ARTICLE

Midkine is a novel regulator of morphine-induced spinal analgesic effects: Evidence for a transcriptional regulation of kappa opioid receptor in midkine knockout mice

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ABSTRACT

Midkine (MK) is a growth factor that exhibits neurotrophic actions and is upregulated at sites of nerve injury. It has been shown that morphine administration significantly regulates MK levels within the brain, suggesting MK could play a role in morphine-induced pharmacological effects. To test this hypothesis, we have now studied morphine-induced antinociceptive effects in MK genetically deficient (MK-/-) and wild type (WT+/+) mice. We found that basal pain responses do not differ between MK-/- and WT+/+ mice in the hot-plate and tail immersion tests, suggesting MK is not involved in the regulation of nociceptive transmission at the supraspinal and spinal levels. We did not find differences among genotypes using different doses of morphine (2.5, 5 and 10 mg/kg) in the hot-plate test. In contrast, we found that morphine (5 mg/kg) significantly delayed pain responses in MK-/- mice compared to WT+/+ mice in the tail-immersion test, an effect that greatly correlates with a significant increase in the levels of expression of the κ -opioid receptor in the dorsal root ganglia (DRG) of MK-/- mice compared to WT+/+ mice in normal condition. The data strongly suggest that MK is an endogenous modulator of morphine antinociceptive effects at the spinal, but not at the supraspinal, level. The data support the hypothesis that concomitant administration of known midkine inhibitors and morphine could result in potentiation of the opioid spinal antinoceptive effects, which may be of critical importance in patients that are unresponsive to opioid analgesia in palliative care.

Keywords: Pleiotrophin; Midkine; Opioids; Hot-plate; Tail flick; Nerve injury.

RESUMEN

La Midkina es un nuevo regulador de los efectos analgésicos de la morfina a nivel espinal: Evidencias sobre la regulación transcripcional del receptor opioide kappa en ratones knockout de midkina

La Midkina (MK) es un factor de crecimiento conocido por sus acciones neurotróficas y que se encuentra sobre-expresado en los lugares donde acontece daño neuronal. La administración de morfina regula los niveles de expresión cerebrales de MK lo que sugiere que la MK podría modular los efectos farmacológicos de la morfina. Para probar esta hipótesis, hemos estudiado los efectos antinociceptivos de la morfina en ratones knockout de MK (MK-/-) y en ratones salvajes (WT+/+). Hemos comprobado que las respuestas basales al dolor en ambos genotipos son semejantes tanto en el test de la placa caliente como en el test de la retirada de la cola, lo que sugiere que la MK no es un factor fundamental para la regulación de la transmisión dolorosa a nivel supraespinal, ni a nivel espinal. Tampoco encontramos diferencias en los efectos analgésicos de la morfina en el test de la placa caliente con ninguna de las dosis que utilizamos (2.5, 5 and 10 mg/kg). Sin embargo, la morfina a la dosis de 5 mg/Kg provocó un efecto analgésico significativamente mayor en ratones MK-/- en el test de la retirada de la cola. Este mayor efecto de la morfina podría estar relacionado con el aumento significativo de los niveles de expresión del receptor opioide κ en los ganglios dorsales de ratones MK-/- comparado con los ratones WT+/+ en condiciones normales. Los datos sugieren que la MK es un nuevo regulador endógeno de los efectos antinociceptivos de la morfina a nivel espinal, pero no a nivel supraespinal. Además, los resultados apoyan la hipótesis de que la administración conjunta de inhibidores conocidos de la MK y morfina podría resultar en una potenciación de los efectos analgésicos del opiáceo lo cual podría resultar importante para el tratamiento de pacientes que no responden a los opiáceos.

Palabras clave: Pleiotrofina; Opioides; Placa caliente; Latigazo de la cola; Daño neural.

