The heparin binding growth factors midkine and pleiotrophin regulate the antinociceptive effects of morphine through α_2-adrenergic independent mechanisms

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Abstract

Genetic deletion of pleiotrophin (PTN) impairs spinal nociceptive transmission suggesting this heparin binding growth factor could play roles in acute pain processing. Despite the high functional redundancy between PTN and midkine (MK), the only other member of this family of growth factors, we now demonstrate that genetic inactivation of MK does not alter acute nociceptive transmission since pain responses of female MK genetically deficient (MK-/-) and wild type (WT+/+) mice were found to be similar in the hot-plate and tail-immersion tests. It has also been shown that morphine administration significantly regulates MK levels within the brain, suggesting MK could play a role in morphine-induced antinociceptive effects. To test this hypothesis, we have now studied morphine-induced antinociceptive effects in female MK-/- and WT+/+ mice. 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 significantly delayed pain responses in MK-/- mice compared to WT+/+ mice in the tail-immersion test. In confirmation of previous results from our group, we also found significantly enhanced morphine-induced antinociceptive effects in PTN-/- mice in the tail-immersion test. In addition, we now demonstrate that enhanced morphine analgesic effects in PTN-/- and MK-/- mice are not caused by a different contribution of descending noradrenergic inhibitory pathways since the α_2-adrenergic antagonist yohimbine failed to alter morphine-induced analgesia in all genotypes. The data demonstrate that MK is an endogenous modulator of morphine antinociceptive effects, identify significant differences between PTN and MK in the control of pain processing at the spinal level, and support the hypothesis that inhibitors of the PTN/MK signaling pathway could potentiate opioid analgesia which may be relevant in opiod-refractory pain cases.

Keywords: Injury, pain, opioid, hot-plate, tail-flick.

1. Introduction

Midkine (MK) is a heparin binding growth factor (Kadomatsu et al. 1988) that shares over 50% identity in amino acid sequence with pleiotrophin (PTN), the only other member of the PTN/MK developmentally regulated gene family (Deuel et al. 2002, Herradon et al. 2005, Li et al. 1990, Milner et al. 1989). Both PTN and MK have been found to play important roles in the development of the nervous system through their actions on neuronal differentiation (Herradon and Ezquerra 2009, Gramage and Herradon 2011). 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 (Blondet et al. 2005, Herradon et al. 2009, Kikuchi-Horie et al. 2004, Mi et al. 2007, Sakakima et al. 2004a, 2004b, Yeh et al. 1998).

In the central nervous system, PTN and MK have been found to be key factors for survival of the injured dopaminergic neurons in vitro and in vivo (Gramage et al. 2010b, Hida et al. 2003, 2007, Jung et al. 2004, Marchionini et al. 2007, Prediger et al. 2011), functions that may be relevant in the substantia nigra of patients with Parkinson's disease where this type of heparin binding growth factors were found to be upregulated and suggested to play reparatory roles (Marchionini et al. 2007). In addition, PTN and MK expression levels have been found to be significantly upregulated in different brain areas in humans and rodents after administration of different drugs of abuse such as amphetamine (Gramage and Herradon 2011), which neurotoxic effects in dopaminergic pathways of the central nervous system are limited by endogenous PTN and MK (Gramage et al. 2011, 2010a, 2010b). In addition, MK has been shown to reduce the brain infarcted area after different types of stroke (Ooboshi 2011) and has been shown to counteract amyloid beta-induced cytotoxicity suggesting MK may be expressed in senile plaques of Alzheimer's

disease patients (Yasuhara et al. 1993) to limit the progression of this disease (Muramatsu 2011). Based on the robust evidences compiled on the reparatory roles of PTN and MK in different diseases of the central nervous system during the last two decades, both PTN and MK have been proposed as therapeutic targets in different central nervous system disorders (Gramage and Herradon 2011, Herradon and Ezquerra 2009, Muramatsu 2011, Ooboshi 2011).

