## Title

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## Authors

Ferrari, Luiz F
Araldi, Dioneia
Bogen, Oliver
et al.

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# Systemic Morphine Produces Dose-dependent Nociceptor-mediated Biphasic Changes in Nociceptive Threshold and Neuroplasticity

**Luiz F. Ferrari**[a], **Dioneia Araldi**[a], **Oliver Bogen**[a], **Paul G. Green**[b], **Jon D. Levine**[a]

[a.]Departments of Medicine and Oral & Maxillofacial Surgery, and Division of Neuroscience, University of California at San Francisco, 513 Parnassus Avenue, San Francisco, CA 94143, USA

[b.]Departments of Preventative & Restorative Dental Sciences and Oral & Maxillofacial Surgery, and Division of Neuroscience, University of California at San Francisco, 513 Parnassus Avenue, San Francisco, CA 94143, USA

## Abstract

We investigated the dose dependence of the role of nociceptors in opioid-induced side-effects, hyperalgesia and pain chronification, in the rat. Systemic morphine produced a dose-dependent biphasic change in mechanical nociceptive threshold. At lower doses (0.003–0.03 mg/kg, s.c.) morphine induced mechanical hyperalgesia, while higher doses (1–10 mg/kg, s.c.) induced analgesia. Intrathecal (i.t.) oligodeoxynucleotide (ODN) antisense to mu-opioid receptor (MOR) mRNA, attenuated both hyperalgesia and analgesia. 5 days after systemic morphine (0.03–10 mg/kg s.c.), mechanical hyperalgesia produced by intradermal (i.d.) prostaglandin $E_2$ ($PGE_2$) was prolonged, indicating hyperalgesic priming at the peripheral terminal of the nociceptor. The hyperalgesia induced by i.t. $PGE_2$ (400 ng/10 μl), in groups that received 0.03 (that induced hyperalgesia) or 3 mg/kg (that induced analgesia) morphine, was also prolonged, indicating priming at the central terminal of the nociceptor. The prolongation of the hyperalgesia induced by i.d. or i.t. $PGE_2$, in rats previously treated with either a hyperalgesic (0.03 mg/kg, s.c.) or analgesic (3 mg/kg, s.c.) dose, was reversed by i.d. or i.t. injection of the protein translation inhibitor cordycepin (1 μg), indicative of Type I priming at both terminals. Although pretreatment with MOR antisense had no effect on priming induced by 0.03 mg/kg morphine, it completely prevented priming by 3 mg/kg morphine, in both terminals. Thus, the induction of hyperalgesia, but not priming, by low-dose morphine, is MOR-dependent. In contrast, induction of both hyperalgesia and priming by high-dose morphine is MOR-dependent. The receptor at which low-dose morphine acts to produce priming remains to be established.

**Author's contribution LFF:** designed research and performed experiments, analyzed the data and wrote the manuscript; **DA:** performed experiments; **OB:** conducted the Western blot analysis; **PGG:** wrote the manuscript; **JDL:** designed research, wrote the manuscript. All authors read and approved the final version of the manuscript.

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**Keywords**

nociceptor; opioid-induced hyperalgesia; morphine; hyperalgesic priming; mu-opioid receptor (MOR)

## Introduction

Chronic pain, which has been reported in up to 30–40% of United States and European populations (Andersson et al., 1993, Johannes et al., 2010, Gaskin and Richard, 2012, Nahin, 2015, van Hecke et al., 2013), and which impacts quality of life and generates immense healthcare costs, remains poorly controlled by currently available therapy. While opioids are the most effective and frequently used treatment for moderate to severe pain, their side effects remain obstacles to adequate pain management in a substantial number of patients (Ballantyne and Shin, 2008, Trescot et al., 2008, Turk et al., 2011, Franklin and American, 2014). In the present experiments we evaluated the dose dependence of two such side effects of a clinically important opioid analgesic, morphine: opioid-induced hyperalgesia (Bannister, 2015, Trang et al., 2015, Roeckel et al., 2016) and pain chronification (e.g., the transition from acute to chronic migraine) (Ossipov et al., 2005, Meng and Cao, 2007). We have recently shown that the clinically used mu-opioid receptor (MOR) agonist, fentanyl (Araldi et al., 2018b), as well as highly MOR selective opioid, DAMGO (Araldi et al., 2015, Araldi et al., 2017, Araldi et al., 2018a), produce opioid-induced hyperalgesia and a persistent increase in responsivity of nociceptors to pro-algesic mediators, a phenomenon referred to as hyperalgesic priming (Joseph et al., 2003, Joseph and Levine, 2010, Araldi et al., 2015, Araldi et al., 2016a, Araldi et al., 2017). While the analgesic effect of morphine is thought to be due to its action as a MOR agonist in the central nervous system (Mayer and Price, 1976, Yaksh and Rudy, 1978), recent evidence suggests that MOR agonists, even when administered systemically, produce some of their side-effects by action at the level of the primary afferent nociceptor (Corder et al., 2017, Machelska, 2011, McDougall, 2011, Stein et al., 2009). We tested the hypothesis that signaling mechanisms mediating the side-effects of a systemic MOR agonist, morphine, vary as a function of dose (Cherny et al., 2001, Villars et al., 2007, Zhao et al., 2004), and evaluated the role of nociceptors in morphine's dose-dependent effect on nociceptive threshold (i.e., hyperalgesia and analgesia) and nociceptor neuroplasticity (i.e., hyperalgesic priming) and their dependence on nociceptor MOR.

## Experimental procedures

### Animals

Experiments were performed on 216 male Sprague–Dawley rats (Charles River Laboratories, Hollister CA). Animals were housed 3 per cage, under a 12-hour light/dark cycle (lights on 7 am), in a temperature- and humidity-controlled room in the animal care facility of the University of California, San Francisco (UCSF). Food and water were available ad libitum. Experimental protocols, approved by the Institutional Animal Care and Use Committee of UCSF, adhere to the National Institutes of Health Guidelines for the Care

and Use of Laboratory Animals. Every effort was made to minimize the number of animals used and their suffering.

## Evaluation of mechanical nociceptive threshold

The Randall-Selitto paw-withdrawal test for mechanical nociceptive threshold was performed using an Ugo Basile Analgesymeter® (Stoelting, Chicago, IL). The Analgesymeter® applies a linearly increasing mechanical force to the dorsum of the rat's hind paw, as previously described (Randall and Selitto, 1957, Taiwo et al., 1989). Nociceptive threshold is defined as the force in grams at which the rat withdraws its paw. Baseline paw-pressure threshold is the mean of the 3 readings taken before injection of a test agent. Experiments were performed between 10 am and 5 pm. Data are presented as the mean percentage change from baseline nociceptive threshold.

## Drugs and their administration

The following drugs were used in this study: morphine sulfate pentahydrate, prostaglandin E2 (PGE2), the protein translation inhibitor cordycepin 5′-triphosphate sodium salt, the Src family kinase inhibitor SU6656, and the MAPK/ERK inhibitor U0126, all from Sigma-Aldrich (St. Louis, MO). Drug doses were selected based on previous studies (Araldi et al., 2016b, Dina et al., 2007, Ferrari et al., 2015). Stock solutions of PGE2 in absolute ethanol (1 μg/μl), were further diluted in 0.9% NaCl to give a final concentration of 0.0–02 μg/μl for the intradermal (i.d.) injections, or 0.04 μg/μl, for the intrathecal (i.t.) injections. The ~2% ethanol concentration in the administered PGE2 solution (5 μl), does not affect the mechanical nociceptive threshold (Ferrari et al., 2016). Morphine and cordycepin were dissolved in saline just prior to an experiment; SU6656 and U0126 were dissolved in DMSO (Sigma-Aldrich) and further diluted in saline (to give 2% DMSO) for a final concentration of 0.5 μg/μl each for i.d. injections, or 2 μg/μl, for i.t. administrations. 2% DMSO does not affect mechanical nociceptive threshold (Ferrari et al., 2016).

For i.d. injections, on the dorsum of the hind paw, we used a beveled 30-gauge hypodermic needle attached to a 50-μl microsyringe (Hamilton Company, Reno, NV) by a short length of polyethylene (PE-10) tubing. The i.d. administration of cordycepin or the combination of SU6656 and U0126 was preceded by a hypotonic shock (1 μl of distilled water separated by an air bubble to avoid mixing, in the same syringe) to facilitate the permeability of the cell membrane to these agents, to enhance their entry into the nerve terminal (Borle and Snowdowne, 1982, Burch and Axelrod, 1987).

