Neuropharmacology 171 (2020) 108110

Contents lists available at ScienceDirect

Neuropharmacology

journal homepage: www.elsevier.com/locate/neuropharm

In vitro and in vivo pharmacological characterization of the synthetic opioid MT-45

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HIGHLIGHTS

- MT-45 behaves as a potent selective mu agonist in DMR assay.
- MT-45 and morphine dose-dependently increased mechanical and thermal analgesia.
- MT-45 progressively impaired the motor-sensorimotor responses in mice as morphine.
- MT-45 altered cardiorespiratory responses in mice at higher doses.

ARTICLE INFO

Keywords:
MT-45
Morphine
Naloxone
DMR assay
Novel psychoactive substances
Synthetic opioids

ABSTRACT

MT-45 is a synthetic opioid that was developed in the 1970s as an analgesic compound. However, in recent years MT-45 has been associated with multiple deaths in Europe and has been included in the class of novel psychoactive substances known as novel synthetic opioids (NSOs). Little is known about the pharmaco-toxicological effects of MT-45. Therefore, we used a dynamic mass redistribution (DMR) assay to investigate the pharmacodynamic profile of this NSO in vitro compared with morphine. We then used in vivo studies to investigate the effect of the acute systemic administration of MT-45 (0.01–15 mg/kg i.p.) on motor and sensorimotor (visual, acoustic and tactile) responses, mechanical and thermal analgesia, muscle strength and body temperature in CD-1 male mice. Higher doses of MT-45 (6–30 mg/kg i.p.) were used to investigate cardiorespiratory changes (heart rate, respiratory rate, SpO_2 saturation and pulse distention). All effects of MT-45 were compared with those of morphine. In vitro DMR assay results demonstrated that at human recombinant opioid receptors MT-45 behaves as a potent selective mu agonist with a slightly higher efficacy than morphine. In vivo results showed that MT-45 progressively induces tail elevation at the lowest dose tested (0.01 mg/kg), increased mechanical and thermal antinociception (starting from 1 to 6 mg/kg), decreased visual sensorimotor responses (starting from 3 to 6 mg/kg) and reduced tactile responses, modulated motor performance and induced muscle rigidity at higher doses (15 mg/kg). In addition, at higher doses (15–30 mg/kg) MT-45 impaired the cardiorespiratory functions. All effects were prevented by the administration of the opioid receptor antagonist naloxone. These findings reveal the risks associated with the ingestion of opioids and the importance of studying these drugs and undertaking more clinical studies of the current molecules to better understand possible therapeutic interventions in the case of toxicity.

Abbreviations: MT-45, 1-cyclohexyl-4-(1,2-diphenylethyl)piperazine; Morphine, (4R,4aR,7S,7aR,12bS)-3-methyl-2,4,4a,7,7a,13-hexahydro-1H-4,12-methanobenzofuro[3,2-e]isoquinoline-7,9-diol; NSO, Novel Synthetic Opioids; Naloxone, (4R,4aS,7aR,12bS)-4a,9-dihydroxy-3-prop-2-enyl-2,4,5,6,7a,13-hexahydro-1H-4,12-methanobenzofuro[3,2-e]isoquinolin-7-one

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https://doi.org/10.1016/j.neuropharm.2020.108110
Received 23 December 2019; Received in revised form 31 March 2020; Accepted 15 April 2020
Available online 25 April 2020

1. Introduction

Designer drugs have become a major public issue worldwide. These substances are known as novel psychoactive substances (NPSs), synthetic alternatives to traditional drugs of abuse, such as cannabis, cocaine, morphine and heroin, that are specifically designed to evade international drug controls and laws. More than 730 new psychoactive substances have been identified by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), 55 of which were detected for the first time in Europe in 2018 (EMCDDA, 2019). The NPS market includes a wide range of drugs, such as synthetic cannabinoids, stimulants, novel synthetic opioids (NSOs), phenethylamines, dissociative anaesthetics and benzodiazepines (EMCDDA, 2019).

The group of NSOs is involved in most lethal overdoses not only in Europe but also in North America, which is currently facing an opioid epidemic (Increases in drug and opioid overdose deaths—United States, 2000–2014 http://www.cdc.gov/mmwr). Overall, 49 NSOs have been detected on Europe's drug market since 2009 (EMCDDA, 2019), including fentanyl, its analogues used in medical therapy (e.g. sufentanil, alfentanil and remifentanil; Lemmens, 1995), novel non-pharmaceutical fentanyl derivatives (e.g. ocfentanil, furanylfentanyl, acetylfentanyl, carfentanil, acryloylfentanyl, tetrahydrofuranylfentanyl, etc.) and other NSOs with different chemical structures, such as U-47,700, U-51,754, AH-7921 and MT-45 (Prekupec et al., 2017; Zawilska, 2017; Armenian et al., 2018; Solimini et al., 2018). Despite the popularity of fentanyl and its derivatives, the recreational abuse of non-fentanyl compounds is becoming a serious problem in terms of fatalities and abuse. In fact, recent reports of abuse highlight the emerging trend of the use of these NSOs, particularly MT-45 (Schneir et al., 2017; Domanski et al., 2017; Frisoni et al., 2018).

Commonly known by the abbreviation MT-45, 1-cyclohexyl-4-(1,2-diphenylethyl) piperazine is a NSO that is chemically distinct from other opioid agonists, with an N,N′-disubstituted piperazine (Fig. 1). It is one of a series of 1-(1,2-diphenylethyl) piperazine analgesics created in the early 1970s by the Dainippon Pharmaceutical Company in Japan as an alternative to morphine (Natsuka et al., 1978; Natsuka et al., 1987). It is sometimes known by the abbreviation I–C6 (Natsuka et al., 1978; Helander et al., 2014). MT-45 contains an asymmetry centre; therefore, it is a chiral molecule. Scientific studies have shown that the modes of action of MT-45 and its enantiomorphs are somewhat different from those of morphine (Nakamura and Shimizu, 1976; Fujimura et al., 1978). The stereoisomeric composition of the MT-45 on the European drug market is unknown, but evidence from Japan suggests that the products sold in Europe are most likely racemic (EMCDDA, 2015).

In 2014, a Japanese study by Kikura-Hanajiri and colleagues classified MT-45 as a new type of psychoactive substances (Kikura-Hanajiri et al., 2014). It has been found in Japan, the United States and Europe (Sweden, Belgium and Germany) and has been sold online as a 'research chemical'(ECDD:Expert Committee on Drug Dependence Thirty-seventh Meeting Geneva, 2015)

The administration routes of MT-45 are typically oral or by nasal aspiration but also include intravenous, sublingual, intrarectal and inhaled (vaporised) administration. Typical doses reported by users are 15–30 mg for insufflation and 25–75 mg for oral administration; in particular, light oral doses range from 30 to 45 mg, typical oral doses range from 45 to 60 mg and strong oral doses are > 60 mg. The onset of action is 30–45 min, the duration of effects is 4–6 h and the duration of after effects is 2–3 h (Zawilska and Andrzejczak, 2015; Zawilska, 2017).

The most common desired effects reported by users on internet discussion forums include getting 'high', feeling sedated, euphoria, feeling 'trippy', having a sense of well-being, 'quietness of mind', feeling 'drunk' and disorientated and 'feeling a strange mixture of simultaneous peace and irritation'. However, unwanted effects, including itchiness, dizziness, nausea, vomiting, insomnia, respiratory depression, incoordination, muscle twitches, anxiety, sweating and disorientation have been described. Specific unwanted effects include 'nasal burn' and 'nasal drip' (after nasal insufflation) and a 'bitter taste' (after oral ingestion). Some users have also reported 'withdrawal symptoms' after use, including restlessness, dehydration and 'feeling hungover' (Siddiqi et al., 2015).A total of 18 non-fatal intoxications (12 analytically confirmed) associated with MT-45 have been reported in Sweden; patients commonly presented with opioid-like symptoms, such as a decreased level of consciousness and respiratory depression, cyanosis, miosis, neurological disturbances (paresthesia in hands and feet), difficulty gripping and coordinating hand movements, balance disturbances and vision impairment (e.g. blurred and double vision; Helander et al., 2014). In some cases, depigmentation, adverse skin symptoms and neurological disturbances, such as paresthesia, blurred vision and bilateral hearing loss, have also been reported (EMCDDA, 2015; Helander et al., 2017). Moreover, from November 2013 to July 2014, 28 deaths (all analytically confirmed) associated with MT-45 have been reported in Sweden (Helander et al., 2014; EMCDDA, 2015). In eight cases, other substances were found (contributing to death), including other opioids, benzodiazepines (authorised and unauthorised medicines), antipsychotics, antidepressants and anticonvulsants (EMCDDA, 2015). This trend of the co-consumption of drugs (especially opioids and benzodiazepines) has recently been confirmed by a fatality in Sweden involving MT-45 and etizolam (Papsun et al., 2016). Also in Sweden, MT-45 has been analytically confirmed in biological samples of two cases of acute intoxication, with one person being suspected of a crime (Coppola and Mondola, 2015).

Preclinical studies suggest that the modes of action of MT-45 and its enantiomorphs are somewhat different. Indeed, the racemate and the S (+)-isomer produced a characteristic morphine-like Straub tail, increased core temperature (~1 °C) in rats when given at a dose of 10 mg/kg s.c and caused respiratory depression by 59% and 57% at 1 mg/kg, respectively. However, the R(-)-isomer had no effect up to 5 mg/kg (Nakamura and Shimizu, 1976; ECDD, 2015). It has also been reported that MT-45 and its enantiomers reproduce analgesic activity as potent as that of morphine except for chemical pain, which was less potent in mice. In rats, MT-45 was more effective than morphine against mechanical pain but less effective against thermal pain (Nakamura and Shimizu, 1976; ECDD, 2015). However, the hyperglycemic and miotic activities of MT-45 and its S (+)-isomer in rabbits were negligible or very low, although they showed potent morphine-like activity (ECDD, 2015).

More recently, it has been reported that racemic MT-45 at 6 mg/kg produces similar effects as morphine in mechanical analgesia and respiratory depression, suggesting a similar potential for acute toxicity (Montesano et al., 2017). Otherwise, in vivo and in vitro studies of NSOs are very limited, and some conflicting results have been reported. For example, in a very recent pharmacological study of NSOs, Baumann and colleagues reported that MT-45 displayed lower binding affinity than morphine (44 nM vs. 5 nM). Interestingly, however, in vivo use of the tail flick test showed MT-45 to be equipotent to morphine in inducing analgesic effects (Baumann et al., 2018).

The aim of this study is therefore to investigate the pharmacodynamic profile of the synthetic opioid MT-45 compared with morphine. A dynamic mass redistribution (DMR) assay was used in vitro; this

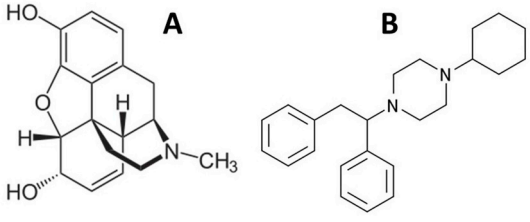

Fig. 1. Chemical structures of morphine (A) and MT-45 (1-Cyclohexyl-4-(1,2-diphenylethyl) piperazine; B).

label-free approach is based on optical biosensor technology (Grundmann and Kostenis, 2015; Schroder et al., 2011) that allows to measure receptor-dependent cellular responses as wavelength shift of an incident light in real time. Using the resonant waveguide grating, DMR measures changes in the refractive index of the bottom portion of the cell layer. The refractive index depends on several different intracellular events including protein recruitment, receptor internalization and recycling, second messenger alternation, cytoskeletal remodeling, and cell adhesion changes. DMR studies have been already performed to investigate the pharmacological profile of several GPCRs (Schroder et al., 2011), including opioid receptors (Codd et al., 2011; Morse et al., 2011, 2013). Moreover, we investigated the acute effects of MT-45 in vivo on motor and sensorimotor responses (to visual, acoustic and tactile stimulation), acute mechanical and thermal analgesia, muscle strength, body temperature and cardiorespiratory changes (heart rate, respiratory rate, SpO_2 saturation and pulse distention) in CD-1 male mice. All the MT-45 effects were compared with those of morphine (at the same doses) and were monitored for over 5 h. Opioid receptor specificity was investigated in vivo using naloxone pretreatment for all the tests.

2. Materials and Methods

2.1. In vitro studies

2.1.1. Drugs and reagents

MT-45 and morphine were purchased from LGC Standards (Sesto San Giovanni, Milan, Italy. Naloxone was purchased from Tocris (Bristol, UK). Bovine serum albumin (BSA) and 4-(2- hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were purchased from Sigma Aldrich (St. Louis, MO, USA). Stock solutions of DPDPE, morphine, dynorphin A and dermorphin were solubilised in bidistilled water at a final concentration of 1 mM. MT-45 was solubilised in DMSO at a final concentration of 10 mM and kept at −20 °C until use. Serial dilutions were made in the assay buffer (Hanks' Balanced Salt solution (HBSS)/HEPES 20 mM buffer containing 0.01% BSA and 0.1% DMSO).

2.1.2. Cells

Chinese hamster ovary (CHO) cells stably expressing the human mu or kappa (provided by L Toll (Torrey Pines Institute for Molecular Studies, Port St. Lucie, USA) or, delta (provided by E Varga (The University of Arizona, USA), opioid receptors were maintained in culture medium consisting of Dulbecco's Modified Eagle Medium (DMEM): F12(1:1) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine and geneticin (G418, 200 μg/ml). The cells were cultured at 37 °C in 5% CO_2 humidified air.

2.1.3. Dynamic mass redistribution assay

When confluence was reached, cells were sub-cultured as required using trypsin/EDTA and were used in experiments. Cells were seeded into Enspire™-LC 384-well fibronectin-coated plates and cultured for 20 h to form a confluent monolayer in the cell culture medium. Cells were seeded at a density of 20,000 cells/well/30 μl. On the day of the experiment, cells were manually washed twice and maintained with the assay buffer for 90 min before the DMR experiment. DMR was monitored in real time with a temporal resolution of 44 s throughout the assay. The present study was performed at 37 °C using the EnSight Multimode Plate Reader (PerkinElmer). A 5-min baseline was established. Next, compounds were added manually in a volume of 10 μl and compound-triggered DMR signals were recorded for 60 min. Maximum picometer (pm) modification (peak) was used to determine the agonist response after baseline normalisation.

2.1.4. Data analysis and terminology

All data were elaborated using Graph Pad Prism 6.0 (La Jolla, CA, USA). Concentration—response curves to agonists were fitted to the classical four-parameter logistic nonlinear regression model, as follows:

$$\text{Effect} = \text{Baseline} + \frac{(E_{max} - \text{Baseline})}{\left(1 + 10^{(LogEC_{50} - Log[compound]Hillslope)}\right)}.$$

Data are expressed as mean ± standard error of the mean (sem) of n experiments performed in duplicate and were analysed using one-way analysis of variance (ANOVA) followed by a Dunnett's test for multiple comparisons. Agonist potency was expressed as pEC_{50}, which is the negative logarithm to base 10 of the agonist molar concentration that produces 50% of the maximal possible effect of that agonist.

