Synergism between oral paracetamol and nefopam in a murine model of postoperative pain

Running head: *Oral paracetamol-nefopam for post-surgical hypersensitivity*

D. Cabañero[1,a] and R. Maldonado[1,2]

[1] Neuropharmacology Laboratory, Department of Experimental and Health Sciences, Pompeu Fabra University, Barcelona, Spain

[2]IMIM (Hospital del Mar Medical Research Institute), Barcelona, Spain

[a] Present address: Institute of Research, Development and Innovation in Healthcare Biotechnology of Elche (IDiBE), Universidad Miguel Hernández. Elche, Alicante, Spain

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Author responsible for correspondence:

Rafael Maldonado

Laboratory of Neuropharmacology, Health and Life Sciences School, University Pompeu Fabra, PRBB, c/Dr. Aiguader 88, 08003 Barcelona, Spain

Phone: 34-93-3160824; E-mail: rafael.maldonado@upf.edu

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Conflicts of interest:

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Significance:

Early postoperative pain is currently undertreated and has been recognized as a relevant source of chronic post-surgical pain. Oral efficient treatments could facilitate fast-track surgeries and patient recovery at home. Here we identify in a mouse model of postoperative pain a potent synergistic oral combination consisting of low paracetamol and nefopam doses that provides relief of post-surgical hypersensitivity to mechanical and thermal stimuli. Oral multimodal paracetamol-nefopam mixtures represent a potential clinically-available pharmacological strategy for the relief of incisional sensitivity and the promotion of patient recovery.

Abstract

Background: The use of paracetamol or nefopam for postoperative pain control is limited by the need of high doses associated with unwanted effects. Previous works suggest positive interactions between both compounds that may be exploited to obtain potentiation of antinociception.

Methods: Mechanical and heat antinociception induced by oral doses of paracetamol, nefopam or their combination was studied by isobolographic analysis in a murine model of post-surgical pain. The effective doses that produced 50% antinociception (ED_{50}) were calculated from the log dose-response curves for each compound. Subsequently, the effects of $ED_{8.7}$s, $ED_{12.5}$s, $ED_{17.5}$s and ED_{35}s of nefopam and paracetamol combined were assessed.

Results: Oral paracetamol induced dose-dependent relief of postoperative sensitivity and showed higher efficacy reducing mechanical hypersensitivity (ED_{50} 177.3±15.4 mg/kg) than heat hyperalgesia (ED_{50} 278.6±43 mg/kg). Oral nefopam induced dose-dependent antinociception with similar efficacy for mechanical and heat hypersensitivity (ED_{50}s 5.42±0.81 vs 5.83±0.72). Combinations of increasing isoeffective doses revealed that combined $ED_{17.5}$s (85.76 mg/kg paracetamol and 1.9 mg/kg nefopam) and ED_{35}s (132.67 mg/kg and 3.73 mg/kg) showed synergistic effects leading to 75% and 90% mechanical antinociception, respectively. These mixtures were defined by interaction indexes of 0.43 and 0.41 and ratios 45:1 and 35:1 paracetamol:nefopam, respectively. The same combinations showed additive effects for the inhibition of incisional thermal hyperalgesia.

Conclusions and limitations: This work describes a synergistic antinociceptive interaction between low doses of nefopam and paracetamol for the treatment of postoperative hypersensitivity to peripheral stimuli. The promising results obtained on reflexive nociceptive responses of young male mice subjected to plantar surgery highlight the interest of further research evaluating the effects of this mixture on the affective-motivational component of pain and in females and additional age groups. Confirmation of pain-relieving efficacy and safety of this oral combination clinically available in European and Asian countries could provide a useful tool for post-surgical pain management.

1. Introduction

Safe and efficient treatment of early post-operative pain is essential for the recovery of surgical patients (Kehlet and Wilmore, 2002). Such statement acquires special relevance taking into consideration that difficulties in controlling acute postoperative pain increase the odds of developing chronic post-surgical pain (Glare et al., 2019). Opioid drugs are efficient for the management of moderate to severe postoperative pain (Desai et al., 2018). However, an overreliance on prescription opioids has produced devastating effects in USA that call for limiting opioid use and finding therapeutic alternatives (DeWeerdt, 2019). Furthermore, current needs for hospitalization space demand novel and optimized pharmacological strategies that allow timely discharge of patients (Nurok and Kahn, 2020; Poeran et al., 2020). The combined use of different groups of approved analgesic medications, namely multimodal analgesia, represents a safe and advantageous strategy for domiciliary pain management of the surgical patient (Fiore et al., 2019). In this scenario, the murine model of incisional pain has been widely used for the study of the manifestations and mechanisms of post-surgical hypersensitivity to mechanical and thermal stimulation, and it also provided translational and anticipated information for improving postoperative pain management in the clinics (Aguado et al., 2013; Cabañero et al., 2009a, 2009b; Célérier et al., 2004; Richebé et al., 2005).

Paracetamol is an analgesic and antipyretic drug available worldwide that shows efficacy for mild to moderate pain and is used for postoperative pain control. It inhibits cyclooxygenase activities, but other mechanisms of action have also been involved in its antinociceptive effects, including inhibition of nitric oxide production, interaction with non-canonical cyclooxygenase isoforms, stimulation of cannabinoid receptor 1 and transient receptor potential TRPV1 or TRPA1 and activation of serotoninergic pathways (Chandrasekharan et al., 2002; Bonnefont et al., 2003; Mallet et al., 2008; Andersson et al., 2011; Przybyła et al., 2020). However, there are safety concerns involving the use of high doses of paracetamol (Roberts et al., 2016) associated with decreases in platelet function (Munsterhjelm et al., 2005) and liver toxicity, being this one of the leading causes of hospital admission for acute liver failure (Ghanem et al., 2016; Kolodziejczyk et al., 2020; Munsterhjelm et al., 2005; Zhang et al., 2002). Among other non-opioid alternatives for the control of moderate pain, nefopam is a prescription drug available in European and Asian countries, mainly administered by parenteral routes in perioperative settings. It is a

benzoxazocine compound that inhibits monoamine reuptake and engages opioidergic mechanisms at spinal and supra-spinal sites (Fuller and Snoddy, 1993; Gray et al., 1999; Gregori-Puigjané et al., 2012). It is used for the treatment of postoperative pain with varying success (Evans et al., 2008; Kakkar et al., 2009), but also presents unwanted effects that limit its use such as nausea, vomiting, tachycardia, somnolence or injection-related pain that can be observed at therapeutic doses (Evans et al., 2008). Both drugs, paracetamol and nefopam have shown efficacy in rodent models of pain after injection (Girard et al., 2004, 2008, 2009; van den Hoogen et al., 2016; Minville et al., 2011) and experiments combining both molecules suggested positive interactions that may allow decreasing the doses needed to obtain effective relief of postoperative sensitization (Girard et al., 2011; Li et al., 2018)

The purpose of this study is to evaluate the acute interaction between paracetamol and nefopam given orally on the expression of post-surgical mechanical and thermal hypersensitivity in a murine model that fairly reproduces the clinical settings (Aguado et al., 2013; Cabañero et al., 2009a, 2009b; Célérier et al., 2004; Pogatzki and Raja, 2003; Richebé et al., 2005). A side-by-side comparison of the antinociceptive effects of paracetamol and nefopam is used to determine the median effective doses (ED50) for each compound. Subsequently, the efficacy of graded combinations of EDs is investigated through isobolographic analysis (Tallarida, 2006) to determine possible interactions.

2. Methods

2.1. Animals

Outbred male CD1 albino mice (Charles Rivers, Lyon, France) 8 to 10 weeks old at the beginning of the experiment were used, based in our previous works on post-incisional pain (Cabañero et al., 2009; Campillo et al., 2010; Célérier et al., 2006). Mice were group-housed (2-4 animals), maintained in cages with free access to food and water and kept on a 12-h light-dark cycle (light on at 08.00h, light off at 20.00h) in a controlled temperature (21 ± 1 °C) and humidity (55 ± 10%) environment. All animal procedures were conducted in accordance with standard ethical guidelines (European Communities Directive 2010/63/EU and NIH Guide for Care and Use of Laboratory Animals, 8th Edition) and approved by autonomic (Generalitat de Catalunya, Departament de Territori i Sostenibilitat) and local (Comitè Ètic d'Experimentació Animal, CEEA-PRBB) ethical committees. Mice were randomly assigned to treatment groups and experiments were performed blinded for pharmacological conditions.

