Published in final edited form as:

Behav Brain Res. 2009 January 30; 197(1): 84–89. doi:10.1016/j.bbr.2008.08.005.

Transgene-mediated enkephalin expression attenuates signs of naloxone-precipitated morphine withdrawal in rats with neuropathic pain

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Abstract

Chronic morphine exposure induces physical dependence and tolerance. Previous studies have shown that there is a decrease in met-enkephalin levels in states of morphine physical dependence, and that increasing enkephalin during opiate physical withdrawal ameliorates the severity of the morphine withdrawal syndrome. In order to investigate the role of spinal opioid peptide in the phenomenon of naloxone-precipitated withdrawal we examined the effect of herpes simplex virus vector-mediated overexpression of proenkephalin in lumbar dorsal root ganglia in rats with neuropathic pain treated with morphine. The morphine physical dependence was induced by chronic administration of intraperitoneal (IP) morphine for 2 weeks. Rats with neuropathic pain inoculated subcutaneously with the vector-mediated overexpression of proenkephalin showed a significant reduction in jumps, ‘wet-dog’ shakes, diarrhea and ptosis precipitated by naloxone after 2 weeks of morphine treatment. The global withdrawal score was also reduced significantly by vector-mediated overexpression of proenkephalin. These studies demonstrate a role for opioid peptide in the spinal cord in mediating some of the withdrawal response.

Keywords

Morphine; Physical withdrawal; Gene therapy; Herpes simplex; Enkephalin

1. Introduction

Opioid drugs are potent analgesic agents but the use of these drugs is complicated by the development of tolerance and physical dependence [24]. Dependence is characterized by the presence of a withdrawal reaction (physical dependence) and/or the presence of a “drug-craving” component (psychic dependence) [35]. The unpleasant effects of withdrawal may occur when administration of the opiate drug is stopped suddenly or when an antagonist is administered [6,24]. The neuroanatomic sites involved in physical dependence include nuclei in the central nervous system such as the locus coeruleus (LC), periaqueductal gray (PAG) and the ventral tegmental area [10,25]. In addition to central nuclei, neuroadaptation in the spinal cord also appears to play a role in generating many of the signs of physical dependence [4, 11,18,34]. Pharmacologic [11] and electrophysiologic studies [1] suggest that the opioid-dependent spinal cord is capable of generating withdrawal symptoms in the absence of participation of supraspinal centers. Dorsal root lesions block the expression of opioid

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withdrawal [4] and withdrawal can be elicited in spinal-transected rats [28]. Both spinal *N*-methyl-D-aspartate (NMDA) receptors and nitric oxide (NO) are involved in the expression of behavioral and autonomic components of the naloxone-precipitated morphine withdrawal syndrome [5]. The pattern of lumbar spinal cord Fos expression following systemic withdrawal suggests that it results primarily from increased activity in opioid receptor-containing circuits intrinsic to the dorsal horn [31]. Activation of CGRP and substance P receptors at the spinal level contributes to the induction and expression of systemic opioid physical dependence [36] and that this activity may be partially mediated through the actions of prostaglandins [37].

Withdrawal syndromes in animals chronically treated with morphine and in human heroin addicts can be reduced by the administration of endogenous opioid peptides [45,46]. Peripheral administration of peptidase inhibitors [13,14,20] such as the enkephalinase inhibitor acetorphan attenuates some aspects of the opioid withdrawal syndrome [26]. In addition, the peptidase-resistant enkephalin analogs FK 33-824 and metkephamid completely suppress opiate withdrawal in monkeys [16]. Together, these results suggest that the strength of the opiate withdrawal syndrome may be related to the level of opioid receptor occupancy either by exogenously administered or endogenous agonists. Therefore, it would be logical to expect that increase in the concentration of enkephalin during opiate withdrawal should ameliorate the severity of the syndrome by restoring this balance in a more physiological manner.