2.2. In vivo studies

2.2.1. Animals

Three hundred thirty-six male ICR (CD-1®) mice weighing 25–30 g (Harlan Italy; S. Pietro al Natisone, Italy) were group housed (8–10 mice per cage; floor area per animal was 80 cm^2; minimum enclosure height was 12 cm), exposed to a 12:12-h light-dark cycle (light period from 6:30 a.m. to 6:30 p.m.) at a temperature of 20–22 °C and humidity of 45–55% and were provided ad libitum access to food (Diet 4RF25 GLP; Mucedola, Settimo Milanese, Milan, Italy) and water. The experimental protocols performed in the present study were in accordance with the U.K. Animals (Scientific Procedures) Act of 1986 and associated guidelines and the new European Communities Council Directive of September 2010 (2010/63/EU), a revision of the Directive 86/609/EEC. Experimental protocols were approved by the Italian Ministry of Health (license n. 335/2016-PR) and by the Animal Welfare Body of the University of Ferrara. According to the ARRIVE guidelines, all possible efforts were made to minimise the number of animals used, to minimise the animals' pain and discomfort and to reduce the number of experimental subjects. For the overall study were used 336 mice. In the battery of behavioral tests used in the safety pharmacology studies (see material and methods) for each treatment (vehicle, 6 different MT-45 or morphine doses, 0.01, 0.1, 1, 3, 6 and 15 mg/kg, naloxone and naloxone + MT-45 or morphine) were used 8 mice (total mice used: 144); in the analysis of spontaneous locomotion in the open field test for each treatment (vehicle or 5 different MT-45 or morphine doses, 0.01, 0.1, 1, 6 and 15 mg/kg) were used 8 mice (total mice used: 96); in the cardiorespiratory studies for each treatment (vehicle, 3 different MT-45 or morphine doses, 6, 15 and 30 mg/kg, naloxone and naloxone + MT-45 or morphine) were used 8 mice (total mice used: 96).

2.2.2. Drug preparation and dose selection

Drugs were dissolved in saline solution (0.9% NaCl) that was also used as the vehicle. The opioid receptor antagonist naloxone (6 mg/kg, i.p.) was administered 15 min before MT-45 and morphine injections. Drugs were administered by intraperitoneal (i.p.) injection at a volume of 4 μl/g. The range of doses of MT-45 and morphine tested (0.01–15 mg/kg i.p.) was chosen based on our previous study (Montesano et al., 2017; ; Bilel et al., 2019a; b).

2.2.3. Behavioural studies

The effects of MT-45 and morphine were investigated using a battery of behavioural tests widely used in pharmacology safety studies for the preclinical characterization of new psychoactive substances in rodents (Vigolo et al., 2015; Ossato et al., 2015, 2018; Canazza et al., 2016; Fantinati et al., 2017; Marti et al., 2019). All experiments were performed between 8:30 a.m. and 2:00 p.m. Experiments were conducted blindly by trained observers working in pairs (Ossato et al.,

2016). Mouse behaviour (sensorimotor responses) was videotaped and analysed offline by a different trained operator who gives test scores.

2.2.3.1. Major neurological changes and aggressive response. Neurological changes in the mice, such as tail elevation, hyperreflexia, myoclonus, convulsions and aggressive responses, were evaluated as previously described (Vigolo et al., 2015; Ossato et al., 2015, 2016; Canazza et al., 2016). Neurological changes are expressed as frequency (percent of animals that develop symptoms), duration (total time in minutes), latency (time in minutes of symptom onset) and score (degree of tail elevation and number of bites connected to spontaneous and stimulated aggressiveness). Tail elevation was measured during the observation of the freely moving mice in a square area (score 0/4 not tail elevation, score 4/4 Straub tail). Spontaneous aggressive response was measured based on the number of times a mouse bit a gray cloth put in front of its snout. During the test, each mouse was free to move within the cage. In the case of stimulated aggressiveness, each mouse was manually restrained and held in a supine position following which an object was brought near the mouth. For both spontaneous and stimulated aggressive behaviour tests, a gray cloth was placed in front of the nose of each mouse 10 consecutive times (score 0/10 not aggressive, score 10/10 very aggressive).

2.2.3.2. Sensorimotor studies. We studied the voluntary and involuntary sensorimotor responses of the mice resulting from different reactions to visual, acoustic and tactile stimuli (Ossato et al., 2015).

2.2.3.2.1. Evaluation of the visual response. Visual response was verified by two behavioural tests that evaluated the ability of the mice to capture visual information when they are moving (the visual placing response) or when they are stationary (the visual object response). The *visual placing response* test is performed using a tail suspension modified apparatus able to bring the mouse towards the floor at a constant speed of 10 cm/s (Ossato et al., 2015). The downward movement of the mouse is videotaped by a camera. A frame-by-frame analysis allows one to evaluate the beginning of a mouse's reaction while it is close to the floor. When the mouse starts to react, an electronic ruler evaluates the perpendicular distance in millimetres from the eyes of the mouse to the floor. The untreated control mouse perceives the floor and prepares to come into contact with it at a distance of 27 $\pm$ 4.5 mm. The visual placing response was measured at 0, 15, 35, 70, 125, 185, 245 and 305 min post injection. A *visual object response* test was used to evaluate the ability of the mouse to see an object approaching from the front or the side, thus inducing the animal to shift or turn its head or retreat it (Ossato et al., 2015). For the frontal visual response, a white horizontal bar was moved in front of the mouse's head; the manoeuvre was repeated three times. For the lateral visual response, a small dentist's mirror was moved into the mouse's field of view in a horizontal arc until the stimulus was between the mouse's eyes. The procedure was conducted bilaterally and was repeated three times. A score of 1 was assigned if there was a reflection in the mouse movement; otherwise, a score of 0 was assigned. The total value was calculated by adding the scores obtained for the frontal and lateral visual object responses (overall score 9). The visual object response was measured at 0, 10, 30, 60, 120, 180, 240 and 300 min post injection.

2.2.3.2.2. Evaluation of acoustic response. Acoustic response measures the reflex of the mouse in response to an acoustic stimulus produced behind the animal (Koch, 1999). In particular, four acoustic stimuli of different intensities and frequencies were tested (see Ossato et al., 2015). Each sound test was repeated three times. A score of 1 was given if there was a response and a score of 0 was given if there was no response, for a total score of 3 for each sound. The acoustic total score was calculated by adding scores obtained in the four tests (overall score 12). The acoustic response was measured at 0, 10, 30, 60, 120, 180, 240 and 300 min post injection.

2.2.3.2.3. Evaluation of tactile response. The tactile response of each mouse was verified through vibrissae, pinna and corneal reflex, as previously described (Ossato et al., 2015). Data is expressed as the sum of the three above-mentioned parameters. The vibrissae reflex was evaluated by touching the vibrissae (right and left) with a thin hypodermic needle once per side. A score of 1 was given if there was a response (turning the head to the side of the touch) or a score of 0 was given if there was no response (overall score 2). The pinna reflex was assessed by touching the pavilions (left and right) with a thin hypodermic needle. First the interior pavilions and then the external pavilions were stimulated. This test was repeated twice per side. A score of 1 was given if there was a response and a score of 0 was given if there was no response (overall score 4). The corneal reflex was assessed by gently touching bilaterally the cornea of the mouse with a thin hypodermic needle and evaluating the response. A score of 1 was given if the mouse moved only its head, 2 if it only closed the eyelid and 3 if it both closed the eyelid and moved the head (overall score 6). Each tactile response was measured at 0, 10, 30, 60, 120, 180, 240 and 300 min post injection.

2.2.3.3. Evaluation of core and surface body temperature. To better assess the effects of the drugs on thermoregulation, we measured both changes in the core (rectal) and surface (ventral fur) temperature. The *core temperature* was determined using a probe (1 mm diameter) that was gently inserted after lubrication with liquid Vaseline into the rectum of the mouse (to about 2 cm) and left in position until the temperature stabilised (about 10 s; Vigolo et al., 2015). The probe was connected to a Cole Parmer digital thermometer, model 8402. The *surface temperature* was measured using a Microlife FR 1DZ1 digital infrared thermometer placed 1 cm from the surface of the abdomen (Vigolo et al., 2015). Core and surface mouse body temperatures were measured at 0, 30, 50, 85, 140, 200, 260 and 320 min post injection.

2.2.3.4. Evaluation of pain induced by a mechanical and a thermal stimulus. *Acute mechanical nociception* was evaluated using the tail pinch test (Vigolo et al., 2015). A special rigid probe connected to a digital dynamometer (ZP-50N, IMADA, Japan) was gently placed on the tail of the mouse (in the distal portion), and progressive pressure was applied. When the mouse flicked its tail, the pressure was stopped and the digital instrument recorded the maximum peak of weight supported (g/force). A cut off (500 g/force) was set to avoid tissue damage. The test was repeated three times, and the final value was calculated by averaging the three obtained scores. *Acute thermal nociception* was evaluated using the tail withdrawal test (Vigolo et al., 2015). The mouse was restrained in a dark plastic cylinder and half of its tail was dipped in 48 °C water, Then, the length of time (in s) the tail was left in the water was recorded. A cut off (15 s) was set to avoid tissue damage. Acute mechanical and thermal nociception was measured at 0, 35, 55, 90, 145, 205, 265 and 325 min post injection.

2.2.3.5. Evaluation of skeletal muscle strength (grip strength). This test was used to evaluate the skeletal muscle strength of the mice (https://www.sciencedirect.com/science/article/pii/S0014488610000191?via%3Dihub; Viaro et al., 2010).The grip-strength apparatus (ZP-50N, IMADA) is comprised of a wire grid (5 $\times$ 5 cm) connected to an isometric force transducer (dynamometer). In the grip-strength test, mice were held by their tails and allowed to grasp the grid with their forepaws. The mice were then gently pulled backward by the tail until the grid was released. The average force exerted by each mouse before losing its grip was recorded. The mean of three measurements for each animal was calculated, and the mean average force was determined. The skeletal muscle strength is expressed in gram force (gf) and was recorded and processed using IMADA ZP-Recorder software. The grip strength was measured at 0, 15, 35, 70, 125, 185, 245 and 305 min post injection.

2.2.3.6. Motor activity assessment. Alterations of motor activity induced by MT-45 and morphine were measured using the bar, the drag and the accelerod tests and an analysis of spontaneous locomotor activity (Vigolo et al., 2015; Ossato et al., 2015). In the *bar test*, the mouse's forelimbs were placed on a plastic bar (height 6 cm). The time spent on the bar was measured (immobility cut off: 20 s), and akinesia was calculated as the total time spent on the bar after three consecutive trials (total maximal time of catalepsy: 60 s; Canazza et al., 2016; Bilel et al., 2019a). The bar test was performed at 0, 20, 40, 70, 140, and 195 min post injection. In the *drag test*, the mouse was lifted by the tail, leaving the front paws on the table, and was dragged backward at a constant speed of about 20 cm/s for a fixed distance (100 cm). The number of steps performed by each paw was recorded by two different observers. For each animal, five to seven measurements were collected. The drag test was performed at 0, 45, 70, 105, 160, 220, 280 and 340 min post injection. In the *accelerod test*, the animals were placed on a rotating cylinder that automatically increases in velocity in a constant manner (0–60 rotations/min in 5 min). The time spent on the cylinder was measured. The accelerod test was performed at 0, 40, 60, 95, 150, 210, 270 and 330 min post injection. *Spontaneous locomotor activity* was measured using the ANY-maze video-tracking system (Ugo Basile, application version 4.99g Beta). The mouse was placed in a square plastic cage (60 × 60 cm) located in a sound- and light-attenuated room, and motor activity was monitored for 240 min. Four mice were monitored at the same time in each experiment. The parameters measured were distance travelled (m) and immobility time (s; an animal is considered immobile when 95% of its image remains in the same place for at least 2 s). The distance travelled and the time of immobility were analysed every 15 min for a maximum of 240 min. To avoid mice olfactory cues, cages were carefully cleaned with a diluted (5%) ethanol solution and washed with water between animal trials. All experiments were performed between 9:00 a.m. and 1:00 p.m.

2.2.3.7. Cardiorespiratory analysis. The experimental protocol to detect the cardiorespiratory parameters used in this study is designed to monitor awake and freely moving animals with no invasive instruments and with minimal handling (Ossato et al., 2018). A collar was placed around the neck of the animal; this collar has a sensor that continuously detects the heart rate, respiratory rate, oxygen saturation and pulse distention with a frequency of 15 Hz. While running the experiment, the mouse moves freely in the cage (with no access to food and water) monitored by the sensor collar using the software MouseOx Plus (STARR Life Sciences® Corp. Oakmont, PA). In the first hour, a collar was placed around the animal's neck to simulate the real one used in the test, thus minimising the possible effects of stress during the experiment. The real collar (with sensor) was then substituted, and baseline parameters were monitored for 60 min. Subsequently, the mice were given MT-45 or morphine by i.p. injection, and data was recorded for 5 h.

2.2.4. Data and statistical analysis

Core and surface temperature values are expressed as the difference between control temperature (before injection) and temperature following drug administration (Δ°C). Antinociception (tail withdrawal and tail pinch tests) and catalepsy (bar test) are calculated as the percent of maximal possible effect {EMax% = [(test - control latency)/(cut off time - control)] X 100}. Data are expressed in absolute values [min in neurological changes, meters (m) for distance travelled, s in immobility time], Δ°C (core and surface temperature), Emax% (tail withdrawal, tail pinch and bar test) and percentage of basal (drag test and accelerod test). In sensorimotor response experiments, data are expressed in arbitrary units (visual objects response; acoustic response; vibrissae, corneal and pinna reflex) or percentage of baseline (visual placing response). Data are expressed in percentage of basal value [maximal muscle strength (expressed as gf), heart rate (expressed as heart beats per min (bpm), pulse distention (vessel diameter changes expressed as μm), respiratory rate (expressed as respiratory rate per minute (rrpm) and SpO2 saturation (oxygen blood saturation expressed as %)]. The statistical analysis of the effects of the individual substances in different concentrations over time and that of antagonism studies were performed using a two-way ANOVA followed by a Bonferroni test for multiple comparisons. The statistical analysis was performed with using Prism software (GraphPad Prism, USA). All analyses were performed using GraphPad Prism software.

3. Results

3.1. In vitro studies

DMR effects on mu opioid receptor - In CHO cells stably transfected with the human mu opioid receptor, the standard agonist dermorphin evoked a robust concentration-dependent DMR response, with a pEC_{50} of 9.07 and a maximal effect of 165 ± 16 p.m. Similar effects were obtained with morphine that showed a pEC_{50} of 7.03 and a maximal effect of 128 ± 11 p.m. MT-45 mimicked the action of morphine with similar potency but greater maximal effects **(**Fig. 2; **panel A).** The shape of the DMR response to dermorphin, morphine and MT-45 was similar **(**Fig. 3**).** Importantly, when these compounds were tested in wild type CHO cells they did not elicit any significant DMR response (Table 1).

DMR effects on kappa opioid receptor - In CHO $_{kappa}$ cells, the standard

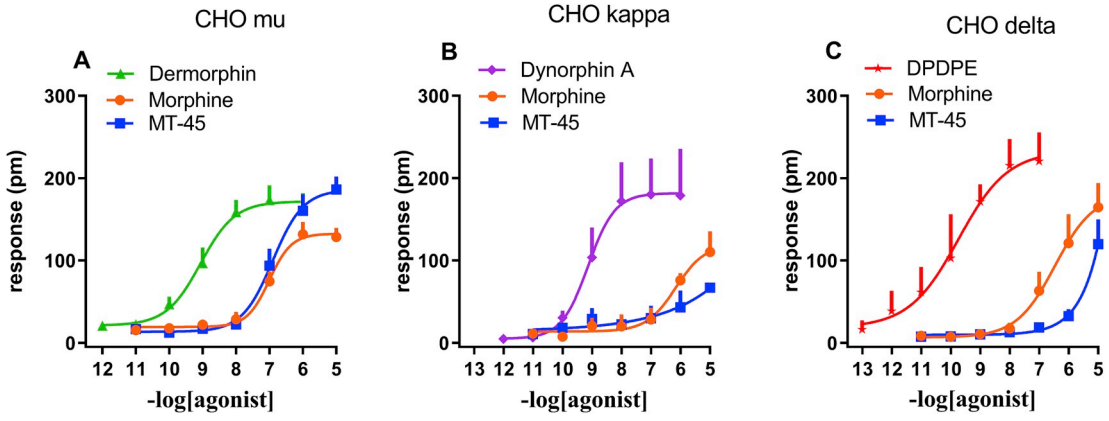

Fig. 2. Concentration response curves to dermorphin, and morphine and MT45 tested in CHO_{mu} cells (**Panel A**). Data are the mean ± s.e.m. of 7 separate experiments made in duplicate. Concentration response curve to the compounds tested in CHOkappa cells (**Panel B**). Data is represented as mean ± s.e.m. of 4 separate experiments made in duplicate. Concentration response curve to the compounds tested in CHOdelta cells (**Panel C**). Data is represented as mean ± s.e.m. of 3 separate experiments made in duplicate.