2.2. Incisional pain model

The incisional pain model that reproduces postoperative sensitization to mechanical and thermal stimuli was adapted from a previous study in rats (Pogatzki and Raja, 2003), with some modifications (Cabañero et al., 2009b). Mice were anesthetized with isoflurane vaporized in oxygen, delivered using a nose mask (induction, 5% V/V; surgery, 2.5% V/V). A 0.7-cm longitudinal incision was made with a number 15 blade through the skin and fascia of the plantar surface of the right hind paw, starting 0.3 cm from the proximal edge of the heel and extending toward the toes. The underlying plantaris muscle then was elevated and incised longitudinally, keeping the muscle origin and insertion intact. After hemostasis with gentle pressure, the skin was closed with two 6-0 silk sutures and the wound was covered with povidone-iodine antiseptic. After surgery, the animals were allowed to recover in cages with sterile bedding under a heat source. Nociceptive sensitivity was evaluated the second day after the surgery to ensure maximal hypersensitivity without interference with surgical anaesthesia.

2.3 Nociceptive behavioural tests

Sensitivity to mechanical and thermal stimuli was measured by using the following behavioural models:

- ***Mechanical sensitivity*** was quantified by measuring the hind paw withdrawal response to von Frey filament stimulation (Chaplan et al., 1994). Briefly, animals were placed in Plexiglas® cylinders (20 cm high, 9 cm diameter) with a wire grid bottom through which the von Frey filaments (bending force range from 0.008 to 2 g) (North Coast Medical, Inc., San Jose CA, USA) were applied by using the up-down paradigm as previously described (Chaplan et al., 1994). The filament of 0.4 g was first applied. Then, the strength of the next filament was decreased when animal responded or increased when animal did not respond. This up-down procedure was stopped 4 measures after the first change in animal responding (i.e. from response to no response or from no response to response). The threshold of response was then calculated by using the up-down excel program generously provided by the laboratory of Dr A. Basbaum (UCSF, San Francisco, USA). Prior to baseline measurements, mice were habituated for 2 hours to the testing environment during 3 days. On the evaluation days, animals were allowed to habituate for 1-2 hours before testing in order to obtain appropriate behavioural immobility. Both ipsilateral and contralateral hind paws were alternatively tested whenever possible, and stimuli were applied at a minimum of 2 min intervals to avoid hypervigilance or sensitization between successive filament applications. Filaments were completely bent before considering responses and hold up to 4-5 s to consider a negative response. Clear paw withdrawal, shaking or licking were considered as nociceptive-like responses.

- ***Thermal sensitivity*** was assessed by using the plantar test (Hargreaves et al., 1988). Paw withdrawal latency in response to radiant heat was measured using the plantar test apparatus (Ugo Basile, Varese, Italy). Briefly, mice were placed in Plexiglas® cylinders (20 cm high, 9 cm diameter) positioned on a glass surface. Prior to baseline measurements, mice were habituated for 2 hours to the testing environment during 3 days. On the evaluation days, animals were allowed to habituate to the environment for 30 min before testing to obtain appropriate behavioural immobility. The heat source was then positioned under the plantar surface of the hind paw and activated with a light beam intensity, chosen in preliminary studies to give baseline latencies from 9 to 11 s in naïve mice. A digital timer connected to the heat source recorded the response latency for paw

withdrawal to the nearest 0.1 s. A cut-off time of 20 s was imposed to prevent tissue damage in absence of response. The mean paw withdrawal latency for the ipsilateral and contralateral hind paws was determined from the average of 3-4 separate trials, taken at 5 min intervals between applications on the same paw, and at least at 2 min intervals between applications on different paws, in order to prevent behavioural disturbances such as hypervigilance or sensitization.

2.4 Experimental protocol

In the first stage, animals were habituated for 2 hours to the testing environment in each paradigm during 3 days. After the habituation period, baseline responses were established during 2 consecutive days for each test in the following sequence: von Frey test and plantar test. On the second day before baseline measurements, mice were habituated to the oral gavage using disposable feeding needles of malleable metal (Agnthos, Lidingö, Sweden). Briefly, mice were gently grasped by the nape of the neck close to the ears and were hold in an upright position. Then, the head of de feeding needle was inserted into the back of their mouth and the needle was turned up to be in line with the esophagus of the mouse. Thus, their head was tipped back to have neck, head and spine in a straight line. Once in this position, the feeding needle was inserted, and the product delivered. Drinking water was used for habituation. After the baseline measurements, incisional injury was induced as previously described. Paracetamol, nefopam or vehicle were administered by oral route with the feeding needles 2 days after surgery. Nociceptive measurements were performed between 30 and 60 min after treatments for the von Frey test and between 75 and 105 min after treatments for the plantar test. Animals were randomly distributed in the different experimental groups, as follows:

Phase I: Head-to-head comparison paracetamol vs nefopam to determine the ED_{50} values. The following experimental groups were included:

Group 1: Incisional injury + Vehicle (n = 10)

Group 2: Incisional injury + Paracetamol (50 mg/kg) (n = 10-11)

Group 3: Incisional injury + Paracetamol (100 mg/kg) (n = 10)

Group 4: Incisional injury + Paracetamol (200 mg/kg) (n = 10)

Group 5: Incisional injury + Paracetamol (300 mg/kg) (n = 10)

Group 6: Incisional injury + Paracetamol (400 mg/kg) (n = 10)

Group 7: Incisional injury + Nefopam (1.5 mg/kg) (n = 10)

Group 8: Incisional injury + Nefopam (3 mg/kg) (n = 11)
Group 9: Incisional injury + Nefopam (6 mg/kg) (n = 10)
Group 10: Incisional injury + Paracetamol (9 mg/kg) (n = 11)
Group 11: Incisional injury + Paracetamol (12 mg/kg) (n = 10)

In this phase, the first doses tested were paracetamol 100 mg/kg and nefopam 3 mg/kg. The following doses were chosen according to the inhibition of nociception previously observed.

Phase II: To quantify possible synergistic effects of paracetamol and nefopam by isobolographic analysis. Dose combinations were calculated from the ED_{50}s obtained in phase I for the anti-allodynic effect using the following experimental groups:
Group 1: Incisional injury + Vehicle (n = 10)
Group 2: Incisional injury + $ED_{8.7}$ paracetamol-nefopam combination (n = 12)
Group 3: Incisional injury + $ED_{12.5}$ paracetamol-nefopam combination (n = 13)
Group 4: Incisional injury + $ED_{17.5}$ paracetamol-nefopam combination (n = 12)
Group 5: Incisional injury + ED_{35} paracetamol-nefopam combination (n = 12)

2.5 Drug preparation

Paracetamol and nefopam were supplied by Aptys pharmaceuticals and were dissolved in a vehicle containing 10% N-Methyl-2-pyrrolidone (Merck, Darmstadt, Germany) and 90% purified water (B Braun Medical Inc., Melsungen, Germany).

2.6 Data Analysis

For each treatment, time-courses of mechanical and thermal sensitivity were analysed with repeated measures ANOVA, with dose as between-subject factor and day as within-subject factor. The Bonferroni post-hoc test was used to compare the thresholds of the hind paws before and after the surgery and to estimate the differences between each dose or the vehicle on the test day. Both baseline responses (days -1 and -2) were considered in the statistical analysis. A p value less than 0.05 was considered statistically significant.

The Percentage Maximum Possible Effect (%MPE) on the nociceptive thresholds of each mouse was calculated as the opposite of the difference between the response of the mouse and its baseline response, divided by the difference of the average response

of vehicle-treated mice and their respective average baseline response, following this formula:

$$\%\,MPE = \left[1 - \left(\frac{Threshold_{D} - Baseline_{D}}{Threshold_{V} - Baseline_{V}}\right)\right] \times 100 \quad \textbf{(Equation 1)}$$

"Threshold $_D$" is the threshold after administration of a drug D, "Baseline $_D$" is the baseline threshold of the mouse, "Threshold $_V$" is the average threshold after administration of vehicle and "Baseline $_V$" is the average baseline of vehicle-treated mice.

