generate depolarizing currents in response to noxious heat stimuli (Tominaga *et al.*, 1998). In contrast, TRPA1 is activated at ~17°C, a temperature that is reported as painfully cold by humans (Story *et al.*, 2003). Thus, we believe that primary afferent activation, through the TRPV1 or TRPA1 by either heat or cold stimulation, may produce action potentials, which in turn results in the phosphorylation of ERK5 in DRG neurons. However, we cannot deny the possibility that ERK5 in DRG neurons may activate these TRP channels themselves by phosphorylation.

Two recent reports of TRPA1 knockout mice gave conflicting results. Bautista *et al.* (2006) reported normal cold responses, whereas Kwan *et al.* (2006) reported impaired cold responses. In fact, further study is needed to reach a consensus on the role of TRPA1 in cold transduction under normal physiological condition. Alternatively, other membrane proteins, such as degenerin/epithelial sodium channels also may be involved in cold transduction (Askwith *et al.*, 2001).

Several studies demonstrated that MEK1/2 inhibitors, such as PD98059 and

U0126, diminish capsaicin-induced hyperalgesia by blockade of ERK1/2 activation in the DRG (Dai *et al.*, 2002; Seino *et al.*, 2006). However, PD98059 and U0126 have been shown to block the activation of ERK5 (Kamakura *et al.*, 1999; Mody *et al.*, 2001). This therefore creates some doubt as to whether the previous conclusion is valid and whether the MEK5-ERK5 module could also be involved in pain hypersensitivity. In the present study, antisense knockdown of ERK5 in the DRG reversed heat hyperalgesia after capsaicin injection, indicating that ERK5 activation in the DRG neurons might affect the sensitivity of the primary afferent itself and consequently affect the pain behavior. Considering that the resolution of the capsaicin-induced heat hyperalgesia is quite short, these results suggest that the ERK5 signaling pathway plays an important role in heat hyperalgesia by posttranslational modifications of kinases, receptors and ion channels, although the specific target proteins are generally unknown. The present study also demonstrated that mechanical allodynia was not changed by selective knockdown of ERK5 expression. Because non-nociceptor-mediated pain reflects a change in the functioning of central neurons, i.e. central sensitization (Julius and Basbaum, 2001; Scholz and Woolf, 2002), we believe that the activation of ERK5 in primary afferents may not be involved in central sensitization.

In the present study, there is a constitutive expression of p-ERK5 in the L4/5 DRG of naive rats (~18%). In contrast, very few neurons label for p-ERK1/2 in the naive DRG (Dai *et al.*, 2002, Mizushima *et al.*, 2005; Seino *et al.*, 2006). Therefore, these results indicate that the activation of ERK5 may not be an accurate reflection of activity, compared to that of ERK1/2. Indeed, further studies are needed to establish the direct relationship between the phosphorylation of ERK5 and the electrophysiological activity in individual neurons. Furthermore, considering that

noxious stimulation induced intensity-dependent p38 MAPK phosphorylation in DRG neurons (Mizushima *et al.*, 2005, 2006), it is possible that there is cross talk between the ERK5 and p38 pathways. However, the characteristics of p-ERK5 labeling in DRG neurons after noxious thermal (both heat and cold) stimulation suggests that the p-ERK5 labeling is, at least in part, correlated with the activation state of the primary afferent neurons through several of the TRP channels, and that examination of not only p-ERK1/2 but also p-ERK5 is useful as an indicator of the activated DRG neurons after noxious, but not innocuous, stimulation *in vivo*.

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Legends for Figures

Figure 1. Stimulus-evoked ERK5 phosphorylation in rat DRG neurons. A. L4 DRG section (10 μ m) immunostained for p-ERK5 in the naive rat. B-D. p-ERK5 labeling in the ipsilateral L4 DRG neurons 2 min (B), 5 min (C) and 10 min (D) after intraplantar injection of capsaicin (10mM, 200 μ l). Scale bars, 100 μ m. E. Time course of capsaicin-evoked p-ERK5 expression in the L4/5 DRG neurons. Data represent mean \pm SD; $n = 4$ each time point. * $p < 0.05$ compared to the naive control. F-H. Colocalization of p-ERK5 (green) and NF200 (red) in the ipsilateral L4 DRG after capsaicin injection. Double staining of p-ERK5 with NF200, a marker for myelinated A-fibers, showed no colocalization in DRG neurons, indicating that ERK5 is predominantly activated in unmyelinated C-fiber nociceptors. Scale bars, 50 μ m.