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 (Sakakima et al. 2009). This is supported by the delay in axonal regeneration uncovered in MK genetically deficient (MK-/-) mice with peripheral nerve injury leading to the hypothesis that MK acts as a reparative neurotrophic factor in damaged peripheral nerves (Sakakima et al. 2009). 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 (Sakakima et al. 2004a, 2004b). 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. Very interestingly, upregulation of the levels of the highly homologous cytokine PTN in the injured dorsal root ganglia (DRG) of rats with Chronic Constriction Injury (CCI) of the sciatic nerve correlates with faster recovery of neuropathic pain states (Ezquerra et al. 2008) suggesting PTN could play a role in nociceptive transmission as well as in neurotrophic processes. Accordingly, whereas supraspinal nociceptive transmission seems to be unaffected in PTN genetically deficient (PTN-/-) mice in acute pain models (Gramage and Herradon 2010), PTN-/- mice showed a significantly delayed response to nociceptive stimulus in the tail-flick test suggesting endogenous PTN modulates nociceptive transmission at the spinal level (Gramage and

Herradon 2010). Despite the well-documented functional redundancy between PTN and MK, the role of MK in nociception has not been assessed in acute pain models. To fill this gap in knowledge, we have now performed comparative behavioural studies 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 chronic administration of morphine (Ezquerra et al. 2007). Whether or not MK is upregulated after acute morphine administration within the brain or in other areas of the central nervous system such as the spinal cord remains to be elucidated. However, the existing data (Ezquerra et al. 2007) suggest the possibility that MK could be involved in the pharmacological effects of morphine within the central nervous system. Given the similarities between PTN and MK functions, this hypothesis is supported as well by the enhanced morphine antinociceptive effects found in PTN-/- mice (Gramage and Herradon 2010). To test this hypothesis in the present work, we also aimed to evaluate morphine-induced analgesia in MK-/- and WT+/+ mice in both the hot-plate and tail-immersion tests. Taking into account that descending noradrenergic inhibitory pathways in the control of pain contribute to opioid-induced analgesia (Herradon et al. 2003, Millan 2002), we also aimed to evaluate potential differences in the contribution of noradrenergic pathways to opioid analgesia in both PTN-/- and MK-/- mice, since both cytokines, PTN and MK, are also known regulators of norepinephrine synthesis (Hida et al. 2003, 2007, Jung et al. 2004).

2. Materials and Methods

Midkine knockout (MK-/-) and PTN knockout (PTN-/-) mice were generated on a 129/Ola x C57BL/6J background by methods essentially identical to those previously described (Amet et al. 2001, Nakamura et al. 1998). The animals used in this study were 8-weeks old female MK-/- and PTN-/- mice that were not littermates of the female WT+/+ mice used as control. All the animals used in this study were maintained according to European Union Laboratory Animal Care Rules (86/609/ECC directive).

2.1. Hot-plate test

To assess supraspinal nociceptive responses in both MK-/- and WT+/+ mice, we used the hot-plate test following the procedure described previously (Gramage and Herradon, 2010). A metal hot-plate was maintained at either 53 ± 0.5°C or 55 ± 0.5°C. The latency 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 was 75 s in the case of the assays carried out at 53ºC and 30 s in the case of the assays carried out at 55ºC.

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 i.p. 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. Three independent experiments in different days were performed using balanced cohorts of animals

per experimental group (genotype and treatment) every day. 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

2.2.1. Morphine-induced antinociceptive effects in MK-/- and WT+/+mice

To assess 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 ± 0.5ºC 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 ± 0.5ºC. 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) were administered i.p. after testing the baseline responses to 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 latency 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.