For i.t. or systemic (subcutaneous, s.c.) drug administration, rats were briefly anesthetized with 2.5% isoflurane (Phoenix Pharmaceuticals, St. Joseph, MO) in O2, as previously described (Alessandri-Haber et al., 2003). The i.t. injections were performed with the rats placed over a small cylinder to elevate and flex their lumbar region. Then, a 30-gauge hypodermic needle was inserted, on the midline, into the subarachnoid space, between the L4 and L5 vertebrae. The volume of the i.t. injection of cordycepin was 10 μl; for the i.t. injection of SU6656+U0126, 5 μl of each inhibitor was combined in the same syringe. PGE2, 400 ng in a volume of 10 μl, was injected 5 min after the inhibitors.

Systemic morphine was given by s.c. injections at the nape of the neck. Animals regained consciousness approximately 1 min after removal from anesthetic.

### Oligodeoxynucleotide (ODN) antisense to MOR mRNA

To evaluate for a participation of MOR on nociceptors in the effects of systemically administered morphine, rats were treated with an oligodeoxynucleotide (ODN) that was antisense to MOR mRNA, previously shown to decrease MOR protein expression in the CNS (Sánchez-Blázquez et al., 1997). The ODN antisense sequence for MOR, 5′-CGC-CCC-AGC-CTC-TTC-CTC-T-3′, was directed against a common region of the rat MOR mRNA sequence [UniProtKB database entry P33535 (OPRM_RAT)] to block translation and downregulate the gene expression of all eight known MOR isoforms. The ODN mismatch sequence, 5′-CGC-CCC-GAC-CTC-TTC-CCT-T-3′, is a scrambled version of the antisense sequence, which has the same bases and GC ratio, with little or no homology to any mRNA sequences posted at GenBank, with four mismatched bases (denoted by bold letters). A nucleotide BLAST search was performed to confirm that the mRNA sequences targeted by the antisense, or its mismatch control, were not homologous to any other sequences in the rat database. The lyophilized ODNs, synthesized by Invitrogen Life Technologies, were reconstituted in nuclease-free saline and then administered i.t. at a dose of 6 μg/μl in a volume of 20 μl (120 μg/20 μl). The treatment with ODNs, performed once a day for 6 consecutive days, did not affect the mechanical nociceptive threshold. Of note, the use of antisense ODNs to attenuate the expression of proteins that play a role in mechanisms of nociceptor sensitization is well supported by previous studies from our group and others (Song et al., 2009, Bogen et al., 2012, Quanhong et al., 2012, Sun et al., 2013, Araldi et al., 2015, Araldi et al., 2016b, Araldi et al., 2017, Ferrari et al., 2016, Oliveira-Fusaro et al., 2017).

### SDS-PAGE and Western blotting

To determine the efficacy of the antisense treatment, MOR expression in rat lumbar DRG was analyzed. 24 h after the last injection of antisense (or mismatch) ODN against MOR, rats were deeply anesthetized with 5% isoflurane, and were then exsanguinated. L4 and L5 DRG were surgically removed and stored at −80 °C until further use. DRGs were transferred into homogenization buffer (100 mM NaCl, 1 mM EDTA, 2% SDS, 50 mM Tris-HCl, pH 7.4) supplemented with a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IL), and manually homogenized with a Teflon pestle. Proteins were solubilized by incubating the DRG homogenates for 2 h at 37 °C and mixed at 1400 rpm in an Eppendorf ThermoMixer (Eppendorf AG, Hamburg, Germany). Solubilized proteins were extracted from insoluble tissue components by centrifugation (15 min at 14000 rpm). Protein concentration was determined using the micro BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL) with bovine serum albumin (BSA) as the standard. Mixtures of 40 μg of protein per sample were denatured by boiling in sample buffer (3% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.025 bromophenol blue, 62.5 mM Tris-HCl, pH 6.8) for 10 min and electrophoresed on 4–15% pre-cast polyacrylamide gels (Biorad, Hercules, CA) in 25 mM Tris containing 192 mM glycine and 0.1% SDS. Proteins were electrophoretically transferred to a nitrocellulose membrane using the semidry method (2 h transfer time at 60 mA/gel with 47.9 mM Tris, 38.9 mM glycine, 0.038% SDS and 20% methanol). The nitrocellulose membranes

were saturated by shaking in antibody dilution buffer (5% BSA in Tris-buffered saline and 0.1% Tween 20 (TBST), pH 7.4) for 1 h at room temperature, cut in half at ~ 45 kDa and probed with either a rabbit polyclonal anti-MOR (AOR-011, 1:500, Alomone labs, Jerusalem, Israel) or a rabbit polyclonal anti-GAPDH (ab9485, 1:1000, Abcam, Cambridge, MA) antibody in antibody dilution buffer at 4 °C overnight. Blots were rinsed with TBST (3 times at room temperature, 15 min each), and probed with a horseradish peroxidase-conjugated anti-rabbit antibody (GE Healthcare LifeSciences, Pittsburgh, PA, 1:2500 in antibody dilution buffer) for 2 h at room temperature. The blotting membranes were rinsed with TBST (3 times at room temperature, 15 min each) and immunoreactivities visualized using the SuperSignal West Femto chemiluminescence detection system (Pierce Biotechnology). Results were analyzed using computer-assisted densitometry and levels of MOR immunoreactivity were normalized with respect to the GAPDH control levels in each sample. The percentage decrease in MOR expression was calculated as: [normalized immunoreactivity for antisense/normalized immunoreactivity for mismatch × 100] − 100.

### Statistical analysis

In all behavioral experiments, the dependent variable was paw-withdrawal threshold, expressed as percentage change from baseline. Only the right paws were used in experiments, which were performed blind. The average paw-withdrawal threshold before the experiments was 140.98 ± 1.7 g; a cut-off value of 240 g was established to avoid tissue injury at the site nociceptive testing on the dorsum of the rat hind paw. Analgesia, defined as an increase in mechanical nociceptive threshold, is plotted in the figures as positive values, directed downward, whereas hyperalgesia (decrease in mechanical nociceptive threshold) is represented in negative values in the figures, and directed upward (see Fig. 1, Fig. 2). Data normality and homogeneity of variance allow for the use of parametric statistical tests. The effect produced by systemic injection of morphine on the mechanical nociceptive threshold on the dorsum of the rat hind paw was analyzed by comparing the baseline (pre-morphine) threshold and the threshold 30 min after morphine, using 2-way repeated measures ANOVA, followed by Bonferroni's multiple comparison test (Fig. 1); 2-way repeated measures ANOVA was also used to compare the magnitude of the mechanical hyperalgesia 30 min and 4 h after i.d. or i.t. injection of PGE2 in the presence or absence of inhibitors (cordycepin or the combination of SU6656 and U0126) in rats previously treated with systemic morphine (Fig. 3, Fig. 4, Fig. 5), or the effect of the treatment with MOR antisense ODN on the induction of hyperalgesic priming by systemic morphine (Fig. 6). To analyze the impact of MOR antisense on the effect of systemic morphine on the mechanical threshold, the mismatch and antisense groups were compared using an unpaired Student's t-test (Fig. 2). Bonferroni test was corrected for multiple comparisons. GraphPad Prism 6.0 (GraphPad Software, Inc, San Diego, CA) was used for the graphics and to perform statistical analyses; $p < 0.05$ was considered statistically significant. Data are presented as mean ± standard error of the mean.

## Results

In experiments evaluating neuroplastic changes (hyperalgesic priming) produced by morphine (Fig. 3, Fig. 4, Fig. 5, Fig. 6), no difference was observed between the baseline

mechanical nociceptive threshold before morphine or vehicle injection and the mechanical threshold 5 d after injection: control (vehicle) and morphine. p = 0.2053 and 0.4348, respectively.

## Systemic morphine produces biphasic dose- and MOR-dependent changes in mechanical nociceptive threshold

To evaluate the nociceptive effect of systemically administered morphine, we injected a wide range of doses (0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3 or 10 mg/kg, s.c.), in separate groups of rats, and evaluated the mechanical nociceptive threshold on the dorsum of the hind paw 30 min later. Morphine produced a biphasic, dose-dependent effect on nociceptive threshold. While no significant difference in the nociceptive threshold before and after morphine injection was observed for vehicle and 0.001 mg/kg morphine, a significant decrease in the nociceptive threshold (i.e., hyperalgesia) was produced by 0.003, 0.01 and 0.03 mg/kg. Conversely, increased mechanical nociceptive threshold (i.e., analgesia) was produced by 1, 3, and 10 mg/kg morphine (Fig. 1).