Figure 2. Heat stimulus intensity-dependent p-ERK5 expression in DRG neurons. A. L4 DRG section (10 μ m) immunostained for p-ERK5 in the naive rat. B-D. p-ERK5 labeling in the ipsilateral L4 DRG neurons 2 min after thermal stimulation at 38°C (B), 42°C (C) and 54°C of the rat's hind paw (D). Scale bars, 100 μ m. E. Quantification of the percentage of p-ERK5-IR neurons of L4/5 DRG neurons after heat stimulation. Data represent mean \pm SD; $n = 4$ each temperature. * $p < 0.05$; ** $p < 0.01$ compared to the naive control. F-H. Colocalization of p-ERK5 (green) and TRPV1 (red) in the ipsilateral L4 DRG after thermal stimulation at 54°C. Open arrows indicate double-labeled neurons with p-ERK5- and TRPV1-IR. Scale bars, 50 μ m.

Figure 3. Cold stimulus intensity-dependent p-ERK5 expression in DRG neurons. A: L4 DRG section (10 μ m) immunostained for p-ERK5 in the naive rat. B-D.

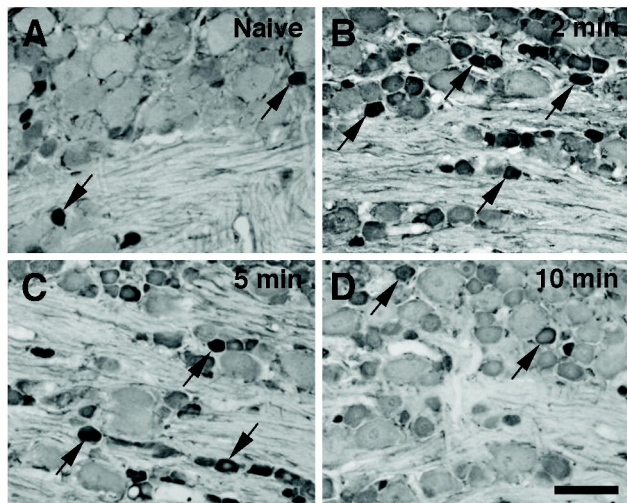
p-ERK5 labeling in the ipsilateral L4 DRG neurons 2 min after thermal stimulation at 20°C (B), 16°C (C) and 4°C of the rat's hind paw (D). Scale bars, 100 μ m. E. Quantification of the percentage of p-ERK5-IR neurons of L4/5 DRG neurons after cold stimulation. Data represent mean \pm SD; $n = 4$ each temperature. * $p < 0.05$; ** $p < 0.01$ compared to the naive control. F-G. Double labeling for TRPA1 mRNA and p-ERK5-IR by a combined method with ISHH and immunohistochemistry in the naive DRG (F) and ipsilateral L4 DRG after thermal stimulation at 4°C (G). Arrows indicate single-labeled neurons for TRPA1 mRNA. Open arrows indicate double-labeled neurons with TRPA1 mRNA and p-ERK5-IR. Scale bars, 50 μ m.

Figure 4. No change in p-ERK5 expression in the spinal cord after noxious stimulation. A-B. p-ERK5 immunostaining in the ipsilateral (A) and contralateral (B) spinal dorsal horn after thermal stimulation at 54°C of the rat's hind paw. Scale bar: 100 μ m. C-D. High-magnification images showing p-ERK5 staining in the ipsilateral (C) and contralateral (D) superficial dorsal horn (lamina I). Scale bar: 50 μ m.

