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Efficacy of multimodal analgesic treatment of severe traumatic acute pain in mice pretreated with chronic high dose of buprenorphine inducing mechanical allodynia

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Author statement

Basile Coutens : Conceptualization, Methodology, Investigation and Formal analysis. **Céline Derreumaux** : Conceptualization, Methodology, Investigation and Formal analysis. **François Labaste** : Conceptualization, Methodology. **Vincent Minville** : Validation, Writing - Review & Editing. **Bruno Pierre Guiard** : Supervision, Writing – Review & Editing. **Lionel Moulédous** : Supervision, Writing – Review & Editing. **Vincent Bounes** : Validation, Writing - Review & Editing. **Anne Roussin** : Supervision, Writing – Review & Editing. **Bernard Frances** : Supervision, Writing – Review & Editing.

Abstract

Managing severe acute nociceptive pain in buprenorphine-maintained individuals for opioid use disorder management is challenging owing to the high affinity and very slow dissociation of buprenorphine from μ -opioid receptors that hinders the use of full agonist opioid analgesics.

In a translational approach, the aim of this study was to use an animal setting to investigate the effects of a chronic high dose of buprenorphine treatment on nociceptive thresholds before and after applying a severe acute nociceptive traumatic surgery stimulus and to screen postoperative pharmacological analgesic strategies.

A chronic treatment of mice with a high dose of buprenorphine (BUP HD, 2 x200 μ g/kg /day; i.p.) revealed significant mechanical allodynia. One and two days after having discontinued buprenorphine administration and having induced a severe nociceptive acute pain by a closed tibial fracture, acute administration of morphine at a dose which has analgesic effects in absence of pretreatment (4.5 mg/kg; i.p.), was ineffective to reduce pain in the BUP HD group. However, mimicking multimodal analgesia strategy used in human postoperative context, the combination of morphine (administered at the same dose) with a NMDA receptor antagonist (ketamine) or an NSAID (ketoprofen) produced antinociceptive responses in these animals.

The mouse model of closed tibial fracture could be useful to identify analgesic strategies of postoperative pain for patients with chronic exposure to opioids and suffering from hyperalgesia.

Keywords: Hyperalgesia, Multimodal analgesia, Opioid, Buprenorphine

1 Introduction

Buprenorphine medication for maintenance treatment of opioid use disorder is available in most countries (INCB 2018). Abuse and misuse of prescription opioids has resulted in an opioid crisis with many patients developing opioid use disorder, particularly in the U.S. where efforts have been recently undertaken to expand access to buprenorphine (Ghertner, 2019). Whereas formulations of buprenorphine at low dosages are marketed for pain relief, there are controversies concerning pharmacological treatments of severe acute pain in patients receiving high doses of buprenorphine for opioid maintenance treatment. Jonan et al (2018) have recently discussed various pharmacological strategies of perioperative pain treatment in patients under buprenorphine for opioid maintenance treatment. These strategies are already described in many clinical guidelines and depend of elective versus emergency surgery and the need of intermediate or high postoperative opioid requirement. Multimodal analgesia is recommended in all scenari with non-opioid adjuvants as Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) or N-methyl-D-aspartate (NMDA) receptor antagonists, regional anesthesia and local anesthetic infiltration. Several pharmacological features of buprenorphine could explain why a multimodal analgesia is required in case of severe acute pain. Buprenorphine is an opioid with a complex pharmacological profile. Indeed, this agent displays mixed agonist-antagonist activity at classical opioid receptors. In particular evidence demonstrates that buprenorphine activates mu and kappa receptors while inhibiting the functional activity of delta receptors (Cowan, 2003). Moreover, the existing data show that buprenorphine displays a 10-fold lower affinity for the delta receptor as compared to the mu and kappa. It should be also noted that buprenorphine has been shown to activate the opioid receptor-like (ORL-1; also known as NOP) receptor (Lufty and Cowan, 2004). These properties hinder the use of full μ -opioid receptor agonists for severe acute pain relief in patients under buprenorphine-maintenance treatment. Moreover, opioid-induced hyperalgesia (OIH) can occur under opioid chronic treatment as well as in patients under opioid-maintenance treatment. OIH refers to the development of hypersensitivity to painful stimuli observed upon chronic opioid administration and includes two major manifestations *i.e.* allodynia and hyperalgesia (Simonnet and Rivat 2003). OIH is highly prevalent (nearly 30%) with a long-term treatment with opioids for chronic pain (Katz et al 2015). For patients under opioid replacement therapy, randomized controlled trials aimed at identifying OIH in nonsurgical setting have been conducted. It has been observed a lower pain tolerance with buprenorphine- or methadone-maintained individuals compared to control (Compton et al 2000; Compton et al 2012; Doverty et al 2001; Athanasos et al 2018). All these experimental studies have been

performed in patients dependent to a full μ -opioid receptor agonist before entry in maintenance therapy. However, whereas buprenorphine can represent the first opioid substance having led to a substance use disorder in some individuals, only few studies have been aimed to investigate if buprenorphine itself could induce hyperalgesia. A pioneer study (Wala and Holtman 2011) examined responsiveness to an ultra-low sub-analgesic dose and a higher analgesic dose of buprenorphine following acute and repeated administration in rat. Animals displayed hyperalgesia with repeated analgesic doses and immediate hyperalgesia at ultra-low dose (Wala and Holtman 2011).

Here, we developed a model of severe acute pain based on a closed tibial fracture in mouse treated during nine days by high dose of buprenorphine (BUP HD) or its vehicle (Saline). The dose range of buprenorphine used for opioid maintenance treatment in human is higher than the one used to treat pain. In mouse, the range for analgesic dose does not exceed 100 $\mu\text{g}/\text{kg}$. In this way, we chose to assess a higher buprenorphine dose (i.e. 2x200 $\mu\text{g}/\text{kg}$). The efficacy of multimodal analgesic approach was then tested in this original model to relieve postoperative pain.

2 Materials and methods

2.1 Animals

The study was approved by the French ethical committee in animal experimentation of the Region Midi-Pyrenees (France) project n° 2015101214598332 and conducted according to the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, 1986), in an accredited laboratory under the supervision of an authorized researcher. The animals were housed in cages with solid floor covered with 3 cm of soft bedding and were fed and watered ad libitum. There were five animals in each cage, living on a 12 h light-dark cycle. Three-four months old C57BL/6 male mice weighting between 20-25g (Janvier Labs, Saint-Berthevin, France) were used for all the experiments.

