

Reversal of Ongoing Thermal Hyperalgesia in Mice by a Recombinant Herpesvirus that Encodes Human Preproenkephalin

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Herpesvirus-mediated transfer of the human preproenkephalin gene to primary afferent nociceptors prevents phasic thermal allodynia/hyperalgesia in mice. It is not known, however, whether similar viral treatments would reverse ongoing or chronic pain and allodynia/hyperalgesia. To this end, mice were given intrathecal injections of pertussis toxin (PTX), which produces a weeks-long thermal hyperalgesia apparently by uncoupling certain G proteins from inhibitory neurotransmitter receptors. This treatment produced profound thermal hyperalgesia in both A δ and C-fiber thermonociceptive tests lasting at least 6 weeks. However, treatment of skin surfaces with an enkephalin-encoding herpesvirus, but not control virus or vehicle, completely reversed this hyperalgesia. This profound antihyperalgesia was observed for both A δ - and C-fiber-mediated responses. Interestingly, however, while the antihyperalgesic effect of the enkephalin-encoding virus on C-fiber-mediated responses was reversed by intrathecal application of μ or δ opioid antagonists, only δ antagonists reversed the effect of this virus on A δ hyperalgesia. Thus, virus-mediated delivery of the preproenkephalin cDNA reverses thermal hyperalgesia produced by PTX-induced ribosylation of inhibitory G proteins by an opioid-mediated mechanism. These results suggest that herpesvirus vectors encoding analgesic peptides may be useful in attenuating centrally mediated, ongoing neuropathic pain and/or hyperalgesia.

INTRODUCTION

We have previously demonstrated that cutaneous application of a herpesvirus encoding the gene for human preproenkephalin (hPPE) induces the expression of this gene in primary sensory afferents [1]. This gene is transcribed and the precursor peptide is processed to enkephalins as demonstrated immunohistochemically in primary afferent processes and spinal terminals. These peptides appear to be released upon activation of the afferents, causing a profound, naloxone-reversible, antihyperalgesic effect, consistent with the analgesic properties of the Met- and Leu-enkephalins [2]. These effects persist for at least 13 weeks after application of the virus, indicating long-term expression using the herpes vector. These effects have been demonstrated for phasic pain models, including cutaneous C-fiber sensitization with capsaicin [1], A δ sensitization with dimethyl sulfoxide [1], formalin-induced inflammatory responses [3], and capsaicin-induced blad-

der pain responses [4]. In addition, similar vectors have demonstrated antihyperalgesic effects in an arthritic rat model [5] and have demonstrated antiallodynic effects in a rat spinal nerve injury model for 3 weeks [6]. Thus, the antinociceptive effects of enkephalin-encoding herpes vectors can be robust and long lasting.

A single intrathecal injection of pertussis toxin (PTX) has been demonstrated to produce a robust, polymodal allodynic/hyperalgesic effect that lasts for at least 100 days in mice [7]. This hyperalgesia is thought to be mediated by the ADP-ribosylation of G_i and G_o proteins linked to neurotransmitter receptors that normally inhibit nociception. Thus, the increase in nociceptive sensitivity is thought to be mediated by a loss of tonic inhibitory tone. Similar mechanisms have been proposed for certain types of chronic, and particularly chronic neuropathic, pain [8], making PTX injection a novel, and potentially important, model of human chronic pain.

The experiments described here tested the hypothesis that cutaneous application of a herpes vector encoding hPPE could reverse the thermal hyperalgesia induced by intrathecal PTX. The initial experiments investigated the effects of the toxin on A δ -mediated and on C-fiber-mediated thermoreception using a model that allows for selective assessment of these two nociception types [9]. Thereafter, animals that had received PTX injections were treated unilaterally with the test virus, while other animals were treated with control virus to determine whether the virus could attenuate the effects of the PTX and the duration of this attenuation. In addition, to examine opioid dependence of any observed antihyperalgesic effects, either the μ opioid antagonist CTAP or the δ -selective antagonist Naltrindole was administered intrathecally in some animals.