2.2.2. Effects of Yohimbine on Morphine-induced antinociceptive effects in MK-/-, PTN-/- and WT+/+ mice

To test the hypothesis that morphine-induced analgesia could be mediated by α_2-adrenergic mechanisms in MK-/- and WT+/+ mice, we tested the effects of morphine (5 mg/Kg) in mice pretreated with the α_2-adrenergic antagonist yohimbine (Sigma, Madrid, Spain). For this purpose, after determination of basal responses, mice were injected (i.p.) with yohimbine (2 mg/Kg) or saline (10 ml/Kg; control) 10 minutes before morphine (5 mg/Kg) or saline (10 ml/Kg; control) administration. The tail-flick latency was recorded 25, 50, 75 and 125 min after morphine (or saline) injection. Since we previously showed that morphine-induced analgesia is significantly enhanced in the tail-immersion test in PTN-/- mice (Gramage and Herradon 2010), we included experimental groups of PTN-/- mice as a reference to compare with MK-/- and WT+/+ mice and, also, to evaluate the possibility that the enhanced morphine-induced analgesia in PTN-/- mice could be mediated by α_2-adrenergic mechanisms. Three independent experiments in different days were performed using balanced cohorts of animals per experimental group (genotype and treatment) every day. Therefore, the experimental groups and total number of animals included in each of those were as follows:

Saline (10 ml/Kg) + Saline (10 ml/Kg): WT+/+, n = 10; MK-/-, n = 7; PTN-/-, n = 9.

Saline (10 ml/Kg) + morphine (5 mg/Kg): WT+/+, n = 14; MK-/-, n = 9; PTN-/-, n = 14.

Yohimbine (2 mg/Kg) + Saline (10 ml/Kg): WT+/+, n = 10; MK-/-, n = 8; PTN-/-, n = 6.

Yohimbine (2 mg/Kg) + morphine (5 mg/Kg): WT+/+, n = 15; MK-/-, n = 11; PTN-/-, n = 12.

2.3. Statistical Analysis

The statistical significance of changes within the same treatment was determined by 2-way repeated-measures ANOVA considering as factors the genotypes and the 4 or 5 time points after injection depending on the experimental model. 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 PTN-/-, MK-/- and WT+/+ mice, and basal values of genotypes were analyzed using student's t test when two genotypes were compared and by 1-way ANOVA followed by Bonferroni's post hoc tests when the three genotypes were compared. $P < 0.05$ 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 of mice from both genotypes used in all experiments performed (WT+/+, n = 67; MK-/-, n = 46). The latency to the first sign of nocifensive behaviour in MK-/- mice was found to be essentially similar to that recorded in WT+/+ mice (Fig. 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 (Fig. 1B).

Nociceptive responses after saline administrations were not significantly altered compared to baseline values of either genotype (Fig. 1C). Morphine-induced antinociceptive effects were found to be clearly dose-dependent (Figs. 1D-F). The analgesic effect of morphine was maximal at 25 min after morphine injection at the highest dose used (10 mg/Kg) (Fig. 1F). The data demonstrate that the analgesic effects of morphine are effectively similar in both genotypes at all doses of the opioid tested (Fig. 1D-F).

3.2. Tail-immersion test

3.2.1. Morphine-induced antinociceptive effects in MK-/- and WT+/+mice

First, we assessed baseline responses of MK-/- and WT+/+ mice (n = 5/genotype) to the tail-immersion test using a bath maintained at a moderate temperature (50 ± 0.5 ºC). MK-/- mice showed a similar latency to a tail-flick compared to WT+/+ mice (Fig. 2A). In the studies designed to test morphine antinociceptive effects, we aimed to use a higher bath temperature (55 ± 0.5 ºC) to allow direct comparisons with studies performed previously in PTN-/- and WT+/+ mice (Gramage and Herradon, 2010). 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 nociceptive response to this heat stimulus of higher intensity compared to WT+/+ mice (Fig. 2B). Nociceptive responses after saline administrations were not altered compared to baseline values of either genotype (Fig. 2C). Differences between MK-/- and WT+/+ mice were clearly observed after morphine (5 mg/kg) administration (Fig. 2D). In this case, 2-way repeated-measures ANOVA showed a significant effect of time ($F(4,68)=30.67$, $P<0.001$) and a significant effect of the genotype ($F(1,68)=4.657$, $P=0.045$) in morphine (5 mg/Kg)-treated mice. In confirmation, we also found a significantly higher AUC value in MK-/- compared to WT+/+ mice treated with 5 mg/kg morphine (Fig. 2E).