2.2 Protocol (Fig. 1)

Groups

A control group was included in all experiments; i.e. Saline (NaCl 0.9%, twice a day for 9 days; i.p.). Wala and Holtman (2011) showed that buprenorphine at a dose of 2x100 µg/kg for ten days enhanced pain sensitivity in mice. Since opioid substitution treatment by buprenorphine involves higher daily doses than those used for analgesia, we decided to focus on a higher dosage (2x200 µg/kg / day for 9 days) of buprenorphine to generate the BUP HD group. For all drug administrations, the volume of injection was the same: 100 µl of solution were injected for 10g of mice.

The experimental protocol was defined in order to evaluate both allodynia and hyperalgesia during a chronic treatment, and hyperalgesia following a painful surgical intervention. It was therefore divided in three parts:

The first part (named “pre-fracture”) lasted 9 days and involved the chronic treatment of mice, divided in two groups: Saline and BUP HD. Pain tests were performed before any treatment (baseline, D0) and thirty min after saline or buprenorphine administration on the 5th (D5) and the 9th day (D9). On the 10th day, a bone fracture was performed (see Fig. 1).

The second part of the protocol (named “post-fracture”) involved pain tests conducted 2 (H2), 4 (H4) and 24 h (H24) after the fracture. An antinociceptive effect of 3 mg/kg morphine one day after the same consolidated tibial fracture has been previously observed in mice (Minville et al 2010). In the present study, morphine (4.5 mg/kg; i.p.) was administered 30 min before the H4 and H24 tests.

In order to avoid the possibility of a “cage effect”, treatments were randomly assigned to each mouse before the beginning of first Von-Frey Test. Mice were then housed by group of five, so each cage contained mice injected with Saline and Buprenorphine treatment.

The third part was devoted to multimodal analgesia, 48h after surgery. Each group was divided in 3 subgroups: morphine (4.5mg/kg i.p.), morphine (4.5mg/kg i.p.) + ketamine (10mg/kg i.p.) or morphine (4.5mg/kg i.p.) + (ketoprofen 50mg/kg i.p.). Ketamine was chosen owing to its ability to antagonize NMDA receptors and have an opioid-sparing effect by reducing pain transmission and by preventing the development of OIH (Crumb et al 2018). Ketoprofen was chosen as representative of the class of Non-Steroidal Anti-Inflammatory Drugs (NSAIDs).

2.3 Surgery: Bone fracture

A bone fracture was performed in all mice according to the validated technique described by Minville et al. (2008). Mice were anesthetized with 4% sevoflurane delivered via cone nose. After antiseptic preparation of the right paw using povidone iodine, an unilateral closed fracture was made in the right tibia using a blunt guillotine (specially designed fracture apparatus). The fracture apparatus consists of four parts: a frame, an animal support system, a guillotine ramming system, and a 300-g weight. The support anvil was made with an adjustable foot rest that ensures that all of the fractures are at the same level by positioning the mouse leg on the anvil with the foot against the foot rest. For the intramedullary pinning using a sterile technique, a hole was made above the tibial tuberosity percutaneously using a 27-gauge needle (Becton Dickinson and Company, Drogheda, Ireland). Then the needle was directed into the medullary canal. By rotating the needle, the canal was reamed to 5 mm up to the ankle joint. The end of the needle was cut as short as possible so that the skin could roll over and cover it. No suture was used. Then, the mouse was placed with the leg on the anvil so that the blunt guillotine lined up with the proximal third of the tibia. The 300-g weight was dropped from a height of 9–10 cm, fracturing the tibia shaft. Radiography confirmed the fracture.

2.4 Mechanical nociception

Animals were placed on an elevated mesh floor surrounded by a clear plastic enclosure (10 X 10 X 10 cm) and allowed to acclimate to this environment for at least 15 min before testing. We used the manual VonFrey filaments, according to the protocol described by Hache et al. (2015). Mechanical sensitivity was then assessed using three VonFrey filaments with bending

force of 0.6g, 1.4g and 4g (Bioseb Inc., Vitrolles France). Each filament was applied in a growing strength for a duration of 2 sec to the mid-plantar area of each hind paw ten times, with 3 sec between each application. Rapid retraction, shaking and/or licking of the hind paw were considered to represent nociceptive specific behaviors and only one of these responses needed to be displayed to be considered as a positive withdrawal response. Applications were done to both hind paws, counted and then expressed as an overall percentage of paw withdrawal response (i.e. 50% for five paw withdrawals after 10 stimuli).

In basal conditions, filament inducing less than 40% of paw withdrawals is considered as painless while filament inducing more than 40% is considered as painful. Filaments were chosen in order to assess allodynia (painless filament 0.6g and 1.4g) and hyperalgesia (painful filament 4.0g) in basal condition (pre-fracture experiment). However, after bone fracture (post-fracture experiment) , all filaments were considered as painful.

2.5 Statistical analysis

Data were presented as mean \pm S.E.M. Statistical significance was set at $P < .05$. For data presented on Fig. 2-4, two-way analyses of variance (ANOVAs) [Time x Treatment] were applied. Significant main effects were followed by Tukey post hoc analysis. For data presented on Fig. 4, one-way ANOVA follow by Dunnett's test were also realized in order to compare results to H2 tests. Comparisons and significant results are summarized in Supplemental Tables 1 and 2.

3 Results

In the VonFrey filament test, the 0.6g and the 1.4g filaments were used to search for eventual allodynia whereas the application of the 4.0g filament which represents a painful stimulus, was used to reveal a potential hyperalgesia.

3.1 Pre-fracture: A prolonged treatment with a high dose of buprenorphine induced mechanical allodynia

Before the initiation of the different treatments (Saline and BUP HD), groups of mice were balanced and their average percentages of hind paw retraction/withdrawal were similar (Fig. 2 A, B, C). As expected, the nociceptive response induced by the application of the 0.6g (Fig. 2A), 1.4g (Fig. 2B) and 4.0g (Fig. 2C) filaments did not vary with the day of treatment in the Saline group. In comparison with D0 values and also with those obtained with saline group, for the group of mice treated by BUP HD, the application of the 0.6g filament revealed significant allodynia from D5 (Fig. 2A – Suppl Table 1). The application of the 1.4g filament gave the same results since a significant increase in paw withdrawal percentage, evoking allodynia, was observed at D5 and D9 (Fig. 2B – Suppl Table 1). The application of the 4.0g filament induced intense response (about 60% of paw withdrawal) throughout the Saline and BUP HD. At D9, a significant increase in paw withdrawal percentage was observed in the mice from the BUP HD group in comparison with the Saline group (Fig. 2C – Suppl Table 1). These observations indicate that chronic buprenorphine treatment led to the development of mechanical allodynia and a potential hyperalgesia.

