Nociceptive-specific activation of ERK in spinal neurons contributes to pain hypersensitivity

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We investigated the involvement of extracellular signal-regulated protein kinases (ERK) within spinal neurons in producing pain hypersensitivity. Within a minute of an intense noxious peripheral or C-fiber electrical stimulus, many phosphoERK-positive neurons were observed, most predominantly in lamina I and IIo of the ipsilateral dorsal horn. This staining was intensity and NMDA receptor dependent. Low-intensity stimuli or A-fiber input had no effect. Inhibition of ERK phosphorylation by a MEK inhibitor reduced the second phase of formalin-induced pain behavior, a measure of spinal neuron sensitization. ERK signaling within the spinal cord is therefore involved in generating pain hypersensitivity. Because of its rapid activation, this effect probably involves regulation of neuronal excitability without changes in transcription.

The extracellular signal-regulated kinases ERK1 and ERK2 are mitogen-activated protein kinases that transduce extracellular stimuli into intracellular post-translational and transcriptional responses[1,2]. The ERKs are activated by an upstream kinase, MEK[2,3], and in the nervous system produce short-term functional (non-transcriptional) changes by phosphorylating kinases, receptors and ion channels and long-term adaptive changes by activating transcriptional factors such as CREB[1]. Depolarization and calcium influx stimulate ERK phosphorylation via Ras in PC12 cells[4], and a pathological and physiological activity-dependent activation of ERK occurs in the brain[5,6]. ERK is phosphorylated in the hippocampus during induction of long-term potentiation (LTP)[7] and seems to be necessary for enhanced synaptic transmission and associative long-term memory[1,8–10].

Activation of high-threshold C-fibers by peripheral noxious stimuli causes not only an immediate sensation of pain but also an increased responsiveness of neurons in the dorsal horn of the spinal cord that outlasts the initiating stimulus. This use-dependent regulation of neuronal excitability, known as central sensitization[11,12], is involved in the heightened pain sensitivity that follows injury. Activation of C-fiber nociceptors by capsaicin—the pungent ingredient in chili pepper—for example, leads to pain in response to normally innocuous stimuli and a spread of pain sensitivity beyond the area of injury/stimulation as a result of central sensitization[13,14]. The behavioral response to intraplantar formalin[15] is also a model for spinal neuronal plasticity[16–18].

The mechanisms responsible for C-fiber-induced, activity-dependent plasticity in the spinal cord include activation of threonine/serine and tyrosine kinases with subsequent phosphorylation of membrane bound receptors, particularly the NMDA receptor[12,19–21]. Given the similarities between synaptic plasticity in the hippocampus and central sensitization in the spinal cord[12], we have now explored whether ERK activation is involved in the generation of nociceptive-specific functional pain plasticity.

Results

ERK activation in dorsal horn neurons

To test whether peripheral noxious stimuli induce ERK activation in the dorsal horn, we injected the chemical irritant capsaicin into the hindpaw of anesthetized rats. Capsaicin is a ligand for the VR1 receptor, which is expressed only in C-nociceptors[22], and produces an intense but short-lived burning sensation when injected into the skin[13,14].

In normal non-stimulated lumbar spinal cord (L4–L5), phospho ERK (pERK) levels are low. However, 2 minutes after an intraplantar capsaicin injection (75 μg in 25 μl), pERK immunoreactivity (IR) was detected within many dorsal horn neurons (**Fig. 1a**). No pERK IR was evoked contralateral to the capsaicin injection. The pERK label was topographically located in the area of the dorsal horn devoted to inputs from the hindlimb, which is the medial half of the dorsal horn in L4 and L5 lumbar segments[23] (**Fig. 1a**). Staining for pERK was present in many, but not all neurons in laminae I and IIo of the superficial dorsal horn, with smaller numbers in more ventral laminae (laminae IIi, III–VI), and was located in the cytoplasm of the soma and neurites as well as the nucleus (**Fig. 1b** and **c**). No labeled cells were found in the ventral horn (**Fig. 1a**). Western blots showed an increase in both forms of ERK, especially Erk2, in the dorsal horn (**Fig. 1d**).

To test when pERK is induced after a noxious stimulus and how long it is maintained, we studied the time course of capsaicin-induced ERK activation, from one minute to two hours. Capsaicin evoked ERK activation one minute after stimulation (**Fig. 2a**). This reached a peak level at two minutes, was maintained at five minutes but decreased at ten minutes, with a return toward basal level at two hours (**Fig. 2a**).

