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SYSTEMICALLY ACTIVE HUMAN OPIORPHIN IS A POTENT YET NON-ADDICTIVE ANALGESIC WITHOUT DRUG TOLERANCE EFFECTS

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Human opiorphin QRFSR-peptide protects enkephalins from degradation by human neutral endopeptidase (hNEP) and aminopeptidase-N (hAP-N) and inhibits pain perception in a behavioral model of mechanical acute pain (1). Here, using two other pain rat models, the tail-flick and the formalin tests, we assess the potency and duration of the antinociceptive action of opiorphin with reference to morphine. The occurrence of adverse effects with emphasis on the side-effect profile at equi-analgesic doses was compared. We demonstrate that opiorphin elicits minimal adverse morphine-associated effects, at doses (1-2 mg/kg, i.v.) that produce a comparable analgesic potency in both spinally controlled thermal-induced acute and peripheral chemical-induced tonic nociception. The analgesic response induced by opiorphin in the formalin-induced pain model preferentially requires activation of endogenous μ-opioid pathways. However, in contrast to exogenous μ-opioid agonists such as morphine, opiorphin, does not develop significant abuse liability or antinociceptive drug tolerance after subchronic treatment. In addition, anti-peristaltism was not observed. We conclude that opiorphin, by inhibiting the destruction of endogenous enkephalins, which are released according to the painful stimulus, activates restricted opioid pathways specifically involved in pain control, thus contributing to a greater balance between analgesia and side-effects than found with morphine. Therefore, opiorphin could give rise to new analgesics endowed with potencies similar to morphine but with fewer adverse effects than opioid agonists. Its chemical optimization, to generate functional derivatives endowed with better bioavailability properties than the native peptide, could lead to a potent class of physiological type analgesics.

Key words: *enkephalin-inactivating neutral endopeptidase inhibitor, aminopeptidase-N inhibitor, opioid pathway, pain, addiction, tolerance*

INTRODUCTION

There are two basic types of pain differing in their etiology and physiopathology: acute pain and chronic pain. Acute pain is self-limiting, usually concordant with the degree of on-going tissue damage, and remitting with resolution of the injury. It is nociceptive in nature. Chronic pain is not self-limiting and, if it is inadequately treated, is predominantly neuropathic in nature, leading to peripheral and/or central nociceptive sensitization. The most efficient drugs to alleviate severe pain are opioid receptor agonists, such as morphine or its surrogates. However, their clinical usefulness is limited by the development of tolerance and dependence that occurs after long-term treatment, while constipation and respiratory depression remain the dose-limiting adverse effects following systemic administration (2-4).

The physiological opioid pathways are a predominant part of an endogenous nociceptive-modulating system that counterbalances the activity of pain transmission pathways. The most important endogenous opioid peptides, the enkephalins, play a major role in the dynamic control of pain perception (5). Enkephalins interact with high affinity, with both the mu (μ)- and delta(δ)-opioid receptors, present on synaptic membranes of opioid and target neurons (6). Because of their high intrinsic efficacy, enkephalins need to occupy a smaller proportion of opioid receptors than morphine to elicit the same antinociceptive responses. Central administration of enkephalins appears to trigger a strong, but brief, analgesic responses due to their rapid inactivation by the concomitant action of two membrane-bound metallo-ectopeptidases which are co-located with opioid receptors, namely, neutral endopeptidase (NEP EC3.4.21.11) and aminopeptidase-N (AP-N EC3.4.11.2) (7-9). Increasing the lifetime of circulating enkephalins, released in response to nociceptive stimuli, by inhibiting their degradation is, therefore, an effective method to increase their bioavailability and thus to enhance their physiological actions and particularly their analgesic potency (10).

In a multi-level approach, using genetic, biochemical, molecular and behavioral pharmacology methodologies, we discovered the existence of physiological dual inhibitors of both enkephalin-inactivating Zn-ectopeptidases, NEP and AP-N in mammals. We named one of these compounds rat Sialorphin. The corresponding QHNPR-pentapeptide, was identified using an integrative post-genomic approach (11-13). Human opiorphin, the QRFSR-pentapeptide, was subsequently discovered using a functional biochemical approach (1, 14). The discovery of such novel endogenous upstream regulators in human, is of crucial

interest from physiological and therapeutic points of view. Indeed, endogenous human opiorphin appears to intervene in the process of adaptation mediated by enkephalins, which are associated with nociception and emotion-related behaviors (5, 15, 16).

We previously demonstrated that human opiorphin protects met-enkephalin from degradation by human NEP and AP-N *in vitro* (1). Remarkably, using a centrally integrated behavioral rat model of mechanical acute pain, the pin pain test, we found that the native opiorphin-peptide (1 mg/kg, i.v.) inhibits the perception of sharp painful stimuli and is as efficient in its pain-inhibitory potency as morphine (1). Thus, because of its exciting *in vivo* properties, we concluded that opiorphin may have therapeutic implications in the field of analgesia. Consequently, it was essential to investigate its ability to induce analgesia in other pain models. With this objective, in the present study we analyzed behavioral responses induced by opiorphin using two rat pain models, the formalin and the tail-flick models. The formalin test, a model of progressive inflammatory pain, measures the behavioral response to a chemical-induced tonic pain. The tail flick test, a model of acute thermal nociception measures the behavioral response caused by a phasic pain (17). In both models, the time course of the analgesic behavioral response of opiorphin was analyzed with reference to morphine. In addition, we investigated the specific involvement of μ-, δ- or κ- opioid receptor-dependent pathways, to further understand the endogenous events triggered by opiorphin.

Addiction liability is known to be associated with opioid administration (18). Thus, in the present study, the potential abuse liability induced by opiorphin was studied with reference to morphine, using the conditioned place preference behavioral model, which is commonly used to investigate the reinforcing effects of drugs (19). In addition to addictive side effects, analgesic morphine tolerance also often develops following repeated treatment (3, 4). Therefore, using the tail flick test, we evaluated, again with reference to morphine, the potential induction of opiorphin tolerance when subchronically administered at equi-analgesic doses. Finally, in the present study we also focused on a third morphine-associated side effect, constipation, following systemic administration of a single analgesic dose of opiorphin.