One-way ANOVA of %MPE followed by Bonferroni post-hoc test was used to compare the efficacy of the different doses with the vehicle and among themselves, and significance levels of paired comparisons were used to construct heat maps for the dose-response curves *(*Figs. 3C, 4C). Similarly, one-way ANOVA followed by Bonferroni was used to compare the efficacy of the different dose combinations and construct the heat maps showing the significant differences among them or versus the vehicle (Figs. 6D, 8D, upper heat maps). For the heat maps representing the significance of the difference between dose combinations and each individual dose of paracetamol or nefopam (Figs. 6D, 8D, lower panels), paired t-tests were used. Shapiro-Wilk tests were conducted prior to parametric analyses to assess the normality of the data. GraphPad Prism Version 7.04 (GraphPad Software, Inc., La Jolla, CA, USA) was used to conduct non-linear regression analysis with the %MPE values to obtain the median effective dose 50 (ED_{50}) for each compound and the hill slopes of sigmoidal dose-response curves. Subsequent effective doses for paracetamol and nefopam were calculated following the formula (Motulsky and Christopoulos, 2004):

$$ED_{X} = \left(\frac{X}{100 - X}\right)^{\frac{1}{H}} \times ED_{50} \quad \textbf{(Equation 2)}$$

"*X*" is the studied effective dose and "*H"* is the hill slope of the dose-response curve.

An interaction index (γ) was calculated as previously described (Tallarida, 2002):

$$\frac{a}{A} + \frac{b}{B} = \gamma \quad \textbf{(Equation 3)}$$

Where "a" and "b" are the doses of the mixture needed to induce the studied % MPE and "A" and "B" the respective EDs of the individual drugs. γ describes synergy when it is <1, absence of interaction when is equal to 1, or antagonism when it is >1. Error bars

from isoboles were obtained through extrapolation from ED standard error values provided by GraphPad Prism. The source data file contains the raw data and results of the statistical analyses shown in the manuscript.

3. Results

3.1. Phase I: Head-to-head comparison paracetamol vs nefopam to determine ED_{50} values

3.1.1. Effects of paracetamol on incisional mechanical sensitivity

Mechanical sensitivity was measured before surgery (Days -2 and -1) and 2 days later (Day 2), 30-60 min after administration of vehicle or paracetamol (50, 100, 200, 300 or 400 mg/kg). Evaluation of mechanical thresholds of the hind paws ipsilateral to the incision (ipsilateral) revealed significant effects of the day of evaluation ($F=225.4$, $P<0.001$), the dose of paracetamol ($F=12.4$, $P<0.001$) and their interaction ($F=16.3$, $P<0.001$). Mice of all groups showed stable nociceptive responses to mechanical stimulation before the surgery (Fig. 1A-F, days -2 and -1). After plantar incision on day 0, mice receiving vehicle showed prominent decrease of mechanical thresholds in the paw ipsilateral to the incision (decreased to 29±3% of average baseline threshold, $P<0.001$ vs. baseline, Fig. 1A). Mice receiving paracetamol at 50 mg/kg did not show significant relief of mechanical hypersensitivity (40±3%, $P<0.001$ vs. baseline, N.S. vs. vehicle, Fig. 1B). Animals treated with doses of 100, 200 and 300 mg/kg showed increasing recoveries of their mechanical thresholds (to 49±3%, 65±5% and 85±4% of baseline thresholds, respectively, $P<0.05$ vs. vehicle, Figs. 1A-E), although the difference vs. baseline sensitivity was still significant ($P<0.05$, Figs. 1A-E). On the contrary, mice treated with 400 mg/kg paracetamol showed complete restoration of the mechanical thresholds (96±6% of baseline sensitivity, $P<0.001$ vs. vehicle, N.S. vs. baseline, Fig. 1F). No significant effects were observed in the contralateral paws (contralateral).

3.1.2. Effects of nefopam on incisional mechanical sensitivity

Mechanical sensitivity was measured before surgery (Days -2 and -1) and 2 days later (Day 2), 30 min after administration of vehicle or nefopam (1.5, 3, 6, 9 or 12 mg/kg). Evaluation of mechanical thresholds of the hind paws ipsilateral to the incision (ipsilateral) revealed significant effects of the day of evaluation ($F=179$, $P<0.001$), the dose of nefopam ($F=7.6$, $P<0.001$) and their interaction ($F=7.7$, $P<0.001$). Mice of all groups showed stable nociceptive sensitivity to mechanical stimulation before the surgery (Fig. 1A,1G-1K, days -2 and -1). The vehicle group

constituted the same vehicle group described in section 3.1.1, since nefopam and paracetamol doses were evaluated in parallel. Mice receiving nefopam at 1.5 mg/kg did not show significant relief of mechanical hypersensitivity (44±3%, P<0.001 vs. baseline, N.S. vs. vehicle, Fig. 1G). On the contrary, animals treated with doses of 3, 6 and 9 mg/kg showed increasing recoveries of their mechanical thresholds (54±6%, 60±5% and 74±5% respectively, P<0.01 vs. vehicle, Figs. 1H-J), although the difference vs. baseline sensitivity remained significant (P<0.01, Fig. 1H-J). Full restoration of mechanical thresholds was observed when mice were treated with 12 mg/kg nefopam (89±8% of baseline response, P<0.001 vs. vehicle, N.S. vs. baseline, Fig. 1K). No significant effects were found in the contralateral side (contralateral).

3.1.3. Effects of paracetamol on incisional thermal sensitivity

Sensitivity to noxious heat stimulation was measured before surgery (Days -2 and -1) and 2 days later (Day 2), 75 min after administration of vehicle or paracetamol (50, 100, 200, 300 or 400 mg/kg). Evaluation of thermal thresholds of the hind paws ipsilateral to the incision (ipsilateral) revealed significant effects of the day of evaluation (F=135.3, P<0.001), the dose of paracetamol (F=3.3, P<0.05) and their interaction (F=3.6, P<0.001). Mice of all groups showed stable nociceptive sensitivity to heat stimulation before the surgery (Figs. 2A-F, days -2 and -1). Vehicle-treated mice showed a marked decrease of the thresholds to heat stimulation in the paw ipsilateral to the lesion (36±3% of average baseline threshold, P<0.001 vs. baseline, Fig. 2A). Mice receiving paracetamol at 50, 100, 200 or 300 mg/kg showed increasing average responses (35±3%, 45±6%, 60±9%, 72±13%, Fig. 2A). These responses were not statistically different from the vehicle-treated group and the difference vs. baseline responses remained significant (P<0.001 for 50, 100 and 200 mg/kg, P<0.01 for 300 mg/kg, Fig. 2A). Paracetamol at 400 mg/kg was the only dose that elicited significant recovery when compared to the vehicle-treated group (76% of baseline responses, P<0.01 vs. vehicle, Fig. 2A), albeit the difference vs. baseline sensitivity (P<0.05) revealed incomplete recovery of normal heat sensitivity. No significant effects after drug administration were observed in the contralateral side (contralateral).

3.1.4. Effects of nefopam on incisional thermal sensitivity

Sensitivity to noxious heat stimulation was measured before surgery (Days -2 and -1) and 2 days later (Day 2), 75 min after administration of vehicle or nefopam (1.5, 3, 6, 9 or 12 mg/kg). Evaluation of thermal thresholds of the hind paws ipsilateral to the incision (ipsilateral) revealed significant effects of the day of evaluation ($F=112.5$, $P<0.001$), the dose of nefopam ($F=4.2$, $P<0.01$) and their interaction ($F=5$, $P<0.001$). Mice receiving nefopam at 1.5 or 3 mg/kg did not show significant relief of heat hyperalgesia (37±3% and 59±5% of baseline, respectively, $P<0.001$ vs. baseline, N.S. vs. vehicle, Fig. 2G-H). Animals treated with 6 and 9 mg/kg showed significant recoveries of their latencies to heat stimulation (69±5% and 75±7% of baseline responses respectively, $P<0.01$ vs. vehicle, Fig. 2I-J), although the difference vs. baseline sensitivity remained significant ($P<0.01$, Fig. 2I-J). Only mice treated with 12 mg/kg nefopam showed complete restoration of the thresholds to heat stimulation (86±10% of baseline response, $P<0.01$ vs. vehicle, N.S. vs. baseline, Fig. 2K). No significant drug effects could be evidenced in the contralateral side after drug administration (contralateral).