RESULTS

Immunohistochemistry

Whereas Leu-enkephalin immunoreactivity was not seen in dorsal root ganglia cell bodies after topical application of KHZ control virus (Fig. 1A), application of KHPE induced the expression of Leu-enkephalin in neuronal cell bodies (Fig. 1B). These results are consistent with our previous findings of human preproenkephalin expression in primary afferent terminals in the dorsal horn of KHPE-infected mice [1]. As the mouse DRG are essentially devoid of endogenous enkephalins, these results are likely indicative of viral induction of enkephalin transgene expression, and processing to functional analgesic enkephalin peptides, in primary afferent neurons.

Behavior

Baseline response latencies to heat were similar to those previously reported [1,9]. Intrathecal injection of PTX produced a profound and significant ($P < 0.05$, ANOVA) thermal hyperalgesia by the first postinjection test session compared to the pre-PTX session as well as to vehicle-treated and untreated hind-paw responses (Fig. 2). We saw similar decreases in response latencies for both A δ -mediated (Fig. 2A) and C-fiber-mediated

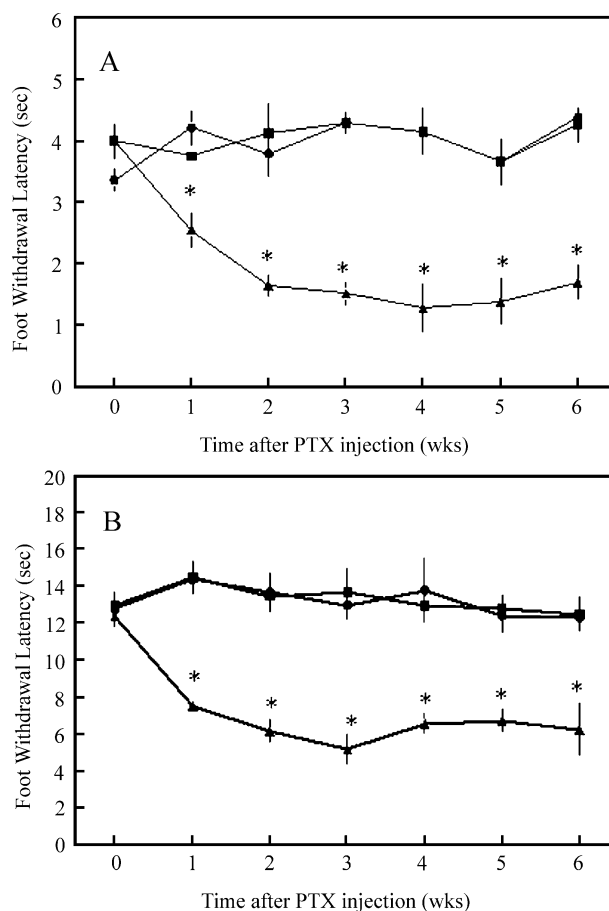


FIG. 2. PTX injection produces a persistent thermal hyperalgesia. (▲) Responses with PTX injection at time 0 ($n = 6$ mice); (■) responses with vehicle injection ($n = 6$); (●) untreated animals. (A) Responses to A δ activation. (B) Responses to C nociceptor activation ($n = 6$). *Significant ($P < 0.05$) difference from control.

(Fig. 2B) responses. Animals that had received KHZ (control) virus continued to demonstrate similar significant decreases in response latencies for both A δ - and C-fiber-mediated responses over the entire 6-week test-