We report here that opiorphin presents minor adverse side effects at 1-2 mg/kg, i.v doses that produced analgesia in both experimental models of morphine-sensitive acute and tonic pain.

MATERIALS AND METHODS

Animals

Male Wistar rats (Harlan, France) weighing 250-280 g were used in this study. After 7-day acclimatization period, they were weighed and randomly housed according to the treatment groups in a room with a 12 hours alternating light/dark cycle (9:00 pm/9:00 am) and controlled temperature (21±1°C) and hygrometry (50±5%). Food and water were available *ad libidum.* Animals were experimentally only tested once.

Behavioral tests, care and euthanasia of study animals were in accordance with guidelines of the European Communities Directive 86/609/EEC and the ASAB Ethical Committee for the use of laboratory animals in behavioral research (Animal Behaviour, 2006; 71: 245-253).

Chemicals

Two different batches of opiorphin synthesis (Genosphere Biotechnologies, France) were used: the first one was used for the formalin and conditioned placed preference tests and the second one for the tail-flick test and tolerance induction. opiorphin was dissolved in vehicle solution (55% of PBS 100 mM–45% of acetic acid 0.01N) and systemically (i.v. tail vein delivery) injected, 5 to 15 min prior to the behavioral tests, at doses ranging from 0.5 to 2 mg/kg body weight. Morphine HCl (Francopia, France) was dissolved in physiological saline solution (0.9% NaCl) and injected *via* the i.v. route 15 min before the behavioral test, at 1-2 mg/kg doses. Naloxone (a centrally and peripherally acting opioid antagonist) was purchased from Sigma Chemical (France), dissolved in 0.9% NaCl and subcutaneously administered at 3 mg/kg, 15 min before opiorphin administration. Naltrindole (δ-opioid antagonist), nor-binaltorphimine (κ-opioid antagonist) and CTAP (μ-opioid antagonist) were purchased from Sigma Chemical, dissolved in 0.9% NaCl and administered at 10 mg/kg i.p., 20 min; 5 mg/kg i.p., 3 hours and 0.8 mg/kg i.v., 25 min before tests, respectively. All drugs were administered in a volume of 1 ml/kg body weight.

The formalin test

The previously prescribed protocol (1) was used to assess the analgesic potency of opiorphin native peptide on a chemical-induced inflammatory pain model. Groups of 8 rats were used to perform each experiment. 50 μl of a 2.5% formalin solution was injected under the surface of the left hind paw 15 min after i.v. injection of opiorphin, morphine or vehicle. The duration of formalin-injected paw licking and the total number of inflamed paw flinches and body tremors were recorded over a period of 60 min after formalin administration. The behavioral scores were expressed as means ± standard error of the mean (S.E.M.) for n=8 rats.

The tail flick test

The tail-flick test measures the time required to respond to a painful radiating thermal stimulus. A standardized tail-flick apparatus (Harvard Apparatus LTD, England) with a radiant heat source connected to an automated tail-flick analgesymeter was used. Rats were accustomed to the contentious situation with two 2-min sessions on the day prior to the test. On the experimental day, they were gently restrained by hand so that the radiant heat source was focused onto the distal dorsal surface of the tail. The previously adjusted intensity of the thermal stimulus was set at 30% to obtain a basal tail-flick latency ≤2-3 sec. Under these experimental conditions, and with reference to morphine, a 5 sec cut-off time was established to prevent tissue damage. For each behavioral test the rat was used as its own control and two consecutive measurements (30 sec interval) were carried out to assess the baseline tail-flick latency. Then rats received an i.v. administration of freshly prepared solutions: – vehicle; – 1 mg/kg morphine or – 2 mg/kg opiorphin. For tolerance induction, rats received daily i.v. administration of freshly prepared solutions: – vehicle; – 1 mg/kg morphine or – 2 mg/kg opiorphin, for seven consecutive days. Results were expressed as means±S.E.M. of tail-flick latency (s) for n=6 rats.

Conditioned place preference paradigm

The conditioned placed preference test provides a robust measure of drug addiction in rats. In this model, the experimental apparatus consisted of a two-compartment plexiglas open field (50 cm x 25 cm x 40 cm) separated by a mobile door. Two distinctive sensory cues differentiated the compartments: the wall coloring and the floor texture. The combination was as follows: white wall-metal grid floor and black wall-smooth floor. In the dimly lit test room, a CCD-TV

camera allows the movement and location of rat to be observed and recorded from a neighboring room. The protocol consisted of three different phases:

1- Preconditioning phase: drug naive rats had free access to both compartments for 30 min over a 3-day period and the time spent in each compartment was recorded for 15 min on day 3 to assess the preferred compartment.

2- Conditioning phase: during this phase each chamber was closed. Rats were treated during a 10-day period alternatively – on odd-numbered days (days 1, 3, 5, 7 and 9) with either morphine (2 mg/kg, i.v.) or opiorphin (1 mg/kg, i.v.) or vehicle, prior to individual placement into the non-preferred conditioning compartment for 45 min (compartment paired to drug injection). On the even-numbered days (days 2, 4, 6, 8 and 10), rats were given saline prior to individual placement into the alternate preferred compartment for 45 min.

3-Testing or postconditioning phase. In this phase, conducted 24 hours after the last conditioning session (day 11), the rats were placed between the two compartments, with free access to each. The time spent in each compartment was recorded during a 15-min session and compared with that obtained in preconditioning session. Results were expressed as means±S.E.M. for n=8 rats of time spent in the non-preferential drug-paired compartment.