Next we examined if ERK activation is specific to noxious stimuli. Three different noxious stimuli, an intense mechanical punctate stimulus, a hot (50°C) stimulus and a cold (4°C) stim-

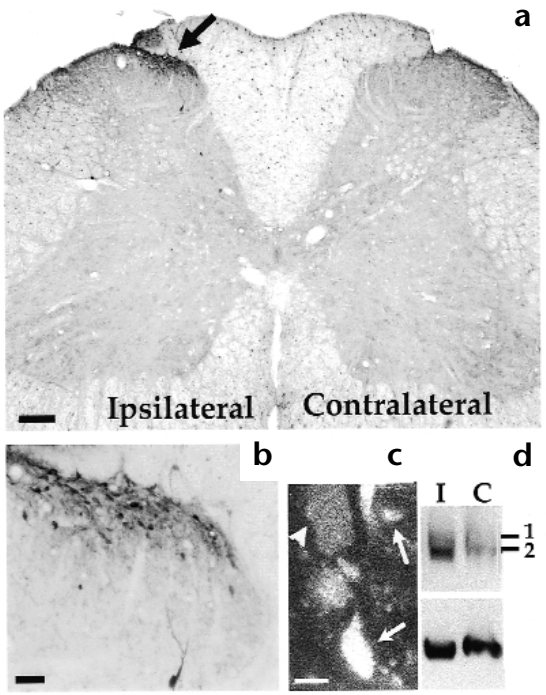

Fig. 1. Induction of ERK phosphorylation in the ipsilateral dorsal horn by intraplantar capsaicin injection. (**a**) Transverse section of the L5 lumbar spinal cord showing increased pERK immunoreactivity (IR) in the ipsilateral medial superficial dorsal horn (arrow) two min after capsaicin injection into the hindpaw. Scale bar, 200 μm. (**b**) High-magnification image from (**a**) showing pERK-IR in many small postsynaptic neurons in the superficial dorsal horn and a solitary deeper neuron. Scale bar, 50 μm. (**c**) Confocal image showing induced pERK-IR in cytoplasm (arrowhead) and nucleus (arrows). Scale bar, 5 μm. (**d**) Western blot obtained from ipsilateral (I) and contralateral (C) spinal dorsal horn, indicating that ERK2 (p42 MAPK) and ERK1 (p44 MAPK) are phosphorylated after capsaicin stimulation. Bottom bands are controls for non-phosphorylated ERK2. 1, ERK 1; 2, ERK 2.