3.1.5. Side by side comparison paracetamol-nefopam on incisional mechanical sensitivity

Percentage Maximum Possible Effect (%MPE) was calculated for the different doses of paracetamol and nefopam, taking into account the decrease of mechanical thresholds of ipsilateral paws after each dose and the decrease of mechanical thresholds of vehicle-treated mice vs. their respective baseline responses (see Data analysis subsection in Methods). One-way ANOVA of %MPE values revealed significant dose effect for both paracetamol ($F=29.8$, $P<0.001$) and nefopam ($F=14.1$, $P<0.001$), with significant inhibition of mechanical hypersensitivity at doses starting from 200 mg/kg for paracetamol ($P<0.001$ vs. vehicle, Fig. 3A) and starting from 6 mg/kg for nefopam ($P<0.01$ vs. vehicle, Fig. 3B). Fig. 3C represents significant differences among the doses tested or with the vehicle for each of the compounds, revealing stronger effects inhibiting mechanical hypersensitivity after paracetamol doses. Non-linear regression analysis of MPE values (Fig. 3D) revealed ED_{50}s of 177.3 ± 15.4 mg/kg for paracetamol, and of 5.83 ± 0.72 mg/kg for nefopam, depicting

the higher potency of nefopam. The dose-effect curves had different hill slopes (2.1 ± 0.3 for paracetamol and 1.4 ± 0.3 for nefopam), indicating nonparallel logarithmic dose-effect curves and variable potency ratio of the drugs depending on the dose (Tallarida, 2006). Thus, isoeffective doses of paracetamol and nefopam had different proportions depending on the degree of effectivity (Table 1).

3.1.6. Side by side comparison paracetamol – nefopam on incisional thermal sensitivity

One-way ANOVA analysis of %MPE values for thermal hyperalgesia revealed significant dose effect for both paracetamol ($F=5.9$, $P<0.001$) and nefopam ($F=10.9$, $P<0.001$), with significant inhibition of hypersensitivity at doses starting from 300 mg/kg for paracetamol ($P<0.05$ vs. vehicle, Fig. 4A) and starting from 3 mg/kg for nefopam ($P<0.05$ vs. vehicle, Fig. 4B). Fig. 4C represents significant differences among the doses tested and with the vehicle for each of the compounds, illustrating stronger effects for nefopam on the inhibition of incisional thermal hypersensitivity. Non-linear regression of MPE values (Fig. 4D) revealed ED_{50}s of 278.6 ± 43 for paracetamol, and of 5.42 ± 0.81 for nefopam, proving the higher potency of nefopam inhibiting thermal sensitivity. Thus, the doses of paracetamol needed to inhibit thermal hypersensitivity (ED_{50} 278.6 ± 43, Fig. 4D) were higher than the doses needed to alleviate mechanical sensitivity (ED_{50} 177.3 ± 15.4, Fig. 3D), whereas nefopam showed similar efficacy for the inhibition of thermal (ED_{50} 5.42 ± 0.81, Fig. 4D) and mechanical sensitivity (5.83 ± 0.72, Fig. 3D). The dose-effect curves for the inhibition of thermal hypersensitivity had also different hill slopes (2.4 ± 0.5 for paracetamol and 1.35 ± 0.3 for nefopam), indicating nonparallel logarithmic dose-effect curves and variable potency ratio of the drugs depending on the dose (Tallarida, 2006).

3.2. Phase II: To quantify possible interactions between paracetamol and nefopam by isobolographic analysis.

In the second phase of the study, doses of paracetamol and nefopam equieffective for the inhibition of incisional mechanosensitivity were co-administered on day 2 after paw incision. The use of isoeffective doses based on the plantar test results was

disregarded because the evaluated doses did not reach complete inhibition of thermal sensitivity and ED estimation may be suboptimal in this case. Based on the ED_{50}s of paracetamol and nefopam for the effect on mechanical sensitivity (Phase I of the study), $ED_{8.7}$s, $ED_{12.5}$s, $ED_{17.5}$s and ED_{35}s were calculated (*Equation 2*, Table 1) and co-administered.

3.2.1. Effect of paracetamol-nefopam ED combinations on incisional mechanical hypersensitivity.

Two days after surgery, mice received vehicle or different equieffective ED combinations of paracetamol and nefopam ($ED_{8.7}$, $ED_{12.5}$, $ED_{17.5}$ or ED_{35}). Evaluation of mechano-sensitivity of the hind paws ipsilateral to the incision (ipsilateral) revealed significant effects of the evaluation day ($F=126.7$, $P<0.001$), the ED combination ($F=3.8$, $P<0.01$) and their interaction ($F=13.1$, $P<0.001$). Mice showed stable nociceptive responses to mechanical stimulation before plantar incision (Figs. 5A-E, days -2 and -1). Two days after surgery, vehicle-treated mice showed pronounced decrease of mechanical thresholds in the ipsilateral paw (decreased to 32±4% of baseline threshold, $P<0.001$ vs. baseline, Fig. 5A). Mice receiving paracetamol-nefopam $ED_{8.7}$s combination did not show significant alleviation of mechanical nociception (42±3% of baseline threshold, $P<0.001$ vs. baseline, N.S. vs. vehicle, Fig. 5B). In contrast, mice receiving combinations of $ED_{12.5}$, $ED_{17.5}$ or ED_{35} of paracetamol and nefopam showed significant inhibition of mechanical hypersensitivity ($P<0.001$ vs. vehicle, Figs. 5C-E) with the thresholds increasing to 69±6%, 84±7% and 93±4% of baseline values. While $ED_{12.5}$ and $ED_{17.5}$ combinations induced partial recovery of basal sensitivity ($P<0.001$ and $P<0.05$ vs. baseline, respectively), baseline sensitivity was completely restored after the mixture of ED_{35} of paracetamol and nefopam (N.S. vs. baseline thresholds, Fig. 5E). No significant effects were observed in the contralateral paws (contralateral).

3.2.2. Isobolographic analysis of paracetamol-nefopam combinations on incisional mechanical hypersensitivity.

One-way ANOVA analysis of %MPE values for mechanical nociception revealed significant dose effect for the equieffective combinations of paracetamol-nefopam

(F=24.9, P<0.001). No antinociception was observed after administration of ED8.7 mixtures (N.S. vs. vehicle, Figs. 6A), and the modification in mechanical thresholds was not significantly different than expected at this dose level (Fig. 6B). Significant antinociceptive effects were found after combinations of $ED_{12.5}$s (55±9% of %MPE, P<0.001 vs vehicle, Fig. 6C), $ED_{17.5}$s (75±10 %MPE P<0.001 vs vehicle, Fig. 6A) and ED_{35}s (90±5 %MPE P<0.001 vs vehicle, Fig. 6A). Thus, combinations of $ED_{12.5}$s, $ED_{17.5}$s and ED_{35}s, with expected combined effects of 25, 35 and 70 % showed empirical %MPEs of 55, 75 and 90% (Fig. 6B) suggesting significant potentiation between the two compounds.

Analysis of the dose-effect curve considering the obtained %MPEs and the combined EDs (∑EDs,) yielded an ED_{50} equal to an $\sum ED_{25}$ of the paracetamol-nefopam mixture (∑ED 25.2 ± 1.4, Figure 6B). Assuming additivity and isoeffectivity, a mixture of an $ED_{12.6}$ of paracetamol and an $ED_{12.6}$ of nefopam would yield an effective dose with 25.2 %MPE. However, this combination achieved 50 %MPE (71 mg/kg paracetamol and 1.4 mg/kg nefopam, 50:1 combination, see Table 1). Such combination showed an interaction index of 0.65, revealing drug synergism. Based on the results obtained in the Phase I of the Study (ED_{50} of 177.3 ± 15.4 mg/kg for paracetamol and 5.83 ± 0.72 mg/kg for nefopam) an isobologram for the median effective dose was constructed (Figure 6C), where a line of theoretical additivity (isobole) illustrates theoretical equieffective combinations of paracetamol and nefopam producing a 50 %MPE. The data point obtained with the dose-response curve of paracetamol-nefopam combinations illustrates that the 50% MPE could be achieved by a theoretical combination of $ED_{12.6}$s (71.59 mg/kg paracetamol and 1.44 mg/kg nefopam, 50:1, Equation 2). The position of the efficacy of the dose pair below the line of additivity (Figure 6C) indicates synergism of the paracetamol-nefopam combination.