Abbreviations

hNEP, human neutral endopeptidase; hAP-N, human aminopeptidase-N; Kruskal-Wallis Test, KWT; Mann-Whitney Test, MWT; Wilcoxon Test, WT;

Statistical evaluation

The significance of differences between groups was evaluated using the Kruskal-Wallis one-way analysis of variance (KWT, a non-parametric method) for comparison between several independent variables across the experimental conditions. When a significant difference among the treatments was obtained, the Mann-Whitney *post hoc* test (MWT) was applied to compare each treated group to the control one. The non-parametric Wilcoxon matched pairs test (WT) was used to compare repeated measures in each treatment group. For all statistical evaluations, the level of significance was set at $p<0.05$. All statistical analyses were carried out using the software StatView®5 statistical package (SAS, Institute Inc., USA).

RESULTS

Human opiorphin inhibits nociception in acute and tonic phases of the formalin test by primarily activating μ-opioid receptors

Using the behavioral formalin-induced pain rat model, we investigated the anti-nociceptive potency, the duration and the mechanism of action of the opiorphin native peptide. The formalin test measures the behavioral response to a chemical-induced inflammatory nociception, which induces two distinct nociceptive phases separated a stationary interphase: a early acute phase (first 20 min after formalin injection) followed by a late phase (30-60 min after formalin injection) in which a more

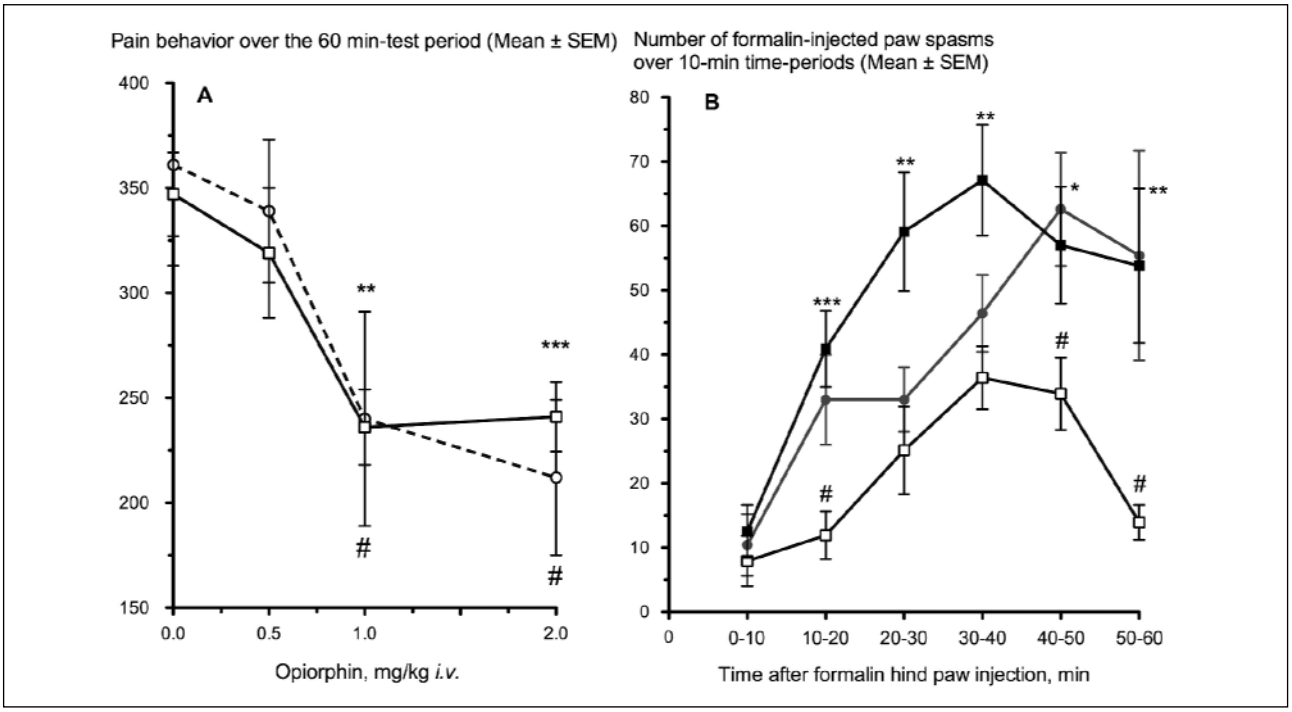

Fig. 1. Human opiorphin displays analgesic activity in the formalin pain model. A: Dose-dependent anti-nociceptive effects of opiorphin on the duration of formalin-injected paw licking (sec, open circle) and number of paw spasms (open square) during the 60 min period of the formalin test. Results are expressed as means±S.E.M. of 8 rats. Asterisk indicates ** $p<0.01$, *** $p<0.001$ and $^{\#}p<0.05$ *vs.* vehicle by MWT. B: Evaluation of the pain response as a function of time to noxious chemical stimuli following i.v. administration of opiorphin, in the absence or presence of the opioid antagonist, naloxone. Effects of opiorphin (open square; 1 mg/kg) compared to vehicle (grey circle) and opiorphin in the presence of naloxone (black square), on the number of formalin-injected paw flinches and body tremors over the six successive 10-min periods after formalin hind paw injection. Results are expressed as means±S.E.M. of 8 rats. Asterisk indicates ** $p<0.01$ and *** $p<0.001$ *vs.* opiorphin and $^{\#}p<0.05$ *vs.* vehicle by MWT.

tonic pain is elicited (*Fig. 1B*, referred under vehicle-treatment conditions, grey circle). The duration of formalin-injected paw licking (sec) and the total number of inflamed paw flinches and body tremors, which determine the behavioral score, were recorded over the 60 min-test period.