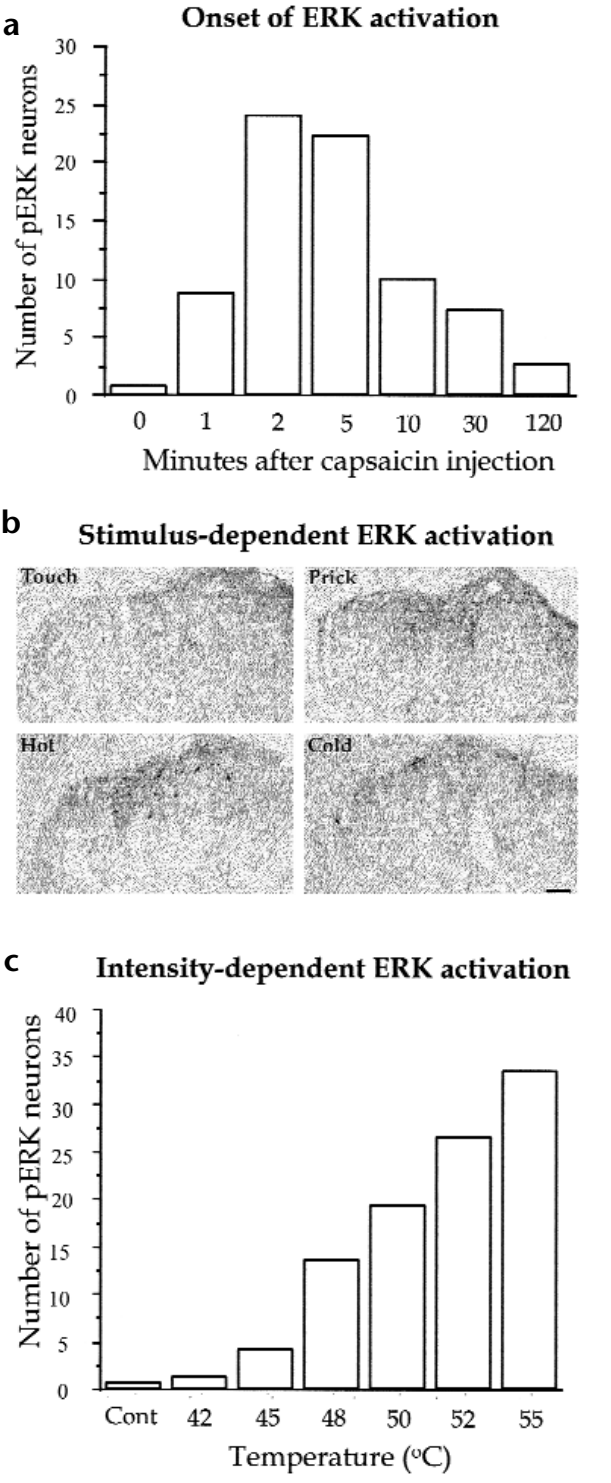

Fig. 2. Activation of pERK in the dorsal horn. (**a**) Rapid onset and decline of ERK phosphorylation after capsaicin administration measured by the number of pERK-positive neurons in the superficial laminae (I–II) of the ipsilateral dorsal horn. (**b**) ERK activation is nociceptive specific. Innocuous tactile stimulation (light touch for 2 min) does not induce pERK. However ERK activation is induced by noxious stimuli: prick (100 g for 2 min), heat (50°C for 1 min) or cold (4°C for 1 min). Scale bar, 50 μm. (**c**) ERK activation measured by the number of pERK-positive neurons in the superficial laminae (I–II) increases with increasing stimulating temperature.

ulus were applied to the hindpaw. All three induced pERK in superficial dorsal horn neurons two minutes later (**Fig. 2b**). However, repeated light touch to either the dorsal or plantar surface of the hindpaw did not induce pERK in the spinal cord (**Fig. 2b**). Activation of ERK in response to graded heat stimuli was intensity dependent (**Fig. 2c**). There was no ERK activation at 42°C, an innocuous warm stimulus. At the threshold for activation of heat-sensitive nociceptors (45°C), a few pERK immunostained cells appeared, and the numbers increased with increasing temperature from 48 to 55°C (**Fig. 2c**).

Calcium entry into neurons via ionotropic glutamate receptors may initiate the ERK signaling cascade[24,25]. We therefore examined the effect of the NMDA receptor channel blocker MK-801 on capsaicin-induced ERK activation. MK-801 was injected into the subarachnoid space of the spinal cord 20 minutes before capsaicin administration. MK-801 at 1.5 nmol reduced the number of pERK-positive neurons in the superficial dorsal horn by 44% ($p < 0.01$), and at 15 nmol by 50% ($p < 0.01$; **Fig. 3**).

Electrical stimulation was used to selectively recruit different types of primary afferents in an adult rat spinal cord slice preparation with an attached dorsal root[26]. Cells positive for pERK were not induced in the dorsal horn after Aβ-fiber stimulation

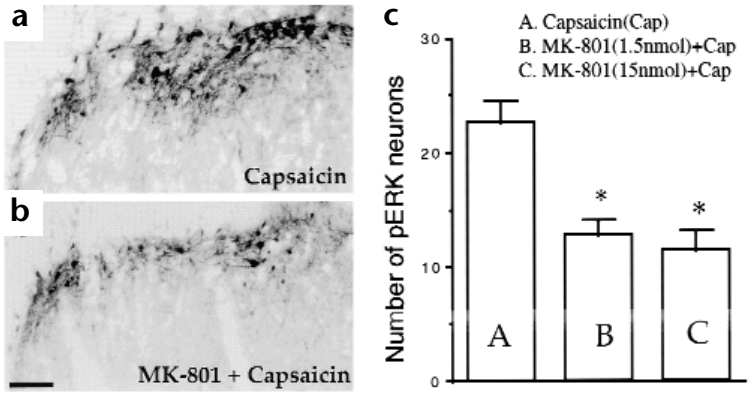

Fig. 3. ERK activation is NMDA receptor dependent. Capsaicin-induced pERK in the ipsilateral dorsal horn (**a**) is partially suppressed by MK 801 (1.5 nmol) intrathecally injected into spinal cord 20 min before the capsaicin (**b**). Scale bar, 50 μm. (**c**) Effect of MK-801 on capsaicin-evoked ERK activation, measured by the number of pERK-positive neurons in the superficial laminae (I–II) of the ipsilateral dorsal horn. *$p < 0.01$, as compared to capsaicin group, $n = 4$.