3.2.2. Effects of Yohimbine on Morphine-induced antinociceptive effects in MK-/-, PTN-/- and WT+/+ mice

Confirming previous work (Gramage and Herradon 2010), baseline responses of PTN-/- mice included in the 4 experimental groups outlined in the methods section of this manuscript were significantly increased compared to WT+/+ and MK-/- mice. 20 % of the total PTN-/- mice used reached experimental cutoff time (15s) in basal condition and, thus, were excluded of the pharmacological tests. The remaining PTN-/- mice still showed a 1-2s significant increase in their tail-flick latencies compared to MK-/- and WT+/+ mice (Fig. 3A) and were accordingly distributed in the four experimental groups in order to minimize baseline differences between the different genotypes (Fig. 3B,C).

Two-way repeated-measures ANOVA showed a significant effect of time ($F(4,136)=31.63$, $P<0.001$) and a significant effect of the genotype ($F(2,136)=5.708$, $P=0.0073$) in saline-pretreated, morphine (5 mg/Kg)-treated mice (Fig. 3C). PTN-/- mice pretreated with saline and treated with morphine exhibited the highest significant increase in

tail-flick latencies followed by MK-/- mice, which also showed significantly increased morphine-induced antinociceptive effects compared to WT+/+ mice (Fig. 3C). In confirmation, we also found a significantly higher AUC value in PTN-/- and MK-/- mice compared to WT+/+ mice pretreated with saline and treated with 5 mg/kg morphine (Fig. 3D). Importantly, pretreatment with the α_2-adrenergic antagonist yohimbine (2 mg/Kg) failed to prevent morphine-induced analgesia in any of the three genotypes (Figs. 4A-C) suggesting differences between genotypes are not caused by differences in the mediation of morphine antinociceptive effects by α_2-adrenergic mechanisms. In control studies, animals of the three genotypes pretreated with yohimbine and treated with saline showed similar responses to those only treated with saline in the tail-immersion test (Figs. 4D-F).

4. Discussion

The present study uncovers for the first time that genetic deletion of MK does not influence acute pain processing at the supraspinal or spinal level. Compared to the spinal reflexive behaviours measured by other acute pain models such as the tail-immersion test, the behaviours tested in the hot plate test require supraspinal sensory processing, which is apparently unaffected in MK-/- mice as well as the spinal reflexes. Interestingly, genetic deletion of PTN, the only other member of the MK family of developmentally regulated growth factors, which is highly redundant in function with MK, impairs spinal nociceptive transmission as it was previously shown (Gramage and Herradon 2010) and confirmed in the present work. Thus, the data presented here identify for the first time previously unexpected differences between MK and PTN in the control of nociceptive transmission in acute pain models.

It was previously found that MK deficiency delays the recovery from chronic neuropathic pain states after peripheral nerve injury (Sakakima et al. 2004a, 2004b, 2009), 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 (Kadomatsu and Muramatsu 2004, Ooboshi 2011), 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. However, when a clinically relevant dose of morphine (5 mg/kg) was tested on the tail-immersion test, we found greater

analgesic effects of the opioid in MK-/- mice compared to WT+/+ mice. Interestingly, it was previously shown that chronic administration of morphine upregulates MK expression levels in the rat hippocampus (Ezquerra et al., 2007). Whether or not an acute administration of morphine as the one used in the present work could regulate MK expression levels remains to be elucidated. However, our data identify, for the first time, MK as an endogenous modulator of morphine-induced analgesic effects at the spinal level. Importantly, similar results were obtained in PTN-/- mice although, in this case, enhanced morphine analgesic effects were observed as well in the hot-plate test, suggesting PTN regulates morphine antinociceptive actions at both spinal and supraspinal levels (Gramage and Herradon 2010). On the other hand, descending noradrenergic inhibitory pathways in the control of pain contributes to opioid-induced analgesia (Millan 2002, Herradon et al. 2003). Interestingly, MK and PTN have been shown to regulate the norepinephrine biosynthetic pathway in vitro and in vivo through their ability to regulate the expression levels of tyrosine hydroxylase, the rate-limiting enzyme of this pathway (Hida et al. 2003, 2007, Jung et al. 2004, Prediger et al. 2011), suggesting potential differences in the contribution of noradrenergic pathways to opioid analgesia in PTN-/- and MK-/- mice. Thus, we aimed to evaluate this contribution in PTN-/-, MK-/- and WT+/+ mice. Our data demonstrate that enhanced morphine analgesic effects in PTN-/- and MK-/- mice are not related to the differential ability of morphine to trigger noradrenergic descending inhibitory pathways in the control of nociceptive responses in these mice since the selective α_2-adrenergic antagonist yohimbine failed to block morphine-induced analgesia in PTN-/-, MK-/- and WT+/+ mice.