First, we determined that opiorphin inhibits, in a dose-dependent manner, the pain behavior induced by long-acting chemical stimuli with significant antinociceptive effect at 1-2 mg/kg i.v. doses (batch of synthesis=1, *n*=8 rats/group, *Fig. 1A*). Thus, compared to controls, the opiorphin-treated rats at 1 and 2 mg/kg doses exhibited a significant reduction in the total number of formalin-injected paw flinches and body tremors from 347±20 (vehicle) to 236±18 (1 mg/kg, p=0.006) and 241±16 (2 mg/kg, p=0.003), as morphine-treated rats at 2 mg/kg i.v. dose (147±27, p=0.001 by Mann-Whitney *U*-test, MWT). Whereas systemic administration of 0.5 mg/kg opiorphin did not significantly decrease the pain behavior exhibited by rats during the 60 min-test period (p=0.3 *vs.* vehicle; $p \leq 0.05$ *vs.* opiorphin 1 and 2 mg/kg doses, by MWT).

In a second experimental set, the time course of opiorphin antinociceptive response to chemical-induced pain was analyzed in the absence and presence of the broad-spectrum opioid receptor antagonist, naloxone (*n*=8 rats/group). The number of formalin-injected paw flinches and body tremors was recorded over six successive 10-min periods of the test (*Fig. 1B*). Systemic administration of opiorphin (1 mg/kg, batch of synthesis =1) significantly reduced the pain behavior during the early phase (time-period from 0 to 20 min) of the test from 43±7 (vehicle) to 20±4 ($p \leq 0.05$ by MWT). It also significantly decreased the pain behavior exhibited by rats throughout the second phase (time-period from 40 to 60 min) of the test from 118±16 (vehicle) to 48±6 ($p \leq 0.05$). The opiorphin-treated rats also spent more than 60% less time in inflamed paw licking over this second pain period: 21±9 sec compared to vehicle-treated rats 55±16 sec ($p \leq 0.05$ by MWT). As shown on *Figure 1B*, the reduction in the chemical-induced pain response by opiorphin during both acute and tonic pain was abolished by pre-treatment with naloxone. The total number of formalin-injected paw flinches and body spasms over the 60 min time-period was also determined (*Fig. 2A*). The following results were obtained: opiorphin plus naloxone 290±32 *vs.* vehicle 242±35 (p=0.17) and *vs.* opiorphin alone 129±15 (p=0.005 by MWT), demonstrating that the endogenous opioid receptors are required for full hypoalgesia induced by opiorphin at 1 mg/kg i.v. dose.

A third series of experiments was undertaken in order to define which specific endogenous opioidergic pathway contributes to the antinociceptive effects induced by opiorphin in the formalin test, either μ-, δ- or κ- opioid receptor-dependent pathway (*Fig. 2B*, *n*=8 rats/group). Pretreatment with selective μ-opioid receptor antagonist (CTAP) blocked the antinociceptive effect exerted by opiorphin over the 60-min time-period of the test. The total number of inflamed paw flinches and body tremors were 285±36 *vs.* vehicle 287±31 (p=1) and *vs.* opiorphin alone 149±28 (p=0.02 by MWT). In contrast, administration of either selective κ-receptor antagonist, nor-binaltorphimine (169±26, P=0.75 *vs.* opiorphin alone) or δ-opioid receptor antagonist, naltrindole (176±29, p=0.56 *vs.* opiorphin alone by MWT) did not significantly affect the antinociceptive response exhibited by opiorphin. Hence, the antinociceptive effect induced by opiorphin in the formalin test was primarily dependent on endogenous μ-opioid receptors.

Together these 3 series of data clearly indicate that opiorphin at 1 mg/kg i.v. (batch of synthesis=1) reliably inhibits nociception induced by acute and long-acting chemical stimuli in the rat model. Furthermore, our data are consistent with the involvement of the endogenous opioidergic pathway,