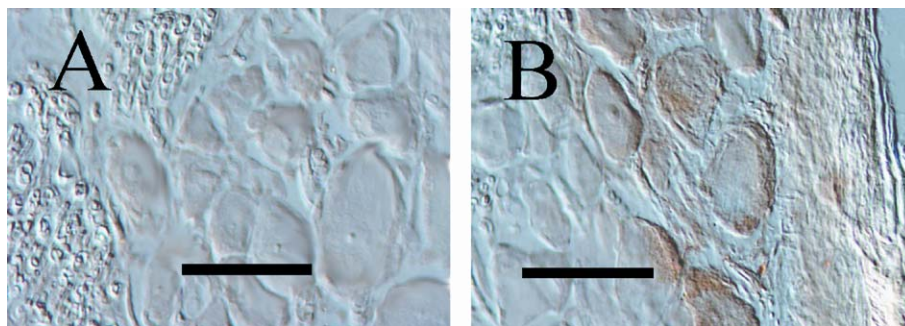


FIG. 1. Nomarski light micrographs of examples of Leu-enkephalin immunoreactivity in mouse dorsal root ganglia. (A) Section through mouse lumbar DRG ipsilateral to KHZ hind-paw skin inoculation. Note lack of Leu-enkephalin immunoreactivity. Scale bar, 20 μ m. (B) Section through mouse lumbar DRG ipsilateral to KHPE skin inoculation. Note group of small to medium-sized DRG cell bodies immunoreactive (brown staining) for Leu-enkephalin to the right of the section. Scale bar, 20 μ m.

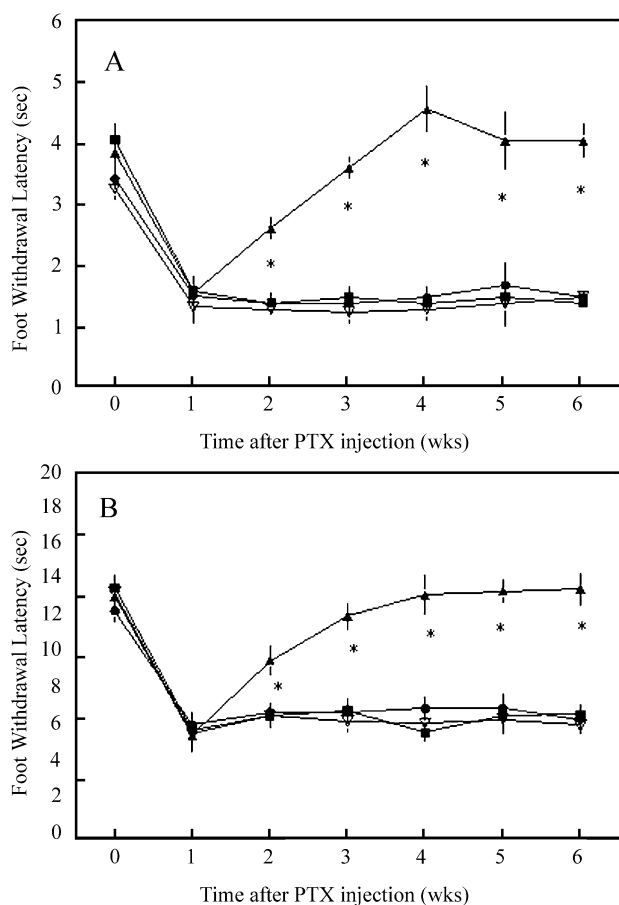


FIG. 3. Proenkephalin (KHPE) virus reverses thermal hyperalgesia induced by PTX injection at time 0. (\blacktriangle) Ipsilateral responses following KHPE administration to one hind paw 1 week after PTX injection ($n = 6$); (\blacksquare) ipsilateral responses following control virus (KHZ) administration to one hind paw 1 week after PTX injection ($n = 6$); (\bullet) ipsilateral responses with vehicle administration to the hind paw 1 week after PTX injection ($n = 6$); (∇) responses of hind paw contralateral to KHPE treatment (same $n = 6$ as KHPE group). (A) Responses to A δ activation. (B) Responses to C nociceptor activation. *Significant ($P < 0.05$) difference from vehicle control.

ing period (Figs. 3A and 3B). Similarly, the hind paw contralateral to the paw infected with KHPE was profoundly hyperalgesic to heat for both A δ - and C-mediated responses throughout the entire 6-week experiment (Figs. 3A and 3B, respectively). In contrast, although the hind paw infected with the KHPE virus demonstrated a similar hyperalgesia at 1 week after application of PTX, the hyperalgesia decreased over 2 weeks after application of KHPE, such that response latencies were not significantly different from pre-PTX latencies by 3 weeks. Thus, foot withdrawal latencies for paws treated with KHPE were significantly different from both KHZ-treated and uninjected paws for each of the weeks after viral application (ANOVA, $P < 0.05$).