3.2 Post-fracture: Loss of anti-allodynia and of analgesic effects of morphine in BUP HD group 24h and 48h after surgery

A closed tibial fracture was performed in all mice on the tenth day in order to induce severe acute pain. An acute injection of morphine (4.5 mg/kg; i.p.) was performed 30 min before the application of the 0.6g, 1.4g and 4.0 g filaments at H4, H24 and H48 post-fracture. The application of the 0.6g filament induced significant mechanical allodynia at H2 after surgery (significant increase of percentage of paw withdrawal in comparison with D0) that was no more observed in saline group after acute administration of morphine at H4 and H24 and H48

and also not observed in the BUP HD group but only at H4 (Fig. 3A – Suppl Table 1). After acute administration of morphine, the percentage of paw withdrawals was significantly higher in the BUP HD group at H24 and H48 post-fracture compared to Saline group. Overall, the application of the 1.4g filament provided the same results (Fig. 3B – Suppl Table 1). The application of the 4.0g filament induced a high level of pain (about 50% of paw withdrawal) since the beginning of the experiments and whatever the group of mice. As for the 0.6g and 1.4g Von Frey filaments, the percentage of paw withdrawals was significantly higher in the BUP HD group compared to Saline group at H24 and H48 after surgery, suggesting a loss of antiallodynic/analgesic effect of morphine (Fig. 3C – Suppl Table 1).

3.3 Post-fracture: multimodal analgesia is efficient to reduce pain in the BUP HD group

The third part of the protocol (named “multimodal analgesia”) involved mechanical pain tests at H48 after the closed tibial fracture in mice from the two groups. Before testing, mice were injected with morphine (4.5 mg/kg; i.p.) alone or in combination with either ketamine (10 mg/kg; i.p.) or ketoprofen (50 mg/kg; i.p.).

In the Saline group, the evoked response was very low with the three filaments in the VonFrey test (similar to pre-injection values) and thus significantly lower than the pain sensitivity at H2 (Fig. 4A-C – Suppl Table 2). Addition of ketamine or ketoprofen to morphine did not change the nociceptive response.

In the BUP HD group, after injection of morphine alone, pain was also similar to the post-fracture value, whereas a reduced pain response was observed in comparison to H2 when ketamine or ketoprofen was combined to morphine-(Fig. 4A-C – Suppl Table 2). In addition, two-way ANOVA revealed that addition of ketoprofen is efficient to reduce pain response for both 0.6g and 1.4g filaments (Fig. 4A-B). In the same way, addition of ketamine reduced pain response only for the 0.6g filament (Fig. 4A).

4 Discussion

The main findings of this study are that a daily treatment of mice with a high dose of buprenorphine (2x200 µg/kg / day; i.p.) induces a significant mechanical allodynia evidenced at five and nine days of treatment. In a marked contrast, changes in thermal sensitivity were not observed in response to buprenorphine (see Fig. S1A-C). Interestingly, morphine exerts anti-allodynic and analgesic effects 24h and 48h after a severe pain induced by traumatic orthopedic surgery in the buprenorphine treated mice for nine days compared to saline controls on mechanical nociception. Reduced pain response was also observed when ketamine or ketoprofen was combined with morphine two days after the surgery. Finally, an increase in pain sensation was observed one and two days after a closed tibial fracture despite a single injection of morphine (which was analgesic in the Saline group).

Prescriptions of buprenorphine have markedly increased of 75.2% between 2011 and 2016 in the USA (Piper et al 2018). Buprenorphine is now the most prescribed substitution drug for opioid addicts. In these patients, the management of intense acute pain as in case of orthopedic surgery is difficult and controversial. This is likely due to the high affinity and very low dissociation of buprenorphine from µ-opioid receptors that hinders the use of common opioid analgesics.

In this study, we pretreated mice during nine days with a high dose of buprenorphine (2x200 µg/kg / day; i.p.) to be as close as possible to clinical conditions of substitution treatment. We based our study on the work of Wala and Holtman (2011) showing that an acute administration of 100 to 200 µg/kg buprenorphine produced anti-nociception in rats.

One of the most original results of our work is that a chronic treatment with buprenorphine induced mechanical allodynia in-pain-free mice (pre-fracture experiments). Whereas not well known, as a subset of OIH, allodynia represents a potential complication of opioid therapy and has been first described after opioid intrathecal administration in rats and also in humans (Hayes and Painter, 2017). Several mechanisms of action of buprenorphine could have been involved in the emergence of allodynia. Activation of the mu opioid receptor, resulting in a classical form of OIH, is of course the primary suspect. However, buprenorphine is a partial agonist on mu opioid receptor but also an antagonist on kappa receptor, and, although with a lower affinity, can also activate nociceptin receptor at high doses. The fact that buprenorphine also binds and activates the nociceptin (NOP) receptor (Lufty and Cowan, 2004) raises the possibility of a contribution of this system to the allodynia observed herein. Indeed, nociceptin has been shown to induce tactile allodynia following intrathecal administration in mice (Hara 1997). Moreover, it has been recently suggested that buprenorphine could also act

as an inverse agonist on CB1 receptor and also as an activator of monoamine transporters (Olson et al 2019). The modulation of these targets could also participate in the observed allodynia through a reduction in endocannabinoid (Booker et al., 2012) and monoamine (Nagakura et al., 2009) tone.

Of particular note, chronic buprenorphine treatment did not affect thermal sensitivity using the Thermal Place Preference or the Hargreaves tests (Fig. S1) suggesting that OIH could be selective to mechanical stimuli. Such a distinction between mechanical and thermal sensitivity has been already reported in response to acutely administered buprenorphine (Siemian et al., 2016). It was also reported that a single administration of buprenorphine produced a greater analgesic effect on mechanical nociception compared to thermal nociception in a polyneuropathic rat model (Christoph et al., 2005). It is well known that sensory nerve fibers differ with respect to their sensory modalities and conduction velocities but also to their relative roles for pain hypersensitivity (Draxler et al., 2014). Based on the present results, it is plausible that pharmacological agents specifically influence mechanical or thermal sensitivity. Some few clinical experimental data suggest that buprenorphine maintenance subjects display hyperalgesia and are resistant to analgesic effects of high doses of morphine (Athanasos et al 2018). In the latter study, pain-free subjects were recruited and maintained under daily buprenorphine treatment (dose range = 2-22 mg; no dose change for 1.5-12 month). Buprenorphine subjects were hyperalgesic in the cold pressor test in comparison with controls. Subjects were tested at about the time of putative trough plasma buprenorphine concentrations. To our knowledge, the publication of Athanasos et al (2018) is the first work examining also the effect of added morphine to buprenorphine-substituted patients who were pain-free at the time of study. Very high doses of morphine (55 mg) failed to provide anti-nociception in either the electrical stimulation or cold pressor tests, irrespective of maintenance buprenorphine dose.