Since morphine is an agonist at the MOR (Matthes et al., 1996), a receptor that is expressed in nociceptors (Ji et al., 1995), we evaluated whether the effects on the mechanical nociceptive threshold induced by 2 doses of morphine, 0.03 mg/kg, which induced hyperalgesia, and 3 mg/kg, which produced analgesia, were MOR dependent. To knockdown expression of MOR on nociceptors we treated rats with ODN antisense to MOR mRNA (120 μg/day, i.t.), for 3 consecutive days; control rats received ODN mismatch. On the 4th day, the mismatch and the antisense groups received either 0.03 or 3 mg/kg s.c. morphine. Mechanical nociceptive threshold was evaluated 30 min after morphine, and both the hyperalgesia induced by 0.03 mg/kg of morphine and the analgesia induced by 3 mg/kg of morphine were significantly attenuated in the ODN antisense-compared to the mismatch-treated groups (Fig. 2).

## Systemic morphine induces hyperalgesic priming

To determine if systemic morphine induces hyperalgesic priming, 5 d after s.c. morphine (Fig. 1), when mechanical nociceptive thresholds were not different from the pre-morphine baseline, rats received PGE2 (100 ng, i.d.), on the dorsum of one hind paw. Mechanical hyperalgesia was present in all groups 30 min after PGE2, and was unattenuated 4 h after injection, in the groups receiving 0.03, 0.1, 0.3, 1, 3 or 10 mg/kg (PGE2-induced hyperalgesia 30 min vs. 4 h, p = 0.950, 0.879, >0.99, >0.99, >0.99, >0.99, respectively), indicating that doses of systemic morphine ranging from 0.03 to 10 mg/kg induce priming at the peripheral terminal of the nociceptor (Fig. 3A).

Since we have previously demonstrated that systemic fentanyl, another MOR agonist, also induces priming at the central terminal of the nociceptor (as determined by prolongation of hyperalgesia induced by i.t. PGE2) (Araldi et al., 2018b), we next investigated whether systemic morphine also produced priming at the central terminal. Rats previously treated with systemic morphine, 0.03 mg/kg, which induced hyperalgesia and was the smallest dose to induce priming at the peripheral terminal of the nociceptor, or 3 mg/kg, which induced analgesia and priming, received PGE2 (400 ng/20 μl, i.t.). Hyperalgesia induced

by i.t. PGE2 was still present, without attenuation, 4 h after injection, in both the 0.03 and 3 mg/kg morphine groups, while in a vehicle pretreated control group PGE2-induced hyperalgesia was significantly less at the 4th h compared to 30 min. Thus, 0.03 and 3 mg/kg morphine given systemically induced priming at both central (Fig. 3B) and peripheral (Fig. 3A) terminals of nociceptors.

### Systemic morphine induces Type I hyperalgesic priming

Two types of hyperalgesic priming have been described, Type I is dependent on local protein translation in both the peripheral and central terminals of the nociceptor (Ferrari et al., 2013, Ferrari et al., 2014, Araldi et al., 2016a), and Type II is maintained by the combined activation of Src and MEK/Erk kinase (Araldi et al., 2016b, Araldi et al., 2017), in both nociceptor terminals (Araldi et al., 2018b). We have recently shown that opioids can induce both types of priming, as systemic administration of fentanyl produced Type I priming at peripheral and Type II at central terminals (Araldi et al., 2018b). To determine which type of priming is produced by systemic morphine, we investigated if the protein translation inhibitor cordycepin, or the combination of a Src and a MEK/Erk kinase inhibitor, would impact hyperalgesic priming (prolongation of PGE2 hyperalgesia) at peripheral and/or central terminals of nociceptors. To evaluate the type of priming induced by systemic morphine at the peripheral terminal (Fig. 4, upper panels), vehicle, cordycepin (1 μg) or a combination of the Src and MEK/Erk inhibitors, SU6656 and U0126, respectively (both 1 μg), was administered i.d., at the site of nociceptive testing, on the dorsum of the hind paw of rats that had received systemic morphine (0.03 or 3 mg/kg, s.c.) 5 d prior. Five minutes after injection of vehicle or the inhibitors, PGE2 (100 ng, i.d.) was injected at the same site on the dorsum of the hind paw. In vehicle- and SU6656+U0126-treated groups there was no attenuation of PGE2 hyperalgesia at the 4th h, when compared to the 30-min time point. However, in the cordycepin-treated groups the hyperalgesia was no longer present at the 4th h in groups treated with systemic morphine with the 0.03 and 3 mg/kg doses. Moreover, when PGE2 was injected again at the same site in which vehicle or cordycepin had been injected 1 week before, the PGE2-induced hyperalgesia was present at the 4th h only in the vehicle-treated group (lower panels). These findings support the suggestion that cordycepin reversed the priming induced by both 0.03 and 3 mg/kg systemic morphine, indicating presence of Type I priming (Fig. 4).

Next, we investigated whether 0.03 and 3 mg/kg systemic morphine induced priming in the central terminal of the nociceptor and, if so, is it Type I or II. Rats that had previously been treated with systemic morphine received i.t. vehicle, cordycepin (4 μg/10 μl) or the combination of SU6656+U0126 (both 10 μg/5 μl) and 5 min later, PGE2 (400 ng/10 μl) was also injected i.t.. In the vehicle- and SU6656+U0126-treated groups, hyperalgesia induced by i.t. PGE2 was present, unattenuated, 4 h after injection, but PGE2 hyperalgesia was not present at the 4th h in the cordycepin-treated groups (Fig. 5). One week later, when PGE2 was again administered i.t., hyperalgesia was present at the 4th h in the group that previously received vehicle, but not in the group that had previously received cordycepin, demonstrating that priming at the central terminal had been reversed by i.t. cordycepin. Collectively, these results demonstrate that both low (hyperalgesic)- and high (analgesic)-dose systemic morphine produce Type I priming in both nociceptor terminals (Fig. 4, Fig. 5).

### Role of MOR in the induction of priming by systemic morphine

To investigate whether the induction of priming by systemic morphine is due to its action at MOR, we evaluated for the prolongation of PGE2-induced hyperalgesia induced by systemic morphine in rats that had been pretreated with ODN antisense or mismatch for MOR mRNA (Khasar et al., 1996, Araldi et al., 2017, Araldi et al., 2018a). ODN antisense (120 μg/day, i.t.) was administered daily for 6 consecutive days, and either 0.03 or 3 mg/kg morphine administered s.c. on day 4. On day 6, PGE2 was injected i.d. on the dorsum of the hind paw or i.t. (Fig. 6, lower panels). In the 0.03 mg/kg morphine group, MOR antisense treatment did not attenuate either i.d. or i.t. PGE2 hyperalgesia at the 4th h, however in the 3 mg/kg morphine group, MOR antisense treatment significantly attenuated PGE2 hyperalgesia at the 4th h at both the peripheral and central terminals; in contrast, in mismatch groups no attenuation of PGE2 hyperalgesia was seen at either terminal. In addition, one week after 3 mg/kg s.c. morphine, hyperalgesia produced by administration of PGE2 to the peripheral or the central terminal was not prolonged in groups previously treated with MOR antisense, but was in the group previously treated with MOR mismatch, indicating that the knockdown of MOR prevented the induction of priming by the higher, but not the lower, dose of morphine (Fig. 6).

## Discussion

While morphine is one of the most widely prescribed analgesics for the treatment of moderate-to-severe pain (Wiffen et al., 2016), its side effects, such as opioid-induced hyperalgesia (Bekhit, 2010, Lee et al., 2011), and ability to produce pain chronification (Wilkinson et al., 2001, Wilder-Smith and Arendt-Nielsen, 2006, Okada-Ogawa et al., 2009, Rivat and Ballantyne, 2016), limit the clinical usefulness of all opioid analgesics. While recent studies have implicated a role for primary afferent nociceptors in some of the non-analgesic effects of systemically-administered opioids (Corder et al., 2017), few studies have addressed the dose dependence and underlying cellular mechanisms in the nociceptor-dependent effects of systemic opioids (Araldi et al., 2018b). In the present study, we evaluated the dose-dependence of systemically administered morphine on the contribution of the nociceptor to two side effects: opioid-induced hyperalgesia and hyperalgesic priming, a model of pain chronification.