1. INTRODUCTION

Midkine (MK) is a heparin binding growth factor (14) that shares over 50% identity in amino acid sequence with pleiotrophin (PTN), the only other member of the PTN/MK developmentally regulated gene family (2, 18, 23). Both PTN and MK have been found to play important roles in the development of the nervous system through their actions on neuronal differentiation (see for example review 7). In addition, it has been hypothesized that both PTN and MK may play important roles on survival of different cell types and wound repair since both cytokines are

upregulated at sites of injury and repair in inflammatory macrophages, microglia, dermal fibroblasts, endothelial cells and other cells (1, 8, 16, 22, 30, 33).

In the central nervous system, PTN and MK have been found to be a key factor for survival of the injured dopaminergic neurons in vitro and in vivo (5, 10-12, 19), results that became promising when this type of heparin binding growth factors were found to play reparatory roles in the substantia nigra of patients with Parkinson´s disease (19). Interestingly, the role of MK in wound repair in the periphery has been recently linked to its potential role in the guidance of neural axon regeneration in peripheral nervous system (31). This is supported by the delay in axonal regeneration uncovered in MK genetically deficient (MK-/-) mice with peripheral nerve injury (31). That report significantly increased the relevance of previous findings demonstrating the consistent upregulation of MK expression levels after traumatic injury of the spinal cord and following sciatic nerve injury (29, 30). Thus, the data strongly suggest MK is synthesized, released, and taken up in neurons in order to play a role in degeneration and regeneration after peripheral nerve injury and, importantly, in the subsequent chronic neuropathic pain state. However, despite the existing evidences, the role of MK in nociception has not been assessed in acute pain models directed to assess pain processing at either supraspinal or spinal level. To fill this gap in knowledge, we have now studied the behaviour of MK-/- and wild type (WT+/+) mice in the hot-plate and tail-immersion tests.

In addition, it is interesting to note that MK expression levels have been found to be significantly upregulated in the rat hippocampus following morphine administration (3). Whether or not MK is upregulated after morphine administration in other areas of the central nervous system such as the spinal cord remains to be elucidated. However, the existing data (3) strongly suggest the possibility that MK could be mediating the pharmacological effects of morphine within the central nervous system. To test this hypothesis, we also aimed to evaluate morphine-induced analgesia in MK-/- and WT+/+ mice in both the hot-plate and tail-immersion tests.

2. MATERIALS AND METHODS

Midkine knockout (MK-/-) mice were generated on a 129/Ola x C57BL/6J background by methods essentially identical to those previously described (24). The animals used in this study were female MK-/- and WT+/+ mice at 8 weeks of age. All the animals used in this study were maintained according to European Union Laboratory Animal Care Rules (86/609/ECC directive).

The genotypes of the MK-/- mice were confirmed prior to sacrifice with the polymerase chain reaction using as primers 5'-ATC GGT TCC AAG TCC TCC CTC

CGT C-3' forward and 5'-CAC CTT CCT CAG TTG ACA AAG ACA AGC-3' reverse to generate from genomic DNA extracted from tails of MK-/- and WT+/+ mice a cDNA of ~ 0.7 kb.

2.1. Hot-plate test

To asses supraspinal nociceptive responses in both MK-/- and WT+/+ mice, we used the hot-plate test. A metal hot-plate was maintained at either 53 ± 0.5 °C or 55 ± 0.5 °C. The time to when the mouse first exhibited nocifensive behaviour (licked its hind paw or jumping) was determined. The cutoff time for the first sign of nocifensive behaviour were 75 s in the case of the assays carried out at 53 ºC and 30 s in the case of the assays carried out at 55 ºC.

To study the effect of morphine on hot-plate response, saline (10 ml/kg) or morphine sulphate (2.5, 5 and 10 mg/kg; Alcaliber, Madrid, Spain) were administered intraperitoneally after testing the baseline response for the hot-plate maintained at 53 ± 0.5 °C. In additional studies, we also tested the baseline responses for the hot-plate maintained at 55 ± 0.5 °C (n = 11/genotype) in order to determine possible differences between genotypes depending on heat stimulus. To study the time course of the effect of morphine, the hot-plate latency was recorded 25, 75 and 125 min after morphine (or saline, as a control) injection. Total number of animals used in every experimental group was as follows:

Saline (10 ml/kg): WT+/+, n = 26; MK-/-, n = 16.