Close inspection of the heat map showing the differences between efficacy of ED combinations and EDs of the individual drugs (Figure 6D) reveals that combinations of $ED_{17.5}$ (Paracetamol 85.76 mg/kg plus nefopam 1.9 mg/kg, 45:1), with expected additive effect of 35% MPE, had significantly higher effect (75±10 %MPE, Fig. 6A) than individual doses of 200 mg/kg paracetamol (equivalent to paracetamol ED_{56}, 56% MPE, Fig. 6A) and 6 mg/kg nefopam (equivalent to nefopam ED_{51}, 51% MPE, Fig. 6B), giving an empirical evidence of the synergistic effect of this paracetamol-nefopam combination. Indeed, the interaction index of this combination of $ED_{17.5}$ was

equal to 0.44, revealing stronger synergism than the combination of $ED_{12.5}$s. The combination of ED_{35}s showed more robust synergism, with an index equal to 0.41, however the effect of this combination was not significantly higher than expected (Fig. 6D)

3.2.3. Analysis of paracetamol-nefopam ED combinations on incisional thermal hypersensitivity

The dose combinations equieffective for the inhibition of mechanical hypernociception ($ED_{8.7}$s, $ED_{12.5}$s, $ED_{17.5}$s and ED_{35}s) that were administered, were equivalent to different EDs for the inhibition of incisional thermal hypersensitivity. The respective EDs corresponded to $ED_{8,}$ $ED_{10.4,}$ $ED_{13.5}$ and $ED_{23.7}$ for paracetamol (Table 2), and to ED_{10}, $ED_{14.2}$, $ED_{19.6}$ and $ED_{37.6}$ for nefopam. Hence, the expected additive effects of the paracetamol-nefopam combinations for thermal hypersensitivity were generally lower than those predicted for mechanical antinociception, with %MPEs of 18, 24.6, 33.1 and 61.3, respectively.

Mice showed stable nociceptive responses to heat stimulation before plantar incision (Figs. 7A-E, days -2 and -1). Two days after the surgery on day 0, mice received vehicle or the ED combinations of paracetamol and nefopam ($ED_{8.7\ (VF)}$, $ED_{12.5\ (VF)}$, $ED_{17.5\ (VF)}$ or $ED_{35\ (VF)}$). Assessment of heat sensitivity of the hind paws ipsilateral to the incision (ipsilateral) revealed significant effects of the evaluation day ($F=173.56$, $P<0.001$) and the interaction between the day and the ED combination ($F=2.83$, $P<0.01$). Two days after plantar incision, vehicle-treated mice showed marked decrease of the thresholds to heat stimulation in the ipsilateral paw (reduced to 36±6% of vehicle baseline threshold, $P<0.001$ vs. baseline, Fig. 7A). Mice receiving mixtures of paracetamol and nefopam $ED_{8.7(VF)}$s, $ED_{12.5(VF)}$s or $ED_{17.5(VF)}$s kept showing decreased thresholds (37.7±3%, 55.1±5%, 58.2±5% of baseline, respectively; $P<0.001$ vs. baseline, Fig. 7B-D). While withdrawal latencies tended to be longer, these were not significantly different from vehicle-treated mice. Only the combination of $ED_{35(VF)S}$ induced substantial alleviation of thermal hyperalgesia (63.5±6% of baseline threshold; $P<0.001$ vs. baseline, Fig. 7E), showing significant differences when compared to vehicle-treated mice ($P<0.05$). No significant drug effects were observed in contralateral paws (contralateral).

3.2.4. Isobolographic analysis of Paracetamol-Nefopam combinations on incisional thermal hyperalgesia

One-way ANOVA analysis of %MPE values for thermal hyperalgesia revealed significant dose effect for the combinations of paracetamol and nefopam ($F=5.92$, $P<0.01$). No significant effect was observed after administration of $ED_{8.7(VF)}$ or $ED_{12.5(VF)}$ mixtures (27±8 %MPE; 27±8 %MPE; N.S. vs. vehicle, Fig. 8A), and significant antinociception was found after combinations of $ED_{17.5(VF)}$ (39±8 %MPE $P<0.05$ vs vehicle) and $ED_{35(VF)}$s (46±9 %MPE $P<0.001$ vs vehicle, Fig. 8A). Thus, mixtures of $ED_{17.5(VF)}$s and $ED_{35(VF)}$s, with expected combined effects of 33.1 and 61.3% (Table 2) showed %MPEs of 39±8 % and 46±9 % (Fig. 8A), suggesting absence of potentiation between these two compounds for the inhibition of incisional thermal hypersensitivity. Analysis of the dose-response curve taking into account the obtained %MPEs yielded a median effective dose equal to an ED_{61} of the paracetamol-nefopam combination (61.27±1.3, Figure 8B), although a complete inhibition of thermal hypersensitivity could not be obtained with the dose combinations tested and this could compromise the accuracy of the determination.

Assuming additivity and isoeffectivity, an $ED_{61.2}$ for the paracetamol-nefopam combination would be composed of an $ED_{30.6}$ of paracetamol and an $ED_{30.6}$ of nefopam (165.9 mg/kg paracetamol and 2.9 mg/kg nefopam, 56:1 combination). Based on the results obtained in Phase I of the study showing an ED_{50} of 278.6 ± 43 mg/kg for paracetamol and an ED_{50} of 5.42 ± 0.81 mg/kg for nefopam on thermal hypersensitivity, Figure 8C shows a line of theoretical additivity (isobole) where combinations of paracetamol and nefopam are equieffective. The behavioural data obtained with the dose-response curves of paracetamol and nefopam combinations reveal that a 50% MPE would be obtained after a mixture of $ED_{30.6}$s (165.9 mg/kg paracetamol and 2.9 mg/kg nefopam, 56:1, Equation 2). Thus, the position of the efficacy of the dose pair fell above the line of additivity (Figure 8C), showing an antagonistic trend for these dose combinations. However, inspection of the heat map representing the differences between efficacy of ED combinations and EDs of the individual drugs (Figure 8D) does not show empirical evidence proving that one of the combinations yielded an effect significantly different than expected. Hence, data shows additive effect or absence of synergistic effect of paracetamol-nefopam combinations on incisional heat hypersensitivity.

Discussion

The present study reveals synergistic antinociception between oral paracetamol and nefopam doses in a mouse model of postoperative pain. Doses of both compounds that achieved 17.5 and 35% inhibition of post-surgical mechanical sensitivity when administered individually, reached 75 and 95% relief when combined. These oral mixtures also showed additive effect reducing postoperative thermal hypernociception. Our findings agree with previous basic and clinical studies suggesting potentiation after administration of both compounds (Van Elstraete and Sitbon, 2013; Girard et al., 2011; Li et al., 2018) and support oral administration of this mixture as a suitable route for relieving early postoperative hypersensitivity.

Paracetamol monotherapy in mice induced dose-dependent antinociception with an ED_{50} of 177 mg/kg for the relief of postoperative mechanical sensitivity (effective doses between 100 and 400 mg/kg p.o.). This range of effective doses was similar to the range of oral doses needed to inhibit visceral pain (Miranda et al., 2006; Mititelu Tartau et al., 2014; Qiu et al., 2007). Paracetamol was less effective for the inhibition of thermal hypernociception (ED_{50} of 279 mg/kg), in agreement with previous studies indicating higher requirements to inhibit heat hypersensitivity in a murine model of incisional pain (ED_{50} of 257 mg/kg and significant effects between 400 and 600 mg/kg, (Dogrul et al., 2012). Such high-dose requirements represent a major limitation for single treatments with paracetamol, since a translational study described toxicity in mice detectable 24h after exposure to oral doses of 200 mg/kg and higher (Harrill et al., 2009). Hence, the doses of paracetamol needed to obtain complete relief of postoperative hypersensitivity could be within the range of toxicity and caution should be taken when increasing doses of paracetamol for postoperative relief in humans, especially in patients with impaired liver or platelet function (Munsterhjelm et al., 2005; Zhang et al., 2002).