Opiate Antagonists

Addition of Naltrindole (NTI), but not CTAP, significantly (ANOVA, $P < 0.05$) attenuated the antihyperalgesic effect of KHPE on A δ -mediated responses (Fig. 4A). Specifically, KHPE application decreased the PTX-mediated reduction in latencies by 1.53 (SEM 0.24) s, demonstrating a robust antihyperalgesic effect. Intrathecal injection of NTI significantly ($P < 0.05$) reduced this antihyperalgesia, by bringing latencies back toward the hyperalgesic state such that they were now only 1.08 (SEM 0.14) s longer than the hyperalgesic state after PTX. These data suggest that the antihyperalgesic effect of KHPE on A δ -mediated responses is likely mediated through δ , but not μ , opioid receptors. Intrathecal administration of either the μ opioid antagonist CTAP or the δ opioid antagonist

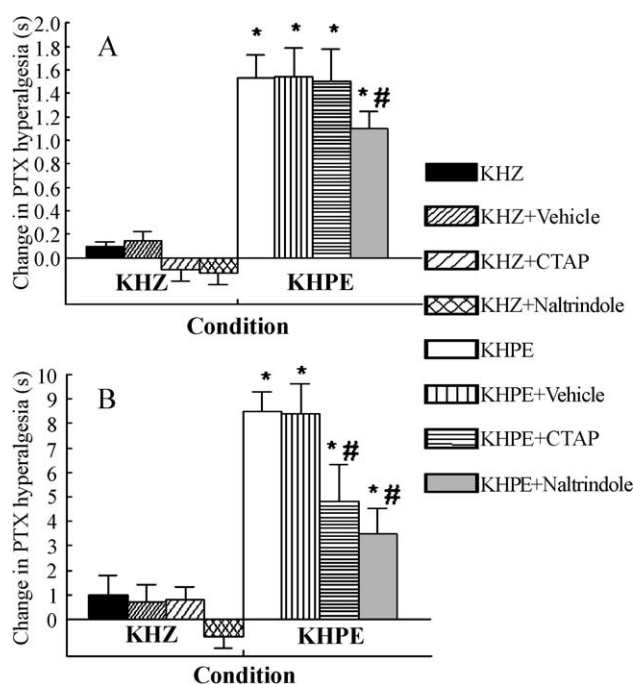


FIG. 4. Antihyperalgesic effect of vector application: reversal by intrathecal application of opiate antagonists. Note different scales for two graphs. Zero line on y axis represents PTX-induced hyperalgesic state. Bars represent extent of antihyperalgesic effect in seconds. Mice were tested for both A δ and C-fiber effects. (A) Effect on A δ -fiber hyperalgesia. Treatment with KHPE, but not KHZ, significantly reversed PTX hyperalgesia. Neither injection of vehicle ($n = 6$) nor the μ antagonist CTAP ($n = 6$) nor the δ antagonist Naltrindole ($n = 6$) affected responses in KHZ-treated mice. Injection of CTAP ($n = 6$), but not Naltrindole ($n = 6$) nor vehicle ($n = 6$), significantly decreased the antihyperalgesic effect of KHPE treatment. (B) Effect on C-fiber hyperalgesia. KHPE treatment, but not KHZ, significantly reversed PTX hyperalgesia. Injection of vehicle, CTAP, or Naltrindole did not significantly affect responses in KHZ-treated animals. Injection of either CTAP or Naltrindole, but not vehicle, significantly reversed the antihyperalgesic effects of KHPE application. *Significant ($P < 0.05$) difference from PTX-induced hyperalgesic response latencies. #Significant ($P < 0.05$) reversal of decrease in the response latency increase caused by vector treatment.