Morphine (2.5 mg/kg): WT+/+, n = 13; MK-/-, n = 8.

Morphine (5 mg/kg): WT+/+, n = 16; MK-/-, n = 12.

Morphine (10 mg/kg): WT+/+, n = 12; MK-/-, n = 10.

2.2. Tail-immersion test

To asses spinal nociceptive responses in both MK-/- and WT+/+ mice, we used the tail-immersion test. In preliminary studies, the latency to a rapid tail-flick in a bath maintained at 50 ± 0.5 ºC was registered in MK-/- and WT +/+ mice (n = 5/genotype) with a cutoff latency of 20 s to prevent tissue damage. We observed that both MK-/- and WT+/+ mice showed normal baseline responses to the heat stimulus (~ 10 s).

To study the effect of morphine on tail-immersion responses we used a bath maintained at a temperature of 55 ± 0.5 ºC. We used the intermediate dose of morphine (5 mg/Kg) that is relevant to the clinically used in humans. Saline (10 ml/kg) or morphine sulphate (5 mg/kg) was administered i.p. After testing the baseline responses for the tail-immersion test using a cutoff latency of 15 s to prevent tissue damage. As shown in the results section, both MK-/- mice (n = 17) and WT+/+ mice (n = 18) used in this study reached normal latencies values (~ 4-

5 s) in basal conditions. To study the time course of the antinociceptive effect of morphine (5 mg/Kg), the tail flick latency was recorded 25, 50, 75 and 125 min after morphine (or saline, as a control) injection. Total number of animals used in every experimental group was as follows:

Saline (10 ml/Kg): WT+/+, n = 8; MK-/-, n = 8.

Morphine (5 mg/kg): WT+/+, n = 10; MK-/-, n = 9.

2.3. Tissue acquisition, RNA extraction and gene chip analyses

After being anaesthetized with halothane, immature MK-/- and WT+/+ mice were sacrificed; spinal cords and dorsal root ganglia (DRG) corresponding to L4 and L5 spinal nerves were rapidly dissected from animals (3/genotype), frozen in dry ice, and stored at -80 ºC pending RNA isolation. Frozen tissues were homogenized in 1ml TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) per 50-100 mg tissue and total RNA extracted following the manufacturer's protocol. The concentration of RNA in each sample was measured by A_{260} and RNA integrity confirmed in 1.25% agarose gels after electrophoresis. RNA samples were treated with a preparation of DNAse enzymes (Ambion, Austin, TX, USA) following manufacturer's protocol and a pool of the three RNA samples from each genotype was used for microarray analysis that were carried out in triplicates.

Affymetrix mouse 14,400 oligonucleotide probe Genome MOE 430A Gene Chips (Affymetrix, Santa Clara, CA, USA) were used according to standard protocols supplied by the manufacturer. Different "housekeeping" genes, including actin, GAPDH, and hexokinase, and other control sequences were included as reference and quality control indicators.

2.4. Statistical Analysis

The statistical significance of changes after every dose of morphine (or saline) injection was determined by 2-way ANOVA considering as factors the genotype (MK-/- and WT+/+) and time point after injection. Bonferroni's post hoc tests were used to detect the sources of group differences revealed by the ANOVAs. Area under the curve (AUC) values obtained from MK-/- and WT+/+ mice, and basal values of both genotypes were analyzed using student's t test. $P < 0.05$ was considered statistically significant.