We and others have shown that transduction of sensory neurons of the dorsal root ganglion (DRG) by footpad inoculation with herpes simplex virus (HSV)-based vectors coding for human proenkephalin results in the release of enkephalin from nerve terminals in the dorsal horn [29], in sufficient amounts to produce an anti-hyperalgesic effect in models of acute thermal nociception [47], subacute inflammatory pain [17], a rodent model of arthritis [3], and neuropathic pain [21]. Transgene-mediated enkephalin release from animals transduced to the HSV vectors is limited to the terminals of the transduced neurons; this restricted distribution of peptide release means the gene transfer can be used to assess the role of spinal opiates and selectively in the phenomena of physical dependence. In the current study we investigated the effect of HSV-mediated expression of enkephalin in lumbar DRG on naloxone-precipitated morphine withdrawal in rats with neuropathic pain treated with continuous morphine. We found that rats inoculated with an HSV vector expressing proenkephalin did not develop physical dependence, and that vector-mediated enkephalin expression attenuated the intensity of the naloxone-induced withdrawal syndrome in rats with neuropathic pain treated chronically with morphine.

2. Methods

2.1. Vectors

The recombinant replication deficient HSV-based vector SHPE was constructed as previously described [17]. Briefly, a cassette containing the human cytomegalovirus immediate-early promoter (HCMV IEp), SV40 intron, human proenkephalin cDNA and SV 40 polyadenylation sequence was inserted into the tk locus of the ICP4-deleted HSV vector d120k-lox [17]. The control vector SHZ was identical to SHPE except that the inserted cassette contained the *E. coli lacZ* reporter gene under the control of the HCMV IEp in the tk locus. Both SHPE and SHZ were isolated by three rounds of limiting dilution, and the final vector purified at 1×10^9 pfu/ml.

2.2. Expression of enkephalin in cultured DRG infected with SHPE or SHZ

DRG neurons from embryonic day 17 rats were cultured on poly-D-lysine-treated coverslips in Neurobasal medium with B27 and NGF supplementation. At 14 days *in vitro* the cells were

infected for 1 h with either SHPE or SHZ at a multiplicity of infection of 1, and the medium was changed to fresh medium without NGF. Twenty-four hours postinfection the medium was collected and the amount of enkephalin determined by HPLC followed by a radioimmunoassay. The supernatant was separated by reverse phase chromatography using a Waters Alliance series HPLC with Empower software and a Vydac C18 column, kept at 25 °C to ensure reproducible separation. A linear gradient with 1 ml/min flow rate consisting of a mobile phase A (water with a 0.1% (v/v) trifluoroacetic acid), and a mobile phase B (acetonitrile with 0.085% TFA) was begun with 5% a mobile phase B for 10 min and increased from 5% to 70% B over 40 min; this was continued at 70% B for 5 min before being reduced to 5% B in 5 min and finally equilibrated in 5% mobile phase B for 5 min. To identify the met-enkephalin containing fractions, a 10 µl sample of standard met-enkephalin (100 µM) was injected onto the column and the retention time (28.001 min) identified. Four fractions eluted at the 28 min retention time were collected, and the amount of enkephalin in the combined fractions was determined by RIA using an RIA kit (Peninsula laboratories San Carlos CA) according to manufacturer' protocol.

2.3. Experimental animals and surgical procedures

Male Sprague–Dawley rats (body weight 225–275 g) were housed one to two per cage approximately 1 week prior to the beginning of the study, with free access to food and water and maintained on a 12:12, light:dark schedule at 21 °C and 60% humidity. All housing conditions and experimental procedures were approved by the University of Michigan IACUC. Under chloral hydrate anesthesia (300 mg/kg, IP), nerve injury was produced by tight ligation of the L5 spinal nerve as previously described [23]. Briefly, the animals were placed in the prone position and under magnification approximately a third of the L6 transverse process was removed. The L5 spinal nerve was identified and carefully dissected free from the adjacent L4 spinal nerve and tightly ligated using 6-0 silk sutures and the incision was closed with wound clips. Animals that showed motor dysfunction of the hind limb after operation were excluded from further analysis. At 1 week after the operation, rats displaying mechanical allodynia were inoculated subcutaneously into the plantar surface of the left paw with 30 µl containing 6×10^6 pfu.