Our data also suggest the possibility 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 or PTN proteins, 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 Smith 2008). In addition, the data presented here support targeting the PTN/MK family of growth factors as a new therapeutic strategy to potentiate the antinociceptive effects of opioids, especially in opiod-refractory pain cases. Fortunately, some of the mechanisms of action triggered by these growth factors have been well described. Both, PTN and MK bind the Receptor Protein Tyrosine Phosphatase (RPTP)β/ζ (Fukada et al. 2005, Meng et al. 2000, Sakaguchi et al. 2003), causing the inactivation of its phosphatase activity. As a result, MK and PTN induce significant increases in the tyrosine phosphorylation levels of the different substrates of RPTPβ/ζ identified so far, β-catenin (Meng et al. 2000), β-adducin (Pariser et al. 2005a,b), Fyn (Pariser et al. 2005c), p190 RhoGAP and membrane-associated guanylate kinase, WW, and PDZ domain containing 1 (Fukada et al. 2005), anaplastic lymphoma kinase (ALK) (Perez-Pinera et al. 2007) and GIT1/Cat-1 (Kawachi et al. 2001). Very interestingly, the gene transcription of one of the key downstream effectors of the PTN/MK signaling pathway, β-catenin (Meng et al. 2000), is significantly increased after treatment with μ-opioid receptor agonists (Ward et al. 2007), effect that has been judged to be very important for opioid effects in neuronal function (Ward et al. 2007). Further studies directed to clarify the possible contribution of each of these substrates and the receptor RPTPβ/ζ to the MK/PTN 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 (Matsui et al. 2010) or modulators of the PTN/MK signaling pathway (Gramage and Herradon 2011) will significantly help to confirm the relevance of our data.

In summary, the data presented here discard a critical role of MK in acute pain transmission in contrast with the known roles of PTN in pain processing. Furthermore, the

data identify MK as a new endogenous modulator of morphine-induced analgesia at the spinal level.

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FIGURES:

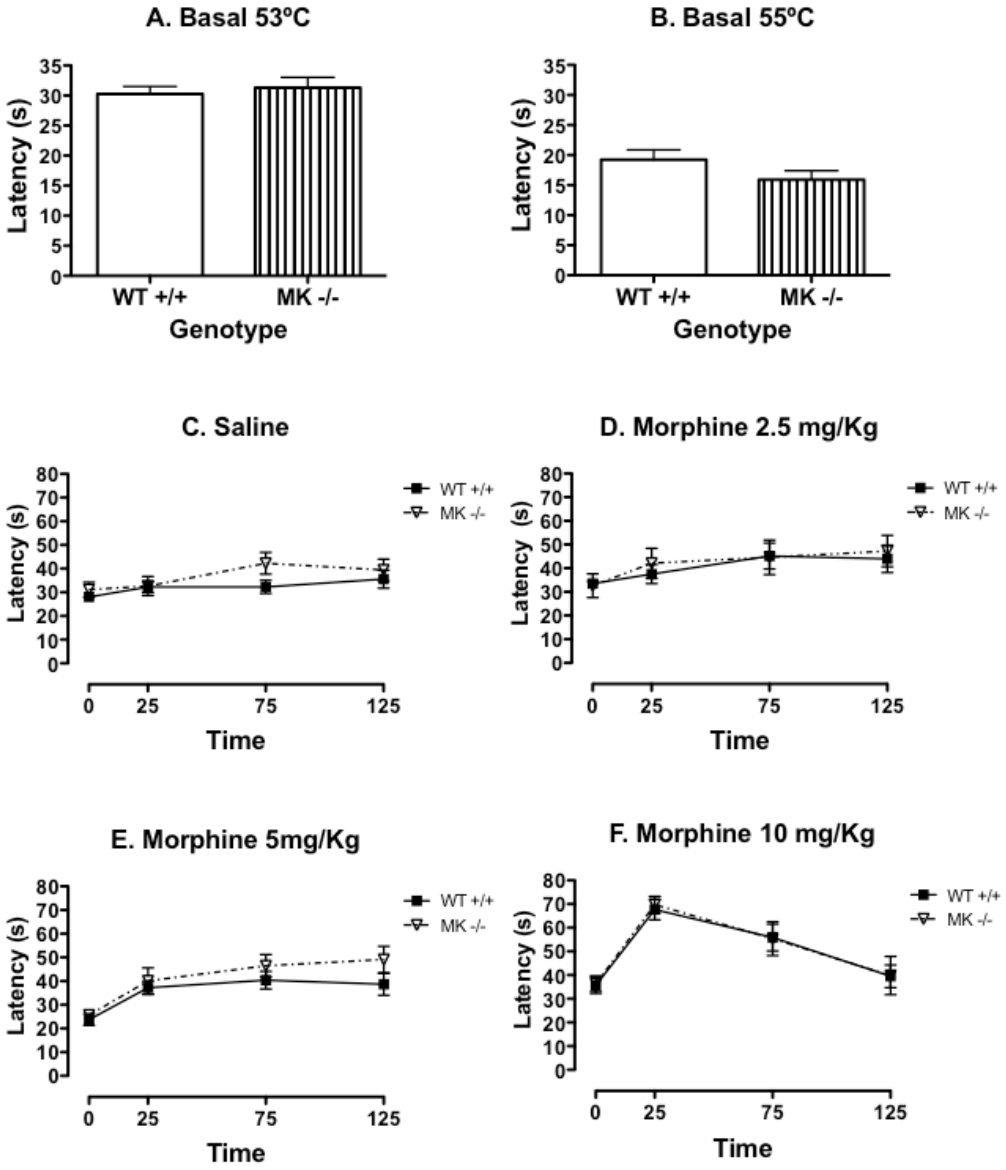

Fig. 1. Comparison of nociceptive behavioural responses and the antinociceptive effect of morphine in WT+/+ and MK-/- mice in the hot-plate test. Basal nociceptive behavioural responses of WT+/+ and MK-/- mice in a hot-plate maintained at 53ºC (A) and

55ºC (B) are shown. The effects of saline administration used as a control (C) and the analgesic effects of morphine at a dose of 2.5 mg/kg (D), 5 mg/kg (E) and 10 mg/kg (F) on the hot-plate test maintained at 53ºC are shown as a function of time. Data show mean ± SEM. 2-way repeated-measures ANOVA showed a significant effect of time in morphine (2.5 mg/Kg)-treated mice ($F(3,57)=2.833$, $P=0.046$), in morphine (5 mg/Kg)-treated mice ($F(3,78)=13.29$, $P<0.001$) and in morphine (10 mg/Kg)-treated mice ($F(3,60)=24.69$, $P<0.001$). No significant effects of the genotype was found in any of the experimental groups.