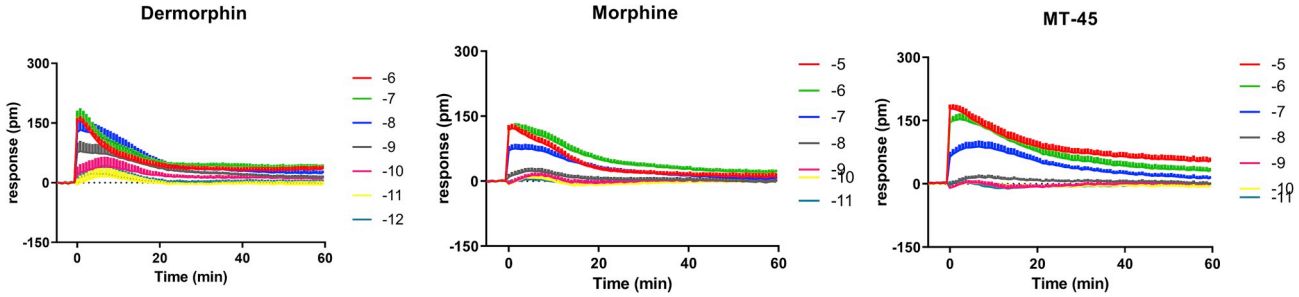

Fig. 3. Representative DMR tracings of mu agonists under investigation in CHO cells expressing mu opioid receptor.

agonist dynorphin A evoked a DMR response with a pEC_{50} of 9.08 and a maximal effect of 180 ± 55 p.m. Morphine and MT-45 were active only at micromolar concentrations (Fig. 2; **panel B)**.

DMR effects on delta opioid receptor - In CHO_{delta} cells, the standard agonist DPDPE evoked a robust concentration-dependent DMR response, with a pEC_{50} of 10.01 and a maximal effect of 220 ± 35 p.m. (Fig. 2; **panel C**). Morphine displayed a pEC_{50} of 6.5, while MT-45 was less potent showing an incomplete concentration response curve. The results of the DMR experiments are summarised in Table 2.

3.2. Behavioural studies

3.2.1. Major neurological changes

Neurological changes, such us convulsions, hyperreflexia, myoclonia and aggressive responses, were not observed after the administration of MT-45 and morphine (0.01–15 mg/kg; data not shown). Both MT-45 and morphine induced tail elevation in mice, and MT-45 was more potent than morphine (Table 3). In fact, MT-45 induced tail elevation at 0.01 and 0.1 mg/kg, while morphine was not effective at the same doses. The pre-treatment with naloxone at 6 mg/kg prevented the tail elevation caused by MT-45 and morphine (6 mg/kg; data not shown).

3.2.2. Sensorimotor studies

3.2.2.1. Evaluation of the visual object response. The visual object response was not affected in mice treated with the vehicle (Fig. 4).

Systemic administration of MT-45 and morphine (0.01–15 mg/kg) decreased the visual object responses of mice. After administration of MT-45, the visual object response was significantly affected by treatment [F6,992 = 115.3; p < 0.0001], time [F7,392 = 12.787; p < 0.001] and treatment × time interaction [F42,392 = 4.678; p < 0.0001] (Fig. 4A). Likewise, the visual object response of mice injected with morphine was also significantly affected by treatment [F6,992 = 305.7; p < 0.0001], time [F7,392 = 94.73; p < 0.0001] and treatment × time interaction [F42,392 = 27.17; p < 0.0001]. The impairment of the visual object responses of the mice induced by both MT-45 and morphine (6 mg/kg) was prevented by pre-treatment with 6 mg/kg naloxone [Fig. 4; **Panel C**: significant effect of treatment ($F_{3,224}$ = 47.54, p < 0.0001), time ($F_{7,224}$ = 15.38, p < 0.0001) and time × treatment interaction ($F_{21,224}$ = 12.72, p < 0.0001). **Panel D**: significant effect of treatment ($F_{3,224}$ = 28.72, p < 0.0001), time ($F_{7,224}$ = 14.76, p < 0.0001) and time × treatment interaction ($F_{21,224}$ = 6.283, p < 0.0001)].

The comparison of the mean effect of MT-45 and morphine in the visual object test, revealed a higher potency of MT-45 respect to morphine only at the dose of 15 mg/kg [significant effect of treatment [$F_{1,84}$ = 5.808, p = 0.0181], dose [$F_{5,84}$ = 47.40, p < 0.0001] and dose × treatment interaction [$F_{5,84}$ = 3.935, p = 0.0030] (Fig. 4 E)].

3.2.2.2. Evaluation of the visual placing response. The visual placing response decreased slightly in the last hours of measurement in mice treated with the vehicle (Fig. 5). The systemic administration of MT-45 and morphine (0.01–15 mg/kg) reduced the visual placing response in mice in a dose-dependent manner. In particular, after administration of MT-45, the visual placing response was significantly affected by treatment [$F_{6,392}$ = 61.32, p < 0.0001], time [$F_{7,392}$ = 13.78, p < 0.0001] and treatment × time interaction [$F_{42,392}$ = 2.112, p = 0.0001] (Fig. 5A). Likewise, the visual placing response of mice injected with morphine was also significantly affected by treatment [$F_{6,392}$ = 27.41, p < 0.0001], time [$F_{7,392}$ = 10.62, p < 0.0001] and treatment × time interaction [$F_{42,392}$ = 1.655, p = 0.0081].The pre-treatment with 6 mg/kg naloxone prevented the impairment of the visual placing response caused by MT-45 and morphine (6 mg/kg) [Fig. 5; **Panel C:** significant effect of treatment ($F_{3,224}$ = 6.431, p = 0.0003), time ($F_{7,224}$ = 3.975, p = 0.0004) and time × treatment interaction ($F_{21,224}$ = 1.539, p = 0.0665). **Panel D**: significant effect of treatment ($F_{3,224}$ = 58.10 p < 0.0001), time ($F_{7,224}$ = 10.86, p < 0.0001) and time × treatment interaction ($F_{21,224}$ = 1.913, p = 0.0113)].

The comparison of the mean effect of MT-45 and morphine in the visual placing test, revealed a similar potency of the two compounds [significant effect of treatment [$F_{1,84}$ = 4.145, p = 0.0449], dose [$F_{5,84}$ = 9.671, p < 0.0001] and dose × treatment interaction [$F_{5,84}$ = 0.988, p = 0.4298] (Fig. 5 E)].

3.2.2.3. Evaluation of the tactile response. The overall tactile responses (pinna, vibrissae, corneal) did not change in vehicle-treated mice over the 5-h observation (Fig. 6). The systemic administration of MT-45 and morphine (0.01–15 mg/kg) decreased the overall tactile responses in mice only at the highest dose tested. In particular, after the administration of 15 mg/kg MT-45, the tactile response was significantly affected by treatment [$F_{6,392}$ = 16.22, p < 0.0001], time [$F_{7,392}$ = 1.892, p = 0.0695] and time × treatment interaction [$F_{42,392}$ = 1.480, p = 0.0316] (Fig. 6A). Similarly, the tactile response of mice treated with the same dose of morphine was also significantly affected by treatment [$F_{6,392}$ = 2.865, p = 0.0096], time ($F_{7,392}$ = 0.6749, p = 0.6934) and time × treatment interaction [$F_{42,392}$ = 0.7260, p = 0.8982] (Fig. 6B); but the effect seemed to be shorter and delayed compared to that induced by MT-45. The pre-treatment with 6 mg/kg naloxone prevented the impairment caused by MT-45 and morphine (15 mg/kg) of the overall tactile responses [Fig. 6; **Panel C**: significant effect of treatment ($F_{3,224}$ = 18.50, p < 0.0001), time ($F_{7,224}$ = 1.420, p = 0.1982) and time × treatment interaction

Table 1
Effects of high concentrations (1 μM for dermorphin, 10 μM for all other compounds) of mu receptor agonists in CHO mu and CHOWT cells in the DMR assay.

	CHO_{mu} pm ± sem	CHO_{WT} pm ± sem
Dermorphin	165 ± 16*	6 ± 4
Morphine	128 ± 11*	10 ± 6
MT-45	186 ± 16*	15 ± 10
Buffer	17 ± 5	49 ± 20

Table 2
Potencies (pEC_{50}) and maximal effects of standard agonists (dermorphin, dynorphin A, and DPDPE for mu, kappa, and delta receptors, respectively), morphine and MT-45 on CHO expressing opioid receptors in the DMR assay. Data are mean of at least 3 experiments performed in duplicate.

	CHO_{mu}		CHO_{kappa}		CHO_{delta}	
Compounds	**pEC_{50} ($CL_{95\%}$)**	**E_{max} ± sem**	**pEC_{50} ($CL_{95\%}$)**	**E_{max} ± sem**	**pEC_{50} ($CL_{95\%}$)**	**E_{max} ± sem**
Standard	9.07 (8.83–9.31)	165 ± 16	9.08 (8.59–9.57)	180 ± 55	10.01 (7.68–12.34)	220 ± 35
Morphine	7.03 (6.72–7.34)	128 ± 11*	6.08 (5.93–6.23)	134 ± 13	6.50 (5.38–7.62)	164 ± 29
MT-45	6.74 (6.37–7.11)	186 ± 16	Crc incomplete		Crc incomplete	

($F_{21,224}$ = 1.420, p = 0.1102). **Panel D**: significant effect of treatment ($F_{3,224}$ = 3.240 p = 0.0229), time ($F_{7,224}$ = 0.6800, p = 06889) and time × treatment interaction ($F_{21,224}$ = 0.6800, p = 0.8508)].

3.2.2.4. Evaluation of the acoustic response. Systemic administration of MT-45 and morphine (0.01–15 mg/kg) does not affect the acoustic responses in mice (data not shown).

3.2.3. Evaluation of the core and surface body temperatures

Systemic administration of MT-45 and morphine (0.01–15 mg/kg) did not affect the core and surface body temperatures of mice (data not shown).

3.2.4. Evaluation of skeletal muscle strength

Muscle strength was not affected in mice treated with the vehicle (Fig. 7). The systemic administration of MT-45 and morphine (0.01–15 mg/kg) increased the pulling force only at the highest dose tested. In particular, after the administration of MT-45 (15 mg/kg), the skeletal muscle strength was significantly affected by treatment [$F_{6,392}$ = 30.25, p < 0.0001], time [$F_{7,392}$ = 3.025, p = 0.0041] and time × treatment interaction [$F_{42,392}$ = 0.9888, p = 0.4948] (Fig. 7A). Similarly, the skeletal muscle strength of mice injected with morphine (15 mg/kg) was also significantly affected by treatment [$F_{6,392}$ = 21.17, p < 0.0001], time [$F_{6,343}$ = 3.387, p = 0.016] and time × treatment interaction [$F_{42,392}$ = 0.9657, p = 0.5355] (Fig. 7B), but the effect seemed to be delayed compared to that induced by the same dose of MT-45. The pre-treatment with 6 mg/kg naloxone prevented the changes in skeletal muscle force caused by MT-45 and morphine at 15 mg/kg [Fig. 7; **Panel C**: significant effect of treatment ($F_{3,224}$ = 63.10, p < 0.0001), time ($F_{7,224}$ = 3.016, p = 0.0048) and time × treatment interaction ($F_{21,224}$ = 1.814, p = 0.0185). **Panel D**: significant effect of treatment ($F_{3,224}$ = 52.88 p < 0.0001), time ($F_{7,224}$ = 1.945, p = 0.0636) and time × treatment interaction ($F_{21,224}$ = 1.840, p = 0.0163)].

3.2.5. Evaluation of pain induced by mechanical and thermal stimuli

Acute mechanical and thermal pain stimuli were not affected in mice treated with the vehicle (Figs. 8 and 9). The systemic administration of MT-45 and morphine (0.01–15 mg/kg) increased the threshold to *acute mechanical pain stimulus* in mice in the tail pinch test. In particular, after the administration of MT-45, the mechanical analgesia was significantly affected by treatment [$F_{6,343}$ = 128.4, p < 0.0001], time [$F_{6,343}$ = 12.82, p < 0.0001] and time × treatment interaction [$F_{36,343}$ = 2.019, p = 0.0007] (Fig. 8A). Likewise, the mechanical analgesia in mice injected with morphine (0.01–15 mg/kg) was significantly affected by treatment [$F_{6,343}$ = 239.0, p < 0.0001], time [$F_{6,343}$ = 42.78, p < 0.0001] and time × treatment interaction [$F_{36,343}$ = 6.533, p < 0.0001] (Fig. 8B).The pre-treatment with 6 mg/kg naloxone inhibited the analgesic effect of both compounds [Fig. 8; **Panel C**: significant effect of treatment ($F_{3,196}$ = 438.8 p < 0.0001), time ($F_{6,196}$ = 8.844, p < 0.0001) and time × treatment interaction ($F_{18,196}$ = 7.263, p < 0.0001). **Panel D**: significant effect of treatment ($F_{3,196}$ = 496.4, p < 0.0001), time ($F_{6,196}$ = 11.16, p < 0.0001) and time × treatment interaction ($F_{18,196}$ = 7.305, p < 0.0001)].

The comparison of the mean effects of MT-45 (ED_{50} = 3.669 mg/kg) and morphine (ED_{50} = 4.603 mg/kg) in the tail pinch test, revealed a similar analgesic potency of the two drugs [significant effect of treatment [$F_{1,84}$ = 3.945, p = 0.0503], dose [$F_{5,84}$ = 49.36, p < 0.0001] and dose × treatment interaction [$F_{5,84}$ = 1.108, p = 0.3626] (Fig. 8 E)]

The systemic administration of MT-45 and morphine (0.01–15 mg/kg) increased the threshold to *acute thermal pain stimulus* in mice in the tail withdrawal test **(**Fig. 9 A,B**)**. In particular, after the administration of MT-45, the thermal analgesia was significantly affected by treatment [$F_{6,343}$ = 146.4, p < 0.0001], time [$F_{6,343}$ = 51.95, p < 0.0001] and time × treatment interaction [$F_{36,343}$ = 8.691, p < 0.0001] (Fig. 9A).Similarly, after the administration of morphine, the thermal analgesia was significantly affected by treatment [$F_{6,343}$ = 87.58, p < 0.0001], time [$F_{6,343}$ = 39.01, p < 0.0001] and time × treatment interaction [$F_{36,343}$ = 8.091, p < 0.0001] (Fig. 9B). The analgesic effect induced by MT-45 and morphine (6 mg/kg) was prevented by the pre-treatment with 6 mg/kg naloxone [Fig. 9; **Panel C**: significant effect of treatment ($F_{3,196}$ = 387.5 p < 0.0001), time ($F_{6,196}$ = 53.36, p < 0.0001) and time × treatment interaction ($F_{18,196}$ = 40.21, p < 0.0001). **Panel D**: significant effect of treatment ($F_{3,196}$ = 92.62, p < 0.0001), time ($F_{6,196}$ = 19.84, p < 0.0001) and

Table 3
Effect of the systemic administration of MT-45 and morphine (0.01–15 mg/kg i.p.) on the elevation tail (neurological changes) of the mouse. Data are expressed as percentage (frequency of animal with this signs), minutes (duration and latency of effect) and score (mean and max, degree of elevation connected to the elevation tail; see materials and methods), represent the mean ± SEM of 8 animals for each treatment.