2.4. Behavioral testing

At the time of testing, rats were placed in transparent plastic cubicles on a mesh floor for a period (at least 30 min) of acclimatization. Mechanical hyperalgesia induced by the L5 spinal nerve ligation was determined by assessing the rate response of paw withdrawal to von Frey hairs of graded tensile strength. A series of 10 calibrated fine von Frey filaments (0.4, 0.7, 1.2, 1.5, 2.0, 3.6, 5.5, 8.5, 11.8, and 15.1 g) was presented serially to the neuropathic hind paw in ascending order of strength with sufficient force to cause slight bending against the paw and held for 6 s. A positive response was defined as a rapid withdrawal and/or licking of the paw immediately upon application of the stimulus. Whenever a positive response to a stimulus occurred, the next finer von Frey hair was applied, and whenever a negative response occurred, the next higher force was applied. In the absence of a response at a pressure of 15.1 g, animals were assigned to this cut-off value. A tactile stimulus producing a 50% likelihood of withdrawal was determined using the up–down method [9,12]. Behavioral testing was performed during the day portion of the circadian cycle (8:00 a.m. to 5:00 p.m.).

2.5. Immunohistochemistry

At 7 days after inoculation of vectors into left paw, rats were anesthetized and perfused through the ascending aorta with saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, 4 °C. After perfusion, the left L4 DRG were removed and postfixed in the same fixative overnight and then replaced with 30% sucrose in 0.1 M PBS for 2 days at 4 °C. Cryostat 20

µm sections were blocked with 2.5% goat serum in 0.3% Triton X-100 for 1 h at RT and incubated for 72 h at 4 °C with mouse anti-enkephalin antibody (Harlan Sera-Lab LTD, Loughborough, England). The sections were then incubated for 2 h at RT with Alexa Fluor® 594 conjugated goat anti-mouse IgG (1:500; Molecular Probes, Eugene, OR). The stained sections were examined with a fluorescence microscope, and images were captured with an Olympus Fluoview BX61 Confocal Microscope.

2.6. Evaluation of morphine physical dependence

We have previously demonstrated that subcutaneous inoculation of SHPE produces an anti-allodynic effect in the neuropathic rats raising the mechanical threshold from 1.8 to 6.8 g [21]. Animals treated with SHPE do not develop tolerance to the continued production of vector-mediated enkephalin over a period of several weeks [21], and animals inoculated with the vector continued to express an analgesic effect despite the induction of tolerance to morphine induced by treatment with intraperitoneal morphine [21]. In this study, we investigated whether HSV vector-mediated enkephalin release reduced chronic morphine physical dependence in the neuropathic rats. Chronic morphine (10 mg/kg, IP) was delivered intraperitoneally twice daily for 2 weeks beginning 1 week after inoculation of SHPE or SHZ into the paw of rats that had undergone spinal nerve ligation 2 weeks earlier and a withdrawal syndrome was precipitated by injection of naloxone (1 mg/kg, IP) 2 h after the final morphine administration. Immediately after naloxone administration, animals were placed individually in test chambers consisting of boxes (50 cm × 35 cm × 45 cm) and withdrawal signs were evaluated over the course of 20 min. Two types of signs were measured during abstinence, as described previously [42]. The number of jumps and ‘wet-dog’ shakes were counted; teeth chatter, ptosis and diarrhea were evaluated over 5 min periods with one point being given for the presence of each sign during each period. The number of periods showing the sign was then counted (maximum score, 4). The body weight of the rats was recorded before the injection of naloxone and then again at 60 min after the naloxone injection. A global withdrawal score was calculated for each rat by assigning a weighting factor to the various physical signs of withdrawal [15] and by adding one point for each 1% decrease in body weight.

To determine if enkephalin produced by SHPE resulted in physical dependence in the neuropathic rats, in a second experiment, rats were inoculated with SHPE or SHZ and 7 days after vector inoculation injected with physiologic saline (1 ml, IP) twice a day for 2 weeks; and a withdrawal syndrome was precipitated by injection of intraperitoneal naloxone (1 mg/kg, IP) 2 h after the final saline administration.

2.7. Drugs and data analysis

Morphine hydrochloride was obtained from Elkins-Sinn, Cherry Hill, NJ. Naloxone hydrochloride was obtained from Sigma. All drugs were dissolved in physiologic (0.9%) saline. The drugs were injected intraperitoneally in a volume of 3 ml per kilogram of body weight. The mechanical threshold determined by the up-down method is presented as mean ± S.E.M. The statistical significances of the differences were determined by ANOVA (StatView J 5.2) followed by *post hoc* comparisons using Scheffe’s *F* test. *P*-values of less than 0.05 were considered to be statistically significant.