Figure 5. A-B. Effect of ERK5 AS-ODN on pain behaviors after capsaicin injection. A. Heat hyperalgesia was examined using the plantar test. Pretreatment with ERK5 AS-ODN significantly changed withdrawal latency to noxious heat after capsaicin injection into the plantar surface of the hind paw. B. Mechanical allodynia was examined using the apparatus called a 'dynamic plantar aesthesiometer.' Antisense knockdown of ERK5 did not show any effect on the threshold of response to mechanical stimulation. Data represent mean \pm SEM; $n = 8$ each group. C-D. Confirmation of a selective blockade of ERK5 expression in the ipsilateral DRG. Western blot analysis reveals inhibition of ERK5, but not ERK1/2, expression in the

ipsilateral L4/5 DRG by ERK5 AS-ODN (C). Quantification of Western blot data is shown at right (D). Data represent mean \pm SD; $n = 4$ each group. # $p < 0.05$; ## $p < 0.01$ compared to the MM-ODN ($0.5 \text{ nmol} \cdot \mu\text{l}^{-1} \cdot \text{h}^{-1}$) group.

Figure 1



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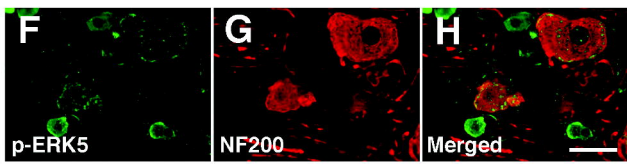
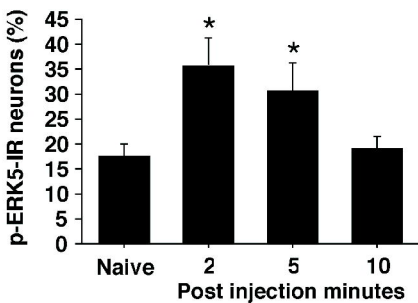


Figure 2

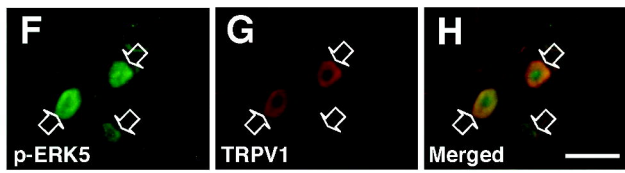
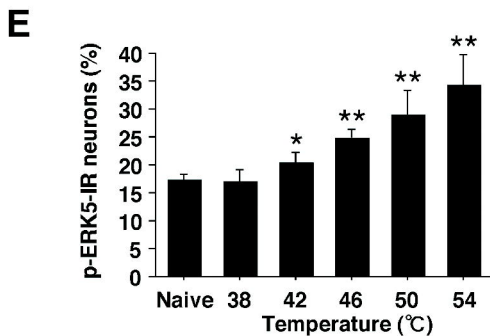
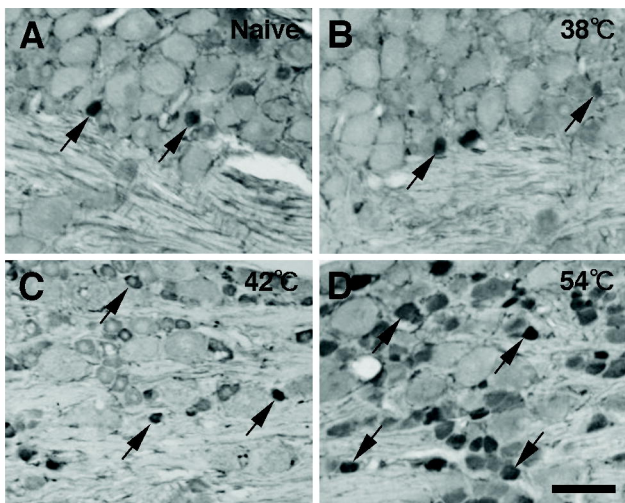