The dose–response relationship for opioid-induced analgesia is, in general sigmoid shaped, when started at a low, just ineffective dose for producing analgesia and progressively increasing to a dose that produces an asymptotic effect, 90–100% of maximum analgesia. Importantly, we observed a pronociceptive effect of systemic morphine at doses starting ~2½ orders of magnitude lower than the lowest analgesic dose. Of note, in this regard, previous studies have reported that ultra-low-dose opioids, such as morphine, administered acutely are pronociceptive (Esmaeili-Mahani et al., 2008, Wala et al., 2011, Wala et al., 2013). While the mechanism mediating the pronociceptive effect of low-dose morphine remains to be fully characterized, we have previously shown that other MOR agonists such as DAMGO and fentanyl induce MOR-dependent hyperalgesia by triggering, respectively, signaling pathways involving Src, focal adhesion kinase and MAPK (Araldi et al., 2018a), or calcium (Araldi et al., 2018b).

Since morphine is a MOR agonist (Matthes et al., 1996), we investigated whether the effects of low- and high-dose systemic morphine on nociceptive threshold are MOR mediated. We observed that i.t. ODN antisense to MOR mRNA, attenuated both hyperalgesia and analgesia induced by 0.03 or 3 mg/kg s.c. morphine, respectively, providing support for the suggestion of a role of MOR in both pro- or anti-nociceptive dose-dependent effects of systemic morphine. These contrasting effects of low and high doses of systemic morphine might result from its action at different neuronal sites, such as spinal cord and sensory neurons, as the MOR antisense treatment was performed by the spinal i.t. route of administration, which would also be expected to knock down MOR expression on spinal cord neurons (Lai et al., 1996). While our experiments do not directly distinguish between these two sites of action, previous studies with different MOR agonists administered to the peripheral terminal of the nociceptor have not produced an elevation in nociceptive threshold (Levine and Taiwo, 1989, Araldi et al., 2015, Araldi et al., 2018b) (although higher doses can reverse hyperalgesia), compatible with the analgesic effect of high-dose morphine, being mediated by its action in the central nervous system rather than by their action on sensory neurons. This interpretation is strongly supported by the recent study of Scherrer and colleagues (Corder et al., 2017) who demonstrated unchanged opioid analgesia in mice in which MOR was deleted from sensory neurons.

Hyperalgesic priming is a model of the transition to chronic pain in which nociceptors undergo neuroplasticity to express a new phenotype characterized by a prolongation of the hyperalgesia produced by multiple direct-acting proalgesic mediators (Aley et al., 2000, Parada et al., 2003, Dina et al., 2008, Ferrari et al., 2010), prototypically PGE2 (Parada et al., 2005, Ferrari et al., 2010, Joseph and Levine, 2010). Recently, we have shown that the administration of low doses of systemic fentanyl, in addition to inducing hyperalgesia by action at MOR, induces Type II hyperalgesic priming (i.e., dependent on Src and MAPK activity (Araldi et al., 2018b)) at nociceptor central terminals and Type I priming (i.e., dependent on translation in the nerve terminal) at the peripheral terminal. To investigate whether the systemic administration of morphine produces hyperalgesic priming (nociceptor neuroplasticity), we evaluated the mechanical hyperalgesia induced by the i.d. injection of PGE2, at the site of nociceptive testing, in the hind paw, in rats that had received 0.001–10 mg/kg, s.c. morphine (Fig. 1). In rats pretreated with doses of morphine ≥0.03 mg/kg, PGE2 hyperalgesia was still present, without attenuation, 4 h after administration, indicating the presence of hyperalgesic priming (Joseph et al., 2003, Parada et al., 2003). Similar to systemic fentanyl (Araldi et al., 2018b), hyperalgesic priming was observed at the central as well as the peripheral terminal of the nociceptor (Fig. 3).

Two types of hyperalgesic priming have been described in nociceptors, Type I and II (Araldi et al., 2016a, Araldi et al., 2016b, Araldi et al., 2017, Araldi et al., 2018b), which are maintained by different cellular mechanisms. While Type I priming is maintained by ongoing protein translation (Araldi et al., 2016a, Araldi et al., 2018b), Type II is maintained by combined ongoing activity of Src and MAPK (Ferrari et al., 2012, Araldi et al., 2017, Araldi et al., 2018a). We have recently shown that i.d. administration of fentanyl produces Type I priming at the peripheral terminal, and Type II at the central terminal; priming is present in the paw previously injected with fentanyl and not the contralateral paw, ruling out a remote site of action in the central nervous system (Araldi et al., 2018b).

Systemic administration of MOR agonists, fentanyl and morphine induces both types of priming in the nociceptor by the. In the present study, we determined the type of priming induced by hyperalgesic and analgesic doses of systemically administered morphine. The prolongation of PGE2 hyperalgesia, at either the peripheral (Fig. 4) or central (Fig. 5) terminal, in rats previously treated with 0.03 mg/kg (hyperalgesic) or 3 mg/kg (analgesic) of s.c. morphine, respectively, were both reversed by treatment with the protein translation inhibitor cordycepin (but not by combined administration of a Src and a MAPK inhibitor), indicating Type I priming in both terminals. This contrasts with systemic fentanyl, which produces Type I priming at the peripheral terminal and Type II at the central terminal. Thus, the type of priming induced by systemically administered clinically used opioid analgesics differs between opioid.

While Type I priming was first described as a change in nociceptor phenotype induced by pro-inflammatory mediators such as tumor necrosis factor alpha or interleukin-6 (Parada et al., 2003, Dina et al., 2008), Type II priming was first described as an effect induced by local administration of DAMGO, a highly potent and selective agonist at MOR, a Gi-protein-coupled GPCR (Joseph et al., 2010, Araldi et al., 2016b, Araldi et al., 2017). However, we recently observed that fentanyl, a clinically important relatively MOR selective agonist (Araldi et al., 2018b) can also produce MOR-dependent Type I priming (Araldi et al., 2018b). To determine if hyperalgesic priming induced by systemic morphine was also MOR dependent (Fig. 2), we evaluated PGE2-induced hyperalgesia elicited at the central and peripheral terminal of the nociceptor in rats that received the 3 mg/kg (analgesic) or 0.03 mg/kg (hyperalgesic) dose of systemic morphine, after being pretreated with ODN antisense to MOR mRNA (Fig. 6). Paradoxically, while MOR antisense prevented the induction of priming by the higher, analgesic dose of morphine, it did not attenuate the induction of priming by the lower, hyperalgesic dose. This finding strongly supports the suggestion that low-dose morphine hyperalgesia, but not priming, is MOR-mediated, while for high-dose morphine both hyperalgesia and priming are MOR-mediated. While the receptor via which low-dose morphine induces priming remains to be elucidated, it may be due to action on MOR splice variants, since the antisense sequence used in the present experiments targets exons 1 and 4 of the MOR-1 clone (Leventhal et al., 1998). Given that MOR is present on spinal cord neurons (Quirion, 1984) as well as primary afferents (Coggeshall et al., 1997), and its expression is knocked down following i.t. MOR antisense (Lai et al., 1996), it is possible that some of the MOR-dependent effects on priming involve MOR-containing intrinsic spinal cord neurons. However, compatible with the suggestion that priming at the level of the spinal cord is due to neuroplastic changes in the central terminal of the nociceptor, identical mechanisms were observed following spinal i.t. administration as observed when morphine was applied at the peripheral terminal of the nociceptor.

In summary, while it has generally been considered that the pro- as well as the anti-nociceptive effects of systemically administered opioid analgesics are produced by their action at MORs on neurons in the central nervous system, recent evidence has provided compelling support for a role of the primary afferent nociceptor in some non-analgesic effects of systemic opioids (Machelska, 2011, McDougall, 2011, Corder et al., 2017). The present study provides further insight into the role of the primary afferent nociceptor in pronociceptive effects of systemically administered morphine. The importance of studies

designed to provide a better understanding of the diverse effects of opioids on nociceptors derives from the fact that opioids are well-established analgesics, often used as first-line therapy in the treatment of moderate-to-severe pain (Marzuillo et al., 2014, Cooper et al., 2017, Wiffen et al., 2017). It is necessary to identify the mechanisms underlying adverse effects of opioid analgesics, such as their pronociceptive effects and ability to produce pain chronification, to drive the development of safer and more effective analgesics for moderate to severe pain.