3. Results

After left L5 spinal nerve ligation, the rats appeared to be in good health, exhibiting normal weight gain, normal toenail growth, and a normal level of activity. They displayed no signs of distress or abnormal aggressive behavior (including jumps, body or head shakes). Following the spinal nerve injury, all rats entered into the study displayed a significant decrease

(mechanical threshold less than 3 g) in the magnitude of the mechanical stimulus necessary to evoke a brisk withdrawal response to von Frey hair stimulation of the injured hind paw.

3.1. Expression of enkephalin after inoculation of SHPE or SHZ *in vitro* and *in vivo*

In our previous study, we demonstrated that inoculation of the footpad with the non-replicating vector SHPE, but not SHZ results in transduction of lumbar DRG and expression of proenkephalin mRNA in both *in vitro* and *in vivo* [17]. In the present study, we examined the expression of met-enkephalin-like immunoreactivity after inoculation of SHPE or SHZ *in vitro* and *in vivo*. DRG neurons infected with SHPE release a substantial amount of enkephalin into the medium, detected by RIA (Fig. 1A). DRG neurons transduced with control vector SHZ were indistinguishable from uninfected cells. Met-enkephalin-like immunoreactivity was seen in lumbar DRG from animals inoculated with SHPE, but not SHZ (Fig. 1B).

3.2. Effect of SHPE on morphine physical dependence

Naloxone (0.25–1 mg/kg) induces a dose-dependent morphine withdrawal syndrome [15]. In this study, we used naloxone of 1 mg/kg to precipitate morphine withdrawal symptoms. Animals receiving SHPE plus chronic saline, but not SHZ showed an anti-allodynic effect of the vector (data not shown) similar to that which we have reported previously [21]. Animals inoculated with SHPE followed by chronic saline treatment did not show a naloxone-precipitated withdrawal similar to animals inoculated with SHZ followed by chronic saline. These results suggest that SHPE inoculation alone does not induce physical dependence, despite a persistent anti-allodynic effect of vector-mediated enkephalin production in this pain model.

In rats treated with chronic morphine, jumps and ‘wet-dog’ shakes usually began about 5 min after injection of naloxone. Animals inoculated with SHZ plus chronic morphine exhibited a significantly greater number of jumps and ‘wet-dog’ shakes following a 1 mg/kg dose of naloxone compared to those inoculated with SHZ plus chronic saline (Fig. 2); the number of jumps and ‘wet-dog’ shakes in rat inoculated SHPE plus chronic morphine was significantly less than that in rats inoculated SHZ plus chronic morphine (Fig. 2). Rats inoculated with SHPE, but not SHZ plus chronic morphine exhibited a significant decrease in diarrhea and sign of ptosis (Fig. 3). Although the score of teeth chattering in rats inoculated with SHPE plus morphine was less than that in rats inoculated with SHZ plus chronic morphine, the change was not statistically significant (Fig. 3).

3.3. Loss of body weight

The principal reason for body weight loss during physical dependence is an increase in the urinary and fecal output; a significant loss of body weight in rats inoculated with SHZ followed by morphine treatment compared to animals inoculated with SHZ followed by saline treatment (Fig. 4A). Weight loss in rats inoculated with SHPE followed by chronic morphine treatment was less than that in animals inoculated with SHZ followed by chronic morphine ($P = 0.061$) (Fig. 4).

3.4. Global withdrawal score

A global withdrawal score was individually calculated for each rat for the observed withdrawal signs, as previously reported [15]. To obtain this global value we gave the entire signs proportional weighting factor. Each 1% above the weight loss contributed 1 point. One or two ‘wet-dog’ shakes or jumps contributed 2 points; three or more did 4 points. Teeth chattering, diarrhea and ptosis contributed 1 point in every 5 min. SHZ-inoculated rats plus chronic morphine showed a higher global score than SHZ-inoculated rats plus chronic saline ($P < 0.001$); global withdrawal score in rats inoculated with SHPE plus chronic morphine was

significantly less than that in animals inoculated with SHZ plus chronic morphine treatment ($P < 0.01$) (Fig. 4B).

4. Discussion

In this study, we reported two findings: (1) animals with persistent allodynia from spinal nerve ligation treated with an HSV vector to achieve local release of enkephalin did not develop physical dependence, and (2) chronic morphine-induced physical dependence was reversed in part by vector-mediated enkephalin released from transduced DRG. To our knowledge, this is the first study to use gene transfer to examine effect of spinal enkephalin on the development of physical dependence on morphine.