NTI significantly (ANOVA, $P < 0.05$) reversed the antihyperalgesic effect of KHPE administration for C-fiber-mediated responses (Fig. 4A). Thus, KHPE reversed the hyperalgesic effect of PTX by 8.5 (SEM 0.8) s, but the addition of CTAP decreased this to 4.8 (SEM 1.5) s, whereas addition of Naltrindole reversed the KHPE effect by decreasing the difference to 3.5 (SEM 0.9) s. These data suggest that the antihyperalgesic effects of KHPE are mediated by both μ and δ opioid receptors.

DISCUSSION

Previous work in our laboratory has demonstrated that application of a TK⁻ herpesvirus that encodes hPPE to the hind paw skin of mice produces an antihyperalgesic effect that lasts for more than 6 weeks [1]. These effects, however, were observed using tests that produce phasic hyperalgesia. To determine whether similar antinociceptive effects would be observed for established, chronic allodynia/hyperalgesia in mice, the same KHPE virus was applied to animals in which an ongoing central hyperalgesia had been established using intrathecal injections of PTX [7].

Injection of PTX produced a profound bilateral hypersensitivity for both A δ - and C-fiber-mediated thermociceptive responses. It has been suggested that hyperalgesia following PTX administration is due to its catalysis of the ADP-ribosylation of the α subunit of G_i/G_o inhibitory G proteins and the subsequent interruption of the function of inhibitory neurotransmitter receptors [7]. Thus, any tonic inhibitory tone is lost, effectively disinhibiting afferent activation of secondary dorsal horn neurons and inducing a chronic facilitation of nociceptive input observed behaviorally as an ongoing hyperalgesia. The duration of this hyperalgesia has provided evidence that some chronic central or neuropathic pain states might be related to the lack of inhibition, rather than tonic facilitation or excitation [8,10].

Unilateral application of the hPPE-encoding virus induced the expression of enkephalins in primary afferent cell bodies (Fig. 1) and neuronal processes in the dorsal horn [1] and reversed the effects of the PTX, returning response latencies to pre-PTX levels, whereas control virus application was ineffective in altering response latencies relative to uninfected skin. In addition, the antinociceptive effects of the virus lasted for at least 4 weeks. Interestingly, no apparent diminution of the effect of KHPE was seen over weeks, possibly indicating a lack of tolerance development. The data presented here clearly indicate that skin application of a herpesvirus encoding hPPE reverses the profound hyperalgesia produced by PTX injection. This suggests that hPPE treatment may be useful in reversing long-established painful and/or hyperalgesic conditions in humans, without the development of tolerance and other side effects of long-term use of opioid analgesics.

As stated above, PTX injection is thought to induce hyperalgesia by way of disabling inhibitory G proteins along with the associated inhibitory neurotransmitter receptors. Thus, tonic inhibition is diminished, effectively disinhibiting nociception. Interestingly, the intracellular signaling mechanisms of opioid receptors, which bind Met- and Leu-enkephalin, are likely to be affected by the PTX treatment. Thus, it seems initially paradoxical that inducing the production of enkephalins would reverse the hyperalgesia induced by PTX. Although it is possible that these effects are mediated by other proenkephalin A gene products, such as BAM22, that bind to nonopiate receptors [11], this seems unlikely as the KHPE reversal of PTX hyperalgesia was inhibited by specific opiate antagonists. A more plausible possibility might be that the opiate receptors underlying KHPE reversal of PTX hyperalgesia may become coupled to G proteins, such as G_z (a member of the G_i subfamily), that are PTX insensitive [12]. G_z, unlike G_{ix} and G_{o α} , does not possess the cysteine residue necessary for PTX-catalyzed modification [13]. Cell lines coexpressing G_z and opioid receptors implicate G_z in the regulation of Ca²⁺ channels critical to neurotransmitter release [14]. Similarly, antisense oligonucleotide strategies have demonstrated the involvement of G_z in μ and δ opioid-mediated antinociception in the central nervous system [15–17]. Thus, although PTX treatment induces a robust hyperalgesia, likely by disabling some G proteins linked to inhibitory neurotransmitter receptors, the reversal of this hyperalgesia by an opioid-encoding herpesvirus suggests that G proteins unaffected by PTX are capable of overcoming this disability. Alternatively, the intrinsic efficacy of the enkephalins may allow for a reversal of the PTX-induced hyperalgesia. Womer and Shannon [18] have shown that full, as opposed to partial, μ opioid agonists can overcome the hyperalgesia resulting from PTX injection, consistent with the hypothesis that full agonists retain efficacy even as receptor reserve decreases [19]. It is unclear whether the enkephalins, which are ligands for both the μ and the δ opioid receptors [20], would act the same. Thus, although PTX disables certain G proteins, resulting in a very persistent hyperalgesia that is reminiscent of human neuropathic pain, introduction of an enkephalin-encoding DNA into primary afferents using herpes vectors apparently reverses this state.