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## Highlights

- Morphine (s.c.) 0.003–0.03 mg/kg is hyperalgesic, while 1–10 mg/kg is analgesic.
- Antisense ODN knockdown of mu-opioid receptors attenuates hyperalgesia and analgesia.
- Morphine induced Type I priming at both central and peripheral nociceptor terminals.
- Hyperalgesia, but not priming, by low-dose morphine, is μ opioid receptor-dependent.
- The receptor at which low-dose morphine acts to produce priming is currently unknown.

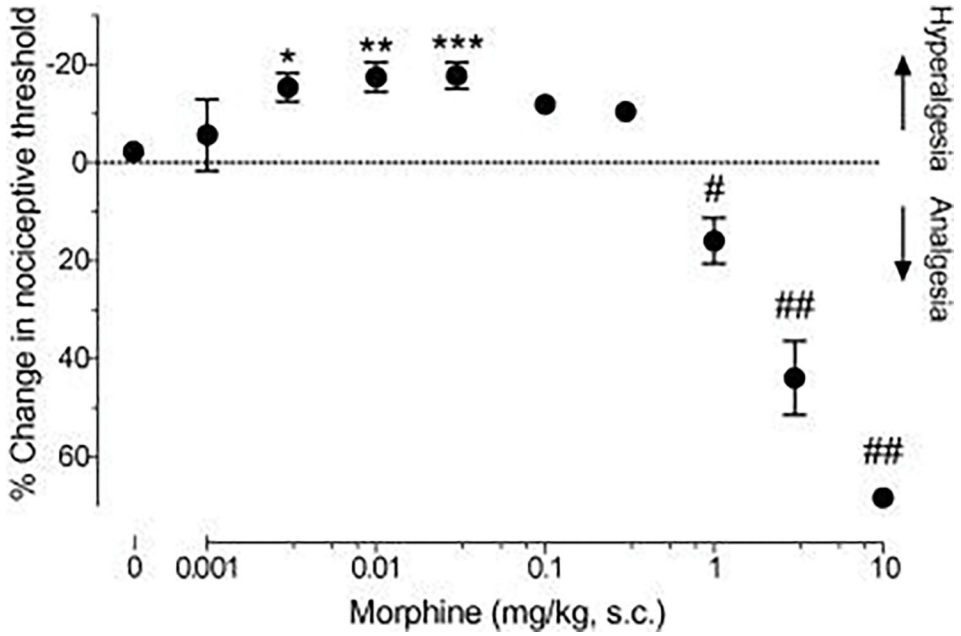


**Fig. 1.**

Dose–response relationship for the effect of systemic morphine on nociceptive threshold. Male rats (280–320 g) received a subcutaneous injection of vehicle or one dose of morphine (0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3 or 10 mg/kg). Mechanical nociceptive threshold was evaluated on the dorsum of the hind paw before and 30 min after injection. While no significant change in mechanical nociceptive threshold was observed in the groups treated with vehicle, comparing mechanical threshold before and after morphine injection using 2-way repeated measures ANOVA there was a significant interaction effect pre- vs. post-morphine at all values of morphine dose ($F_{9,50} = 30.40$, $p < 0.0001$), with morphine dose ($F_{9,50} = 15.86$, $p < 0.0001$) and treatment ($F_{1,50} = 4.28$, $p < 0.05$) significantly affecting the result. Bonferroni's multiple comparison test showing significant *hyperalgesia* at 0.003 mg/kg ($^{*}p = 0.04$), 0.01 mg/kg ($^{**}p = 0.027$) and 0.03 mg/kg ($^{***}p = 0.006$), and significant *analgesia* at 1 mg/kg ($^{\#}p = 0.018$), 3 mg/kg ($^{\#\#}p < 0.0001$) and 10 mg/kg ($^{\#\#}p < 0.0001$), indicating a dose-dependent biphasic effect of systemic morphine on the mechanical nociceptive threshold in the rat hind paw. ($n = 6$ per group).

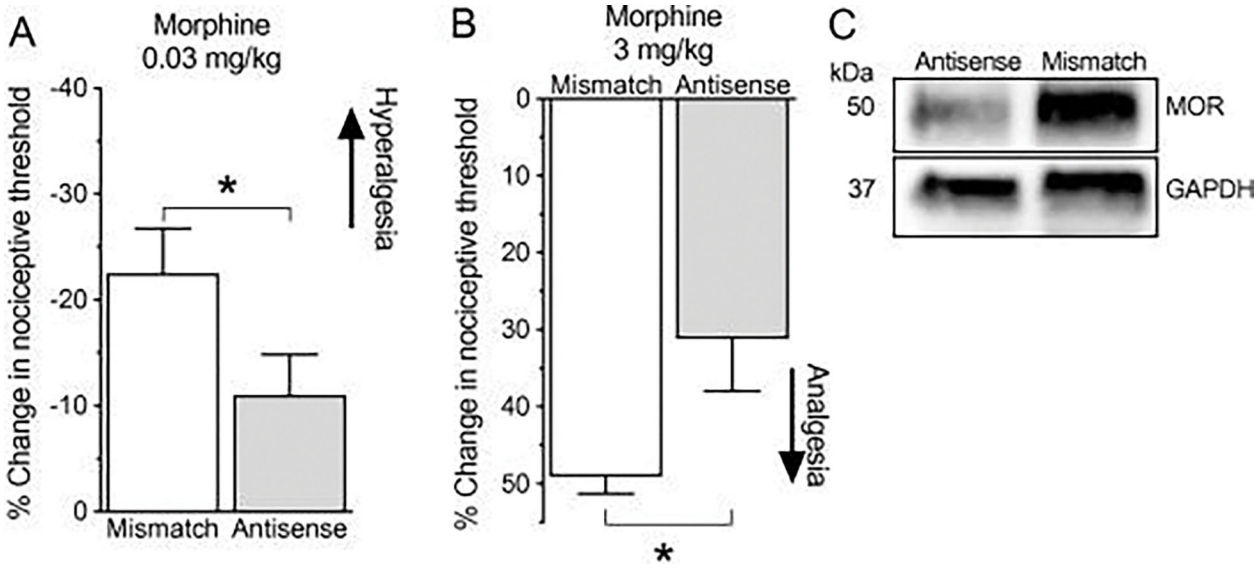


**Fig. 2.**

Nociceptor MOR-dependent effects of systemic morphine on mechanical nociceptive threshold. Male rats (350–400 g) were treated for 6 consecutive days with i.t. injections of ODN antisense or mismatch against MOR mRNA (120 μg/day); ODN treatment did not affect the mechanical nociceptive threshold (data not shown). On the 4th day of ODN treatment, the mismatch and antisense groups were each divided into two subgroups that received subcutaneous injection of 0.03 or 3 mg/kg of morphine.; mechanical nociceptive thresholds were evaluated before and 30 min after injection. A. *0.03 mg/kg:* When compared to the mismatch groups, the magnitude of the hyperalgesia induced by 0.03 mg/kg of morphine in the antisense group was reduced 51% ($t_{10}$ = 2.009, *$p$ = 0.0361, unpaired Student's $t$-test); B. *3 mg/kg:* When compared to the mismatch groups, the magnitude of the analgesia produced by 3 mg/kg was decreased 36% ($t_{10}$ = 2.514, $^{\#}p$ = 0.0307), indicating that the effects of systemic morphine, at both low (hyperalgesic) and high (analgesic) doses on the mechanical threshold are MOR-dependent. ($n$ = 6 per group). C. Western blot analysis of DRG extracts from rats injected with MOR antisense ODN (120 μg/day for three consecutive days) shows a significant decrease in MOR immunoreactivity when compared to extracts from ODN mismatch-treated rats (−23.4 ± 8.4% unpaired Student's t-test, $t$10 = 2.737, p = 0.011, $n$ = 6,). Note that the MOR exist in several isoforms with calculated molecular weights between 43.5 and 52 kDa (UniProtKB database entry P33535). GAPDH, which was used as loading control has a calculated molecular weight of ~36 kDa (UniProtKB database entry P04797).