(**Fig. 4a** and **b**). Aδ-fiber stimulation increased the number of pERK-positive neurons, but significantly less than C-fiber stimulation ($p < 0.01$; **Fig. 4c** and **d**). The C-fiber stimulus-evoked pERK was partially inhibited by the competitive NMDA receptor antagonist APV (35% at 100 μM, $p < 0.01$; **Fig. 4d**).

ERK activation and formalin-induced pain hypersensitivity

Formalin (1.5%, 50 μl) was injected into the plantar surface of the hindpaw of awake rats, and the time the rats spent licking or lifting the injected paw was measured over five-minute intervals for an hour as an index of pain behavior. Control animals showed a biphasic behavioral response. The first phase (0–5 minutes) results from activation of nociceptors. This was followed, after a short recovery, by a second phase (10–60 minutes). Based on its sensitivity to centrally applied NMDA receptor antagonists[18,27] and the differential effects of pre- and post-treatment with intrathecal opioids[18], this second phase has been interpreted as an expression of use-dependent changes in spinal neurons, initiated by activity generated during the first phase. Nevertheless, input from the periphery also develops during the second phase[29,30], which may sustain tonic pain behavior[31]. Like capsaicin, formalin injection into the hindpaw induced unilateral rapid ERK activation in isilateral superficial laminae. The level of pERK peaked at 3 minutes, declined at 8 minutes, but was still higher than baseline at 60 minutes (**Fig. 5a**).

Intrathecal injection of PD 98059, a MEK inhibitor that blocks phosphorylation of the ERKs[32], suppressed the second phase of the formalin test in a dose-dependent manner, without significant effect on the first phase (**Fig. 5b**). Over the entire course of the second phase (10–60 min.), 0.1 μg or 1 μg PD 98059 inhibited pain behavior by 55% ($p < 0.01$) and 71% ($p < 0.01$), respectively (**Fig. 5b**). At these doses, PD 98059 had no effect on basal mechanical or thermal pain sensitivity in naive animals but inhibited pERK immunolabel evoked by intraplantar capsaicin (data not shown).

To investigate if timing of the MEK inhibitor influenced the behavioral response, we administered 1 μg PD 98059 intrathecally 5 minutes after the formalin injection, that is, immediately after the first phase. The response to formalin in the second phase (10–60 min.) was still significantly inhibited (43%, $p < 0.01$) by such post-treatment. Interestingly, in these animals, no significant decrease in pain behavior occurred during the rising component of the second phase; the effect of the drug was delayed relative to its action when given before formalin injection (**Fig. 5b** and **c**). These results imply that the early component of the second phase is driven by input generated in the first phase, and the falling component of the second phase is established after the first phase, presumably by nociceptor afferent input evoked during the second phase. However, both phases involve ERK.

Discussion

Although ERK activation in most cells is driven by growth factors[2,33], activity-dependent activation occurs in many neurons[5,6,10,34]. We have found that ERK 1 and 2 are activated in the spinal cord following peripheral stimulation. The phosphorylation of these kinases, however, is specific to noxious stimuli and highly spatially and temporally organized.

ERK phosphorylation in the spinal cord is not simply activity dependent because natural or electrical input evoked in low-threshold Aβ-fibers does not initiate it, even though such input activates many cells in the dorsal and ventral horn. It is a stimulus-specific, activity-dependent phosphorylation. Only noxious peripheral stimuli (thermal, mechanical or heat) or Aδ-or C-fiber stimulation activate ERK in the dorsal horn, in a manner that encodes stimulus intensity. Moreover, the pERK-labeled neurons have a highly restricted anatomical distribution. The mediolateral and rostrocaudal location of labeled cells in the lumbar spinal cord follows the somatotopic architecture of the central termi-