Since sedation could have represented a bias in the interpretation of the results, we decided to address this point by evaluating the locomotor activity of mice at baseline and after 9 days of saline or buprenorphine injection (see Fig S2). As expected, there was no locomotor activity difference between the two groups before injection of saline or buprenorphine (D0), showing a good balance in term of locomotor activity. Interestingly after 9 days of treatment, we observed a significant increase in locomotor activity following buprenorphine injection thereby ruling out the possibility that this pharmacological compound elicited sedative-like effects. Effect on locomotion was well described after opioid administration as shown by

Allouche (2013) where locomotor sensitization was observed after buprenorphine and morphine injection.

Some limitations of our study must be pointed out. In our model, mice chronically treated with buprenorphine were not pre-treated with an opioid agonist like heroin, so our model is not representative of a substitution treatment of opioid dependence. This choice relies on the fact that heroin is also known to induce hyperalgesia (Compton et al 2012) and we wanted to address the specific effects of buprenorphine.

Another important point that has yet to be solved is to determine whether it is necessary to discontinue/withdraw buprenorphine treatment in patients that undergo major elective surgery. So far, there is no consensus on this question although recommendations exist on perioperative optimal acute pain management strategies for patients taking buprenorphine. However, reduction or even stopping buprenorphine should be differentially considered regarding previous use of buprenorphine for chronic pain treatment or for substance in patients with opioid use disorder (Goel A et al 2019). Anderson et al (2017) suggested to continue buprenorphine in patients undergoing surgeries with only mild pain expected postoperatively, or patients at high risk for perioperative substance abuse relapse, because of the anxiety and possible destabilization caused by the cessation. In patients taking buprenorphine for chronic pain or if it is anticipated that postoperative pain may be difficult to control, buprenorphine may be discontinued preoperatively and replaced with opioid agonists in the interim. In our model, we decided to interrupt buprenorphine treatment before the orthopedic surgery.

Tibial fracture was chosen to induce a major orthopedic pain (Minville et al 2008). Among orthopedic surgery patients, preoperative opioid use is associated with increased postoperative pain, higher postoperative opioid requirements, and continued use of opioids in the weeks to months after surgery (Hayhurst & Durieux 2016; Smith et al 2017; Rozell et al 2017).

In our study, using the VonFrey test, we found that a dose of morphine (4.5 mg/kg) usually analgesic such as in the Saline group, was not efficient to alleviate pain in BUP HD group one and two days after the orthopedic surgery whichever the hind paw stimulated (fractured vs non-fractured paw). However, in the peri-operative period (H4) morphine was effective to produce analgesia, no difference was observed between Saline and BUP HD groups. It cannot also be excluded that at H24 or H48 apparent hyperalgesia (i.e. the loss of morphine effect) could represent withdrawal-induced hyperalgesia, a well-known phenomenon following the abrupt cessation of opioid (Stein, 2018) or tolerance to the antinociceptive effect of morphine. In fact, it is difficult to anticipate whether a higher dose of morphine would have produced

analgesic or hyperalgesic responses. On these grounds, it could have been interesting to compare the pain levels after surgery with and without morphine injection, to know if hyperalgesia is observed or not but also to evaluate the evolution of pain/recovery all along post-fracture experiment. We did not perform this control group for ethical reasons but also to fit with clinical practice in which pain must be relieved as soon as possible. Moreover, we do believe that tests at H2 represent valuable controls in post-fracture experiment and allow us to provide reliable conclusions only by evoking a loss of sensitivity to morphine and not hyperalgesia.

The efficacy of multimodal analgesic approach was then tested to relieve postoperative pain. We tested pharmacological substances now recommended for the perioperative pain treatment in human.

The combination of opioids and nonsteroidal anti-inflammatory drugs (NSAIDs) may provide synergistic analgesia. Evidence suggests a role for COX-2 inhibitors in the modulation of OIH in humans (Angst et al 2003; Koppert et al 2003). Thus, it is suggested that there is a possible role for prostaglandins in sensitizing the nociceptive system before pathologic activation, and that it probably has a less important role than the N-methyl-D-aspartate receptor (NMDA) system, at least in human experimental pain models after acute opioid exposure (Lee et al 2011). Therefore, we assessed multimodal analgesia two days after surgery to alleviate pain observed in VonFrey test despite morphine (4.5 mg/kg) administration. Our results showed that the combination of morphine with ketoprofen, a nonsteroidal anti-inflammatory drug (NSAID), was able to alleviate pain in BUP HD group in comparison with morphine alone. These results suggest that the combination of opioids and NSAIDs may provide additive analgesia and alleviate OIH. Multimodal analgesia with ketoprofen associated to morphine was efficient to reduce pain in post-fracture experiments in the BUP HD group.

Minville and colleagues (2010) investigated the effects of the NMDA-receptor antagonist ketamine on postoperative hyperalgesia in a mouse model of orthopedic pain. These experiments showed that mice given intraoperative opioid analgesia (i.e. sufentanil) and high dose ketamine had significantly less thermal hyperalgesia (measured as the amount of time a hind paw could remain on a hot plate before withdrawal) for the first 4 postoperative days as compared to mice treated with sufentanil alone. Prior research with ketamine had shown similar results. Indeed, ketamine enhanced the earlier response (analgesia) and prevented the development of long-lasting enhancement in pain sensitivity in fentanyl-treated rats (Celerier et al 2000). The NMDA receptor antagonist MK-801 also prevented both heroin-induced long-lasting enhancement in pain sensitivity, suggesting that hyperalgesia is issued from a

neuroadaptive process in which NMDA systems play a critical role (Celerier et al 1999; 2001; Laulin et al 2002). On this context, we assessed the combination of morphine with ketamine. Our results showed that this combination was able to alleviate pain in BUP HD group in comparison with morphine alone. We chose not to evaluate the effect of ketamine and ketoprofen alone because it would have been necessary to split groups and we would have to reduce the number of mice per group. In fact, these results suggest that the combination of opioids and NMDA-receptor antagonist may provide additive analgesia and alleviate OIH compare to morphine alone.

While it is unrealistic to expect that the diversion and misuse of opioid analgesics can be entirely eradicated, the effects of these drugs on public health need to be acknowledged, tracked, and mitigated. Important research gaps exist in such areas and intensive efforts worldwide (from basic science to drug development) must be directed toward understanding the neurobiology of acute/chronic pain and the mechanism of action of related treatments based on the manipulation of opioid receptors. This will likely help provide new therapeutic options with safer and more effective profile. In this prospect, three main strategies are currently under investigation: mu-opioid receptors biased agonists, multiple opioid receptor mixed agonists, and blockade of the catabolism of endogenous peptides (Turnaturi et al., 2019, Noble and Marie, 2018).