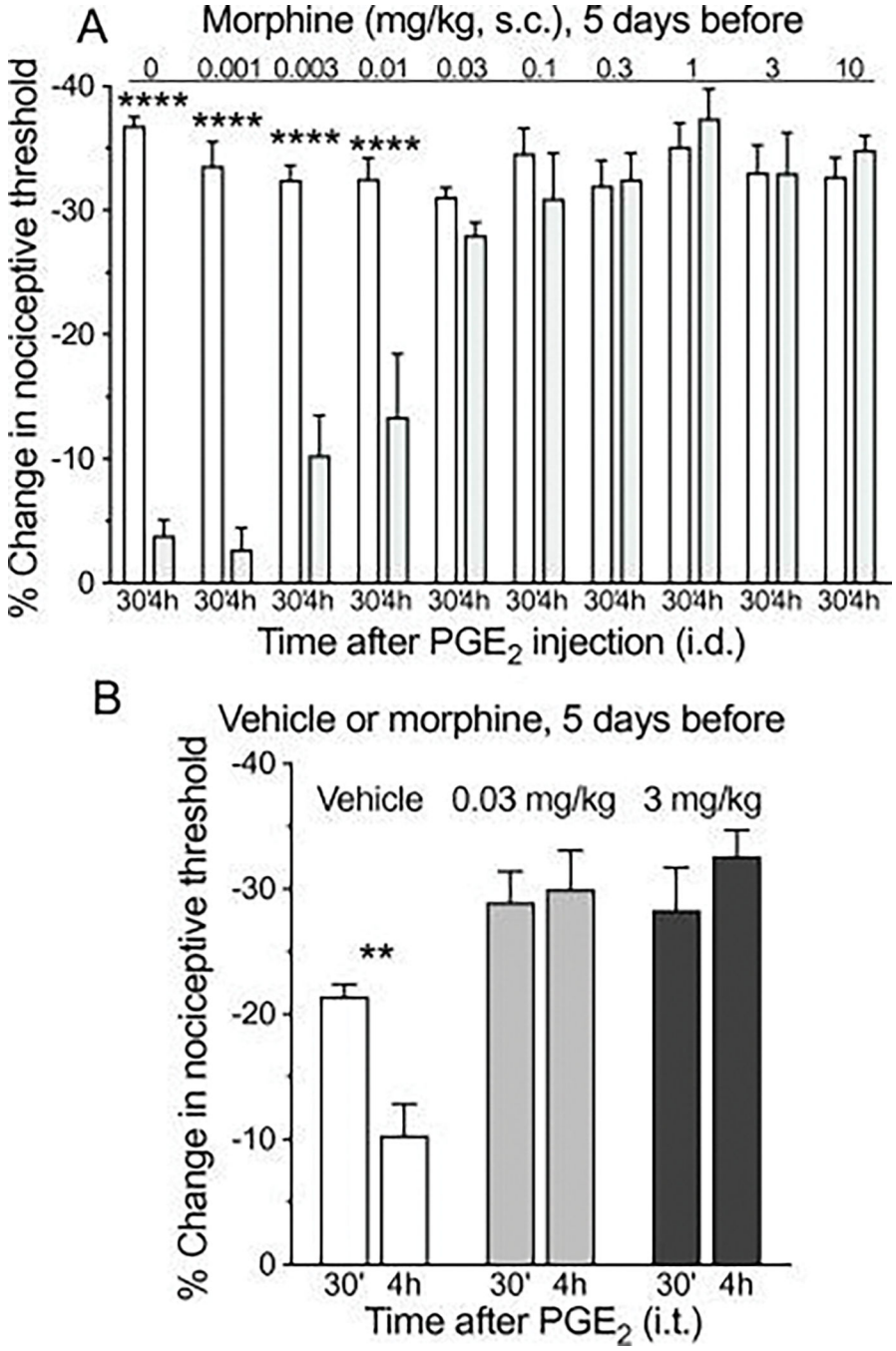


**Fig. 3.**

Systemic morphine induces hyperalgesic priming in nociceptor central and peripheral terminals. Panel A: Groups of rats pretreated with systemic vehicle or different doses of morphine (0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3 or 10 mg/kg, Fig. 1) received an i.d. injection of $PGE_2$ (100 ng) on the dorsum of the hind paw 5 d later, at the site of nociceptive testing. At that time, mechanical nociceptive thresholds were not significantly different from pre-morphine baseline. 2-way repeated measures ANOVA revealed that there is a significant interaction effect of time at all values of morphine dose ($F_{9,50} = 26.47$, $p < 0.0001$), and

significant differences of morphine dose ($F_{9,50}$ = 12.89, $p$ < 0.0001), and of time ($F_{1,50}$ = 153.25, $p$ < 0.0001). Bonferroni's multiple comparison test revealed that in the groups that had received morphine 0.03, 0.1, 0.3, 1, 3 and 10 mg/kg, $PGE_2$-induced hyperalgesia was still present 4 h after injection (i.e. not significantly different from 30 min); in contrast, groups previously treated with vehicle or 0.001, 0.003, and 0.01 mg/kg morphine 5 d before, $PGE_2$ hyperalgesia was significantly less at 4 h than at 30 min ($^{****}p$ < 0.0001 for each of these 3 morphine doses and vehicle). Panel B: Rats that had been pretreated with vehicle, or 0.03 or 3 mg/kg of morphine (s.c.), received an i.t.injection of $PGE_2$ (400 ng/10 μl). 2-way repeated measures ANOVA revealed a significant interaction of time at all values of morphine dose ($F_{2,15}$ = 9.18, $p$ = 0.0025), and a significant effect of the dose of morphine ($F_{2,15}$ = 14.02, $p$ = 0.0004), but time does not affect the result ($F_{1,15}$ = 1.53, $p$ = 0.235). Bonferroni's multiple comparison test revealed that in vehicle group the magnitude of the hyperalgesia induced by $PGE_2$ had decreased significantly by the 4th h ($^{**}p$ = 0.0026), but in the morphine-treated groups, at both doses, it was still present at that time point, without attenuation (0.03 mg/kg $p$ < 0.99; 3 mg/kg $p$ = 0.373). Together, these results show that both low and high doses of systemic morphine induce hyperalgesic priming in both terminals of the nociceptor. ($n$ = 6 per group).