3. RESULTS

3.1. Hot-plate test

In the studies directed to assess morphine-induced antinociceptive effects in the hot-plate maintained at 53 ºC, we first analyzed together basal values from mice from both genotypes used in all experiments performed (WT+/+, n = 61; MK-

/-, n = 45). The latency to the first sign of nocifensive behaviour in MK-/- mice was found to be essentially similar to that recorded in WT+/+ mice (Figure 1A). We also assessed baseline responses of MK-/- mice and WT+/+ mice (n = 11/genotype) to the hot-plate maintained at 55 ºC, determining again similar latencies between both genotypes (Figure 1B).

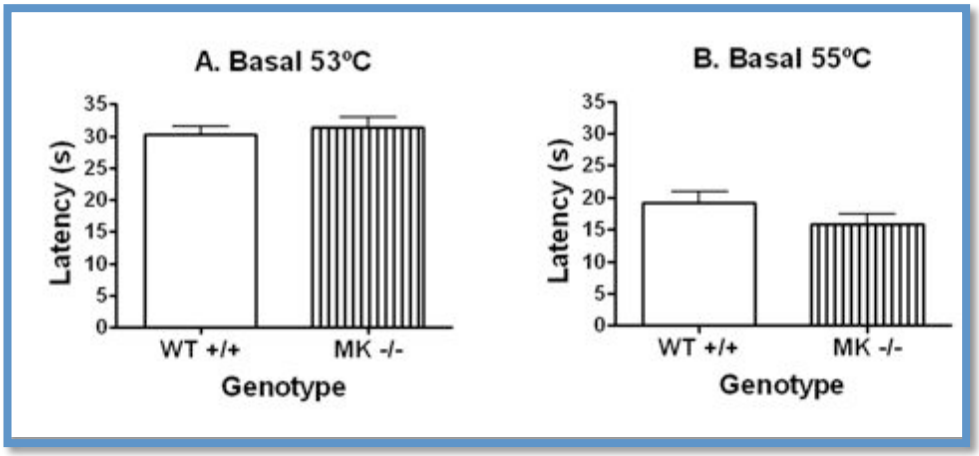

Figure 1.- Basal nociceptive behavioural responses of WT+/+ and MK-/- mice in the hot-plate test. Data show mean ± SEM.

Nociceptive responses after saline administrations were not altered compared to baseline values of either genotype (Figure 2A). Morphine-induced antinociceptive effects were found to be clearly dose dependent (Figure 2). The analgesic effect of morphine was maximal at 25 min after morphine injection at the highest dose used (10 mg/Kg) (Figure 2D). The data demonstrate that the analgesic effects of morphine are effectively similar in both genotypes at all doses of the opioid tested (Figure 2).

3.2. Tail-immersion test

First, we assessed baseline responses of MK-/- and WT+/+ mice (n = 5/genotype) to the tail-immersion test using a bath maintained at 50 ± 0.5 ºC. MK-/- mice showed a similar latency to a tail-flick compared to WT+/+ mice (Figure 3A). In the studies designed to test morphine antinociceptive effects, we aimed to use a higher bath temperature (55 ± 0.5 ºC). Taking together the basal values of all mice from both genotypes used in the studies assessing morphine effects, we found again that MK-/- mice exhibited a similar pain response to this heat stimulus of higher intensity compared to WT+/+ mice (Figure 3B).

To evaluate morphine (and saline, as control)-induced analgesia in this test in MK-/- mice, we only used the bath maintained at 55 ± 0.5 ºC. Nociceptive responses after saline administrations were not altered compared to baseline values of either genotype (Figure 4A). Differences between MK-/- and WT+/+ mice were clearly observed after morphine (5 mg/kg) administration (Figure 4B). In this case, two-way ANOVA showed highly significant effects of the genotype (F(1,

85) = 12.74; P = 0.0006) and significant effects of time (F(4, 85) = 17.36; P < 0.0001). In confirmation, we also found a significantly higher AUC value in MK-/- compared to WT+/+ mice treated with 5 mg/kg morphine (Figure 4C).