Elevation tail										
Compound	MT-45					morphine				
Doses (mg/kg)	0.01	0.1	1	6	15	0.01	0.1	1	6	15
Frequency (%)	25	25	65	75	100	–	–	50	65	100
Mean score (n)	2	2 ± 2	2 ± 2	2.4 ± 1.6	2.5 ± 1.5	–	–	1	2 ± 1	2.3 ± 1.7
Max score (n)	2	4	4	4	4	–	–	1	3	4
Duration (min)	82 ± 18	121 ± 13	138 ± 15	187 ± 20	192 ± 13	–	–	121 ± 1	181 ± 15	216 ± 14
Latency (min)	59.3 ± 11	6 ± 1.2	4.8 ± 0.7	4.5 ± 1.2	3.7 ± 1	–	–	6 ± 1.2	5 ± 0.6	4.7 ± 1.1

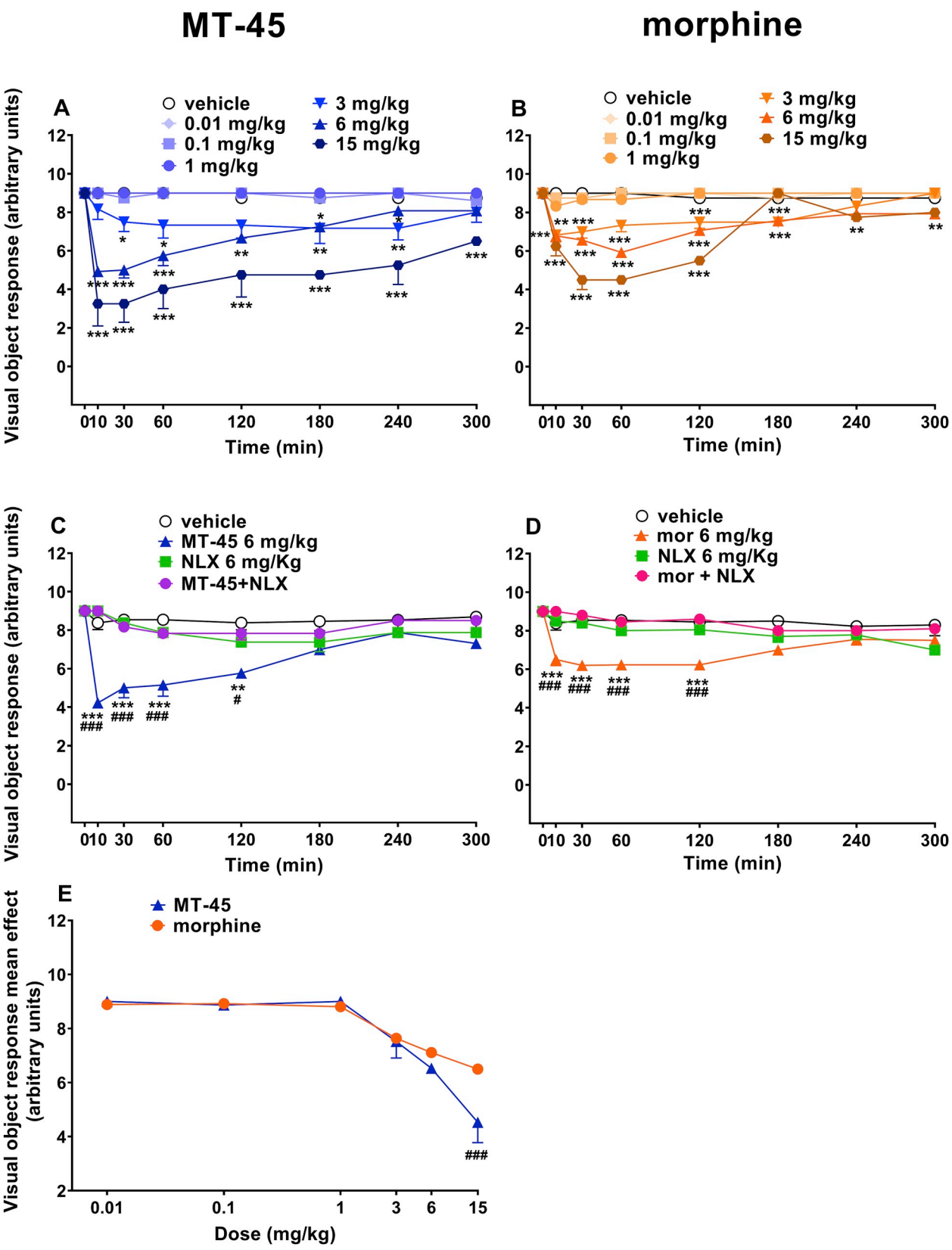

Fig. 4. Effect of the systemic administration (0.01–15 mg/kg i.p.) of MT-45 (**panel A**) and morphine (**panel B**) on the visual object test in the mouse. Interaction of effective dose of MT-45 and morphine (6 mg/kg) with the opioid receptor antagonist naloxone (6 mg/kg, i.p.; respectively **panel C and D**) and the comparison of the mean effect of the two opioids (**panel E**). Data are expressed (see material and methods) as arbitrary units and represent the mean ± SEM of 8 determinations for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons. *p < 0.05, **p < 0.01, ***p < 0.001 versus vehicle; #p < 0.05, ###p < 0.001 versus naloxone + agonist.

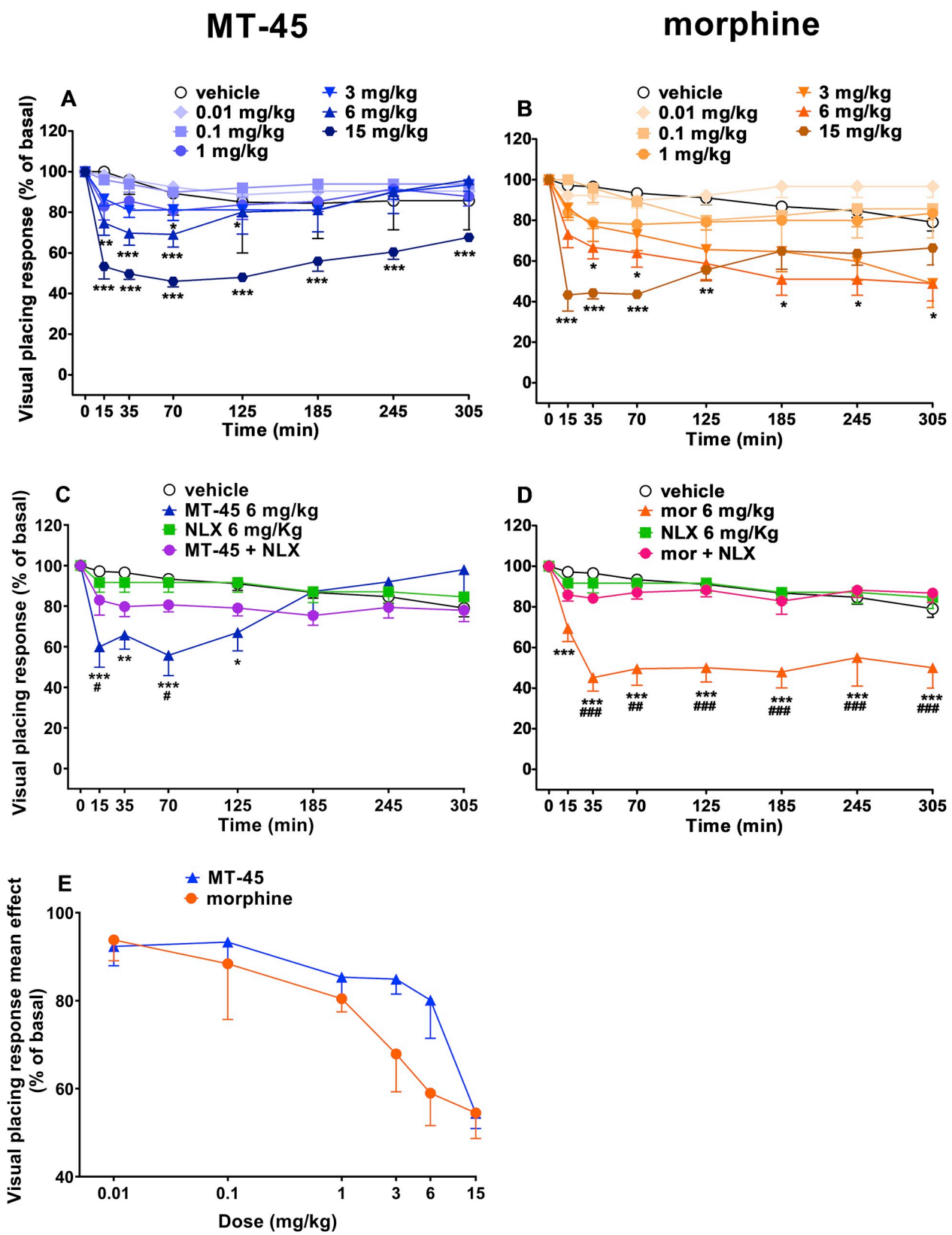

Fig. 5. Effect of the systemic administration (0.01–15 mg/kg i.p.) of MT-45 (panel A) and morphine (panel B) on the visual placing test in the mouse. Interaction of effective dose of MT-45 and morphine (6 mg/kg) with the opioid receptor antagonist naloxone (6 mg/kg, i.p.; respectively panel C and D) and the comparison of the mean effect of the two opioids (**panel E**). Data are expressed (see material and methods) as percentage of baseline and represent the mean ± SEM of 8 determinations for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons $p < 0.0001$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus vehicle; ##$p < 0.01$, ###$p < 0.001$ versus naloxone + agonist.

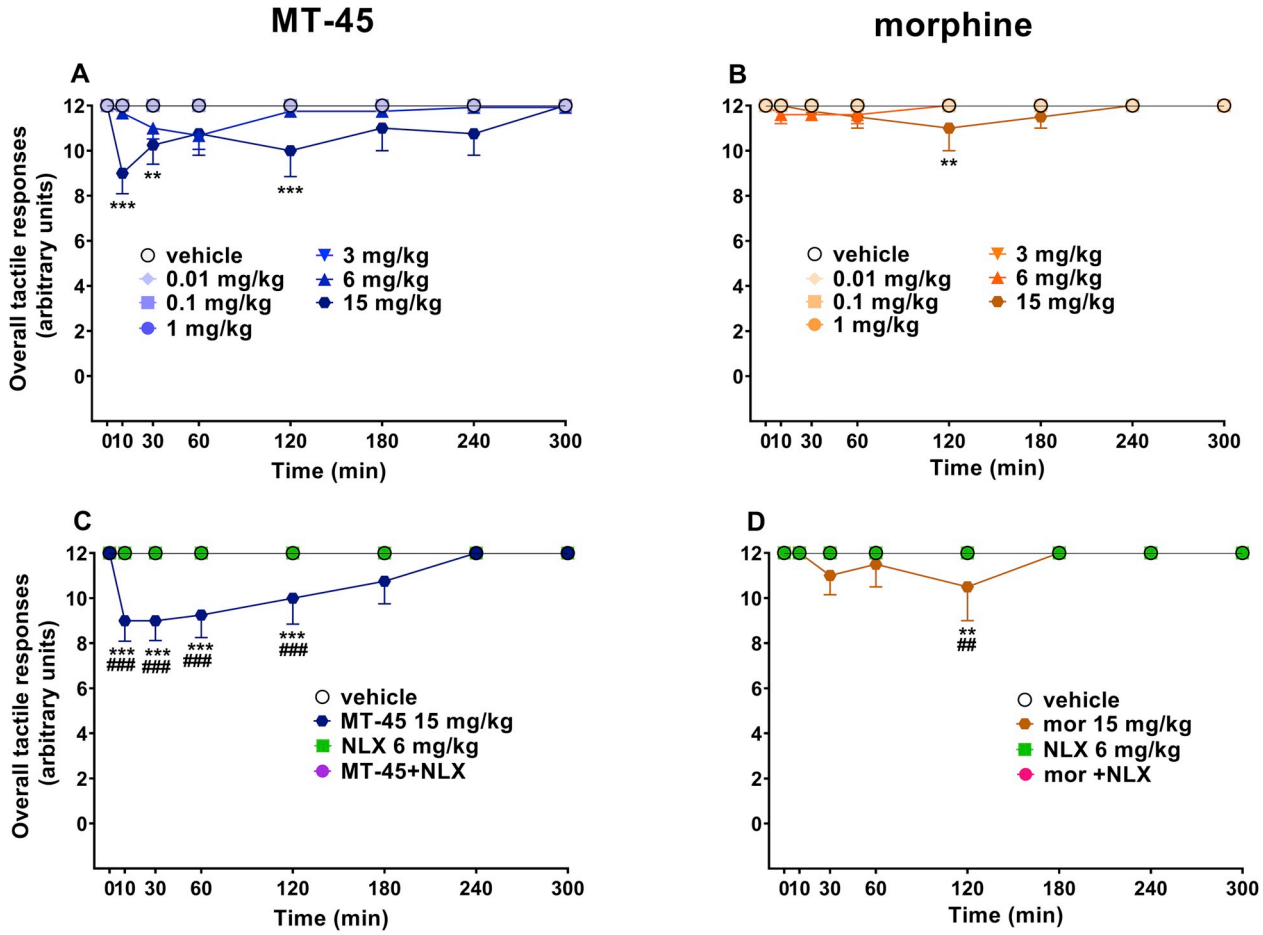

Fig. 6. Effect of the systemic administration (0.01–15 mg/kg i.p.) of MT-45 (panel A) and morphine (panel B) on the overall tactile response (vibrissae, pinna and corneal reflex; see material and methods) in the mouse. Interaction of effective dose of MT-45 and morphine (15 mg/kg) with the opioid receptor antagonist naloxone (6 mg/kg, i.p; respectively **panel C and D**) Data are expressed (see material and methods) as arbitrary units and represent the mean ± SEM of 8 determinations for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for both the dose response curve of each compounds at different times. *p < 0.05, **p < 0.01, ***p < 0.001 versus vehicle; #p < 0.05, ##p < 0.01, ###p < 0.001 versus naloxon + agonist.

time × treatment interaction ($F_{18,196}$ = 14.93, p < 0.0001)]. The comparison of the mean effect of MT-45 (ED_{50} = 11.60 mg/kg) and morphine (ED_{50} = 13.61 mg/kg) in the tail withdrawal test, revealed a similar analgesic potency of the two drugs [significant effect of treatment [$F_{1,84}$ = 0.0241, p = 0.8769], dose [$F_{5,84}$ = 40.26, p < 0.0001] and dose × treatment interaction [$F_{5,84}$ = 0.4481, p = 0.8135] (Fig. 9 E)]

3.2.6. Evaluation of motor activity

3.2.6.1. Bar test. The systemic administration of MT-45 and morphine (0.01–15 mg/kg) did not induce catalepsy in mice (data not shown).