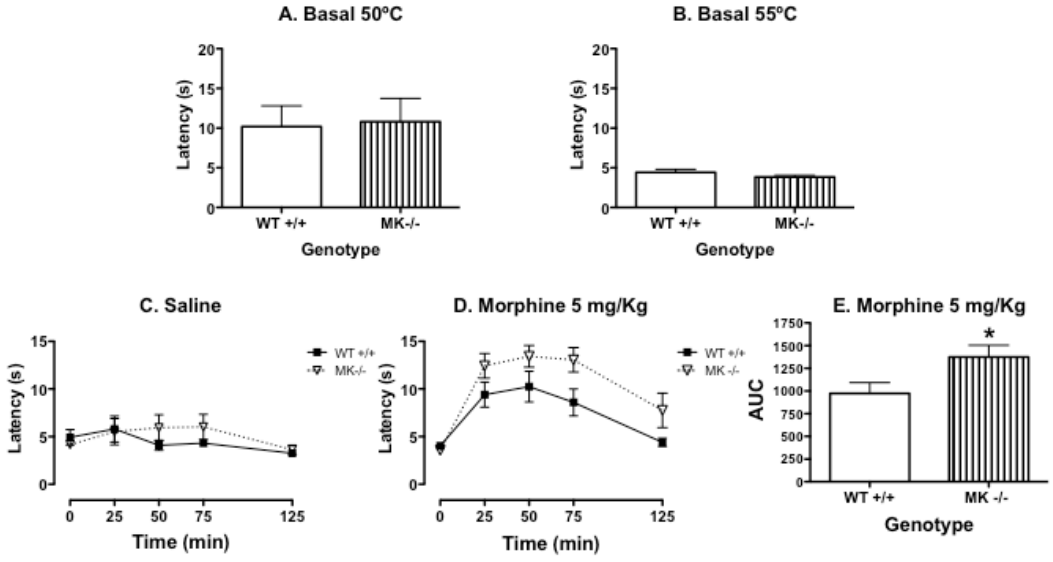

Fig. 2. Comparison of nociceptive behavioural responses and the antinociceptive effect of morphine in WT+/+ and MK-/- mice in the tail immersion test. Basal nociceptive behavioural responses of WT+/+ and MK-/- mice using a bath maintained at 50ºC (A) and 55ºC (B) are shown. The effects of saline administration used as a control (C) and the analgesic effects of morphine at a dose of 5 mg/kg (D) on the tail-immersion test using a bath

maintained at 55ºC are shown as a function of time. 2-way repeated-measures ANOVA showed a significant effect of time ($F(4,68)=30.67$, $P<0.001$) and a significant effect of the genotype ($F(1,68)=4.657$, $P=0.045$) in morphine (5 mg/Kg)-treated mice. Area under the curve (AUC) values for WT+/+ and MK-/- mice treated with morphine (5 mg/kg) are also compared (E). Data show mean ± SEM. * $P < 0.05$ vs. WT+/+.