To conclude, our findings showed for the first time that a chronic treatment with a high dose of buprenorphine is able to induce an allodynia on pain-free mice model and that our model of closed tibia fracture will allow to study new protocols of pain management of peri- and postoperative severe acute pain management for patients suffering from OIH.

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Figure 1 – Experimental protocol. Pain tests: VonFrey filament test.

Figure 2 – Pre-fracture: A prolonged treatment with a high dose of buprenorphine induced mechanical allodynia and hyperalgesia Mechanical nociception test using von Frey filaments with bending force (A) 0.6g, (B) 1.4g, and (C) 4.0g. Tests were performed at baseline (D0), after five and nine days of chronic treatment (D5-D9). Results are expressed in percentage of withdrawal \pm S.E.M. for Saline (n=17, white bar) and BUP group (n=22, black bar). *** P<0.001 significantly different from their respective baseline (D0); \$ P< 0.05 and \$\$\$ P<0.001 significantly different from Saline group at the corresponding day.

Figure 3 – Post-fracture: Loss of anti-allodynia and analgesic effects of morphine in the BUP HD group 24h and 48h after surgery Mechanical nociception test using von Frey filaments with bending force (A) 0.6g, (B) 1.4g, and (C) 4.0g. Tests were performed at baseline (D0); pre-fracture (Fig. 2), two (H2), four (H4), twenty-four (H24) and forty-eight h (H48) after a closed tibial fracture. Results are expressed in percentage of withdrawal \pm S.E.M. for Saline (n=17, white bar) and BUP group (n=22, black bar). For H48 tests, both groups were divided in subgroups for multimodal analgesia (Saline n=8; BUP HD n=6). For each result \$ P< 0.05, \$\$ P< 0.01 and \$\$\$ P<0.001 significantly different from their respective value assessed two h after fracture (H2). * P< 0.05 and *** P<0.001 significantly different from baseline (D0), ° P<0.05, °°P<0.01 and °°° P<0.001 significantly different from their respective Saline group.

Figure 4 - Post-fracture: multimodal analgesia is efficient to reduce pain in the BUP HD group Mechanical nociception test using von Frey filaments with bending force (A) 0.6g, (B) 1,4g and (C) 4.0g. Tests were assessed two h (H2; Fig. 3) and 2 days after a closed tibial fracture (H48). Results were expressed in percentage of withdrawal \pm S.E.M. for Saline (white histogram) and BUP HD group (black bar). Respective results of H2 tests were represented by horizontal solid line (Mean) and discontinuous line (S.E.M.). All groups were divided in subgroups to assess different multimodal analgesia: Saline (Morphine, n=8; Morphine+Ketamine, n=4; Morphine+Ketoprofene, n=5), and BUP HD (Morphine, n=6; Morphine+Ketamine, n=8; Morphine+Ketoprofene, n=10). For each result * p<0.05, ** P< 0.01 and *** P<0.001: significantly different from Morphine condition. For each result \$ P<0.05, \$\$ P< 0.01 and \$\$\$ P<0.001: significantly different from respective H2 tests.