3.2.6.2. Accelerod test. Mice treated with a vehicle did not change their performance in the accelerod test (Fig. 10). The systemic administration of MT-45 and morphine (0.01–15 mg/kg) significantly increased the performance of the mice in accelerod test only at the highest dose tested. In particular, after the administration of MT-45, the performance of mice in the accelerod was affected by the treatment [$F_{6,392}$ = 10.07, p < 0.0001], time [$F_{7,392}$ = 5.578, p < 0.0001]. and time × treatment interaction [$F_{42,392}$ = 1.145, p = 0.2546] (Fig. 10 A). Similarly, the performance of mice treated with morphine was also significantly affected by the treatment [$F_{6,392}$ = 7.970, p < 0.0001], time [$F_{7,392}$ = 3.309, p = 0.0020] and time × treatment interaction [$F_{42,392}$ = 1.052, p = 0.3886]. The pre-treatment with 6 mg/kg naloxone inhibited the motor impairment induced by the two compounds in the accelerod test [Fig. 10: **Panel C**: significant effect of treatment ($F_{3,224}$ = 14.96, p < 0.0001), time ($F_{7,224}$ = 2.765, p = 0.0089) and time × treatment interaction ($F_{21,224}$ = 1.568, p = 0.0585). **Panel D**: significant effect of treatment ($F_{3,224}$ = 13.82, p < 0.0001), time ($F_{7,224}$ = 2.171, p = 0.0378) and time × treatment interaction ($F_{21,224}$ = 3.309, p = 0.1776)].

3.2.6.3. Drag test. The systemic administration of MT-45 and morphine (0.01–15 mg/kg) caused a decrease in the number of steps performed by the front legs of mice. After the administration of MT-45 the number of steps performed by mice was significantly affected by the treatment [$F_{6,392}$ = 19.81, p < 0.0001], time [$F_{7,392}$ = 1.445, p = 0.1858] and time × treatment interaction [$F_{42,392}$ = 1.446, p = 0.0001] (Fig. 11 A).

Likewise, the number of steps of mice injected with morphine was significantly affected by the treatment [$F_{6,392}$ = 4.854, p < 0.0001], time [$F_{7,392}$ = 3.234, p = 0.0024] and time × treatment interaction [$F_{42,392}$ = 0.8778, p = 0.6897] (Fig. 11B). Inhibitory effects induced by MT-45 (15 mg/kg) and morphine (15 mg/kg) were prevented by the pre-treatment with 6 mg/kg naloxone [Fig. 11; **Panel C:** significant effect of treatment ($F_{3,224}$ = 35.21, p < 0.0001), time ($F_{7,224}$ = 1.478, p = 0.1762) and time × treatment interaction ($F_{21,224}$ = 1.643, p = 0.0418). **Panel D:** significant effect of treatment ($F_{3,224}$ = 17.09, p < 0.0001), time ($F_{7,224}$ = 1.982, p = 0.0586) and time × treatment interaction ($F_{21,224}$ = 1.504, p = 0.0776)].

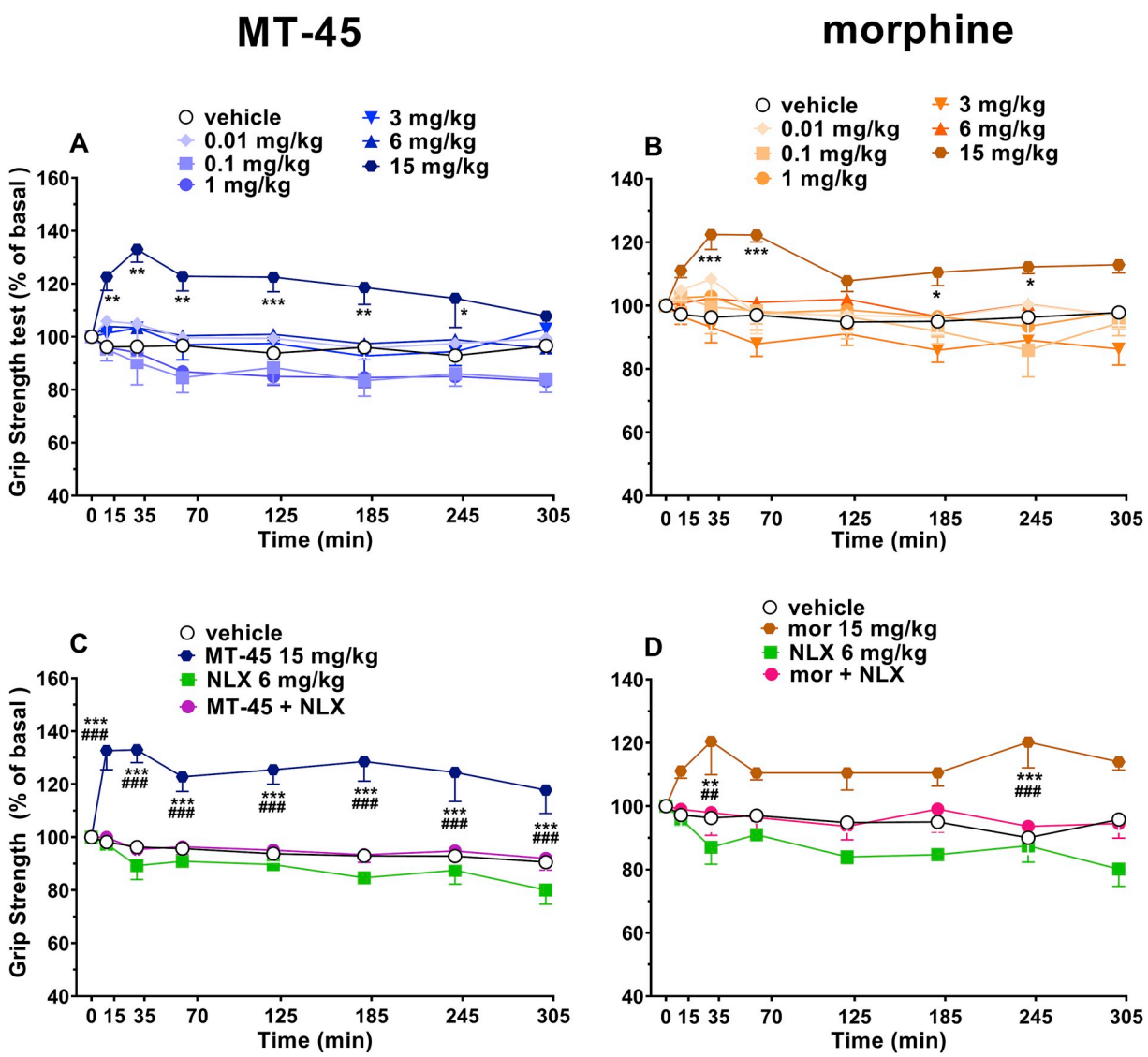

Fig. 7. Effect of the systemic administration (0.01–15 mg/kg i.p.) of MT-45 (panel A) and morphine (panel B) on the Grip strength test. Interaction of effective dose of MT-45 and morphine (15 mg/kg) with the opioid receptor antagonist naloxone (6 mg/kg, i.p.; respectively panel C and D). Data are expressed as percentage of baseline (see material and methods) and represent the mean ± SEM of 8 determinations for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons. *p < 0.05, **p < 0.01, ***p < 0.001 versus vehicle; ###p < 0.001 versus naloxone + agonist.

3.2.6.4. Spontaneous locomotor activity. To exclude the possibility that reduction of sensorimotor responses could be due to the inhibition of motor activity, we investigated the effect of MT-45 and morphine administration (0.01–15 mg/kg) on spontaneous locomotor activity in the mice (Fig. 12). After the administration of MT-45, the total distance travelled by mice was significantly affected by treatment [$F_{2,336}$ = 4.957, p = 0.0076], time [$F_{15,336}$ = 20.89, p < 0.0001] and time × treatment interaction [$F_{30,336}$ = 0.7343, p = 0.8462] (Fig. 12 A). Also, the immobility time was significantly affected by treatment [$F_{2,336}$ = 15.17, p < 0.0001], time [$F_{15,336}$ = 22.10, p < 0.0001] and time × treatment interaction [$F_{30,336}$ = 1.155, p = 0.2681] (Fig. 12C). Similarly, the total distance travelled by mice injected with morphine was significantly affected by the treatment [$F_{2,336}$ = 2.410, p = 0.0914], time [$F_{15,336}$ = 17.40, p < 0.0001] and time × treatment interaction [$F_{30,336}$ = 0.6554, p = 0.9193] (Fig. 12B) and the immobility time was also significantly affected by the treatment [$F_{2,336}$ = 0.5842, p = 0.5581], time [$F_{15,336}$ = 15.73, p < 0.0001] and time × treatment interaction [$F_{30,336}$ = 0.7808, p = 0.7909] (Fig. 12D). The effect of both opioids on the locomotor activity of mice was no longer evident during the last hour of testing. Pre-treatment with naloxone 6 mg/kg completely prevented the effects of MT-45 and morphine both at 15 mg/kg (data not shown).

3.2.7. Cardiorespiratory analysis

To investigate if the treatment with the two opioids can modify the normal cardiorespiratory pattern of mice, we used the MouseOX instrument (see Materials and Methods). The vehicle used in this experiment showed a stable profile during the 6 h of measuring the cardiorespiratory parameters (heart rate, respiratory rate, oxygen saturation and pulse distention) (Fig. 13). The systemic administration of MT-45 and morphine (6–30 mg/kg IP) induced modifications of the cardiorespiratory function of mice in a dose-dependent manner; heart rates decreased 5 min post injection with both substances. In particular, after the administration of MT-45, heart rates was significantly affected by treatment [$F_{3,2016}$ = 45.67, p < 0.0001], time [$F_{71,2016}$ = 10.62, p < 0.0001] and time × treatment interaction [$F_{213,2016}$ = 1.107, p < 0.0001] (Fig. 13 A). Likewise, the heart rate of mice injected with morphine was also significantly affected by treatment [$F_{3,2016}$ = 18.27, p < 0.0001], time [$F_{71,2016}$ = 5.957, p < 0.0001] and time × treatment interaction [$F_{213,2016}$ = 1.567, p < 0.0001] (Fig. 13 B).

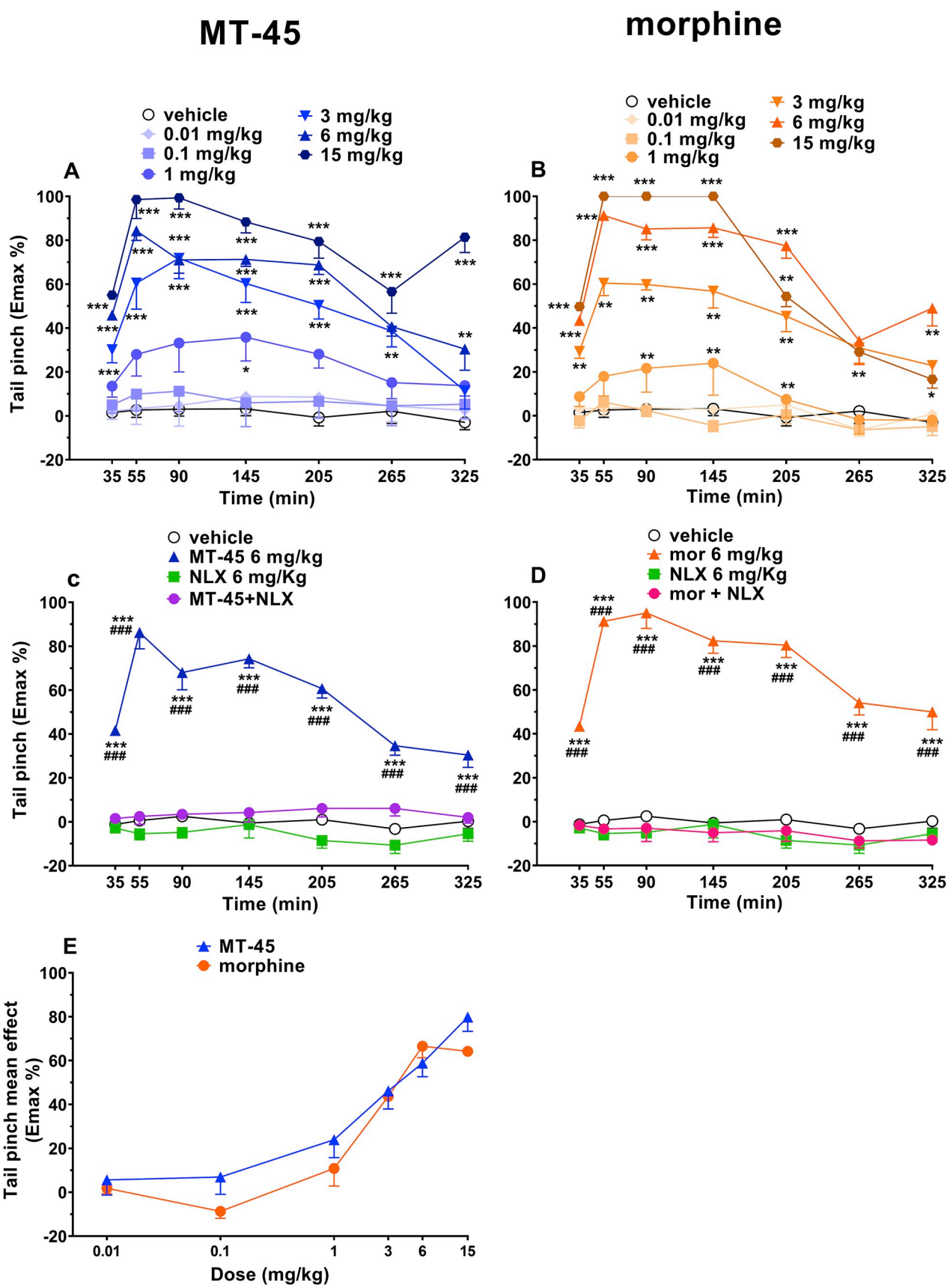

Fig. 8. Effect of the systemic administration (0.01–15 mg/kg i.p.) of MT-45 (panel A) and morphine (panel B) on the tail pinch test of the mouse. Interaction of effective dose of MT-45 and morphine (6 mg/kg) with the opioid receptor antagonist naloxone (6 mg/kg, i.p.; respectively panel C and D) and the comparison of the mean effect of the two opioids (**panel E**). Data are expressed as percentage of maximum effect (see material and methods) and represent the mean ± SEM of 8 determinations for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons. *p < 0.05, **p < 0.01, ***p < 0.001 versus vehicle; ###p < 0.001 versus naloxone + agonist.