Although many studies have focused on the role of supraspinal centers in opioid withdrawal, there is also evidence that spinal centers may be involved in the phenomenon. Pretreatment with intrathecal injection of either the NMDA receptor antagonist or an inhibitor of NO produces a significant reduction in the expression of both naloxone-precipitated cardiovascular and withdrawal symptoms in systemic morphine-treated rats [5]. Chronic morphine-treated rats display clear signs of withdrawal and spinal c-fos expression induced by naloxone despite prior spinal cord transection, and intrathecal naloxone precipitates signs of withdrawal and spinal c-fos expression in rats rendered dependent on systemic morphine [31]. Studies suggest that activation of CGRP and substance P receptors at the spinal level contributes to the induction and expression of opioid physical dependence and that this activity may be partially expressed through the intermediary actions of prostaglandins [37]. Evidence demonstrates that activity of lipoxygenase products at the spinal level contribute to the expression of opioid physical dependence, and that this activity may be expressed through increased sensory neuropeptide release [38]. Recent studies show that both spinal extracellular signal-regulated kinase-1/2 (ERK1/2) and NO contribute to naloxone-precipitated withdrawal and to withdrawal-induced spinal neuronal sensitization in systemic morphine-dependent rats [7,8].

The specific signs of antagonist-precipitated withdrawal from morphine in rats can change qualitatively as well as quantitatively as a function of degree of physical dependence, dose of antagonist, and time after antagonist administration [2]. Previous studies have shown that withdrawal scores are dependent on the time those animals consumed morphine and that the scores rapidly increased and reach a plateau 2 weeks after morphine [15]. In this protocol, chronic morphine was intraperitoneally administered for 2 weeks. Although vector-mediated enkephalin expression alleviated several symptoms of the morphine withdrawal, other signs were unaltered; global withdrawal scores were significantly reduced. The weight loss is a good single indicator of the severity of the precipitated withdrawal syndrome [15], but in the present study, the change of weight loss was not significant between rats that had received SPHE or SHZ ($P = 0.062$). However, the combined withdrawal score is more sensitive in separating the intensity of withdrawal precipitated by naloxone [15].

The mechanisms by which enkephalin released by the vector in spinal cord suppresses morphine withdrawal signs is not apparent. Changes in the CNS met-enkephalin level of animals treated with opioids have been implicated in opioid tolerance and dependence [19]. Animals treated chronically with opioids show a decrease in met-enkephalin level during tolerance and dependence [40]. This decrease in met-enkephalin may be due to a reduction in its biosynthesis [40]. Trujillo et al. have proposed a mechanism for the involvement of endogenous opioid peptides in opioid dependence. Previous studies have shown that diazepam inhibits chronic morphine tolerance and physical dependence and reverses the decrease in met-enkephalin that characterizes morphine tolerance and dependence [33]. Administration of an exogenous neurotransmitter or its agonists lead to a decrease in biosynthesis and release because of feedback inhibition, and chronic administration of exogenous opioids such as

morphine might down-regulate opioid peptide biosynthesis and release by feedback inhibition [40]. Recent studies demonstrate that preproenkephalin knock-out mice show a significant decrease in chronic morphine or cannabinoid withdrawal [30,41] and that naloxone-precipitated physical withdrawal signs are attenuated in the pro-enkephalin knockout mice relative to wild-type animals [32].

It would be logical to suggest that an increase in the concentration of enkephalin during opiate withdrawal should ameliorate the severity of the syndrome by restoring this balance in a physiologic manner, which may explain the current result. The increase in endogenous opioid peptides (e.g. enkephalin and beta-endorphin) inhibits the binding to opioid receptors of competitive antagonist, naloxone injected to precipitate the morphine withdrawal response [27]. Study demonstrated that delta 9-tetrahydrocannabinol by increasing the release of met-enkephalin-like material significantly reduced naloxone-induced withdrawal syndrome [42]. A change in endogenous opioid activity together with other changes resulting from chronic stimulation of opioid receptors is likely responsible for many of the neural and behavioral changes observed during opiate tolerance and dependence [33,39,40]. Previous clinical studies demonstrated that a met-enkephalin analogue can produce effective opioid detoxification and that acupuncture and electrical stimulation was used in an out-patient detoxification setting via increasing endogenous enkephalin [43,44].