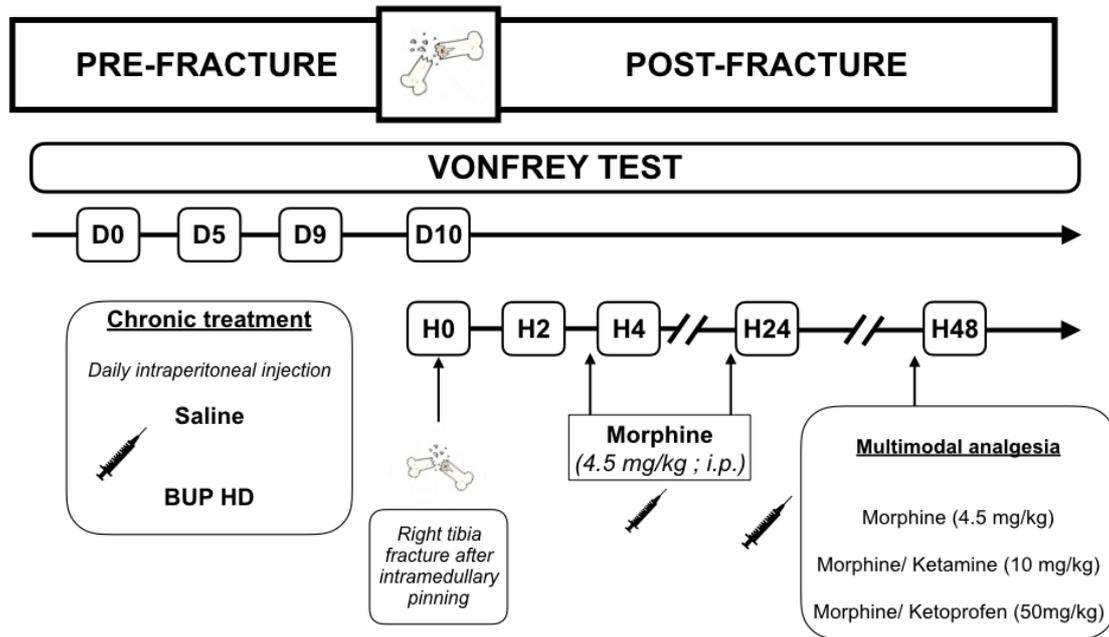


Figure 1

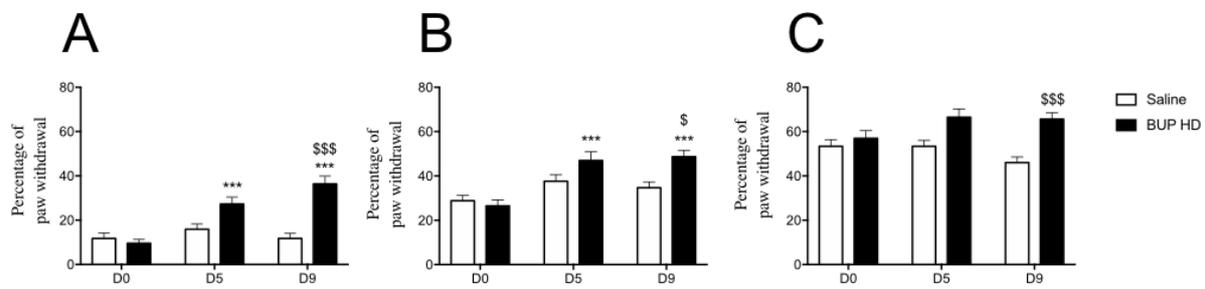


Figure 2

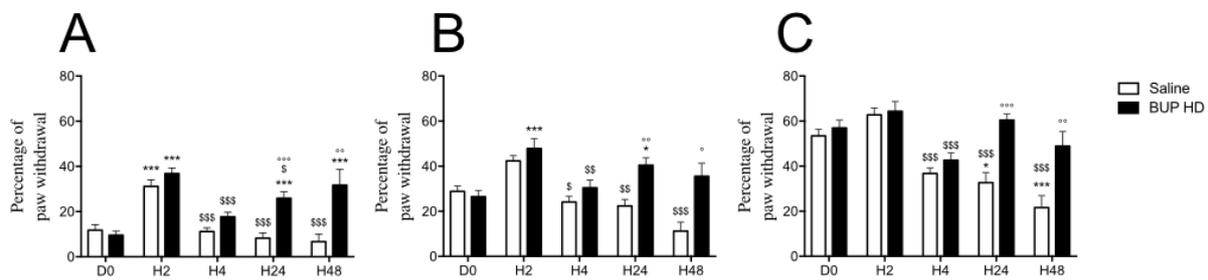


Figure 3

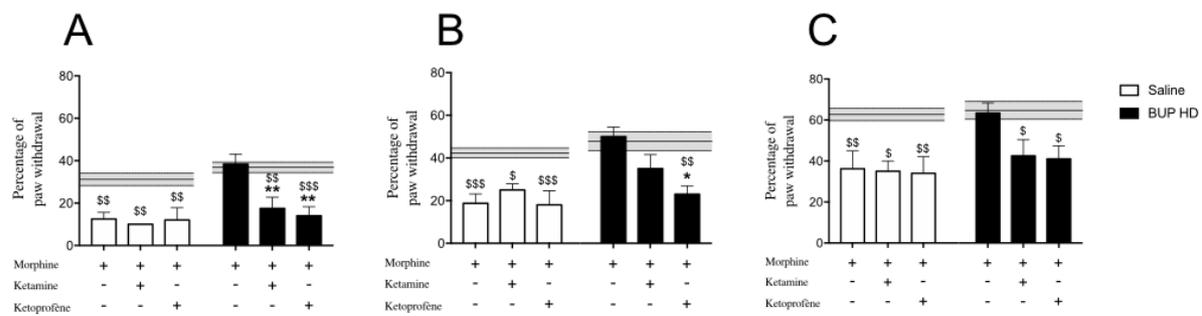


Figure 4

SUPPLEMENTARY DATA

1- Materials and methods

1.1 Thermal nociception

The protocol of the Thermal Place Preference Test was as described by Hache et al (2012). Mice were placed in an enclosure in which the floor is composed by two adjoining thermoelectric computer-managed metal plates (Bioseb Inc., Vitrolles France), and first were allowed to freely explore the area for 5 min daily, for 2 days (habituation). Both plates were enclosed by an opaque Plexiglas box (Bioseb Inc., Vitrolles France). Low illumination was used for each compartment. Preliminary experiments determined that ‘naïve’ animals spent a similar amount of time on the two plates when they were set to 25°C, suggesting that there is no bias towards one compartment. Subsequently, in the present work, the floor of one compartment was maintained at 25°C and the other plate was set at 20°C (moderate cold). This latter temperature is well within the threshold range of channel complexes localized within the terminal endings of sensory neurones that are capable of responding to cooling and cold. The animals were videotaped from above for 5 min, and the time spent on each plate was automatically recorded. The number of transitions between the two plates and the total mobility time were recorded as well, to check the good walking ability of the animals in the post-fracture time.

Paw withdrawal latency in response to radiant heat was measured using the Hargreaves equipment (Ugo Basile, Varese, Italy). Mice were placed in a clear plastic chamber on a glass floor, and allowed to acclimate to this environment for at least 15 min before testing. Then the radiant heat source was positioned under the glass floor directly beneath the right hind paw, and a trial commenced by a switch which activated the radiant heat source and started an electronic timer. The radiant heat source consisted of a high intensity projector lamp bulb located around 12 cm under the glass floor, so as to create a temperature of 45°C on the hind paw. The heat beam was switched off when the mouse lifted or licked its hind paw, signs of pain, and the electronic timer stopped automatically. A cut-off time of 20 sec was used to avoid tissue damage. Two measures, separated by a 5 min interval, were performed and the mean of the two values was kept.

1.2 Locomotor Activity

Mice were tested in the Open Field test in order to evaluate the effect of Saline and BUP HD treatment on locomotion at D0 and D9 in pre-fracture experiment. The circular field dimensions were 40 x 30 cm. The tests were conducted in the morning, following habituation in room experimentation (1 h in the homecage). Mice were tracked by video tracking (Ethovision Noldus, Wageningen, The Netherlands). Before the beginning of chronic treatment and 30 min after the last drug administration, each animal was placed in the center of the open field then locomotor activity was recorded during 10 min. The open field was cleaned with 30% ethanol between animals. Locomotor activity score was expressed as distance travelled (in cm).

2 – Results

2.1 Lack of evidence of allodynia or hyperalgesia in thermal nociceptive tests

The percentages of time spent on the 20°C plate in the Thermal Place Preference Test on D0 were undistinguishable between both groups such as the paw withdrawal latencies in response to radiant heat (Hargreaves' test). After the 9-days chronic treatments (pre-fracture), buprenorphine-treated mice showed a marked decrease of time spent on 20°C plate but it was not significant (Figure S1A) and the paw withdrawal latencies were similar (Figure S1C) in both groups. In this case, the chronic treatment with high doses of buprenorphine did not induce any allodynia or hyperalgesia thereby demonstrating different sensibilities of mice to mechanical or thermal stimuli.

In the Thermal Place Preference Test, the level of pain was higher in both groups two and four h (H2 and H4) after the surgery without highlighting any consequences of the chronic treatments, nor of morphine administration (figure S1B). Two-way ANOVA only showed a significant effect of BUP HD treatment at H2 with an increase of pain sensitivity while 4.5 mg/kg of morphine reversed this effect at H24 (D0 vs H2, $P=0.009$; H2 vs H24, $P=0.021$). As observed previously in mechanical experiment, the chronic treatment with BUP HD did not induce any allodynia/hyperalgesia 24 h after surgery, again demonstrating different sensibilities of mice to mechanical or thermal stimuli. In the Hargreaves test, the pain threshold remained stable throughout the experiments (Figure S1D).