Of importance in discerning the mechanism of the antihyperalgesic effect of KHPE are the results of experiments in which selective antagonists, introduced into the intrathecal space, reversed this antihyperalgesia. Both the selective δ opioid receptor antagonist Naltrindole and the selective μ opioid receptor antagonist, CTAP, effectively reversed KHPE antihyperalgesia for C-fiber thermociceptive responses. These results clearly demonstrate that the observed antihyperalgesia is mediated by both μ and δ opioid receptors, at least some fraction of which must

remain functionally coupled to their signaling pathway(s). On the other hand, δ , but not μ , receptor antagonists attenuated the antihyperalgesic effects of KHPE for A δ -mediated responses. The reason for this disparity is unknown, but may supply clues as to the mechanism of the antihyperalgesic effect. Both μ and δ opioid receptors are located on presynaptic afferent terminals [21–23], as well as on postsynaptic spinal neurons [23–25]. However, some evidence, including preliminary work in our laboratory using antisense μ opioid receptor HSV vectors, suggests that μ receptors may be presynaptic on C but not A δ nociceptors [26]. Thus, the results of the present study may indicate that the effect of spinal PTX injection on μ opioid receptors may be predominantly at postsynaptic endings.

In summary, the results of these experiments suggest that: (a) a TK⁻ HSV-based enkephalin-encoding vector can attenuate profound, ongoing, centrally mediated nociception/hyperalgesia in mice, (b) this effect is mediated by (at least) μ and δ spinal opioid receptors, and (c) these effects display no obvious development of tolerance to the antihyperalgesic effects.

METHODS

All experiments were approved by the University of Illinois at Chicago Animal Care Committee and the Stanford University Institutional Animal Care and Use Committee.

Virus construction and purification. Construction of herpesviruses containing either the human preproenkephalin cDNA (KHPE) or the *Escherichia coli lac-Z* gene (KHZ) under control of the human cytomegalovirus immediate-early promoter/enhancer has been described [1]. The transgenic cassette is inserted into the thymidine kinase viral DNA sequence that essentially prevents viral replication in nondividing cells. Viral stocks were produced by infection of Vero cells and stored in cell culture medium containing 10% glycerol at -80°C .

Intrathecal pertussis toxin. Animals (6-week-old female Swiss-Webster mice) were anesthetized using 350 mg/kg tribromoethanol (ip). PTX was then administered intrathecally [27]. Briefly, a 10- μl Hamilton syringe fitted with a 30-gauge, 0.5-in. needle was inserted between either the fourth and the fifth or the fifth and the sixth lumbar vertebrae at an angle of 10–20 $^{\circ}$ until puncture of the dura resulted in a brief, characteristic “flick” of the tail. Pertussis toxin (0.3 μg in 0.5 μl vehicle) was then injected as described by Womer and Shannon [7]. Injected animals were returned to their home cages.