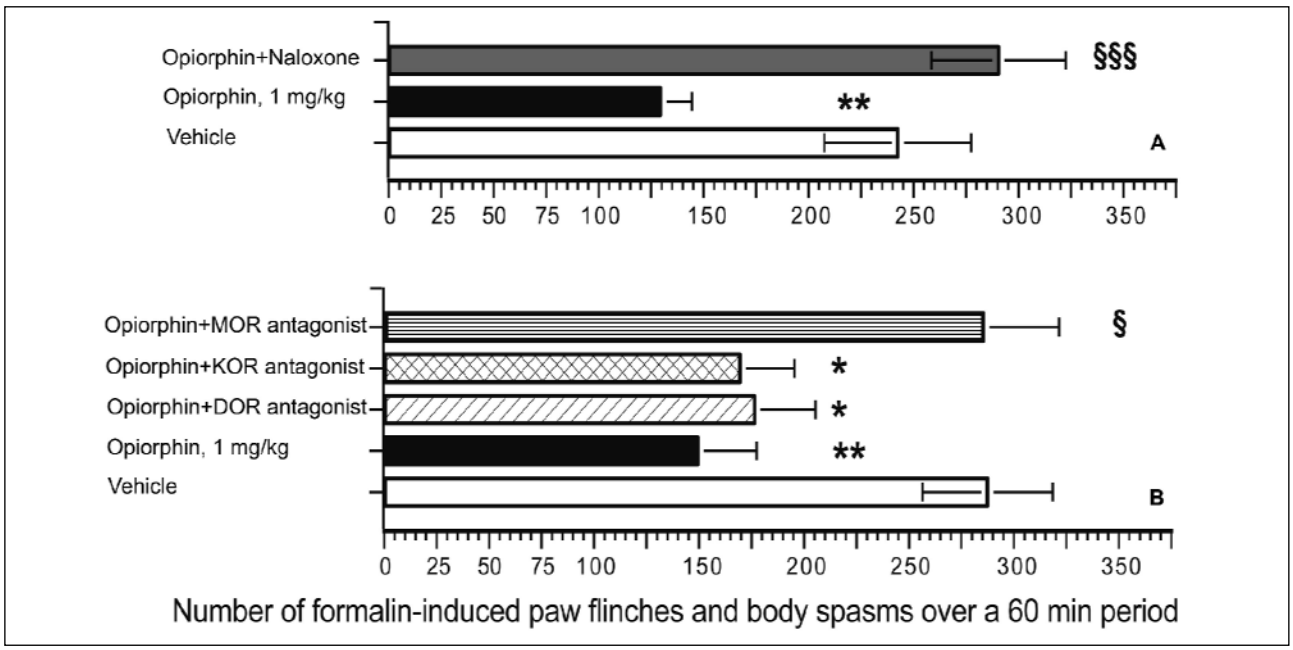

Fig. 2. Human opiorphin displays analgesic activity in the formalin pain model by activating the endogenous μ-opioid pathway. The total number of formalin-induced paw flinches and body spasms over a 60 min time-period following i.v. administration of opiorphin in the absence or presence of the opioid antagonist naloxone and specific μ, δ or κ opioid antagonists are shown. A: open bar=vehicle; black bar=opiorphin 1 mg/kg; gray bar=opiorphin plus naloxone. B: open bar=vehicle; black bar=opiorphin 1 mg/kg; horizontal-striped bar=opiorphin plus CTPA (μ-antagonist); crossed bar=opiorphin plus nor-binaltorphimine (κ-antagonist); diagonal-striped bar=opiorphin plus naltrindole (δ-antagonist). The values represent the mean±S.E.M. of 8 rats for each experimental condition. Asterisk indicates *$p<0.05$ and ** $p<0.01$ *vs.* vehicle and $^{\S}p<0.05$, and $^{\S\S\S}p<0.001$ *vs.* opiorphin by MWT.

preferentially μ-opioid receptor-dependent pathways, in opiorphin-induced pain-reduction in the formalin test.

Human opiorphin displays antinociceptive effect in acute thermal pain, the tail-flick test

The tail-flick test, evaluating the time required to respond to a radiating acute thermal stimulus, preferentially reflects nociceptive spinal reflex. Tail withdrawal latency determines the thermal nociceptive threshold, which is functionally related to pain responsiveness to the noxious stimulus. Using the rat tail-flick test, the aim of the study was to assess the potency and the duration of opiorphin analgesic effect with reference to morphine. A systemic dose of 2 mg/kg opiorphin (batch of synthesis=2) was chosen according to preliminary data showing that opiorphin induced significant antinociceptive responses at 2 mg/kg (p=0.05) while only tended to induce effect at 1 mg/kg as compared to controls (p=0.08 *vs.* vehicle by MWT, n=3 rats/group).

The pain threshold of each animal before and after opiorphin (2 mg/kg, i.v), morphine (1 mg/kg, i.v.) or vehicle administration was evaluated for four different time points: 5, 15, 25 and 60 min post-treatment. *Figure 3A* shows the mean tail flick latency as a function of time under either opiorphin, morphine or vehicle-treatment conditions (n=6 rats/group). The pre-injection baseline and 5 min post-injection values did not significantly differ between the 3 groups (p=0.54 and p=0.34 by Kruskal-Wallis analysis, KWT). However, the statistical analysis revealed a significant effect of treatments 15, 25 and 60 minutes post-injection (p=0.005, p=0.005 and p=0.02, respectively by KWT). Subsequent individual mean comparisons of tail flick latencies by the MWT analysis indicated that the time latency significantly increased 15 and 25 minutes after administration of both opiorphin and morphine: from 2.57±0.10 sec and 2.51±0.06 sec for vehicle to 3.12±0.07 sec and 3.19±0.07 sec (p=0.005 and p=0.004), respectively for opiorphin, and to 3.56±0.32 sec and 3.29±0.15 sec (p=0.01 and p=0.008), respectively for morphine. opiorphin-treated rats did not significantly differ in response latencies to morphine-treated rats at 15, 25 and 60 minutes after treatment (p=0.20, p=0.34 and p=0.26 *vs.* morphine by MWT, respectively). In addition, comparison with respective pre-injection baseline values showed that the tail flick latency significantly increased at 15 and 25 minutes after treatment with opiorphin (p=0.03 and p=0.03 by Wilcoxon test, WT, respectively) similarly to morphine (p=0.03 and p=0.05 by WT respectively). Interestingly, comparison with the corresponding baseline response for the control group revealed a significant progressive decrease in the time latency of tail withdrawal following repeated exposure to the test. The diminution of pain threshold to a repetitive stimulus may reflect painful sensitization or learning to the stimulus leading to a facilitation of the nociceptive response in these animals. Importantly, the enhanced pain responsiveness to repetitive stimulus observed in controls, is totally reversed in rats treated with opiorphin similarly to morphine, still confirming the potent analgesic potency of opiorphin on thermal acute pain.

Thus, in the rat tail-flick paradigm, the analgesic effect of opiorphin at 2 mg/kg, i.v. (batch of synthesis=2) occurs 15 to 25