2.1 Chronic buprenorphine treatment promotes locomotor activity

As expected, the results of locomotor activity test at D0 showed that there is no difference in distance travelled between both groups which were well balanced in term of locomotor activity. Interestingly after 9 days of treatment, two-way ANOVA [Treatment x Day] showed a significant effect of interaction ($F_{(1:16)}=38.6$, $P < 0.001$), Day factor ($F_{(1:16)}=17.2$, $P < 0.001$) and Treatment factor ($F_{(1:16)}=23.6$, $P < 0.001$). Indeed, we observed a significant increase in locomotor activity in buprenorphine-injected mice after nine days of chronic treatment compared to their respective baseline (D9 vs. D0: $P < 0.001$).

FIGURE S1

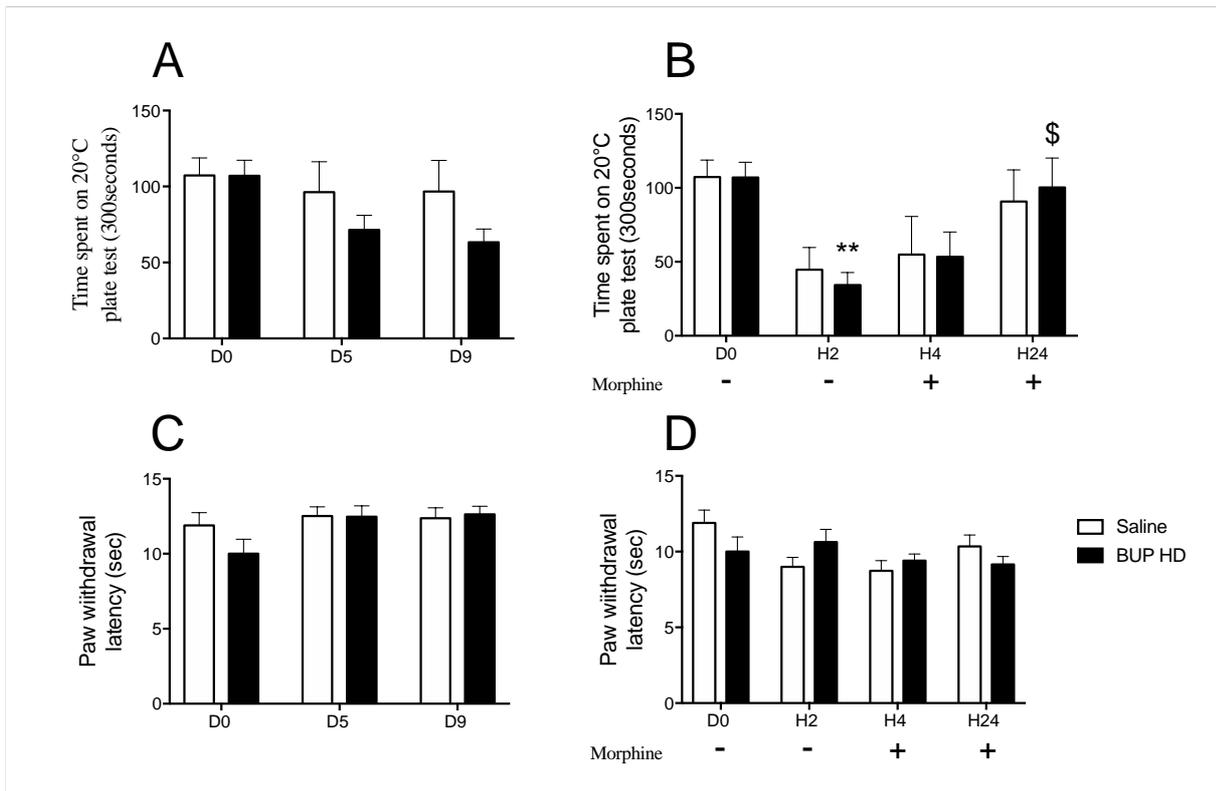
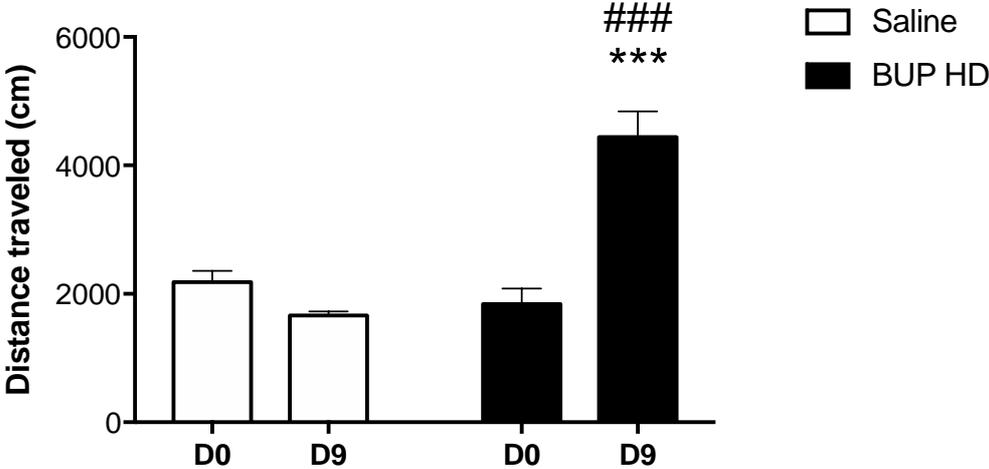


FIGURE S2



SUPPLEMENTARY TABLE 1

| Experiment | Statistical values from two-way ANOVA | Significant P values from post hoc Tukey tests | Figure |
|----------------------|--|---|------------------|
| Pre-fracture | | | Figure 2 |
| 0.6g filament | Interaction F(2,111)=11.4 P<0.0001 Time F(2,111)=12.9 P<0.0001 Treatment F(1,111)=24.2 P<0.0001 | BUP HD vs. Saline: D9 P=0.0001 BUP HD group: D5 vs. D0 P=0.0001; D9 vs. D0 P=0.0001 | Figure 2A |
| 1.4g filament | Interaction F(2,114)=3.63 P=0.0295 Time F(2,114)=14.2 P<0.0001 Treatment F(1,114)=7.59 P=0.0068 | BUP HD vs. Saline: D9 P=0.0228 BUP HD group: D5 vs. D0 P=0.0001; D9 vs. D0 P=0.0001 | Figure 2B |
| 4.0g filament | Interaction F(2,108)=2.87 P=0.0609 (NS) Time F(2,108)=1.18 P=0.3105 Treatment F(1,108)=19.6 P<0.0001 | BUP HD vs. Saline: D9 P=0.001 | Figure 2C |
| Post-fracture | | | Figure 3 |
| 0.6g filament | Interaction F(4,158)=6.24 P<0.0001 Time F(4,158)=26.9 P<0.0001 Treatment F(1,158)=32.5 P<0.0001 | BUP HD vs. Saline: H24 P=0.0001; H48 P=0.003 Saline group: H2 vs. D0 P=0.0001; H4 vs. H2 P=0.0001; H24 vs. H2 P=0.0001; H48 vs. H2 P=0.0001. BUP HD group: H2 vs. D0 P=0.0001; H24 vs. D0 P=0.0001; H48 vs. D0 P=0.0005; H4 vs. H2 P=0.0001; H24 vs. H2 P=0.0289 | Figure 3A |
| 1.4g filament | Interaction F(4,167)=3.83 P=0.0052 Time F(4,167)=11.3 P<0.0001 Treatment F(1,167)=19.8 P<0.0001 | BUP HD vs. Saline: H24 P=0.0054; H48 P=0.0245. Saline group: H2 vs. D0 P=0.17; H48 vs. D0 P=0.1374 H4 vs. H2 P=0.0121; H24 vs. H2 P=0.0035; H48 vs. H2 P=0.0001 | Figure 3B |