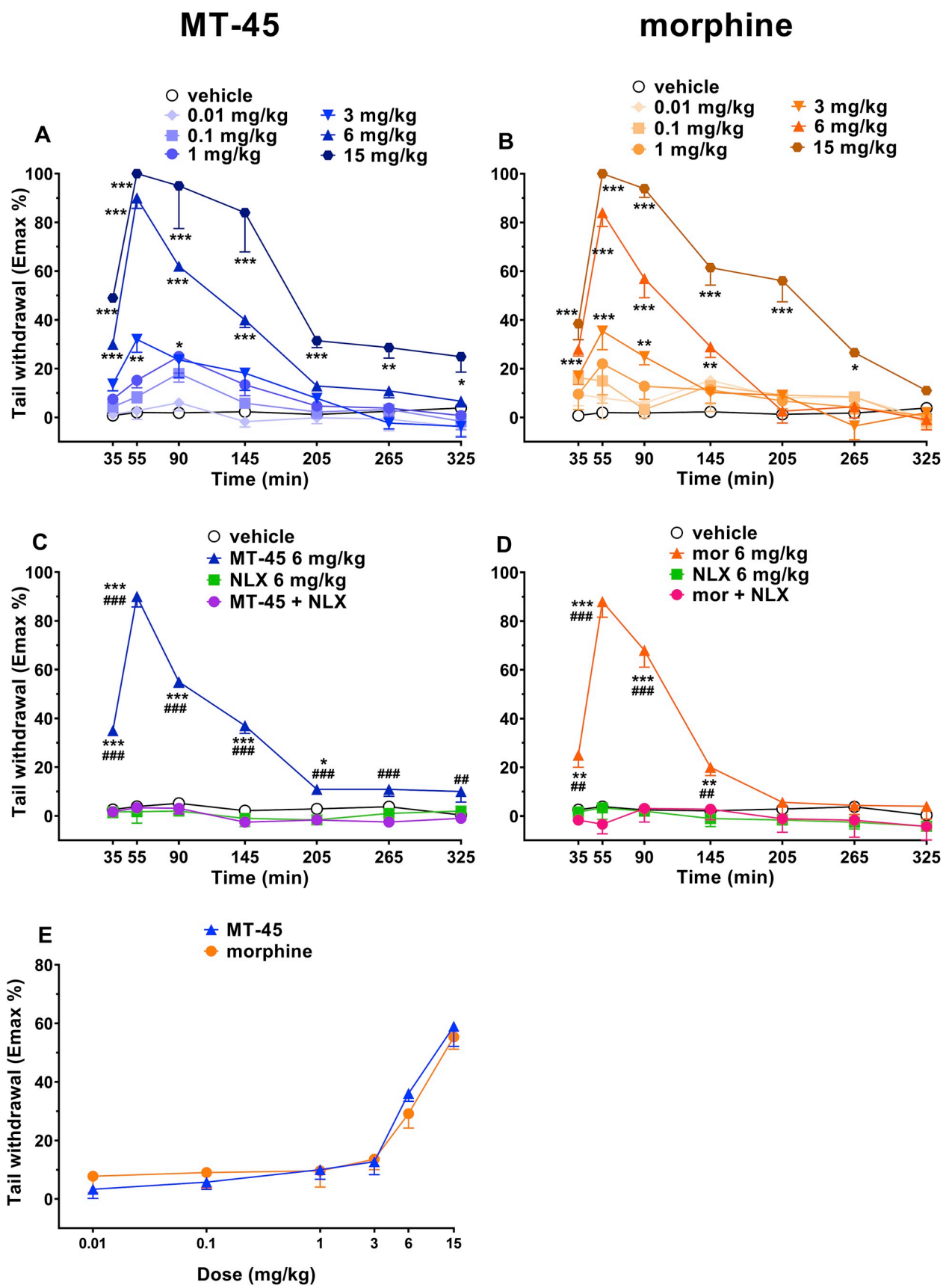

Fig. 9. Effect of the systemic administration (0.01–15 mg/kg i.p.) of MT-45 (panel A) and morphine (**panel B**) on the tail withdrawal test of the mouse. Interaction of effective dose of MT-45 and morphine (6 mg/kg) with the opioid receptor antagonist naloxone (6 mg/kg, i.p.; respectively **panel C and D**) and the comparison of the mean effect of the two opioids (**panel E**). Data are expressed as percentage of maximum effect (see material and methods) and represent the mean ± SEM of 8 determinations for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons. *p < 0.05, **p < 0.01, ***p < 0.001 versus vehicle; #p < 0.05, ##p < 0.01, ###p < 0.001 versus naloxon + agonist.

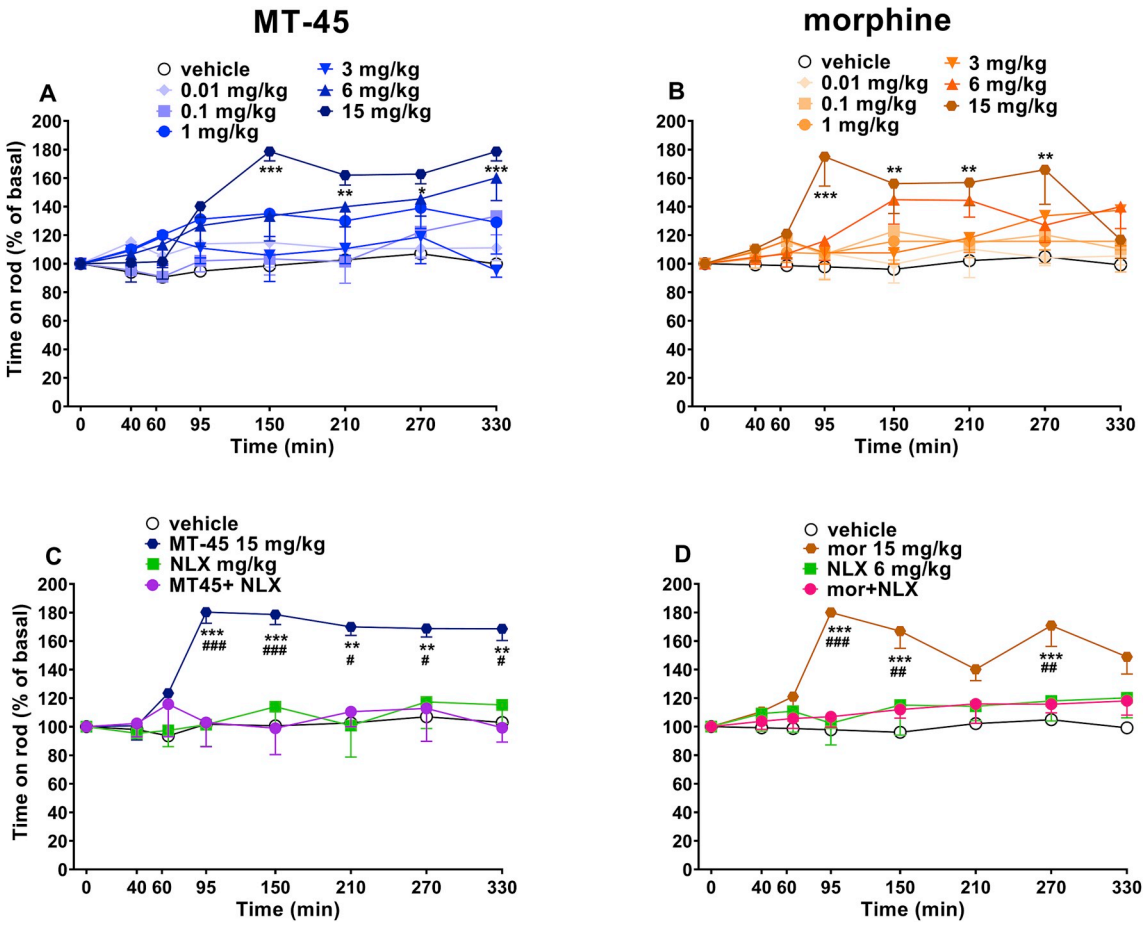

Fig. 10. Effect of the systemic administration (0.01–15 mg/kg i.p.) of MT-45 (**panel A**) and morphine (**panel B**) on the accelerod test of the mouse. Interaction of effective dose of MT-45 and morphine (15 mg/kg) with the opioid receptor antagonist naloxone (6 mg/kg, i.p.; respectively **panel C and D**) Data are expressed as percentage of baseline (see material and methods) and represent the mean ± SEM of 8 determinations for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for both the dose response curve of each compounds at different times. *p < 0.05, **p < 0.01, ***p < 0.001 versus vehicle; #p < 0.05, ##p < 0.01, ###p < 0.001 versus naloxone + agonist.

Similarly, the respiratory rate in the group of mice treated with MT-45 was significantly affected by treatment [$F_{3,2016}$ = 29.02, p < 0.0001], time [$F_{71,2016}$ = 6.849, p < 0.0001] and time × treatment interaction [$F_{213,2016}$ = 1.416, p < 0.0001] (Fig. 13C). After the administration of morphine, the respiratory rate was also significantly affected by treatment [$F_{3,2016}$ = 22.12, p < 0.0001], time [$F_{71,2016}$ = 7.556, p < 0.0001] and time × treatment interaction [$F_{213,2016}$ = 3.496, p < 0.0001] (Fig. 13D). Oxygen saturation also decreased in the mice treated with MT-45 and morphine. In particular, after the injection of MT-45 the oxygen saturation was significantly affected by treatment [$F_{3,2016}$ = 2.386, p = 0.0673], time [$F_{71,2016}$ = 2.105, p < 0.0001] and time × treatment interaction [$F_{213,2016}$ = 0.7081, p = 0.9993] (Fig. 13E). The SpO2 Saturation of mice treated with morphine was also significantly affected by treatment [$F_{3,2016}$ = 72.58, p < 0.0001], time [$F_{71,2016}$ = 3.767, p < 0.0001] and time × treatment interaction [$F_{213,2016}$ = 1.173, p = 0.0521] (Fig. 13 F). The pulse distention of the group of mice treated with MT-45 was significantly affected by treatment [$F_{3,2016}$ = 164.1, p < 0.0001], time [$F_{71,2016}$ = 3.914, p < 0.0001] and time × treatment interaction [$F_{213,2016}$ = 4.668, p < 0.0001] (Fig. 13 G). In the same manner, after the injection of morphine, the pulse distention was significantly affected by the treatment [$F_{3,2016}$ = 97.19, p < 0.0001], time [$F_{71,2016}$ = 2.561, p < 0.0001] and time × treatment interaction [$F_{213,2016}$ = 1.833, p < 0.0001] (Fig. 13 H). The pre-treatment with naloxone (6 mg/kg) prevented all the cardiorespiratory alterations (heart rate, respiratory rate, oxygen saturation and pulse distention) caused after the injection of MT-45 (15 mg/kg) and morphine (15 mg/kg) [Fig. 14; **Panel A**: significant effect of treatment ($F_{3,2016}$ = 45.67, p < 0.0001), time ($F_{71,2016}$ = 10.62, p < 0.0001) and time × treatment interaction ($F_{213,2016}$ = 1.107, p < 0.0001). **Panel B**: significant effect of treatment ($F_{3,2016}$ = 38.46, p < 0.0001), time ($F_{71,2016}$ = 10.62, p < 0.0001) and time × treatment interaction ($F_{213,2016}$ = 2.820, p < 0.0001). **Panel C**: significant effect of treatment ($F_{3,2016}$ = 8.992, p < 0.0001), time ($F_{71,2016}$ = 7.428, p < 0.0001) and time × treatment interaction ($F_{213,2016}$ = 1.641, p < 0.0001). **Panel D**: significant effect of treatment ($F_{3,2016}$ = 39.97, p < 0.0001), time ($F_{71,2016}$ = 10.62, p < 0.0001) and time × treatment interaction ($F_{213,2016}$ = 1.413, p = 0.0002). **Panel E**: significant effect of treatment ($F_{3,2016}$ = 36.29, p < 0.0001), time ($F_{71,2016}$ = 1.757, p < 0.0001) and time × treatment interaction ($F_{213,2016}$ = 1.073, p = 0.2337). **Panel F**: significant effect of treatment ($F_{3,2016}$ = 86.04, p < 0.0001), time ($F_{71,2016}$ = 3.896, p < 0.0001) and time × treatment interaction ($F_{213,2016}$ = 4.378, p < 0.0001). **Panel G**: significant effect of treatment ($F_{3,2016}$ = 6.517, p < 0.0001), time ($F_{71,2016}$ = 2.857, p < 0.0001) and time × treatment interaction ($F_{213,2016}$ = 2.339, p = 0.0002). **Panel H**: significant effect of treatment ($F_{3,2016}$ = 1.439, p = 0.0249), time ($F_{71,2016}$ = 2.857, p < 0.0001) and time × treatment interaction ($F_{213,2016}$ = 1.833, p = 0.2296)].

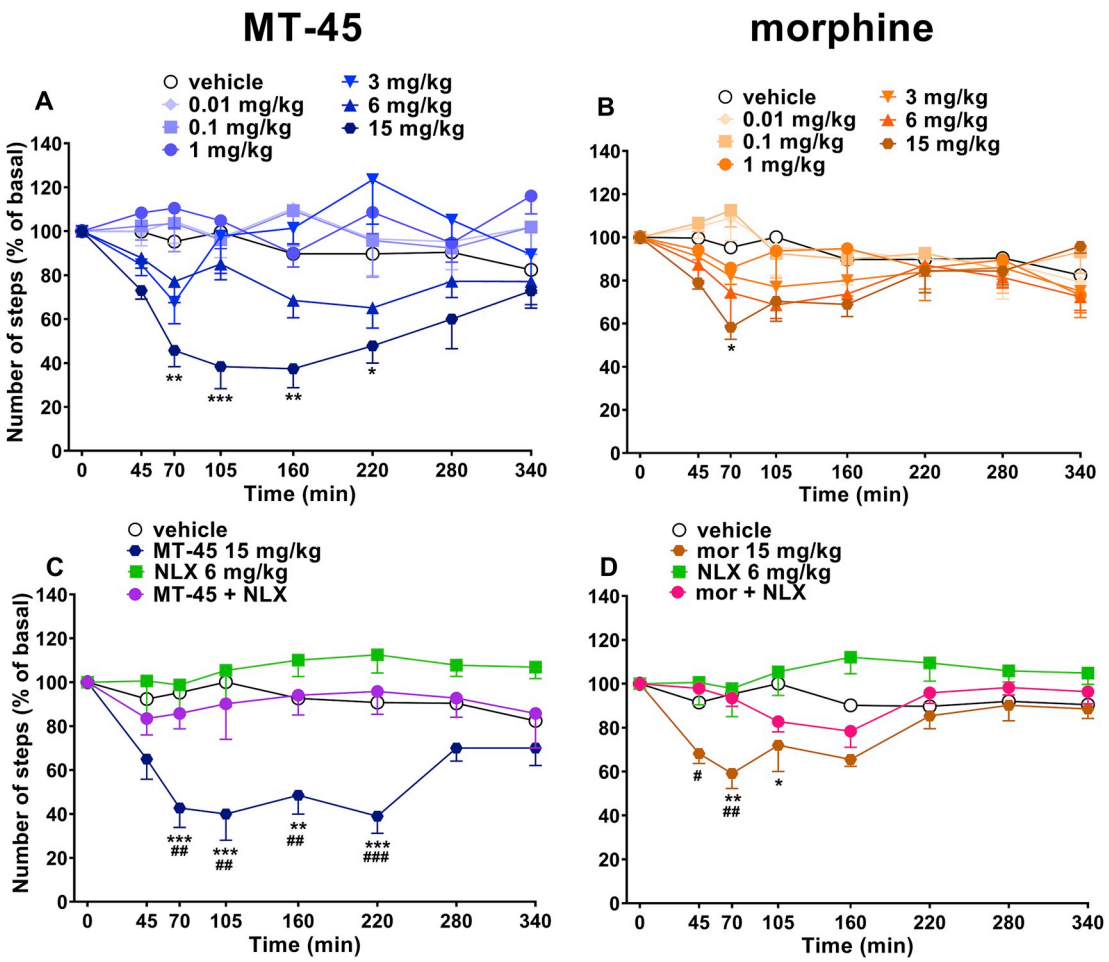

Fig. 11. Effect of the systemic administration (0.01–15 mg/kg i.p.) of MT-45 (**panel A**) and morphine (**panel B**) on the drag test of the mouse. Interaction of effective dose of MT-45 and morphine (15 mg/kg) with the opioid receptor antagonist naloxone (6 mg/kg, i.p.; respectively panel C and D). Data are expressed as percentage of baseline (see material and methods) and represent the mean ± SEM of 8 determinations for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons. *p < 0.05, **p < 0.01, ***p < 0.001 versus vehicle; #p < 0.05, ##p < 0.01, ###p < 0.001 versus naloxon + agonist.