Oral treatment with nefopam had similar efficacy reducing mechanical and heat hypersensitivity (ED_{50}s of 5.8± 0.7 and 5.4±0.8 mg/kg, respectively). Indeed, 12 mg/kg of oral nefopam was virtually sufficient for complete inhibition of postoperative hypernociception in the operated mice. Most of the previous works investigating the antinociceptive effects of nefopam used parenteral routes (Van Elstraete and Sitbon, 2013; Girard et al., 2004, 2008, 2009, 2011). However, a previous work described

efficacy of higher doses of oral nefopam for the inhibition of visceral pain in mice, measured with the acid acetic writhing test (ED_{50} of 3.2 mg/kg, (Yoshitaka et al., 1991). Since the efficacy of nefopam in the same paradigm of visceral pain was described to be higher when administered through parenteral routes (ED_{50}s 1.5-2.5 mg/kg) (Girard et al., 2009), it can be assumed that first-pass metabolism reduces nefopam bioavailability after oral administration, as it is described in humans (Aymard et al., 2003). The oral antinociceptive doses of nefopam tested in our work (3-12 mg/kg) are too low to induce substantial inhibition of noradrenaline or serotonin re-uptake according to previous works investigating these mechanisms in mice (Fuller and Snoddy, 1993; Girard et al., 2009), but could induce antinociception through facilitation of dopaminergic neurotransmission (Yoshitaka et al., 1991) or inhibition of glutamatergic signaling (Czuczwar et al., 2011). Nevertheless, effective oral doses of nefopam monotherapy in humans can induce drowsiness, sweating, nausea and tachycardia, which represent a major limitation in ambulatory patients (Heel et al., 1980). In addition, nefopam elimination could be compromised in patients with renal dysfunction (Mimoz et al., 2010). Hence, a combinatorial strategy to lower oral nefopam doses is desirable to facilitate pain management in surgical patients.

In our study, oral nefopam and paracetamol acted synergistically to inhibit postoperative hypersensitivity in a mouse model with high predictive validity (Aguado et al., 2013; Cabañero et al., 2009a, 2009b; Célérier et al., 2004; Richebé et al., 2005). Each drug, alone or in combination, induced antinociceptive effects selectively in the injured paw, suggesting that the antinociceptive effects of both compounds are related to the mechanisms triggered by the incisional injury. Several works using murine models of inflammatory, visceral and postoperative pain suggested this synergistic interaction after intraperitoneal administration of specific combinations of paracetamol and nefopam (Girard et al., 2011). In addition, a clinical study conducted in patients undergoing tonsillectomy also described synergistic interaction between intravenous doses of both compounds in a 30:1 ratio paracetamol:nefopam (Van Elstraete and Sitbon, 2013). This is in contrast with a recent clinical work that did not find beneficial effects of the combination administered in an overall 8:1 ratio paracetamol:nefopam for major abdominal injury (Cuvillon et al., 2017). Our work reveals for the first time through isobolographic analysis that optimal potentiation of mechanical pain relief can be achieved with oral

mixtures of paracetamol:nefopam administered at 35-45:1 ratios and combinations of $ED_{17.5}$s and ED_{35}s, respectively. The combination of ED_{35}s showed also additive efficacy relieving thermal hypersensitivity, although the antinociceptive effect could be underestimated due to the evaluation at a later time interval after drug administration.

The encouraging results of the paracetamol:nefopam combination should be carefully interpreted, since the data were obtained in young male mice, and the effects on females or other age groups were not investigated. Furthermore, possible effects of the combination on affective-motivational behaviour could be assessed through operant or classical conditioning models (Cabañero et al., 2020; Mogil JS., 2020; Navratilova et al., 2012) to rule out aversive drug effects or affectation of motor responses, although no overt effects on motor behaviour were observed at the doses tested.

Paracetamol and nefopam are currently used through parenteral routes for postoperative pain control (Benhamou et al., 2009; Dualé et al., 2009). However, their effective use is limited by unwanted effects obtained at analgesic doses. The validation of opioid-free treatments that could boost their safety profile through oral routing represents a valuable tool to provide relief of postoperative sensitization after ambulatory surgery and fasten patient recovery. Our data obtained in a murine model of postoperative pain that reproduces post-surgical hypernociception validates the exploitation of these potent multimodal and synergistic interactions between paracetamol and nefopam as a readily available strategy that could facilitate postoperative pain control.

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Figure Legends

Figure 1. Effect of paracetamol doses on post-incisional mechanical sensitivity. Mechanical thresholds of hind paws ipsilateral and contralateral to the plantar incision, before the surgery (Days -2, -1) and 2 days later after treatment with vehicle (A), paracetamol 50 mg/kg (B), paracetamol 100 mg/kg (C), paracetamol 200 mg/kg (D), paracetamol 300 mg/kg (E), paracetamol 400 mg/kg (F), nefopam 1.5 mg/kg (G), nefopam 3 mg/kg (H), nefopam 6 mg/kg (I), nefopam 9 mg/kg (J) or nefopam 12 mg/kg (K). N=10-11 mice per group. Stars are comparisons vs. baseline responses, #s are comparisons vs. vehicle group. *P<0.05, ***P<0.001. Points represent average thresholds and error bars are SEM.

Figure 2. Effect of paracetamol doses on post-incisional hypersensitivity to heat. Heat withdrawal latencies of hind paws ipsilateral and contralateral to the plantar incision, before surgery (Days -2, -1) and 2 days later after treatment with vehicle (A), paracetamol 50 mg/kg (B), paracetamol 100 mg/kg (C), paracetamol 200 mg/kg (D), paracetamol 300 mg/kg (E), paracetamol 400 mg/kg (F), nefopam 1.5 mg/kg (B), nefopam 3 mg/kg (C), nefopam 6 mg/kg (D), nefopam 9 mg/kg (E) or nefopam 12 mg/kg (F). N=10-11 mice per group. Stars are comparisons vs. baseline responses, #s are comparisons vs. vehicle group. *P<0.05, **P<0.01, ***P<0.001. Points represent average latencies and error bars are SEM.

Figure 3. Side by side comparison of paracetamol and nefopam effects on post-incisional mechanical sensitivity. Percentage maximum possible effect (%MPE) of paracetamol (A) and nefopam (B) inhibiting incisional mechanical hypersensitivity 2 days after surgery. C) Heat map representing significant differences vs. vehicle and among the different doses tested for each drug. D) Sigmoidal dose-response curves revealed ED_{50}s of 5.83 ± 0.72 for nefopam and 177.3 ± 15.4 for paracetamol. Hill slopes (H) were of 1.384 for nefopam and 2.135 for paracetamol. N=10-11 mice per group. **P<0.01, ***P<0.001 vs. vehicle. Bars (A, B) and points (D) represent average %MPE, and error bars are SEM.

Figure 4. Side by side comparison of paracetamol and nefopam effects on post-incisional hypersensitivity to heat. Percentage maximum possible effect (%MPE) of paracetamol (A) and nefopam (B) inhibiting incisional thermal hypersensitivity measured 2 days after surgery. C) Heat map representing significant differences among the different doses tested for each drug. D) Sigmoidal dose-response curves revealed ED_{50}s of 5.42 ± 0.81 for nefopam and 278.6 ± 43 for paracetamol. Hill slopes (H) were of 1.35 for nefopam and 1.577 for paracetamol. N=10-11 mice per group. **P<0.01, ***P<0.001 vs. vehicle. Bars (A, B) and points (D) represent average %MPE, and error bars are SEM.

Figure 5. Effect of Paracetamol-Nefopam ED combinations on post-incisional mechanical sensitivity. Mechanical thresholds of hind paws ipsilateral and contralateral to the plantar incision, before surgery (Days -2, -1) and 2 days later after treatment with vehicle (A) or combinations of $ED_{8.7}$s (B), $ED_{12.5}$s (C), $ED_{17.5}$s (D) or ED_{35}s (E) of paracetamol and nefopam. N=10-13 mice per group. Stars are comparisons vs. baseline responses, #s are comparisons vs. vehicle group. *P<0.05, **P<0.01 ***P<0.001. Points represent average thresholds and error bars are SEM.

Figure 6. Isobolographic analysis of ED combinations for the inhibition of post-incisional mechanical sensitivity. (A) Percentage maximum possible effect (%MPE) of combinations of paracetamol and nefopam $ED_{8.7}$s, $ED_{12.5}$s, $ED_{17.5}$s or ED_{35}s. (B) Sigmoidal dose-response curve for the combined EDs ($ED_{17.5}$, ED_{25}, ED_{35} and ED_{70}) vs the obtained %MPE revealed a median effective dose equal to the $ED_{25.2}$ of the paracetamol-nefopam combination (paracetamol 71.4 ± 6 mg/kg – $ED_{12.6}$ and nefopam 1.44 ± 0.12 mg/kg– $ED_{12.6}$). (C) Isobologram for median effective doses of paracetamol and nefopam shows the experimental median effective dose of the combination of paracetamol 71.4 ± 6 mg/kg and nefopam 1.44 ± 0.12 mg/kg, with an interaction index (γ) of 0.65 indicating synergism. The position of the dose pair below the line of additivity illustrates synergistic effect of the paracetamol-nefopam combination. (D) Heat map representing significant differences among the different ED combinations and vs. the tested paracetamol and nefopam EDs. Combination of $ED_{17.5,}$ with expected additive effect of 35 %MPE, yielded 75±10 %MPE, significantly superior to the %MPE obtained after paracetamol ED_{56} and nefopam ED_{51}.