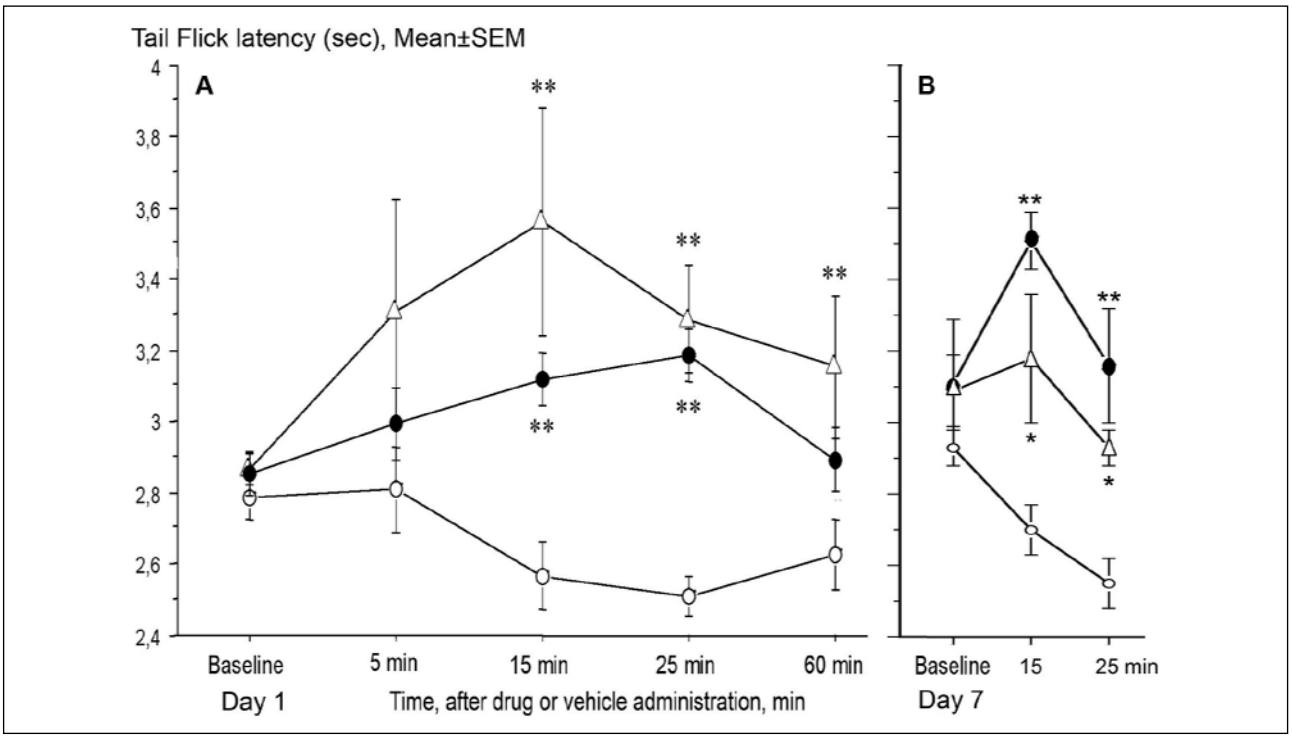

Fig. 3. Human opiorphin displays analgesic activity without drug tolerance in the tail-flick pain model. The tail-flick latencies measured before (baseline values) and after opiorphin, morphine or vehicle bolus i.v. administration are expressed in sec as means±S.E.M. of 6 rats for each experimental condition. A: Evaluation of the pain response, in function of time, to noxious thermal stimuli following single administration (day 1) of vehicle, opiorphin or morphine. Tail-flick latencies were evaluated for four different time points: 5, 15, 25 and 60 min after opiorphin (black circle; 2 mg/kg) or vehicle administration (open circle) with reference to morphine (open triangle; 1 mg/kg). B: Evaluation of the pain response to noxious thermal stimuli following daily i.v. administration for 7 days of vehicle, opiorphin or morphine. On day 7, tail-flick latencies were evaluated for 2 different time points: 15 and 25 min after opiorphin (black circle; 2 mg/kg), vehicle (open circle) or morphine (open triangle; 1 mg/kg) bolus dose. Asterisk indicates *p<0.05 and ** p<0.01 *vs.* vehicle by MWT.

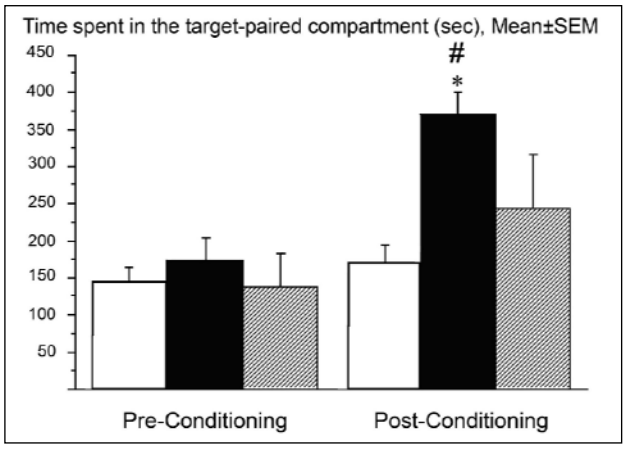

Fig. 4. Human opiorphin behaves as a non-addictive analgesic in the conditioning place preference test. Place preference paradigm is described in *Materials and Methods*: briefly, during a 10-day period rats alternatively received - on odd-days, either morphine or opiorphin or vehicle (i.v.) prior to placement in the aversive non-preferred compartment and - on even-days, saline prior to placement in the preferred one. On day 11, the time spent in the target-paired compartment was measured and compared with that obtained in the preconditioning session on day 0. The Y axis values represent the mean±S.E.M. of time spent (sec) in the non-preferred compartment for 8 rats during the pre-conditioning and post-conditioning sessions: open bar=vehicle; black bar=morphine, 2 mg/kg; hatched-bar=opiorphin 1 mg/kg. Asterisk indicates $*p<0.05$ *vs.* vehicle, $^{\#}p<0.05$ *vs.* pre-conditioning phase by MWT.

min after i.v. administration and is of the similar range of magnitude as morphine (1 mg/kg, i.v.). We conclude that opiorphin inhibits thermal injury-evoked acute pain behavior which is preferentially controlled by spinal opioidergic pathways.

Human opiorphin administration does not result in drug tolerance in the tail-flick test following chronic treatment in rats

We wished to investigate, using the tail-flick test, the potential emergence of opiorphin-induced antinociceptive tolerance with reference to morphine. Rats received daily intravenous administration of opiorphin (2 mg/kg, batch of synthesis=2), morphine (1 mg/kg) or vehicle for seven consecutive days. On day 7, the tail flick latency was measured at peak time of opiorphin analgesic effect to thermal stimulus, *i.e*., 15 min and 25 min after the challenge dose (*Fig. 3*).