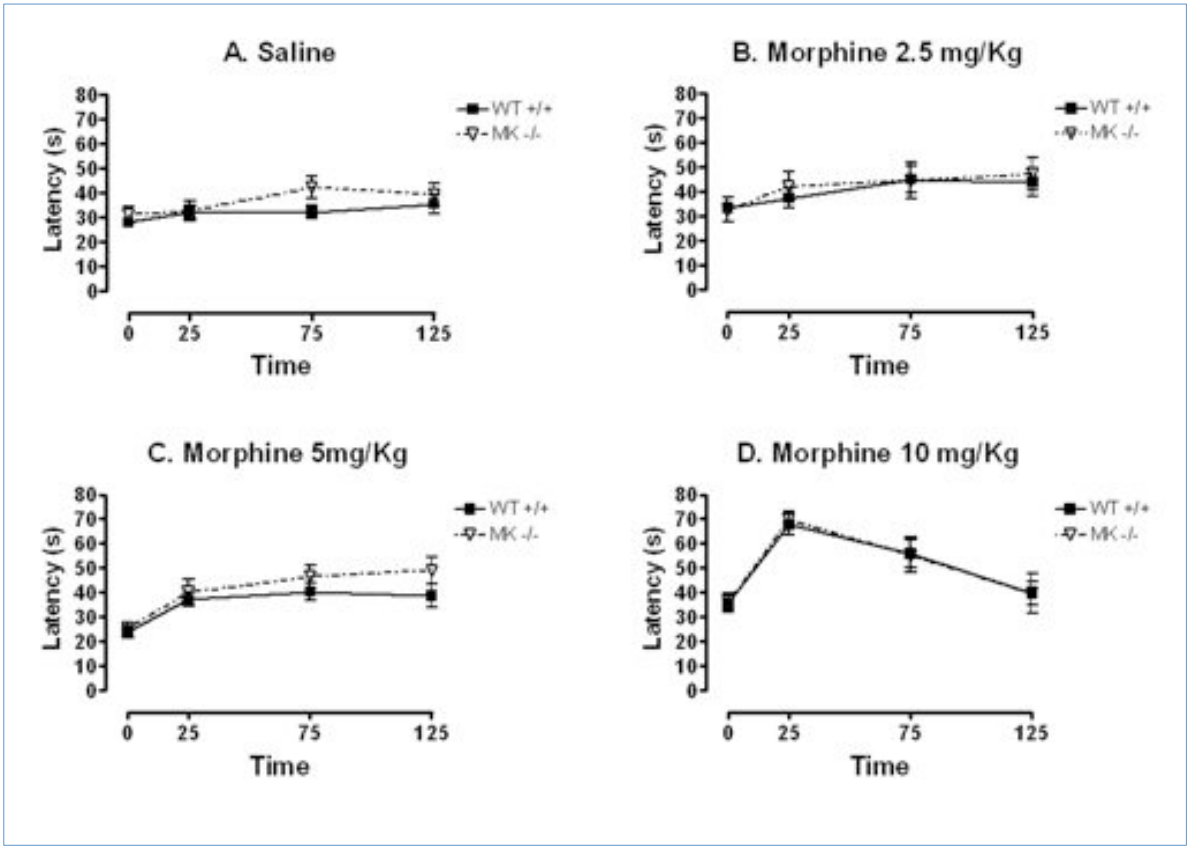

Figure 2.- Comparison of nociceptive behavioural responses and the antinociceptive effect of morphine in WT+/+ and MK-/- mice. Results from hot-plate maintained at 53 ºC are shown. The effects of saline administration used as a control (A) and the analgesic effects of morphine at a dose of 2.5 mg/kg (B), 5 mg/kg (C) and 10 mg/kg (D) on the hot-plate test are shown as a function of time. Data show mean ± SEM.