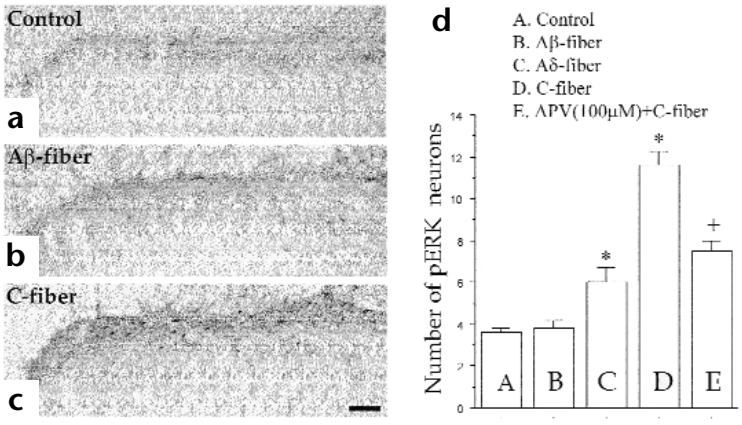

Fig. 4. Aδ- and C-fiber-dependent activation of ERK *in vitro*. Levels of pERK are low in control spinal cord slices (**a**). ERK is not induced by Aβ-fiber stimulation of the attached ipsilateral dorsal root (**b**), but is induced in the most superficial layers of the ipsilateral dorsal horn by C-fiber stimulation (**c**). Scale bar, 50 μm. (**d**) Number of pERK-positive neurons per section in the ipsilateral dorsal horn. Induction of pERK by C-fiber stimulation is partially blocked by the NMDA receptor antagonist APV (100 μM). *$p < 0.01$, compared to control (**a**); $^{+}p < 0.01$, compared to C-fiber stimulation (**d**), $n = 5$.

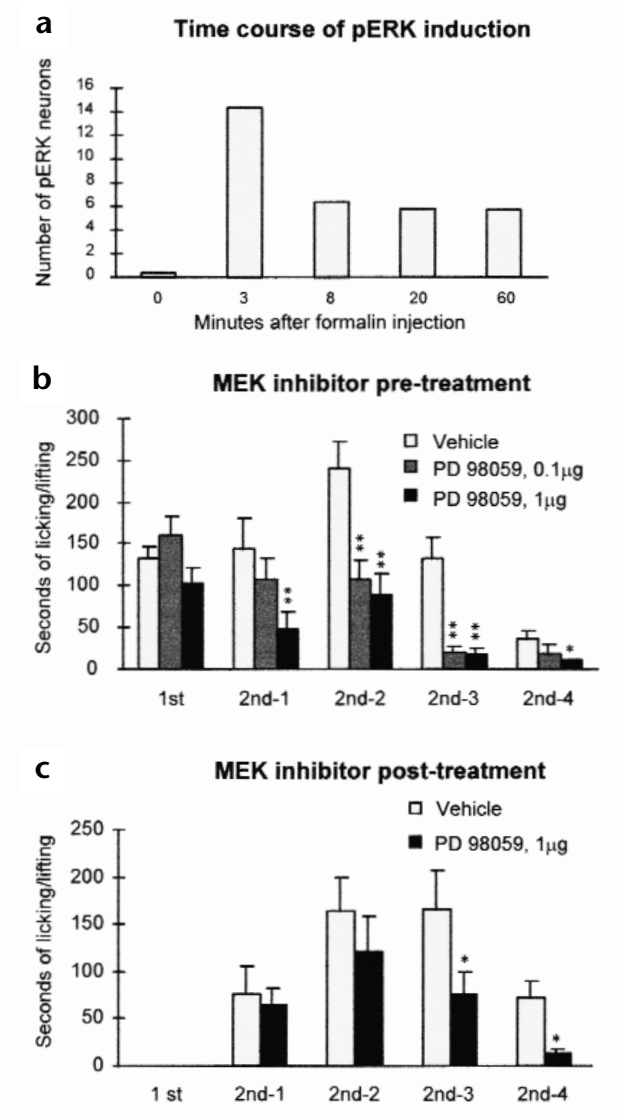

Fig. 5. Pre- and post-treatment with MEK inhibitor PD 98059 attenuates the behavioral response to intraplantar formalin. (**a**) Time course of ERK phosphorylation after intraplantar formalin injection measured by the number of pERK-positive neurons in the superficial laminae (I–II) of the ipsilateral dorsal horn. (**b**) Behavioral response to 1.5% formalin 20 min after intrathecal injection (pretreatment) of vehicle (10% DMSO; $n = 7$), 0.1 μg ($n = 6$) or 1 μg ($n = 6$) PD 98059 was measured by counting the seconds spent licking or lifting the injected paw over 5-min intervals for 1 hour. After the formalin injection, the first phase (0–5 min) and second phase 1 (10–20 min), 2 (20–30 min), 3 (30–40 min) and 4 (40–50 min) are plotted. (**c**) Vehicle ($n = 7$) or 1 μg PD 98059 ($n = 7$) was intrathecally infused 5 min after formalin injection (post-treatment), and formalin response was plotted in the second phase 1 (15–25 min), 2 (25–35 min), 3 (35–45 min) and 4 (45–55 min). *$p < 0.05$, **$p < 0.01$, compared to corresponding vehicle control.