4. Discussion

Our study presents in vitro and in vivo characterization of the synthetic opioid MT-45. Using the DMR assay, we demonstrated in vitro that MT-45 acts as a potent and selective mu agonist at human recombinant opioid receptors. In vivo results showed that the administration of MT-45 progressively (Fig. 15) induces tail elevation, increased mechanical and thermal antinociception, decreased visual sensorimotor responses, reduced tactile responses, modulated motor performance and muscle rigidity in mice. In addition, MT-45 impaired the cardiorespiratory functions causing bradycardia and bradypnea, accompanied by a reduction in SpO2 saturation and vasodilatation. All the effects of MT-45 were prevented by naloxone, demonstrating that the biological actions of MT-45 are exclusively due to opioid receptor (particularly mu) activation.

4.1. In vitro DMR study

The present in vitro study revealed that standard opioid receptor agonists (dermorphin, dynorphin A, and DPDPE) promoted DMR responses in CHO cells expressing the human recombinant opioid receptors with potency and efficacy values in line with those in the existing literature. As expected, morphine behaved as a mu receptor preferring agonists. MT-45 mimicked the actions of the alkaloid, displaying similar potency but slightly higher efficacy and mu selectivity. The DMR assay is now widely used for pharmacological studies on GPCR, including opioid receptors (see the references in the introduction). This assay has been recently set up and validated in our laboratories to investigate the pharmacological profile of the nociceptin/orphanin FQ receptor (Malfacini et al., 2018). Under the present experimental conditions, the standard agonists dermorphin, dynorphin A, and DPDPE elicited DMR responses with potency values similar to those reported in previous DMR studies (Malfacini et al., 2018) and in calcium mobilisation studies performed in cells co-expressing opioid receptors and chimeric G proteins (Camarda and Calo, 2013; Ferrari et al., 2016). Importantly, previous studies (Malfacini et al., 2018) demonstrated that the DMR responses to these standard agonists were sensitive to the universal opioid receptor antagonist naloxone. Morphine displayed moderate potency and selectivity for the mu receptor, which is again in line with previous DMR (Codd et al., 2011; Morse et al., 2011) and calcium mobilisation studies (Camarda and Calo, 2013; Rizzi et al., 2016). MT-45 mimicked the stimulatory effects of morphine, showing similar potency but higher efficacy and selectivity for the mu receptor. In receptor-binding experiments, MT-45 displayed higher affinity for mu compared to kappa and delta receptors and behaved as a mu agonist in GTPγS binding studies (Baumann et al., 2018). However, in binding experiments MT-45 displayed approximately 10-fold lower affinity than morphine for the mu receptor, while a similar

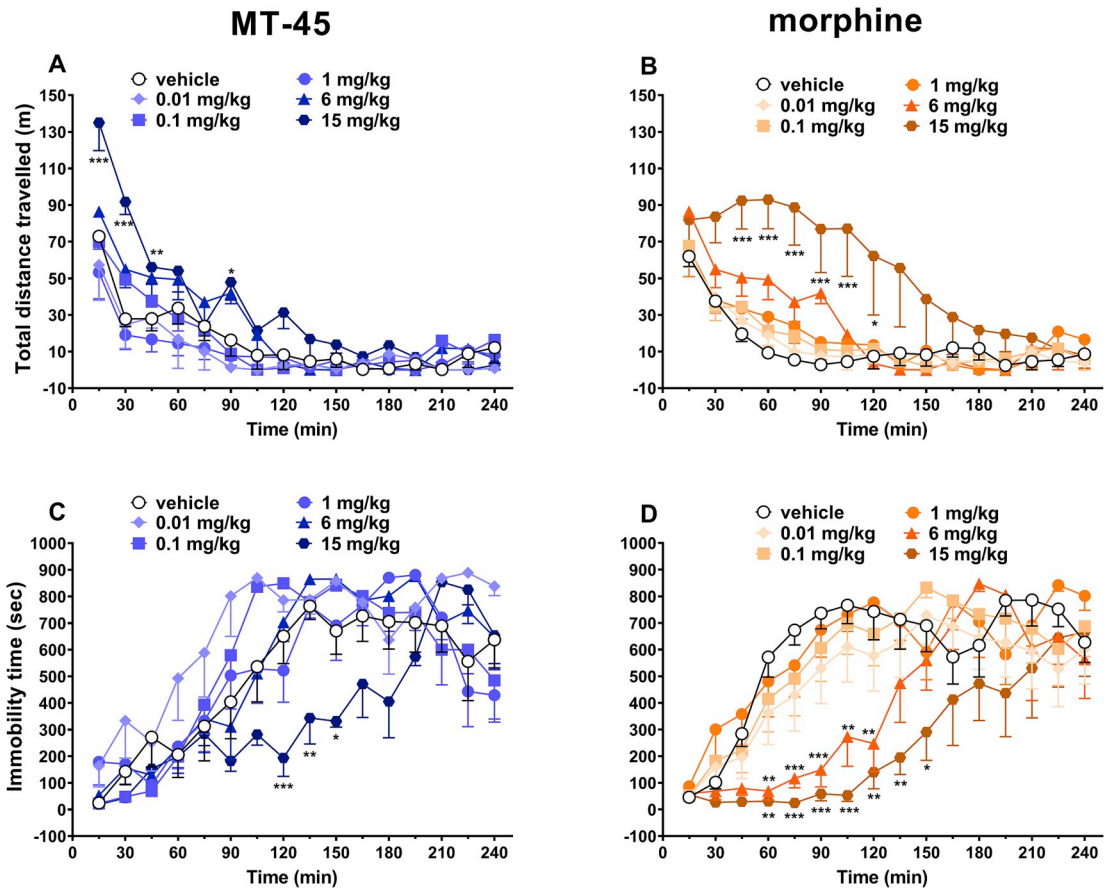

Fig. 12. Effect of the systemic administration (0.01–15 mg/kg i.p.) of MT-45 (**panel A and C**) and morphine (**panel B and D**) on the total distance travelled and the total time immobile of the mouse. Data are expressed as meters travelled (total distance travelled) and as seconds immobile (total time immobile) represent the mean ± SEM of 10 determinations for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for both the dose response curve of each compound at different times. *p < 0.05, **p < 0.01, ***p < 0.001 versus vehicle.

potency was measured in the present experiments. Finally, the DMR response to MT-45 is entirely due to its ability to stimulate the mu opioid receptor as demonstrated by its lack of action in wild type CHO cells.

In conclusion, DMR studies demonstrated that MT-45 behaves as a potent and selective mu full agonist at human recombinant opioid receptors.

4.2. *In vivo studies*

Our data demonstrates that MT-45 and morphine dose-dependently induce tail elevation (Straub tail) in mice in a naloxone-sensitive manner. These results are in accordance with the study by Nakamura and Shimizu demonstrating that MT-45 causes Straub tail in rodents, similar to morphine (Nakamura and Shimizu, 1976). The Straub tail is a typical opioid effect that is mediated by mu receptors. After the existence of two types of mu receptors was proposed, Nath and colleagues speculated that morphine induces Straub tail by acting specifically on the μ_2-opioid receptors (Nath et al., 1994).

Opioids have been widely studied for their analgesic property. Morphine is the most studied opioid due to its anti-nociceptive effect both in clinical and preclinical studies. Our study demonstrates that the systemic administration of MT-45 and morphine increased the threshold to acute mechanical and thermal pain stimulus in mice. In particular, both opioids produced an analgesic effect in mice in a dose-dependent manner, similar to previous studies of rodents treated with morphine (Hill et al., 1957; Hunskaar et al., 1986; Gades et al., 2000; Foroud and Vesal, 2015) and MT-45 (Nakamura and Shimizu, 1976; Montesano et al., 2017; Baumann et al., 2018).

It should be noted that the maximal anti-nociceptive effects were reached 35 min after the administration of both drugs. Our data are in line with recent studies that show the highest latencies in relation to the hot plate and tail flick tests were reached 30 min after the administration of morphine (Ben Haddou et al., 2014) and MT-45 (Baumann et al., 2018) injections, despite differences in the methods and the strain used. Moreover, the analgesic effect induced by both drugs at the dose of 15 mg/kg reached 100% at 55 min into the test. These results confirm those of our previous publication (Montesano et al., 2017) and are similar to those of Nakamura and colleagues who conducted analgesic tests (thermal, mechanical, electrical and chemical pain) in mice and rats. MT-45 showed similar potency as morphine except for chemical pain, for which it was less potent (Nakamura and Shimizu, 1976). Our findings are in line with a recent mouse study using the 50 °C warm water tail withdrawal (WWTW) test to assess the analgesic effect of some mu opioid receptor agonists. The latter study demonstrated that the acute administration of morphine (i.p.) induced analgesia in C57BL6/J mice, and the maximal analgesic effects were induced by doses of around 10 mg/kg (Anand et al., 2018). The pre-treatment with naloxone totally inhibited the analgesic effect induced by MT-45 and morphine. These results confirm the involvement of mu-opioid receptors in MT-45-induced analgesia in mice, as recently established in a study using mu-receptor KO mice (Baumann et al., 2018). The mechanisms by which opioids induce analgesia have been widely studied in animal models (Lazorthes et al., 1988; Besson et al., 1992; Stein et al., 2003; Aoki et al., 2014) and have confirmed that mu agonists produce analgesia through both pre- and postsynaptic mechanisms at

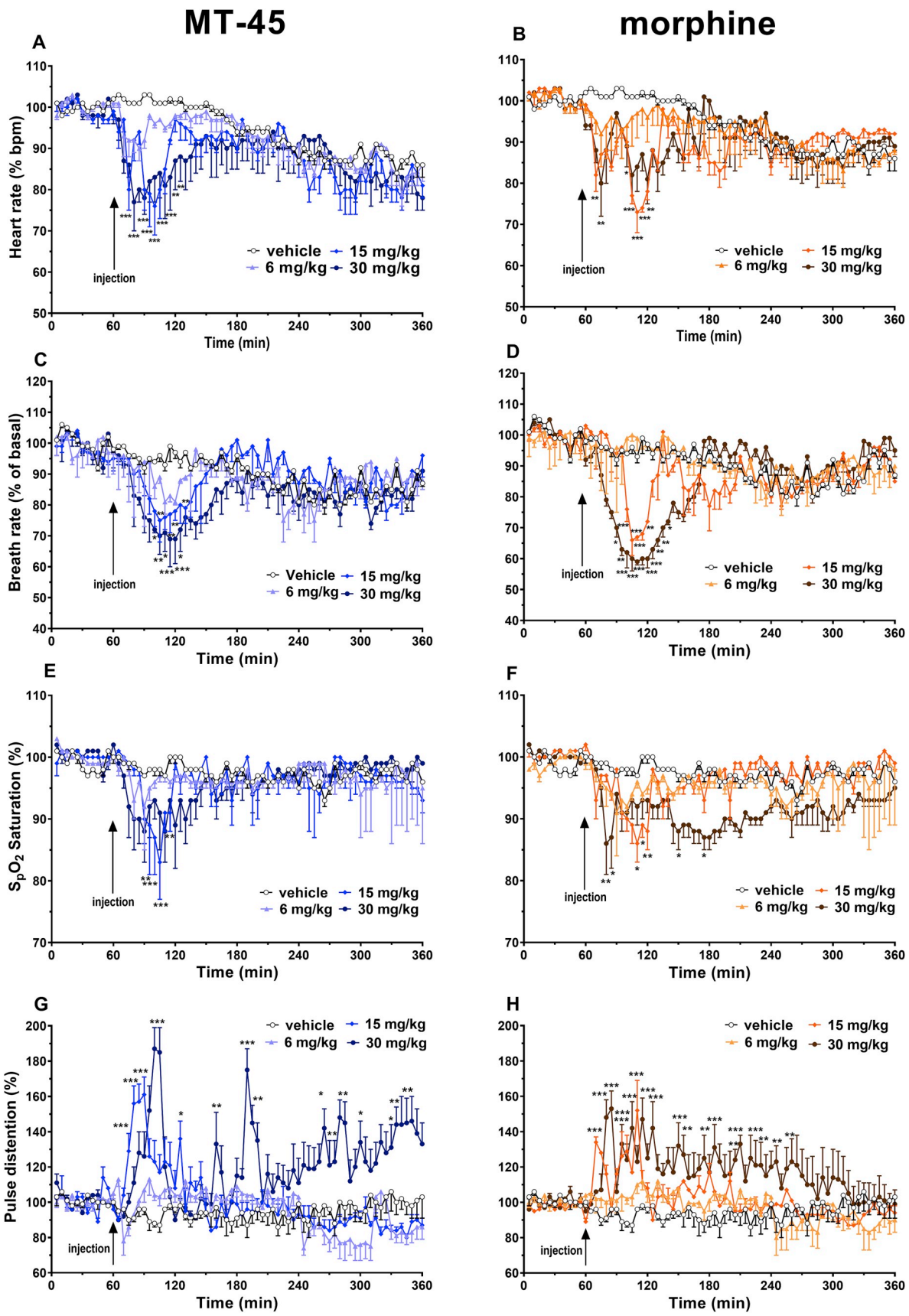

Fig. 13. Effect of the systemic administration (6–30 mg/kg i.p.) of MT-45 and morphine on the cardiorespiratory parameters. In particular, on heart rate (respectively **panel A and B**); on breath rate (respectively **panel C and D**), on arterial saturation (SpO_2; respectively **panel E and F**) and on pulse distention (respectively **panel G and H**). Data are expressed as percentage of basal value (Heart and Breath Rate) while as absolute value for oxygen blood saturation (% SpO_2 saturation) and pulse distention and represent the mean ± SEM of 4 determinations for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for both the dose response curve of each compound at different times. *p < 0.05, **p < 0.01, ***p < 0.001 versus