Comparing pre-injection baseline values, no significant difference between the mean tail flick latency of the 3 groups was observed (p=0.14 on day 7 by KWT, n=6 rats/group). However, a significant treatment effect in tail flick latency among the 3 groups was revealed at 15 and 25-min post-injection time-points (p=0.003 by KWT).

In comparison to chronically treated vehicle rats, morphine and opiorphin appeared to induce a significant increase in response latencies after 7 consecutive daily treatments: from 2.70±0.07 sec and 2.55±0.07 sec at 15 min and 25 min post-injection, respectively for vehicle to 3.18±0.18 sec and 2.93±0.05 sec, respectively for morphine (p<0.05 by MWT) and to 3.51±0.08 sec and 3.16±0.16 sec, respectively for opiorphin (p<0.01 *vs.* vehicle by MWT) (*Fig. 3B*). However, comparison with respective pre-injection baseline values (3.09±0.10 sec for morphine-group and 3.11±0.18 sec for opiorphin-group) showed that the tail flick latencies remain stable or even significantly decrease, after the challenge dose of morphine (p=0.69 at 15 min and p=0.03 at 25 min post-dose by WT), reflecting an absence of morphine antinociceptive potency following chronic treatment. Under the same experimental conditions, the challenge dose of opiorphin significantly increased the tail-flick response at 15-min post-injection (p=0.03 by WT) whereas it seems not to have a significant difference at 25-min post-injection (*Fig. 3B*). Thus, the analgesic intensity of opiorphin was unaltered after subchronic treatment although there seems to be a decrease in its duration of action. Furthermore comparisons by WT analysis of the time-response profiles between day 1 and day 7 after daily repeated treatment revealed that the tail-flick latencies remain stable for the vehicle-treated group (p≥0.99), tended to decrease for the morphine-treated group (p=0.06) while tended to increase for the opiorphin-treated group (p=0.06). These data clearly demonstrate that opiorphin still induced a powerful activity following a 7-day chronic treatment contrary to the loss of morphine-induced analgesia.

In conclusion, unlike morphine (1 mg/kg, i.v.), subchronic systemic administration of opiorphin (2 mg/kg, i.v.) administered once daily, for 7 days, does not induce the development of tolerance to the antinociceptive effect in the tail-flick test.

Human opiorphin has a low potential of abuse liability compared to morphine in the conditioned place preference test

In the conditioned place preference test, after repeated daily exposure to a potential addicting compound paired with placement in distinctive environmental cues, the rat increases the amount of time spent previously associated with the drug-paired environment, as an indicator of preference. Using this model, we studied the eventual opiorphin-paired place preference with reference to morphine. During a paired trial, both compounds were given systemically at doses that produce maximal analgesic effect in formalin-induced pain, 1 mg/kg (opiorphin, batch of synthesis =1) and 2 mg/kg (morphine) and then there followed a confinement in a distinctive aversive environment (*Fig. 4*). Comparison of preconditioning time spent in each compartment confirmed the existence of a pre-existing bias between the two compartments. Indeed, all naive rats exhibited the same preference for the non-aversive (black wall/smooth floor) compartment as they spent about 5-fold more time in the compartment initially defined as preferential than in the adjoining distinct one (white wall/metal grid floor), defined as aversive (p=0.01 by WT, n=24 rats).

Fig. 4 illustrates the pre- and post-conditioning time spent by vehicle-, morphine- and opiorphin-treated groups in this distinctive un-preferential compartment during the 15-min test sessions (n=8 rats/group). Control rats, which received vehicle treatment during conditioning trials, spent an equivalent time in the non-preferential compartment in preconditioning and post-conditioning tests (145±20 sec *vs.* 172±23 sec, respectively p=0.12 by WT). Similarly in opiorphin-treated groups, the time spent in the non-preferential compartment was also not significantly different between the first preconditioning and the final post-conditioning phase (137±45 sec *vs.* 241±73 sec, respectively p=0.12) indicating that these animals did not exhibit significant preference for the opiorphin-paired compartment. In contrast, pairing morphine with the non-preferred compartment significantly increased the time spent in this drug-paired environment: 370±32 sec for the final post-conditioning phase compared to 173±31 sec for the first preconditioning (p=0.01). Furthermore, the KWT analysis, applied to the time spent in the non-preferred compartment during the first phase and the final post-conditioning phase of the test, indicates that the behavior of the three groups did not differ on the first preconditioning phase (p=0.55), while there is a significant difference in preference score

among groups in the final post-conditioning phase (p=0.02). In the latter case, the subsequent *post hoc* MWT analysis shows a significant morphine treatment effect (p=0.001 *vs.* vehicle) whereas, there is no significant difference between opiorphin treated-rats and vehicle ones (p=0.92 *vs.* vehicle).

Morphine-conditioned rats expressed a clear, significant, behavioral preference for their drug-paired environment. Unlike morphine, it seems that opiorphin did not induce a significant conditioned place preference at the dose that produces maximal analgesic effects in formalin pain model. Although, there is a tendency in particular for some rats (3/8) to spend more time in the opiorphin-paired compartment, their behavioral preference was clearly lower than that induced by morphine. Thus we conclude that opiorphin has a low potential of abuse liability, compared to morphine, when they are subchronically administered at equi-effective analgesic doses.

Systemically active human opiorphin is a non-constipating analgesic

Another side-effect associated with opioid therapy, in particular morphine therapy, is constipation which is related to the direct stimulation of μ-opioid receptors negatively regulating intestinal peristaltism. In normally fed rats, a significant decrease in gastrointestinal propulsion was observed with morphine at 6 mg/kg i.p. single dose from 100±19% (vehicle) to 24±8% of feces, p=0.005 by MWT, n=8 rats/group. The coincidence of simultaneous analgesic effects on acute pain and anti-propulsive effects is high for morphine, as previously reported (4). On the contrary, under the same experimental conditions, animals pre-treated with opiorphin did not exhibit any inhibition of bowel functioning, even at the highest analgesic i.v. bolus dose tested: 86±17% (1 mg/kg) and 93±12% (2 mg/kg) percent of feces, p=0.59 and p=0.79 by MWT compared to vehicle-treated ones, respectively (n=8 rats/group). Thus, the coincidence of simultaneous analgesic effects on acute pain and anti-propulsive effects is very low for opiorphin.