nals of primary afferent C-fibers in the dorsal horn, where input from the hindpaw terminates in the medial part of the L4 and L5 segments[23]. The dorsoventral or laminar location of pERK neurons is restricted largely to lamina I and IIo, where NGF-responsive, peptide-containing afferents terminate, and is much less evident in IIi, where the afferents have a different phenotype and respond to the GDNF family of growth factors[35,36]. TrkA- but not GFR-expressing afferent central terminals may release a synaptic neuromodulator, such as BDNF[21], that selectively recruits the ERK cascade. Alternatively, the difference may be postsynaptic. Cells in lamina I and IIo may be more adapted to respond to C-fiber inputs by activating ERK, leading to a differential capacity to dynamically alter neuronal excitability in response to nociceptive inputs.

The peak increase in ERK phosphorylation in the dorsal horn is relatively transient, activated maximally within 2 minutes followed by a slow decline over tens of minutes, although levels remain elevated above baseline for beyond 30 minutes. The inactivation of ERK may result from an activity-dependent activation of specific phosphatases. The immediate early gene MKP-1 (MAPK phosphatase-1), a possible ERK phosphatase[37], is rapidly induced by electrical stimulation in the striatum[34]. Once activated, ERK may set in motion both post-translational and transcriptional changes, thus modifying synaptic function for prolonged periods that outlast both the initiating stimulus and ERK activation. NMDA receptors contribute to ERK activation in the dorsal horn evoked by noxious stimuli and to the behavioral effects of formalin[18,27]. However, in contrast to the almost-complete blockade by NMDA receptor antagonists of ERK activation induced by high-frequency stimulation in the hippocampus[7], these antagonists only partially inhibited pERK in the dorsal horn. The NMDA receptor involvement in ERK activation may not be direct and could be mediated by intervening interneurons. Alternatively something in addition to calcium entry through the NMDA receptor ion channel may be responsible for initiating activation of the ERK cascade. This could be glutamate acting via other ionotropic or metabotropic receptors, a neuropeptide like substance P, CGRP or the growth factor BDNF[21,38].

Central sensitization, a nociceptor-mediated, activity-dependent increase in the excitability of spinal neurons, results from post-translational changes in membrane-bound proteins[12,39]. This increased excitability recruits normally subthreshold synaptic inputs, resulting in an amplification of the responsiveness of the neurons to low- and high-intensity inputs as well as the spread of sensitivity to areas beyond the site of tissue damage[13,14]. We have found that the direct activation of nociceptive mechanisms by formalin (first phase) is not dependent on ERK activation. The MEK inhibitor did, however, block pain-related behavior in the second phase, with maximal action at the peak and late in the second phase. The sensitivity of the second phase to intrathecal NMDA receptor antagonists, which do not alter baseline pain sensitivity[40], points to a contribution of altered central neuronal excitability to the second phase. Post-treatment with PD 98059 still had a significant effect, albeit delayed, indicating that the slowest components of the second phase are likely to be generated by afferent activity elicited after the first phase. The action of PD 98059 is too quick to result from inhibition of any ERK-mediated increase in transcription. ERK activation must contribute to changes in dorsal horn neuronal properties by non-transcriptional means, presumably as a result of directly or indirectly phosphorylating kinases, key receptors and ion channels, thus modifying membrane excitability. A non-transcriptional role for ERK also occurs in the hippocampus, where PD 98059 attenuates the early phase of LTP beginning at 20 minutes[9,10]. As for LTP in the hippocampus[41], many transmitters/modulators seem to contribute to the initiation of central sensitization. One possible explanation for such diversity may be that these players all increase activation of a single convergent intracellular signal transduction pathway, the MAPK cascade.