Unexpected results (synergy) highlighted in white. N=10-13 mice per group. ***P<0.001 vs. vehicle, error bars are SEM.

Figure 7. Effect of Paracetamol-Nefopam combinations on post-incisional hypersensitivity to heat. Paw withdrawal latencies of hind paws ipsilateral and contralateral to the plantar incision, before the surgery (Days -2, -1) and 2 days later after treatment with vehicle (A) or combinations of $ED_{8.7(VF)}$s (B), $ED_{12.5(VF)}$s (C), $ED_{17.5(VF)}$s (D) or $ED_{35(VF)}$s (E) of paracetamol and nefopam. N=10-13 mice per group. Stars are comparisons vs. baseline responses, #s are comparisons vs. vehicle group. *P<0.05, ***P<0.001. Points represent average latencies, error bars are SEM.

Figure 8. Isobolographic analysis of ED combinations for inhibition of post-incisional hypersensitivity to heat. (A) Percentage maximum possible effect (%MPE) of paracetamol-nefopam combinations of $ED_{8.7(VF)}$s, $ED_{12.5(VF)}$s, $ED_{17.5(VF)}$s or $ED_{35(VF)}$s. (B) Sigmoidal dose-response curve for the combined EDs (ED_{18}, $ED_{24.6}$, $ED_{33.1}$ and $ED_{61.3}$) vs the obtained %MPE revealed a median effective dose equal to an estimated $ED_{61.2}$ of the paracetamol-nefopam combination. (C) Isobologram of paracetamol and nefopam ED_{50}s shows that a theorethical combination of 165.9 mg/kg paracetamol and 2.9 mg/kg nefopam would give a 50 % MPE. The position of the dose pair above the line of additivity reveals an antagonistic trend of the paracetamol-nefopam combination. (D) Heat map representing significant differences among the ED combinations and tested EDs of paracetamol and nefopam. None of the comparisons among the doses and dose combinations tested confirm the antagonistic trend, suggesting additivity or absence of synergy. N=10-13 mice per group. *P<0.05, **P<0.01 vs. vehicle, error bars are SEM.

Tables

Table 1. Co-administered Equieffective Doses (EDs) of Paracetamol and Nefopam and expected additive effects.

Tested EDs (von Frey)	Paracetamol (mg/kg)	Nefopam (mg/kg)	Expected Additive Effect (% MPE)	Isoeffective Ratio Paracetamol: Nefopam
8.7	58.95	1.07	**17.4**	**55:1**
12.5	71.27	1.43	**25**	**50:1**
17.5	85.76	1.9	**35**	**45:1**
35	132.67	3.73	**70**	**35:1**

Isoeffective oral doses of Paracetamol and Nefopam (EDs) for inhibition of mechanical allodynia were administered by gavage in a vehicle containing10% N-Methyl-2-pyrrolidone and 90% purified water. Expected additive effect expressed in Maximum Percentage Effect (%MPE) is the sum of the two co-administered EDs.

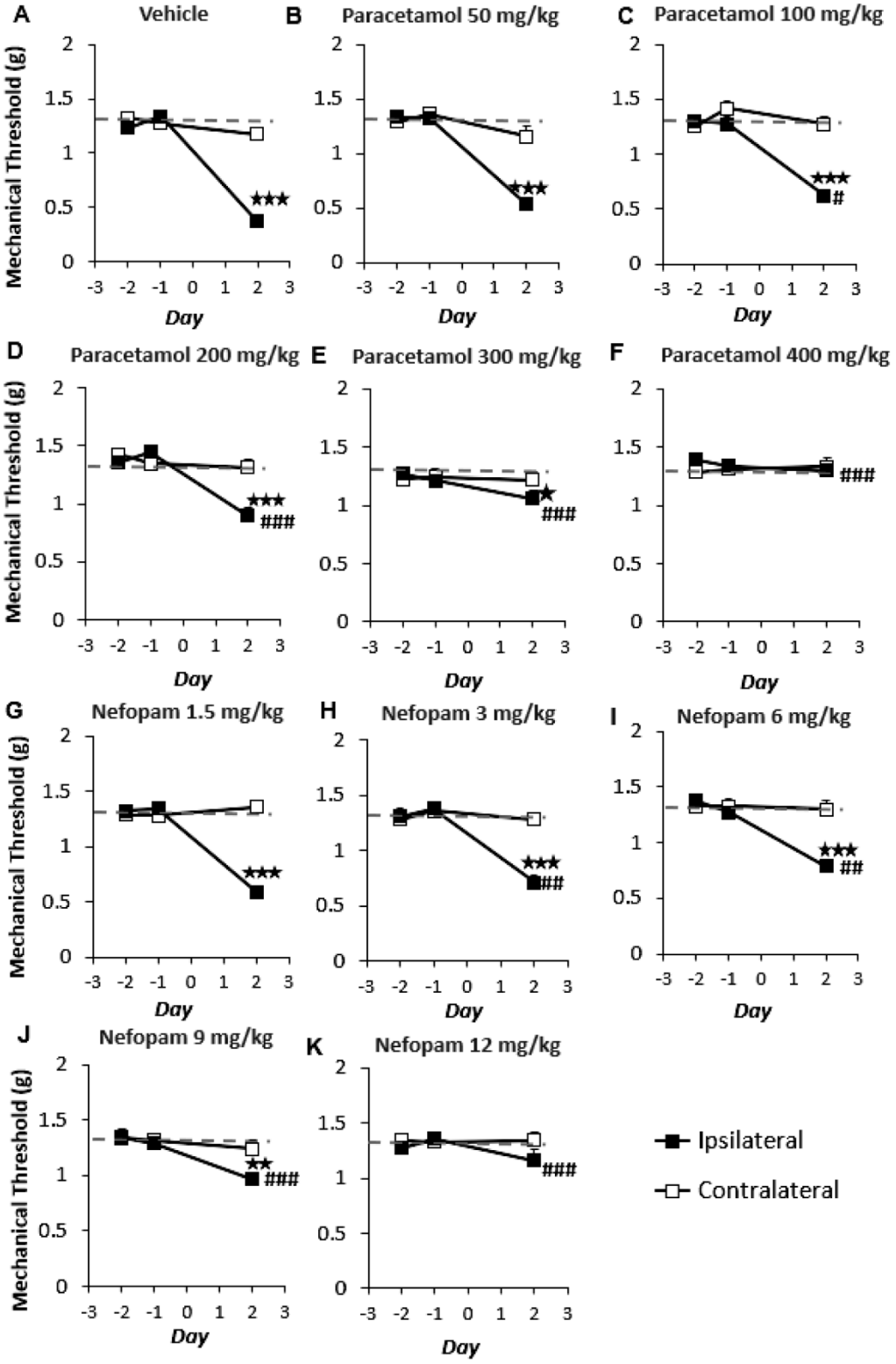
Table 2. Co-administered Doses (EDs) of Paracetamol - Nefopam and expected effects

EDs (Von Frey)	Expected Additive %MPE (Von Frey)	Paracetamol (mg/kg)	Equivalent Paracetamol ED (Plantar)	Nefopam (mg/kg)	Equivalent Nefopam ED (Plantar)	Expected Additive %MPE (Plantar)
8.7	**17.5**	58.95	**8**	1.07	**10**	**18**
12.5	**25**	71.27	**10.4**	1.43	**14.2**	**24.6**
17.5	**35**	85.76	**13.5**	1.90	**19.6**	**33.1**
35	**70**	132.67	**23.7**	3.73	**37.6**	**61.3**

Isoeffective oral doses of Paracetamol and Nefopam (EDs) for inhibition of mechanical allodynia were administered by gavage in a vehicle containing10% N-Methyl-2-pyrrolidone and 90%

purified water. Expected additive effects are expressed in Maximum Percentage Effect (%MPE) as the sum of the two co-administered EDs for the von Frey test.The EDs for von Frey test are equivalent to different EDs in the plantar test (Plantar) and the expected additive effects in the plantar test reach lower magnitudes.