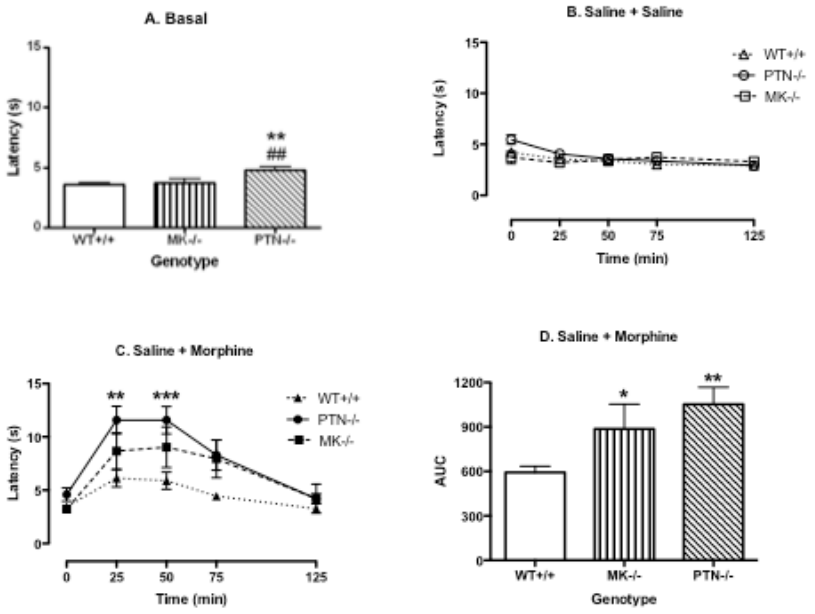

Fig. 3. Comparison of nociceptive behavioural responses and the antinociceptive effect of morphine in WT+/+, MK-/- and PTN-/- mice pretreated with saline. Basal nociceptive behavioural responses of WT+/+, PTN-/- and MK-/- mice in the tail-immersion test (A). 20% of PTN-/- mice used in these studies reached the experimental cut off time (15s), were not used further in these studies and are not included in the represented data (A). Effects of saline administration as a control (B) and analgesic effects of morphine (5 mg/kg) (C) on the tail-immersion test in mice pretreated with saline are shown as a function of time. 2-way repeated-measures ANOVA showed a significant effect of time ($F(4,136)=31.63$, $P<0.001$), a significant effect of the genotype ($F(2,136)=5.708$, $P=0.0073$) and a significant interaction between time and genotype ($F(8,136)=2.933$, $P=0.0047$) in saline-pretreated, morphine (5

mg/Kg)-treated mice. Area under the curve (AUC) values for WT+/+, MK-/- and PTN-/- mice pretreated with saline and treated with morphine (5 mg/kg) are also compared (D). Analysis of AUC data by one-way ANOVA showed a robust effect of the genotype ($F(2,34)=5.396$, $P=0.0092$). Data show mean ± SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. WT+/+.

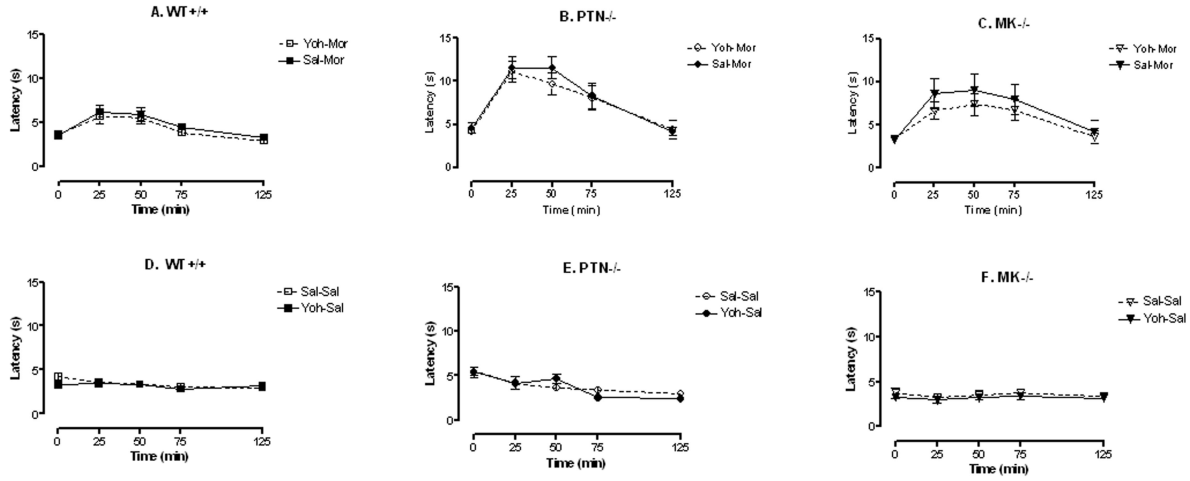

Fig. 4. Comparison of nociceptive behavioural responses and the antinociceptive effect of morphine in WT+/+, MK-/- and PTN-/- mice pretreated with yohimbine. Analgesic effects of morphine (5 mg/kg) on the tail-immersion test are shown as a function of time in WT+/+ (A), PTN-/- (B) and MK-/- (C) mice pretreated with yohimbine (2 mg/Kg). 2-way repeated-measures ANOVA showed a significant effect of time in yohimbine/saline-pretreated and morphine-treated WT+/+ mice ($F(4,108)=13.70$, $P<0.001$), PTN-/- mice ($F(4,96)=33.10$, $P<0.001$) and in MK-/- mice ($F(4,72)=13.62$, $P<0.001$). No significant effect of the treatment (pretreatment with saline or with yohimbine) was found in any of the experimental groups. In control studies, effects of saline are also shown as a function of time in WT+/+ (D), PTN-/- (E) and MK-/- (F) mice pretreated with yohimbine (2 mg/Kg).