The model which we have examined in this study is a very specific one of neuropathic pain treated with chronic opioids. There has been some suggestion that the incidence of withdrawal signs may be higher in rats with neuropathic pain compared to normal rats [22]. We cannot determine from the studies performed whether the regional release of opiates in the spinal cord by gene transfer would reduce withdrawal signs in chronic opioid dependence that occurs in other animal models or clinical situations. Nonetheless, our findings indicate that HSV vector-mediated enkephalin release from transduced lumbar DRG attenuates many of the withdrawal responses in rats with neuropathic pain rendered morphine-dependent by the chronic administration of morphine. The most plausible explanation for this finding would be that increased concentration of enkephalin in the spinal dorsal horn produced by vector-mediated release competes with naloxone, leading to a more intensive saturation of the opiate receptor and corresponding attenuation of the withdrawal syndrome. The results indicate an important role for spinal opiate receptors in the physical manifestations of withdrawal in this model.

Acknowledgements

This work was supported by grants from the Department of Veterans Affairs and the NINDS and NIDDK to D.J.F., and NIDA to S.H. We acknowledge the excellent technical assistance of Vikram Thakur, Shue Liu and Gui Zhang.

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Fig. 1.

(A) Primary DRG neurons in culture infected at a multiplicity of infection of 1 released enkephalin into the medium determined by HPLC followed by RIA, $**P < 0.01$ vs. SHZ, ANOVA. (B) Enkephalin immunoreactivity in DRG 1 week after subcutaneous inoculation of SHPE was increased in both large and small diameter neurons compared to control animals.

Fig. 2.

Jumps (A) and 'wet-dog' shakes (B) measured during naloxone-precipitated morphine withdrawal in rats with chronic morphine or vehicle. Vectors were inoculated 7 days prior to chronic morphine or vehicle. Morphine dependence was induced by repeated injection of morphine (10 mg/kg IP) for 2 weeks. Withdrawal was precipitated by injecting naloxone (1 mg/kg IP) 2 h after the last morphine or vehicle administration. The number of jumps and 'wet-dog' shakes was counted. Expression of enkephalin by SHPE significantly suppressed jumps and 'wet-dog' shakes during naloxone precipitated morphine withdrawal in rats with chronic morphine. Data were presented as mean $\pm$ S.E.M. * $P < 0.05$, ** $P < 0.01$ vs. SHZ, ANOVA. $N = 5-6$ animals per group.

Fig. 3.

The changes of teeth chattering (A), diarrhea (B) and ptosis (C) during naloxone-precipitated morphine withdrawal in rats with chronic morphine or vehicle. Withdrawal was precipitated by injecting naloxone (1 mg/kg, IP) 2 h after the last morphine or vehicle administration. Rats inoculated with SHPE, but not SHZ plus chronic morphine exhibited a significant decrease in diarrhea and sign of ptosis. Decrease in teeth chattering was not significant. Data were presented as mean $\pm$ S.E.M. * $P < 0.05$, *** $P < 0.001$ vs. SHZ, ANOVA. $N = 5-6$ animals per group.

Fig. 4.

(A) The percent body weight loss during naloxone-precipitated morphine withdrawal in rats with chronic morphine or vehicle. Withdrawal was precipitated by injecting naloxone (1 mg/kg, IP) 2 h after the last morphine or vehicle administration. Body weight was measured before naloxone and 1 h after naloxone. Weight loss in rats receiving SHPE plus chronic morphine was less than that in rats receiving SHZ plus chronic morphine but the difference was not statistically significant ($P = 0.061$). Data were presented as mean $\pm$ S.E.M. (B) The global withdrawal scores during naloxone-precipitated morphine withdrawal in rats with chronic morphine or vehicle. Withdrawal was precipitated by injecting naloxone (1 mg/kg, IP) 2 h after the last morphine or vehicle administration. The global withdrawal scores were based on all

signs. To determine the global value we gave all signs proportional weighting factor. The global withdrawal scores in rats receiving SHPE plus chronic morphine were significantly less than that in rats receiving inoculation of SHZ plus chronic morphine. $**P < 0.01$ vs. SHZ, ANOVA. $N = 5-6$ animals per group.