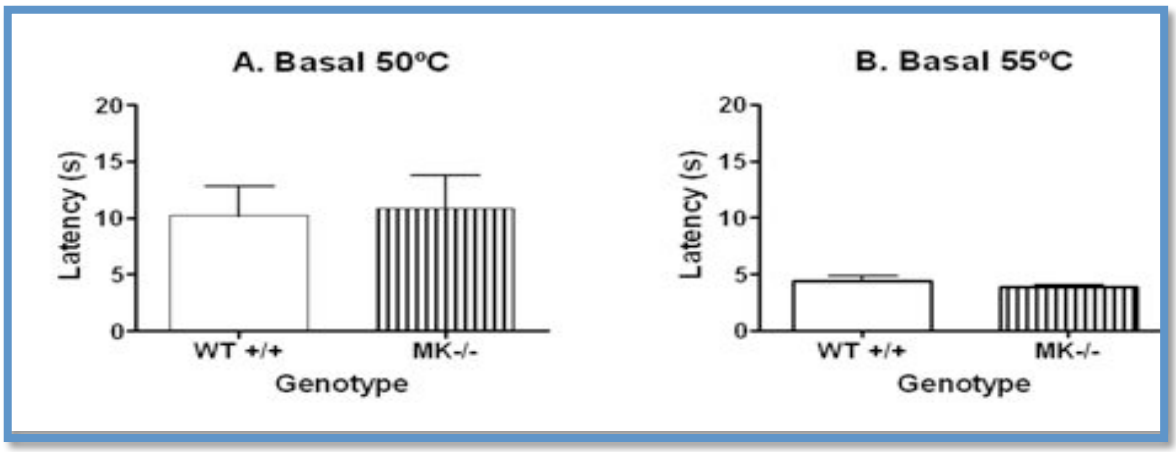

Figure 3.- Basal nociceptive behavioural responses of WT+/+ and MK-/- mice in the tail-immersion test. Data show mean ± SEM.

3.3. Gene chip analyses

In order to better understand how MK influences the spinal analgesic effects of morphine, we compared the levels of the transcripts of the different components of the endogenous opioid system, including opioid receptors and endogenous opioid peptide precursors (see Table 1), in spinal cords and DRGs of immature MK-/- mice in gene chip microarrays. Very interestingly, we only found one gene whose levels of expression were significantly altered in the DRG of MK-/- mice compared to WT+/+, the κ -opioid receptor. It was found a striking 10-fold increase of the levels of expression of κ-opioid receptor in DRG of MK-/- mice in normal condition whereas the rest of genes related to the endogenous opioid system remained unchanged within genotypes (Table 1). The data suggest that a basal greater population of κ-opioid receptors in a key tissue to process nociceptive transmission from the periphery, such as the DRG, could underlie the enhanced morphine antinociceptive effects in the tail immersion test in mice lacking endogenous MK.