| | | | |
|-----------------------------|---|--|------------------|
| | | BUP HD group: H2 vs. D0 P=0.0001; H4 vs. H2 P=0.0029; H24 vs. H2 P=0.77; H48 vs. H2 P=0.4959; H24 v. D0 P= 0.0443. | |
| 4.0g filament | Interaction F(4,164)=5.33 P=0.0005 Time F(4,164)=16.1 P<0.0001 Treatment F(1,164)=27.4 P<0.0001 | BUP HD vs. Saline: H24 P=0.0001; H48 P=0.0045. Saline group: H2 vs. D0 P=0.84; H4 vs. H2 P=0.0006; H24 vs. H2 P=0.0001; H48 vs. H2 P=0.0001; H24 vs. D0 P=0.0181; H48 vs. D0 P=0.0001 BUP HD group: H4 vs. H2 P=0.0003 | Figure 3C |
| Multimodal Analgesia | | | Figure 4 |
| 0.6g filament | Interaction F(2,35)=3.58 P=0.038 Analgesia F(2,35)=4.38 P=0.02 Treatment F(1,35)=8.86 P=0.0053 | Saline group: MOR vs MOR-KETA P=0.939; MOR vs MOR-KETO P=0.9971; MOR-KETA vs MOR-KETO P=0.967. BUP HD group: MOR vs MOR-KETA P=0.0081; MOR vs MOR-KETO P=0.0012; MOR-KETA vs MOR-KETO P=0.8151. | Figure 4A |
| 1.4g filament | Interaction F(2,35)=3.5 P=0.041 Analgesia F(2,35)=3.64 P=0.0366 Treatment F(1,35)=12 P=0.0014 | Saline group: MOR vs MOR-KETA P=0.7355; MOR vs MOR-KETO P=0.9949; MOR-KETA vs MOR-KETO P=0.7254. BUP HD group: MOR vs MOR-KETA P=0.1173; MOR vs MOR-KETO P=0.0014; MOR-KETA vs MOR-KETO P=0.1656. | Figure 4B |
| 4.0g filament | Interaction F(2,35)=1.09 P=0.3463 Analgesia F(2,35)=1.54 P=0.22 Treatment F(1,35)=4.54 P=0.0401 | Saline group: MOR vs MOR-KETA P=0.9942; MOR vs MOR-KETO P=0.9784; MOR-KETA vs MOR-KETO P=0.9969. | Figure 4C |

| | | | |
|--|--|--|--|
| | | BUP HD group: MOR vs MOR-KETA P=0.1415; MOR vs MOR-KETO P=0.0888; MOR-KETA vs MOR-KETO P=0.9861. | |
|--|--|--|--|

SUPPLEMENTARY TABLE 2

| Experiment | Statistical values from One-way ANOVA (Dunnnett's multiple comparisons test) | | |
|---|--|---|------------------|
| | Saline group | BUP HD group | Figure |
| 0.6g filament | F(3,30)=8.928 P=0.0002 <u>Comparison vs. H2</u> MOR P=0.0012 MOR-KETA P=0.0044 MOR-KETO P=0.0049 | F(3, 44)=11.36 P<0.0001 <u>Comparison vs. H2</u> MOR P=0.9928 MOR-KETA P=0.0012 MOR-KETO P<0.0001 | Figure 4A |
| 1.4g filament | F(3,30)=12.26 P<0.0001 <u>Comparison vs. H2</u> MOR P<0.0001 MOR-KETA P=0.0205 MOR-KETO P=0.0004 | F(3,44)=4.03 P=0.0129 <u>Comparison vs. H2</u> MOR P=0.9349 MOR-KETA P=0.3854 MOR-KETO P=0.0083 | Figure 4B |
| 4.0g filament | F(3,30)=7.435 P=0.0007 <u>Comparison vs. H2</u> MOR P=0.0028 MOR-KETA P=0.0169 MOR-KETO P=0.0064 | F(3,44)=4.286 P=0.0097 <u>Comparison vs. H2</u> MOR P=0.9999 MOR-KETA P=0.0412 MOR-KETO P=0.0149 | Figure 4C |
| <i>MOR (Morphine); MOR-KETA (Morphine + Ketamine); MOR-KETO (Morphine + Ketoprofen)</i> | | | |

Figure S1 – Chronic Buprenorphine did not induce allodynia nor hyperalgesia in thermal nociceptive tests (A-B) Thermal place preference test. Results of Saline (n=9), MOR (n=13) and BUP (n=12) groups are represented as time spent on the 20°C plate \pm S.E.M. in pre- (A) and post-fracture (B) experiments in which all animals received a single injection of morphine (4.5 mg/kg; i.p.). (C-D) Hargreaves test. Results of Saline (n=11), MOR (n=12) and BUP (n=13) groups are represented as latency of paw withdrawal \pm S.E.M. in pre- (C) and post-fracture (D) experiments in which all animals received a single injection of morphine (4.5 mg/kg; i.p.). For each result * $P < 0.05$ significantly different from their respective baseline (D0). \$ $P < 0.05$ significantly different from respective H2 test.

Figure S2 – Chronic Buprenorphine increases locomotor activity. Data are mean \pm S.E.M. of distance travelled (in cm) in the open-field for 10 min before (D0) and after (D9) injection of saline (n=5) or BUP (n=5). * $p < 0.001$ significantly different from the corresponding group of mice tested at D0 respective baseline (D0). ### $P < 0.001$ significantly different from the group of mice injected with saline at D9.**