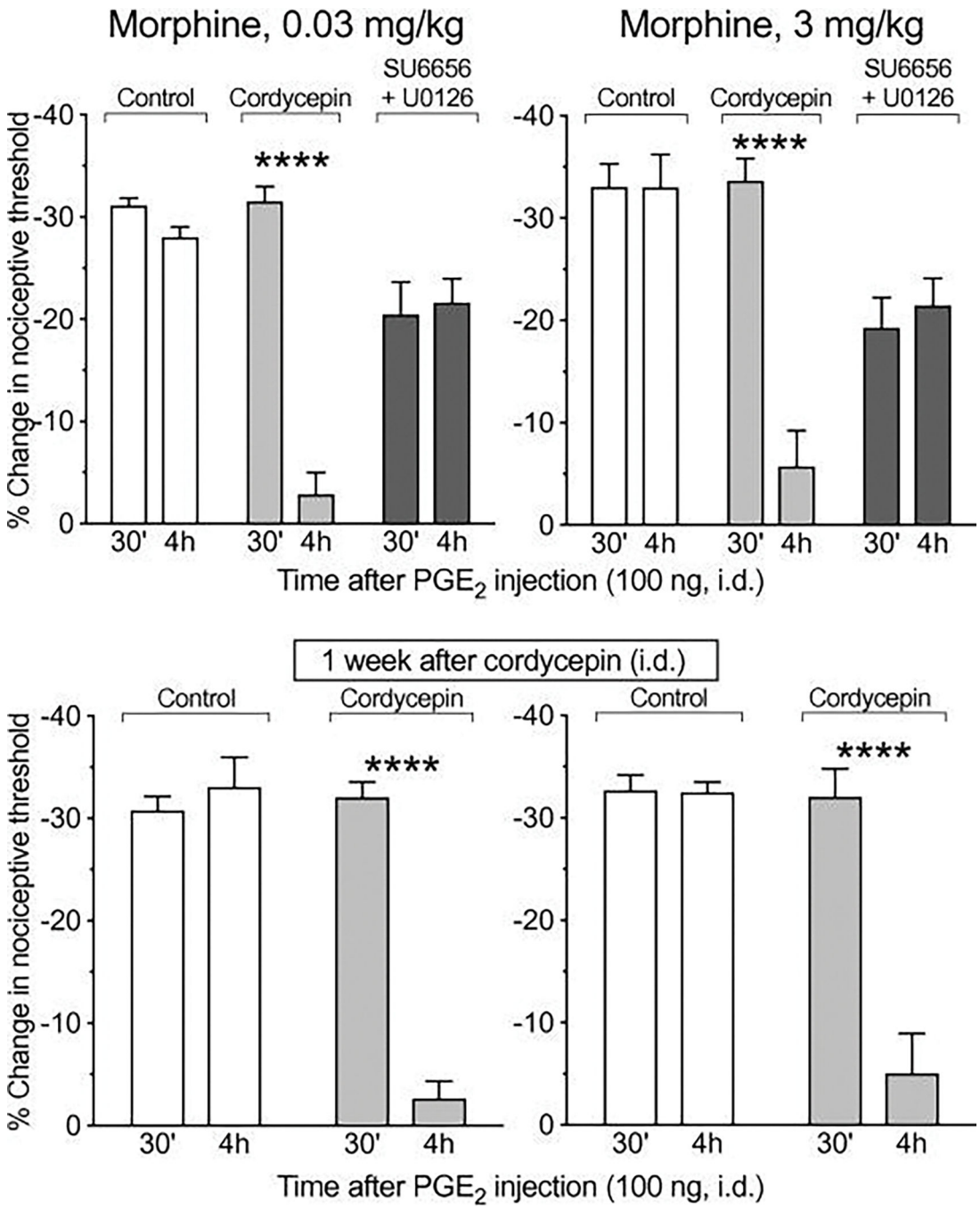


**Fig. 4.**

Systemic morphine induces Type I hyperalgesic priming at the nociceptor peripheral terminal. Upper panels: Groups of rats (350–390 g) were treated with systemic (s.c.) injection of 0.03 or 3 mg/kg of morphine, both of which induce hyperalgesic priming. Five days later, the groups were each divided into 3 subgroups that received i.d. injection of vehicle (control), the protein translation inhibitor cordycepin (1 μg), or a combination of the Src inhibitor SU6656 and the MEK/Erk inhibitor U0126 (both 1 μg), on the dorsum of the hind paw, at the site of nociceptive testing. After 5 min, $PGE_2$ (100 ng), was injected at

the same site. Evaluation of the mechanical nociceptive threshold, before and 5 days after morphine (immediately before the experiment), showed no difference between mechanical nociceptive threshold, pre- and post-morphine (data not shown). In all groups, mechanical hyperalgesia was robust 30 min after $PGE_2$ injection. 2-way repeated measures ANOVA revealed a significant interaction of time at all values of treatment (*0.03 mg/kg:* $F_{2,15}$ = 28.02, $p < 0.0001$; *3 mg/kg:* $F_{2,15}$ = 29.88, $p < 0.0001$), a significant effect of treatment (*0.03 mg/kg:* $F_{2,15}$ = 22.96, $p < 0.0001$; *3 mg/kg:* $F_{2,15}$ = 9.82, $p = 0.0019$) and time (*0.03 mg/kg:* $F_{1,15}$ = 33.70, $p < 0.0001$; *3 mg/kg:* $F_{1,15}$ = 23.51, $p = 0.0002$). Bonferroni's multiple comparison shows that for both doses of morphine there was no attenuation in the control and the SU6656+U0126 groups, but a significantly lower $PGE_2$-induced hyperalgesia in the cordycepin-treated group (both morphine doses $^{****}p < 0.0001$). Lower panels: When $PGE_2$ was injected again, at the same site, one week later, 2-way repeated measures ANOVA revealed a significant interaction of time at all values of treatment (*0.03 mg/kg:* $F_{1,10}$ = 146.19, $p < 0.0001$; *3 mg/kg:* $F_{1,10}$ = 48.83, $p < 0.0001$), a significant effect of treatment (*0.03 mg/kg:* $F_{1,10}$ = 34.75, $p = 0.0002$; *3 mg/kg:* $F_{1,10}$ = 20.90, $p = 0.001$) and time (*0.03 mg/kg:* $F_{1,10}$ = 106.88, $p < 0.0001$; *3 mg/kg:* $F_{1,10}$ = 50.55, $p < 0.0001$). Bonferroni's multiple comparison shows that for both doses of morphine there was no attenuation in the control, but a significantly lower $PGE_2$-induced hyperalgesia at the 4th h in the cordycepin-treated group ($^{****}p < 0.0001$).

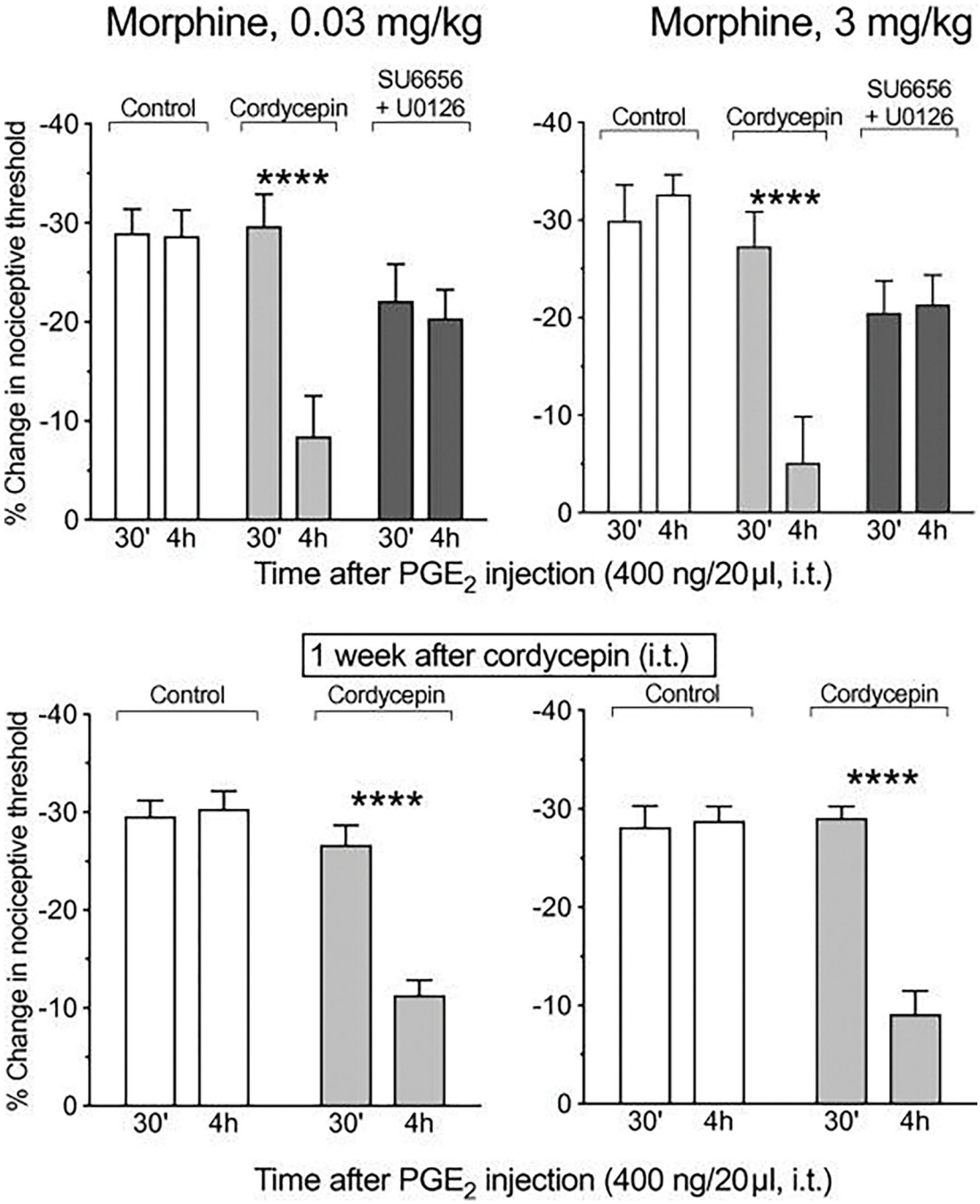


**Fig. 5.**

Systemic morphine induces Type I hyperalgesic priming at the central terminal of the nociceptor. Upper panels: Groups of rats (360–410 g) were treated systemically (s.c.) with morphine, 0.03 or 3 mg/kg, which induces priming at the central terminal of the nociceptor (Fig. 3B). Five days later, the groups were each divided into 3 subgroups that received i.t. injection of vehicle (control), the protein translation inhibitor cordycepin (4 µg/10 µl), or a combination of the Src inhibitor SU6656 and the MEK/Erk inhibitor U0126 (both 10 µg/5 µl). Five min later, $PGE_2$ (400 ng/10 µl), was injected, also i.t.. Mechanical nociceptive