DISCUSSION

The aim of the present study was to compare opiorphin with morphine for analgesic efficacy, in terms of the time course and intensity of the responses, and for the occurrence of major side-effects, with emphasis on the side-effect profile at equi-analgesic doses. A consistent finding was that opiorphin elicited minimal adverse morphine-associated effects at systemically active doses (1-2 mg/kg, i.v.) that produced a comparable analgesic potency in well-established and validated experimental models of morphine-sensitive pain in rats. These models included a supraspinally controlled mechanical-induced acute nociception (1), a spinally controlled thermal-induced acute nociception and a peripheral chemical-induced acute and inflammatory tonic nociception. These data are also consistent with other recent study demonstrating that opiorphin pain-suppressive effect at centrally active dose (5 μg/kg, i.c.v.) is as potent as morphine (10 μg/kg, i.c.v.) on thermal-induced acute pain, *i.e.*, the tail immersion mouse model (20).

Taking together all data, it is important to point out that across these four pain models induced by different stimulus modalities and intensities, opiorphin is as efficient as morphine in its maximal analgesic dose-effect and potency on acute pain (mechanical and thermal). While, it seems less efficient than morphine in its maximal inhibitory potency and duration on chemical induced long-acting inflammatory pain. More generally, opiorphin analgesic potencies vary from 30 to 60% (at 1-2 mg/kg i.v. or 5 μg/kg i.c.v. dose) while varying from 40 to 80% for morphine (at 1-2 mg/kg i.v., 6 mg/kg i.p. or 10 μg/kg i.c.v.). Nevertheless, as previously reported for synthetic dual NEP and APN inhibitors (10, 21), the natural enkephalin-degrading peptidase inhibitor, opiorphin, does not produce the maximum analgesic effects induced by the opioid agonist morphine. This provides evidence that the local levels of endogenous enkephalins, released depending on the noxious stimulus applied and then protected from degradation by opiorphin, is still too low to saturate opioid binding receptors. Such an integrated mechanism would prevent receptor over-stimulation as is the case in the induction caused by exogenous μ-opioid agonists, such as morphine.

We also demonstrate here that the analgesic response induced by systemic administration of opiorphin in peripheral formalin-induced pain in rat preferentially requires activation of endogenous μ-opioid pathways. Mu-receptors are critical components of the opioid system required for opioid-ligand antinociceptive action on spinal and peripheral target neurons (22). Other study showed that the analgesic effect induced by central administration of opiorphin on thermal pain model in mice is exerted *via* activation of endogenous μ- and δ-opioid receptors (20). And, we previously demonstrated that opiorphin exhibits *in vitro* inhibitory potency on enkephalin-inactivating NEP- and AP-N-ectopeptidases (1). Furthermore, using *in vitro* cell binding assays we assessed that in contrast to specific opioid agonists, opiorphin did not directly bind to μ- and δ-opioid receptors even at concentrations inhibiting enkephalin-inactivating ectopeptidases. Thus, all these findings lead us to conclude that opiorphin, is an inhibitor of pain perception by potentiating the endogenous enkephalin-related activation of μ- and δ-opioid pathways.

Interestingly, opiorphin is about 10 fold more potent, in terms of dose-effect, in pain-suppressive efficacy than the synthetic potent dual NEP and AP-N inhibitor RB101 (10-20 mg/kg i.v.) (10, 21). This suggests that the natural dual inhibitor opiorphin may carry a structural signature, adapted *in vivo* in terms of affinity, selectivity and bioavailibility, to the topological and functional characteristics of its targets.

Besides, the low degree of abuse liability and analgesic tolerance, observed after chronic treatment with opiorphin, compared to morphine, might result from limited occupation and specific stimulation of opioid receptor-dependent pathways and from an unaltered mechanism of opioid receptor recycling in a fully active state to the cell surface (23). Consistent with these results opiorphin, by increasing the lifetime of endogenous enkephalins, which are tonically released according to the painful stimulus in pathways specifically involved in control of nociception, might trigger specific and limited opioid receptor-dependent pathways, thus minimizing excessive stimulation of opioid receptors and appearance of corresponding side-effects that are associated with exogenous opiate agonists such as morphine. Many studies have pointed to the mesocorticolimbic dopaminergic systems, in particular neurons that project from the ventral tegmental area to the nucleus accumbens, as a critical site for the initiation of psychological dependence on drugs such as opiates, cocaine and cannabinoid. A large body of data also provides evidence for the existence of bidirectional functional interactions between both endogenous opioid and dopamine transmission to modulate emotionally motivated behaviors (21, 24-26, and reviewed in 27). This is probably one of the major reasons why repeated systemic administration of opiorphin, at a dose that produces analgesic effects by activating endogenous opioid pathways, led to a moderate dependence liability (in 40% of treated rats) but not a lack of opioid-associated rewarding effects. The tendency of rats to exhibit place preference after chronic drug-treatment was also found for the synthetic dual NEP and AP-N inhibitor RB-101 with a response clearly lower than that induced by morphine (10, 21), as it is found for the natural enkephalin-degrading peptidase inhibitor opiorphin.

It has been stated that any painkiller that produces fewer adverse effects than morphine at equi-analgesic doses would be an improvement since it would enhance the quality of life of patients (3, 28). It is clear, from the data presented here, that opiorphin could lead to new analgesics endowed with antinociceptive potency similar to that of morphine but with limited propensity to induce opioid agonist side effects. The chemical optimization of opiorphin to generate functional derivatives endowed with better bioavailability properties (lipophilicity and metabolism resistance) than the native peptide, could lead to a potent class of analgesic compounds.

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