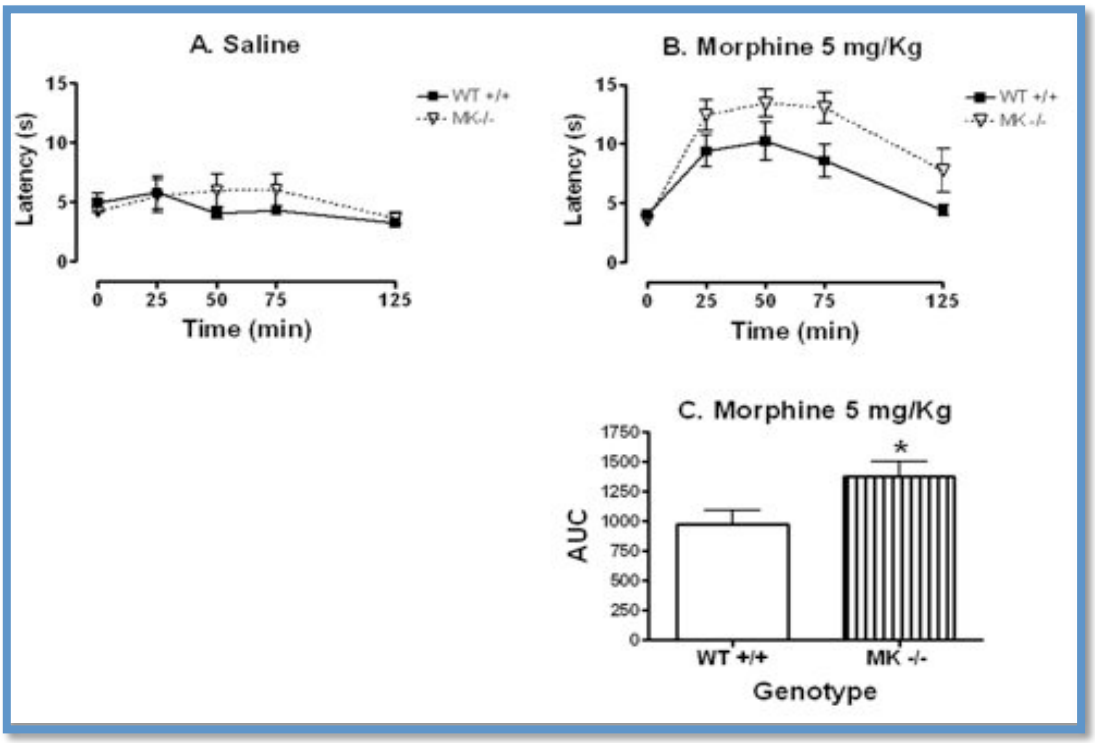

Figure 4.- Comparison of nociceptive behavioural responses and the antinociceptive effect of morphine in WT+/+ and MK-/-mice. Results from tail-immersion test using a bath maintained at 55 ºC are shown. The effects of saline administration used as a control (A) and the analgesic effects of morphine at a dose of 5 mg/kg (B) on the tail-immersion test are shown as a function of time. Area under the curve (AUC) values for WT+/+ and MK-/- mice treated with morphine (5 mg/kg) are also compared (C). Data show mean ± SEM. * $P < 0.05$ vs. WT+/+.

Table 1. Upregulation of expression levels of κ-opioid receptor in DRG of MK-/- mice compared to WT+/+ mice. The Affymetrix MOE 430A chip was used to measure simultaneously the expression levels of 14,400 genes. Total RNA isolated from three spinal cords and DRG corresponding to L4 and L5 spinal nerves of each genotype was used for this study. The values show the result of the ratio of the expression intensities of each gene in MK-/- vs. WT+/+ mice ("~ 1" is used when the result of the ratio performed was 1 ± 0.5).

Gene	Ratio of expression levels in spinal cord MK-/- / WT+/+	Ratio of expression levels in DRG MK-/- / WT+/+
μ-opioid receptor	~ 1	**~ 1**
δ-opioid receptor	~ 1	**~ 1**
κ-opioid receptor	~ 1	**10.12 ± 2.04**
Opioid Receptor Like (ORL)-1	~ 1	**~ 1**
Proopiomelanocortin	~ 1	**~ 1**
Proenkephalin	~ 1	**~ 1**
Prodynorphin	~ 1	**~ 1**
Prepronociceptin	**~ 1**	**~ 1**

4. DISCUSSION

Despite the evidences pointing to a role of MK in the process of chronic (neuropathic) pain discussed in the introduction of this manuscript, the present study reveals for the first time that MK deficiency does not influence acute pain processing at the supraspinal or spinal level. In the hot-plate test, the nocifensive behaviour involves licking, flinching and head, trunk and limb coordination. Compared to the spinal reflexive behaviours measured by other acute pain models such as the tail immersion test, these behaviours are more complex, organized and unlearned behaviours and involve purposeful actions requiring supraspinal sensory processing, being all these qualities apparently unaffected in MK-/- mice as well as the spinal reflexes.