threshold was measured before and 5 d after the administration of morphine (immediately before the experiment), and 30 min and 4 h after $PGE_2$ injection. No difference between pre- and post-morphine mechanical thresholds was observed (data not shown). In all groups, robust mechanical hyperalgesia was observed 30 min after i.t. $PGE_2$. 2-way repeated measures ANOVA revealed significant interaction of time with treatment (*0.03 mg/kg:* $F_{2,15}$ = 13.67, $p < 0.0004$; *3 mg/kg:* $F_{2,15}$ = 25.63, $p = 0.0001$) and a significant effect of treatment for *3 mg/kg* ($F_{2,15}$ = 5.78, $p = 0.0137$) but not for *0.03 mg/kg* ($F_{2,15}$ = 3.26, $p = 0.0668$), and a significant effect of time (*0.03 mg/kg:* $F_{1,15}$ = 18.12, $p = 0.0007$; *3 mg/kg*: $F_{1,15}$ = 15.47, $p = 0.0013$). Bonferroni's multiple comparison shows that for both morphine 0.03 and 3 mg/kg-treated groups there was no attenuation in the control and the SU6656+U0126 groups, but a significantly lower $PGE_2$-induced hyperalgesia in the cordycepin-treated group ($^{****}p < 0.0001$). Lower panels: When $PGE_2$ was injected again, at the same site, one week later, 2-way repeated measures ANOVA revealed a significant interaction of time at all values of treatment (*0.03 mg/kg:* $F_{1,10}$ = 18.29, $p < 0.0016$; *3 mg/kg:* $F_{1,10}$ = 33.98, $p$ = 0.0002), a significant effect of treatment (*0.03 mg/kg:* $F_{1,10}$ = 33.68, $p = 0.0002$; *3 mg/kg:* $F_{1,10}$ = 28.39, $p = 0.0003$) and time (*0.03 mg/kg:* $F_{1,10}$ = 18.52, $p < 0.0016$; *3 mg/kg:* $F_{1,10}$ = 23.65, $p < 0.0007$). Bonferroni's multiple comparison shows that for both doses of morphine there was no attenuation in the control, but a significantly lower $PGE_2$-induced hyperalgesia at the 4th h in the cordycepin-treated group ($^{****}p < 0.0001$).

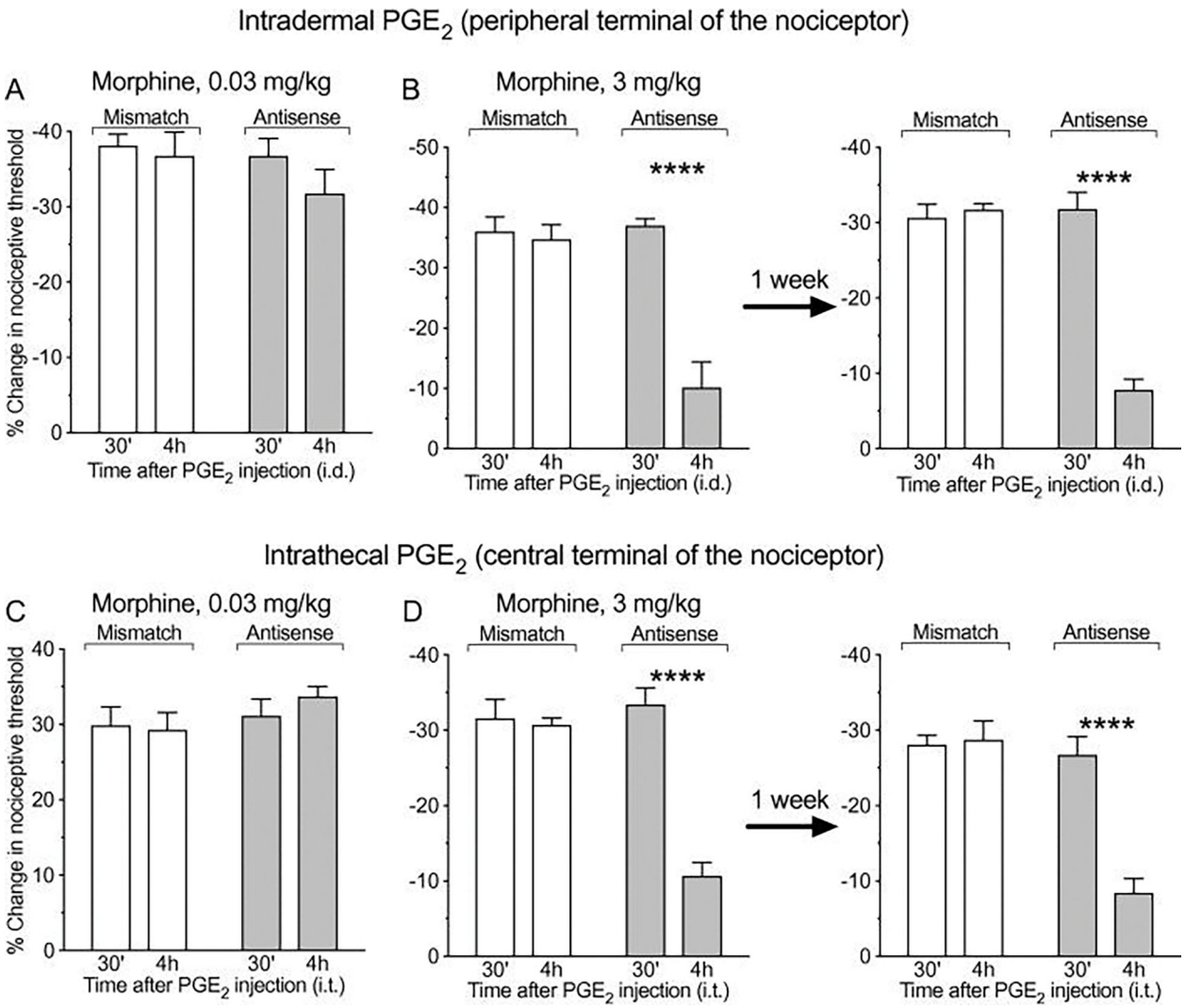


**Fig. 6.**

Role of nociceptor MOR in the induction of priming by systemic morphine. Male rats (370–410 g) were treated for 6 consecutive days with i.t. injections of ODN antisense or mismatch against MOR mRNA (120 μg/day). Mechanical nociceptive threshold was not significantly different pre- and post-ODN treatment (data not shown). In all groups, robust mechanical hyperalgesia was observed 30 min after i.t. $PGE_2$. Upper panels *(Peripheral terminal):*For 0.03 mg/kg morphine, 2-way repeated measures ANOVA revealed no significant interaction of time with treatment ($F_{1,10} = 0.63$, $p = 0.4452$), treatment ($F_{1,10} = 1.88$, $p = 0.1998$), or time ($F_{1,10} = 1.17$, $p = 0.3054$). However, for 3 mg/kg morphine, 2-way repeated measures ANOVA revealed significant interaction of time with treatment ($F_{1,10} = 22.70$, $p = 0.0008$), treatment ($F_{1,10} = 19.28$, $p = 0.0014$), or time ($F_{1,10} = 22.84$, $p = 0.0007$). One week later in the 3 mg/kg-treated group, 2-way repeated measures ANOVA revealed significant interaction of time with treatment ($F_{1,10} = 44.73$, $p < 0.0001$), treatment ($F_{1,10} = 36.69$, $p = 0.0001$), or time ($F_{1,10} = 58.74$, $p < 0.0001$). Lower panels *(Central terminal):* For 0.03 mg/kg morphine, 2-way repeated measures ANOVA revealed no significant interaction of time with treatment ($F_{1,10} = 0.56$, $p = 0.4703$), treatment ($F_{1,10} = 1.86$, $p = 0.2021$), or

time ($F_{1,10}$ = 0.20, $p$ = 0.6607). However, for 3 mg/kg morphine, 2-way repeated measures ANOVA revealed significant interaction of time with treatment ($F_{1,10}$ = 26.47, $p$ = 0.0004), treatment ($F_{1,10}$ = 18.39, $p$ = 0.0016), or time ($F_{1,10}$ = 42.48, $p$ < 0.0001). One week later in the 3 mg/kg-treated group, 2-way repeated measures ANOVA revealed significant interaction of time with treatment ($F_{1,10}$ = 33.85, $p$ = 0.0002), treatment ($F_{1,10}$ = 44.18, $p$ < 0.0001), or time ($F_{1,10}$ = 12.76, $p$ = 0.0051).