Virus application. Animals were anesthetized by administering 350 mg/kg tribromoethanol (ip). Hair was removed from the dorsal surface of both hind paws by application of Nair for 3–5 min followed by rinsing of the paws with water. The dorsal skin of each hind paw was then scarified by light application of a motorized sanding drum. Vehicle or viral suspension containing 10^7 plaque-forming units in 5 μl was then applied to the dorsal surface of the hind paw and gently distributed using the side of a disposable pipettor tip. After 10 min, when the virus suspension had absorbed/dried onto the foot, the animals were returned to their home cages.

Behavioral testing. Thermoception was assessed using the A δ /C foot-withdrawal test [9,28]. Mice were lightly anesthetized with urethane (500

mg/kg). Three sets of withdrawal latency measurements were made when the dorsal hairy surface of the hind paw was heated alternately at either a high (A δ) or a low (C) rate. Measurements were separated by 3-min intervals to minimize sensitization/desensitization that can occur with repeated skin heating. Response latency measurements were made prior to injection of PTX, 1 week later just prior to administration of the herpesvirus, and then weekly thereafter for a total of 6 weeks.

Opioid receptor antagonism. Three groups of six animals received intrathecal injections of opioid antagonists. In these experiments, hyperalgesia was first established by means of intrathecal PTX injection as above. As before, 1 week after administration of the toxin, these animals received unilateral KHPE viral application to one paw. Approximately 3 weeks later, at which time maximum antihyperalgesic effects of the virus were observed, animals were given antagonist injections. After baseline testing of response latencies, either 5 μl vehicle or 0.01 nmol of the μ -selective antagonist CTAP (Sigma) or 5 nmol of the δ -selective antagonist NTI (Sigma) in 5 μl vehicle was administered intrathecally (as above). Response measurements were then again measured at 10 min after injection to determine whether the antagonists attenuated the antihyperalgesic effects of viral application. At the end of these experiments, the animals were deeply anesthetized with urethane (1000 mg/kg) and intracardially perfused with 4% paraformaldehyde in phosphate buffer (pH 7.4) in preparation for immunohistochemical examination for the presence of human proenkephalin A in the spinal cord.

Statistical analysis. Means and standard errors of the latencies were calculated across animals for A δ -mediated and C-fiber-mediated responses. Analyses of variance were performed to examine whether significant differences existed between the following conditions: baseline vs. post-PTX treatment; KHZ vs KHPE; uninfected paw vs. KHPE-infected paw. Individual post hoc analyses (Tukey's) were performed to examine whether significant differences existed at each weekly time point. In addition, the effects of antagonists on KHPE-induced antihyperalgesia were determined by comparing mean peak response latencies after CTAP or Naltrindole injection with latencies after antagonist vehicle injection (Student's *t*). Differences were considered significant if $P < 0.05$.

Immunohistochemical demonstration of gene expression. Lumbar dorsal root ganglia were cryosectioned at 30 μm and mounted on Superfrost Plus slides (VWR Cat. No. 48311-703). Sections were blocked in 10% normal donkey serum (Jackson ImmunoResearch, Cat. No. 017-000-121) at room temperature before being incubated overnight with rabbit anti Leu-enkephalin with minimal cross-reactivity (Peninsula Labs, Cat. No. IHC 8601) at 1:1000 in a humid staining box at 4 $^{\circ}\text{C}$. After being washed in TBS, sections were incubated with biotinylated donkey anti-rabbit IgG (Amersham, Cat. No. RPN 1004) diluted at 1:200 for 1.0 h at room temperature. Subsequently, sections were incubated with streptavidin-biotinylated horseradish peroxidase complex (Amersham, Cat. No. RPN 1051) diluted at 1:100 for 30 min. Localization was visualized after a 10-min incubation in DAB (0.5 mg/ml + 0.1% H₂O₂) in 0.1 M sodium phosphate, pH 7.4. Sections were dehydrated and mounted in DPX (Fluka, Cat. No. 44581) and examined for Leu-enkephalin immunoreactivity using a Leica DMR-XA microscope using Nomarski optics.

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