The ERKs have a major role, via Rsk activation and subsequent CREB phosphorylation, in transcriptional regulation[1,10,42], and this is important for long-term facilitation[43] depending on gene expression in *Aplysia* and for LTP in the hippocampus[10]. Noxious stimulation induces phosphorylation of CREB[44,45] and transcriptional activation of many genes in the dorsal horn such as *c-fos*, *dynorphin*, *enkephalin*, *NPY*, *galanin*, *NK-1*, *TrkB*[12,21,46–50]. ERK activation in the spinal cord after noxious stimulation may regulate the expression of some of these genes via CRE-mediated transcription and contribute to the establishment of persistent pain as well as acute pain hypersensitivity.

Methods

Animals. Adult male Sprague-Dawley rats were used. The animal protocols were approved by the animal use committee of Massachussetts General Hospital. All procedures, except formalin injections, were performed under sodium pentobarbital anesthesia (60 mg per kg, i.p.). Capsaicin (8-methyl-N-vanillyl-6-noneamide, Sigma, 3 mg per ml dissolved in 10% Tween 80) was injected into the plantar surface of the left hindpaw (25 µl, 75 µg). Mechanical stimulation was applied to the dorsal surface of the hindpaw 15 times per minute for 2 minutes either by using a Von Frey filament (100 g) or by manually lightly stroking the skin from the ankle to the toes. Cold (4ºC), warm (42ºC) or hot (45–55ºC) stimuli were produced by immersion of the hindpaw into a water bath for 1 minute. For intrathecal injections, a PP10 catheter was implanted into the intrathecal space of the spinal cord (L3–L4 spinal cord segment), and 15 µl MK-801 or PD 98059 (RBI) was injected. Drugs were administered 20 minutes before capsaicin or formalin injection. After appropriate survival times, rats were perfused with saline followed by 4% paraformaldehyde with 1.5% picric acid, and L4–L5 spinal cord was removed and post-fixed for 90 minutes.

Immunohistochemistry and western blots. Transverse spinal cord sections (30 µm) were cut and processed for immunohistochemistry[49]. Briefly, sections were incubated overnight at 4ºC with polyclonal primary antibody for pERK1/2 (1:200, New England BioLabs). Most sections were stained using the ABC Vectastain kit; in others, immunofluorescence was used and examined with an MRC-600 Confocal microscope (Bio-Rad). Western blots were done as described[44]. Protein samples from spinal cords were separated on SDS-PAGE gel and transferred to PVDF filters. The filters were incubated overnight at 4ºC with anti-pERK1/2 antibody (1:1000) and finally visualized in ECL solution and exposed onto X-films for 10–30 minutes. The filters were then stripped and reprobed with anti-ERK2 antibody (1:1000, New England Biolabs).

Spinal cord slice preparation. The lumbar spinal cord was removed from adult rat and immersed in cold Kreb's solution, and a 700-µm-thick transverse slice with attached L4 dorsal root (15–20 mm long) was prepared[26] and perfused with Kreb's solution saturated with 95% O_2 and 5% CO_2 at 36–37ºC. The L4 dorsal root was stimulated using a suction electrode at 20 µA (0.05 ms, 15 trains) for Aβ fibers, 100 µA (0.05 ms, 5 trains) for Aδ-fibers, and 1000 µA (0.5 ms, 3 trains) for C-fibers. The activation of the different fiber groups was verified by compound action potential recordings[26]. At the Aβ stimulus strength, we estimate 60–70% of Aβ-and no Aδ-fibers were activated. At the Aδ stimulus strength, we saw no evidence of C-fiber activation. Each train consisted of 50 pulses (50 Hz, 1 s) with a 10-s inter-train interval. The slices were perfused with Kreb's solution for two hours before electrical stimulation, fixed two minutes after stimulation, cut on a cryostat and processed for immunohistochemistry. Some slices were incubated with D,L-2-amino-5-phosphonovarelic acid (APV, Sigma) for 15 minutes before stimulation.

Behavioral assessments. Formalin (50 µl of 1.5% solution) was injected into the plantar surface of the left hindpaw of awake rats, and the time (seconds) each animal spent licking or lifting the injected paw was counted over five-minute intervals for an hour.

Quantification and statistics. Eight sections from the L4–L5 lumbar spinal cord were randomly selected, and the numbers of pERK-positive neurons in the superficial laminae (I–II) or dorsal horn (laminae I–VI) were counted. Two to three rats were included in each group for the capsaicin and formalin time course and temperature range studies. Differences between groups were compared with one way ANOVA, followed by Fisher's PLSD using Statview statistical software.

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