As mentioned before, it was previously found that MK deficiency delays the recovery from chronic neuropathic pain states after peripheral nerve injury (29-31), suggesting an important role of MK in the control of chronic pain states. In contrast, the data presented here suggest that MK is not involved in pain transmission in acute pain states. Although these evidences suggest opposite roles of MK in chronic and acute pain states, it seems reasonable to think that attenuation of chronic neuropathic pain responses could result from the neurotrophic actions of MK after injury and its capacity to form functional

neovasculature in the injured area (see review 13), functions of MK that should not influence acute pain responses.

We also found that the antinociceptive effect of morphine was dose-dependent and similar in both MK-/- and WT+/+ mice in the hot-plate test. Interestingly, when a clinically relevant dose of morphine (5 mg/kg) was tested on the tail-immersion test, we found greater analgesic effects of morphine in MK-/- mice compared to WT+/+ mice. Keeping in mind that morphine is known to regulate MK endogenous levels within the central nervous system (3), we could affirm that our data identify, for the first time, MK as an endogenous modulator of morphine-induced analgesic effects at the spinal level. In addition, our results show that the only opioid receptor transcriptionally altered in the MK-/- mice is the κ-opioid receptor, which was found to be strikingly upregulated in the DRG of MK-/- mice compared to WT+/+ mice. The data may be important since the κ-opioid receptor has been found to be involved in morphine-induced peripheral antinociceptive effects together with the μ-opioid receptor, whereas the supraspinal analgesic effects of morphine seems to be mediated almost exclusively by the μ-opioid receptor subtype (17). Thus, the data strongly suggest that endogenous MK modulates morphine-induced spinal, but not supraspinal, analgesic effects in mice through the ability of MK to specifically regulate the levels of expression of the κ-opioid receptor in DRG.

It is interesting to note that the novel neurotrophic factor pleiotrophin (PTN), that is highly redundant in structure and function with MK (9), has been recently proposed as an important regulator of morphine-induced analgesic effects as well (6). As in the case of MK-/- mice, morphine exhibit exacerbated analgesic effects in PTN-/- mice. However, whereas PTN-/- mice are known to exhibit a significant disruption of basal spinal nociceptive transmission (6), the data presented here tend to discard an important role of MK in the control of spinal (or supraspinal) pain processing, thus identifying uncommon functional differences between these two “redundant” neurotrophic factors. The data also suggest that individual differences in the expression levels of MK (and PTN), or the existence of polymorphisms leading as a result to a decreased functionality of the encoded MK protein, could potentially underlie some of the well-documented differences concerning the efficacy of morphine to induce satisfactory analgesic effects in humans (for a review see 32).

From a translational point of view, the data presented here clearly support that MK plays an important role in morphine analgesic effects and, thus, suggest the different molecules involved in the signaling pathways initiated by MK as potential drug targets to potentiate morphine effects in the treatment of pain. Midkine binds the Receptor Protein Tyrosine Phosphatase (RPTP)β/ζ (28), causing the inactivation of its phosphatase activity. As a result, MK induces

significant increases in the tyrosine phosphorylation levels of the different substrates of RPTPβ/ζ identified so far, β-catenin (21), β-adducin (25, 26), Fyn (27), p190 RhoGAP and membrane-associated guanylate kinase, WW, and PDZ domain containing 1 (4) and GIT1/Cat-1 (15). Studies directed to clarify the possible contribution of each of these substrates and the receptor RPTPβ/ζ to the MK mediation of morphine antinociceptive effects could result in very significant contributions to the field in the near future. Also, additional preclinical studies directed to test the analgesic effects of combinations of morphine and known MK inhibitors (20) will significantly help to confirm the relevance of the data presented here.

In summary, the data presented here discard a critical role of MK in acute pain transmission. Furthermore, the data identify previously unexpected roles of endogenous MK in the modulation of morphine-induced analgesia at the spinal level.

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