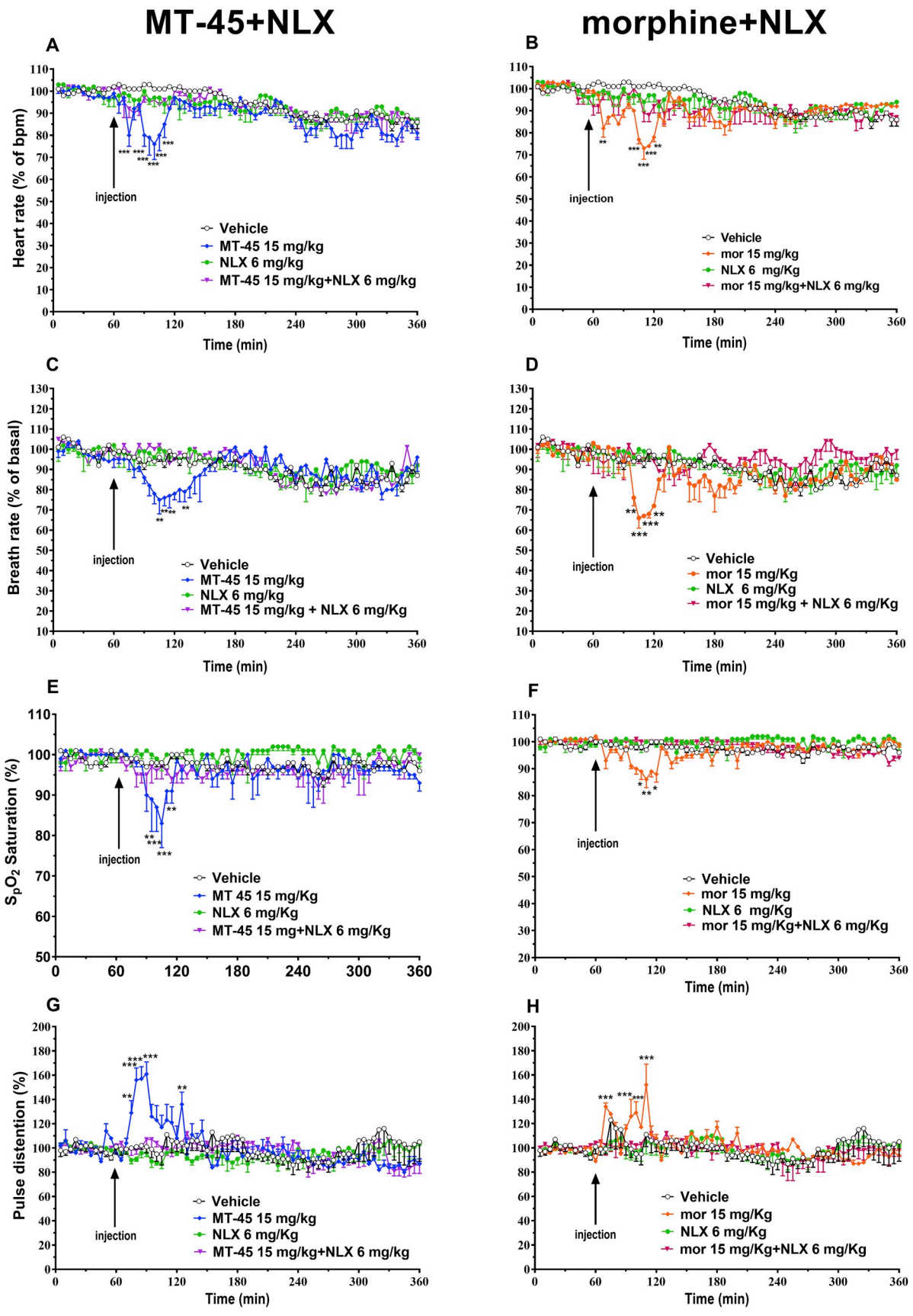

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Fig. 14. Effect of the pre-treament with naloxone (6 mg/kg i.p.) on the modification of the impairement induced by MT-45 (15 mg/kg i.p.) and morphine (15 mg/kg i.p.) on the cardiorespiratory parameters. In particular, on heart rate (respectively **panel A and B**); on breath rate (respectively **panel C and D**), on arterial saturation (SpO_2; respectively **panel E and F**) and the pulse distention (respectively **panel G and H**). Data are expressed as percentage of basal value (Heart and Breath Rate) while as absolute value for oxygen blood saturation (% SpO_2 saturation) and pulse distention and represent the mean ± SEM of 4 determinations for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for both the dose response curve of each compound at different times. *p < 0.05, **p < 0.01, ***p < 0.001 versus vehicle.

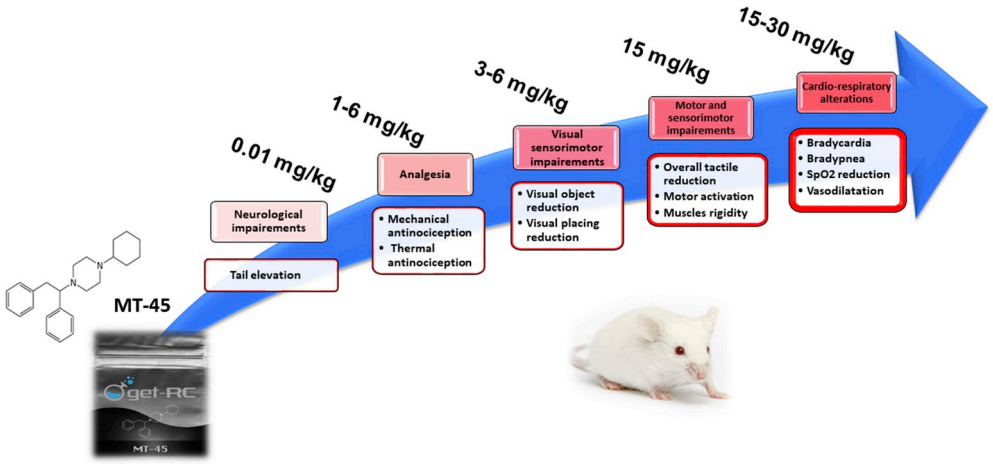

Fig. 15. Schematic illustration of the progressive occurrence of pharmacological and behavioral effects in mice in response to the increasing doses of MT-45 (0.01–30 mg/kg).

multiple CNS sites (Torrecilla et al., 2002). Moreover, it has been well documented that opioids can directly block pain transmission at the spinal cord level, acting on primary afferents (Wall, 1967; Zollner et al., 2003) and nociceptive relay neurons in the dorsal horn (Glaum et al., 1994).

The systemic administration of MT-45 and morphine affects the startle response to visual stimuli in a dose-dependent manner. The pre-treatment with naloxone prevented the inhibitory effects induced by opioids in visual object and visual placing tests. These results demonstrate that opioid receptors may play a central role in modulating the visual responses of mice after opioid injections (Howells et al., 1980; Zhu et al., 1998). In contrast to the visual object test, the visual placing test links the movement of the mouse to its visual perception. In particular, to perform the visual placing test the mouse needs to integrate the visual and tactile stimulus with the vestibular information to be able to correctly extend the muscles of the neck and forelegs to land on the ground (Lambert et al., 2016). Thus, the involvement of the vestibular inputs and the spinal motoneurons in controlling posture and body movement in the face of gravity has been established (Tosolini and Morris, 2012; Tosolini et al., 2013; Angelaki and Cullen, 2008). These findings allow us to suggest that MT-45 and morphine could alter vestibular pathways in addition to impairing visual perception. Iasnetsov and Pravdivtsev reported that the iontophoretic application of the opioid agonists morphine or methionine- and leucine-enkephalin increased the firing rate of neurons in the medial vestibular nucleus (Iasnetsov and Pravdivtsev, 1986). Other studies on the mechanism of action of opioids in the medial vestibular nucleus have proved that opioids can induce direct excitatory actions after GABAergic inhibition (Lin and Carpenter, 1994).

Our in vivo studies showed that the administration of MT-45 and morphine in the dose range of 0.01–15 mg/kg did not alter the thermoregulation of mice. Our results are not in line with previous studies reporting that morphine may produce hypothermia, hyperthermia or a biphasic effect depending upon the dose (Herrmann, 1942; Glick, 1975) and the ambient temperature (Rosow et al., 1980). Nevertheless, we cannot exclude the possibility that the administration of MT-45 and morphine at doses higher than those tested might induce hypothermia. However, the major alterations seen in other parameters prevents us from increasing doses.

Our data demonstrates that MT-45 slightly decreases tactile responses in mice only at the highest dose tested (15 mg/kg) and that this effect was prolonged compared to morphine. The effect of opioids on tactile response is not yet established. However, a study of rats that received a microinjection of morphine in the rostral ventromedial medulla (RVM) showed a decrease in tactile allodynia using the von Frey test. This study speculates RVM opioid signalling plays an important role in controlling the sensory responses of rodents (Gomtsian et al., 2018).

Likewise, our study demonstrates that MT-45, like morphine, increases locomotion in mice in the accelerod test but only at the dose of 15 mg/kg. Similarly, in spontaneous locomotion tests animals have shown an increase in the total distance travelled after a 15 mg/kg injection of both opioids and a decrease in immobility time. Various studies have demonstrated the effect of opioids, particularly morphine, on locomotion. Opioids can induce stimulation and/or depression and a state of catatonia followed by motor excitation, depending on the dose and the time after injection (Babbini and Davis, 1972; Costall and Naylor, 1974; Oka and Hosoya, 1976; Teitelbaum et al., 1979; Szekely et al., 1980; Longoni et al., 1987; Narita et al., 1993; Funada et al., 1994; Belknap et al., 1998; Manzanedo et al., 1999; Rodriguez-Arias et al., 2000). The neurochemical mechanisms by which opioids can induce excitatory and inhibitory motor effects is not fully explained. One of the main mechanisms involved in the facilitatory effects of opioids is related to the stimulation of the mesolimbic dopamine pathway. In fact, it has been demonstrated that systemic or direct infusions of mu agonists into the ventral tegmental area (VTA) increases dopamine release in the dorsal striatum (Di Chiara and Imperato, 1988) and enhances locomotion and motor sensitisation, probably by activating mu receptors located in GABAergic interneurons in the VTA (Matsui et al., 2014). This motor enhancement can be prevented by applying dopamine receptor antagonists to the dorsal striatum area (NAc) (White et al., 1995). Nevertheless, the involvement of the serotonergic system in the sequence of events triggered by mu-opioid receptor excitation cannot be denied, as serotonergic antagonists are able to block hyperlocomotion induced by morphine (Li et al., 2013). Regardless, in our study naloxone was effective in blocking the increase in

locomotion induced by morphine and MT-45, based on the accelerod and spontaneous locomotion tests (data not shown) that prove the central role played by mu-opioid receptors in controlling motor performance in mice (Amalric and Koob, 1985; Gurtu, 1990).

Interestingly, our data demonstrates that in the drag test the administration of 15 mg/kg of MT-45 decreased the number of steps to 50%, while the same dose of morphine was less effective. Our data indicate that locomotion tests cannot always predict the overall effects of opioids on motor activity. For this reason, we hypothesised that the inhibitory effect of opioids in front paws could be related to muscle rigidity. In fact, the results of our grip strength test showed an increase in muscle rigidity caused by both opioids; the effect of MT-45 seemed to be more rapid compared to that of morphine. Naloxone prevented all effects induced by both opioids, which demonstrates again that muscle rigidity is triggered by mu receptors. Our data are in line with many studies investigating morphine for its effects on muscle rigidity in rodents, particularly the gastrocnemius soleus muscle (Wand et al., 1973; Winkler et al., 1982; Kolasiewicz et al., 1987). It has also been reported that morphine-induced rigidity could be mediated by a GABAergic pathway from the substantia nigra to the ventromedial thalamic nucleus (Ossowska et al., 1986).

Regarding the synthetic opioid MT-45, there are no studies demonstrating its effect on muscle rigidity, but it has been reported that four patients taking MT-45 at high doses presented with paresthesia in the hands and feet, difficulty with gripping and hand coordination and balance disturbances (Helander et al., 2014). It is important to note that we also used the bar test to better understand the effect of opioids on motor activity and the possibility of inducing catalepsy; however, the results were negative for both opioids at the dose range of 0.01–15 mg/kg during the 5 h of the test. Our data can be verified by many studies suggesting that the phenomena of catalepsy is separate from muscle rigidity (Costall et al., 1972; Ellenbroek et al., 1984, 1985; Havemann et al., 1980; Winkler et al., 1982; Ossowska et al., 1986). The pretreatment with naloxone prevented the excitatory/inhibitory motor effects and muscle rigidity triggered by morphine and MT-45, confirming the importance of mu receptors in controlling motor behaviours.

Cardiorespiratory alterations in animals following the administration of opioids, particularly morphine (Kennedy and West, 1967; Chen and Ashburn, 2015), fentanils (Yadav et al., 2018) and to a lesser extent MT-45 (Nakamura et al., 1979) have been well established. We have demonstrated through in vivo tests that MT-45 and morphine significantly altered the cardiorespiratory parameters when administered to mice at high doses. Specifically, the dose of 30 mg/kg of both opioids was the most effective in reducing the basal heart rate, respiratory rate and SpO2 and in increasing the pulse distention. The effect of MT-45 on the heart rate could be induced by the inhibition of the sympathetic tone and the enhancement of the parasympathetic tone in the central nervous system, which results in significant bradycardia. In fact, it has been reported that the application of opioids in the cardiac parasympathetic neurons of the nucleus ambiguous elicits an increase in parasympathetic cardiac function, followed by bradycardia (Irnaten et al., 2003). In addition, the increasing evidence of the presence of mu, delta and kappa in myocardial cells (Sobanski et al., 2014) could be related in part to the decrease in heart rate, even if some studies predicted a protective role of these receptors against myocardial ischemia; however, clinical applications have not yet been established (Schultz et al., 1996). Respiratory depression is the main cause of opioid-related death (Dahan et al., 2010). In particular, MT-45 abuse induces cardiorespiratory arrest initiated by respiratory depression (WHO: ECDD, 2015). Yet, of nine MT-45-positive patients included in the STRIDA project in Sweden seven presented in hospital with respiratory depression, decreased oxygen saturation and cyanosis, and two cases presented with apnea (Siddiqi et al., 2015). Our study confirms the effects of MT-45 on the respiratory apparatus. Moreover, the NSO could decrease the respiratory rate like other opioids acting on mu and delta receptors located in the respiratory centres (White and Irvine, 1999) and the nuclei of the pons altering the respiratory drive and also in the periphery through chemoreceptors located in the carotid and aortic bodies where they determine the blood levels of O_2 and CO_2 (Yeadon and Kitchen, 1989; Yeadon and Kitchen, 1989; Gross, 2003). Naloxone was effective in blocking all the cardiorespiratory impairments induced by MT-45 and morphine. Our results prove the efficacy of this molecule as an antagonist for this NSO, as reported in seven cases of intoxication (Helander et al., 2014).

5. Conclusions

This study confirms the in vitro and in vivo findings of Baumann and colleagues and provides more data regarding the pharmaco-toxicological effects of the synthetic opioid MT-45 in comparison with those of morphine. DMR studies demonstrated in vitro that MT-45 behaves as a potent and selective mu agonist at human recombinant opioid receptors. In vivo, MT-45, like morphine, dose-dependently altered sensorimotor and motor responses in mice and impaired the cardiorespiratory functions, thus demonstrating its dangerous pharmaco-toxicological effects. Our findings highlight the importance of studying NSOs and dedicating more research, particularly clinical studies of the current molecules, to better understand possible therapeutic interventions in the case of toxicity.

Funding sources

This research was supported by the Drug Policies Department, Presidency of the Council of Ministers, Italy (project: "*Effects of NPS: development of a multicentre research for the information enhancement of the Early Warning System*" to M. Marti); by local funds from the University of Ferrara (FAR grants to M. Marti and G. Calo'); by FIRB 2012 from the Italian Ministry of Education, University and Research (Grant no. RBFR12LDOW to F. De-Giorgio) and by local funds from the Catholic University of Rome (Linea D1 grants to F. De-Giorgio).

Ethical statements

All applicable international, national and/or institutional guidelines for the care and use of animals were followed. All procedures performed in the studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. Project activated in collaboration with the Presidency of the Council of Ministers-DPA Anti-Drug Policies (Italy).

Author contributions

MM, BS, GC contributed conception and design of the study. BS, TM, AR, FP performed in vivo experimental section. GC and ANJ performed in vitro experimental section. MM, BS, GC wrote the manuscript. BS MM, JA, GC, DEF, NM, FP, TM, AR, SG, NM edited sections of the manuscript. BS, MM, GA, SG, ANJ performed statistical analysis. All authors contributed to manuscript revision, read and approved the submitted version.

CRediT authorship contribution statement

S Bilel: Conceptualization, Writing - original draft, Writing - review & editing, Formal analysis. **NJ Azevedo:** Formal analysis. **R Arfè:** Writing - review & editing. **M Tirri:** Writing - review & editing. **A Gregori:** Formal analysis. **G Serpelloni:** Writing - review & editing, Formal analysis. **F De-Giorgio:** Writing - review & editing. **P Frisoni:** Writing - review & editing. **M Neri:** Writing - review & editing. **G Calò:** Conceptualization, Writing - original draft, Writing - review & editing. **M Marti:** Conceptualization, Writing - original draft, Writing - review & editing, Formal analysis.

Declaration of competing interest

The Authors declare no conflict of interest.

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