Figure 3

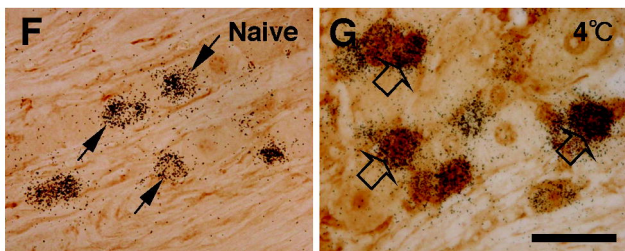
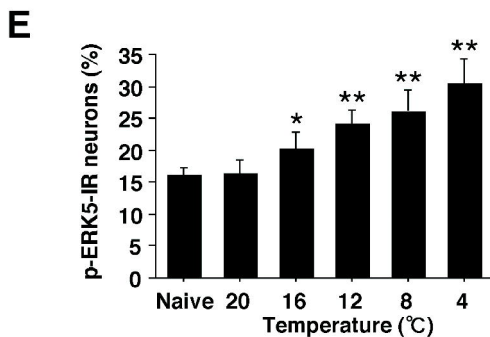
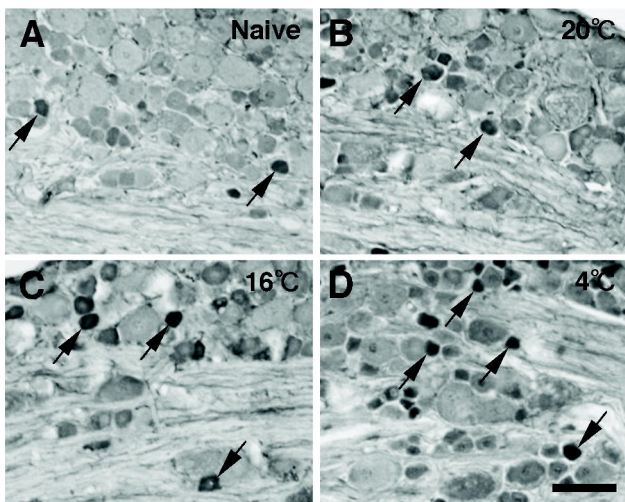


Figure 4

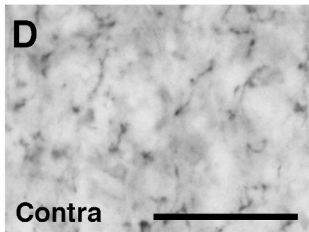
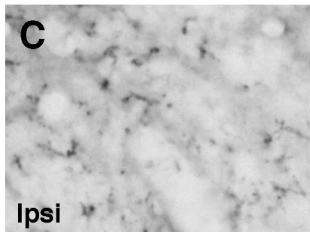
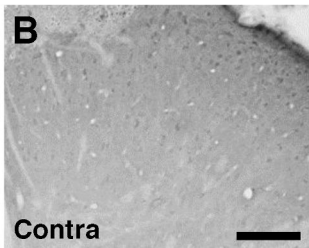
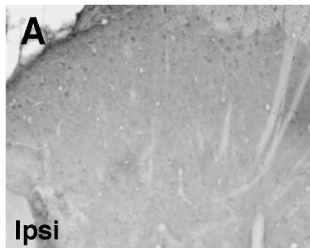
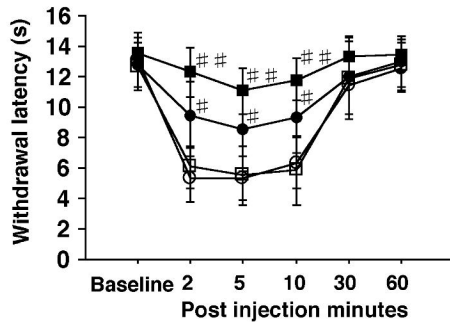
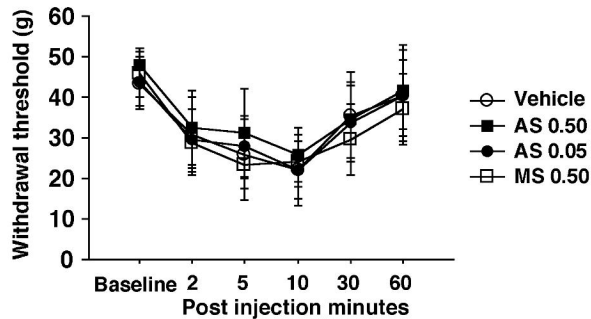
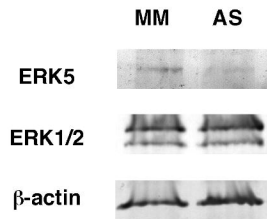


Figure 5

A**B****C****D**