Figure 1


A
Vehicle
B
Paracetamol 50 mg/kg
C
Paracetamol 100 mg/kg
D
Paracetamol 200 mg/kg
E
Paracetamol 300 mg/kg
F
Paracetamol 400 mg/kg
G
Nefopam 1.5 mg/kg
H
Nefopam 3 mg/kg
I
Nefopam 6 mg/kg
J
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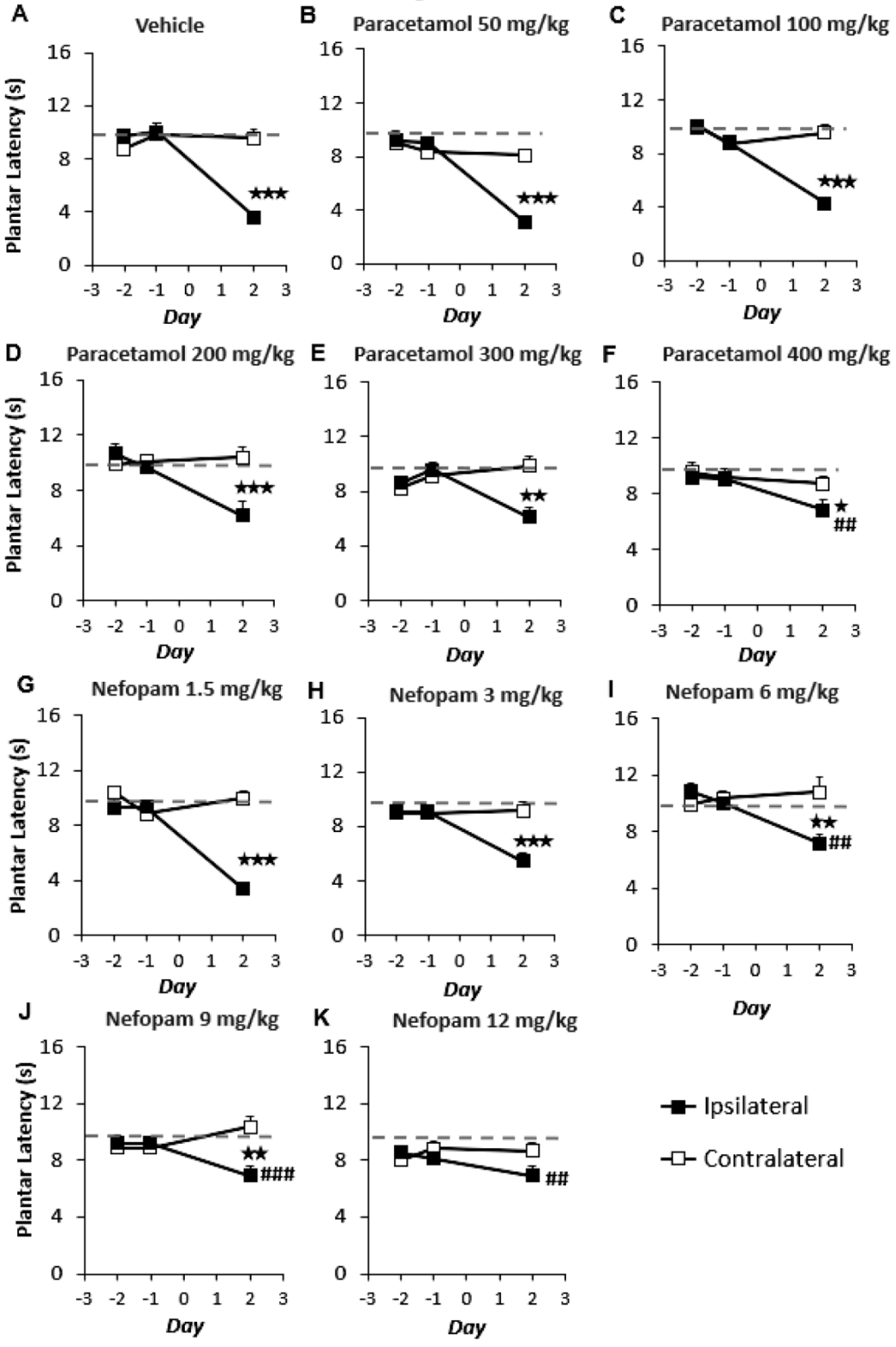
K
Nefopam 12 mg/kg
Mechanical Threshold (g)
Day
Ipsilateral
Contralateral

Figure 2


A
Vehicle
B
Paracetamol 50 mg/kg
C
Paracetamol 100 mg/kg
D
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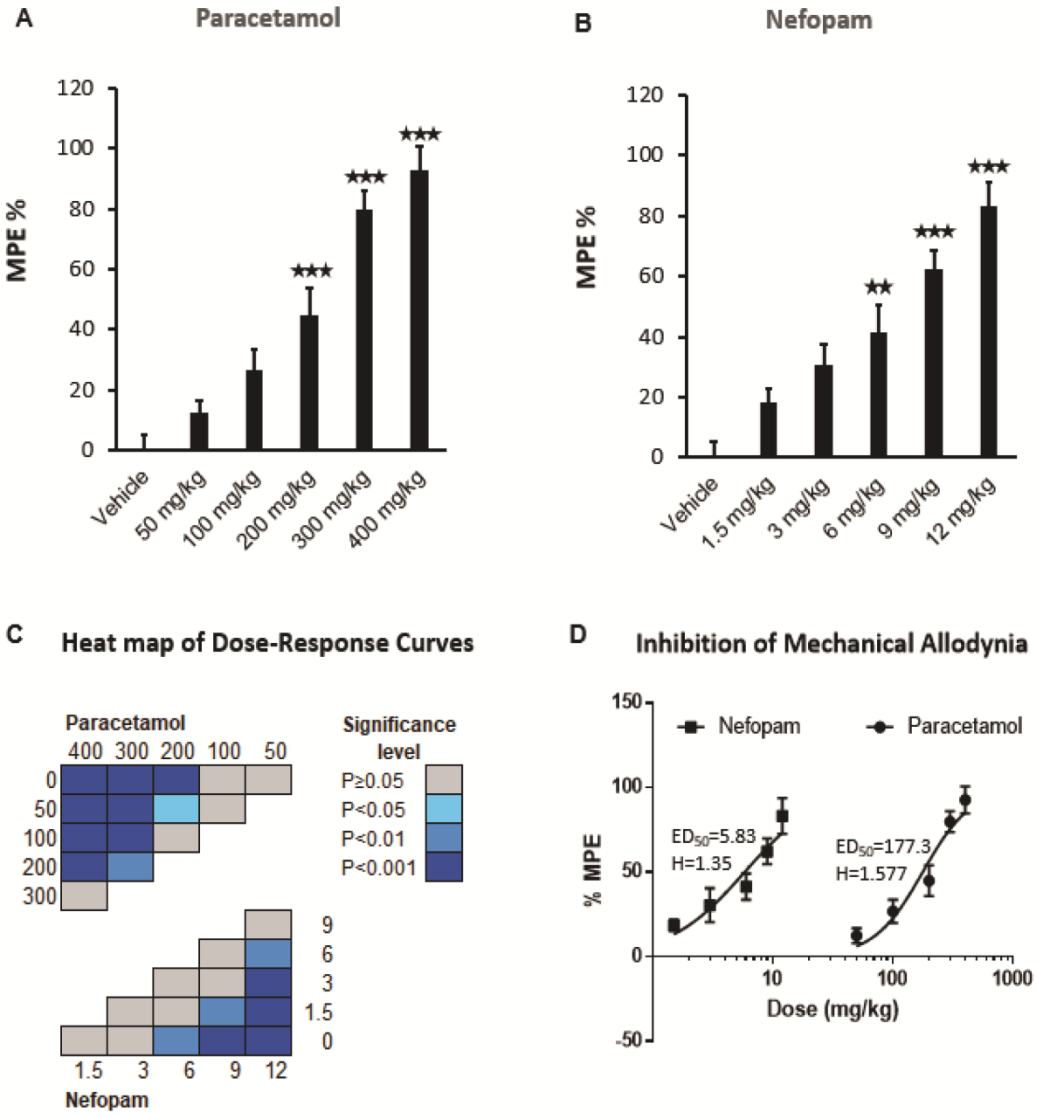
E
Paracetamol 300 mg/kg
F
Paracetamol 400 mg/kg
G
Nefopam 1.5 mg/kg
H
Nefopam 3 mg/kg
I
Nefopam 6 mg/kg
J
Nefopam 9 mg/kg
K
Nefopam 12 mg/kg
Plantar Latency (s)
Day
Ipsilateral
Contralateral

Figure 3



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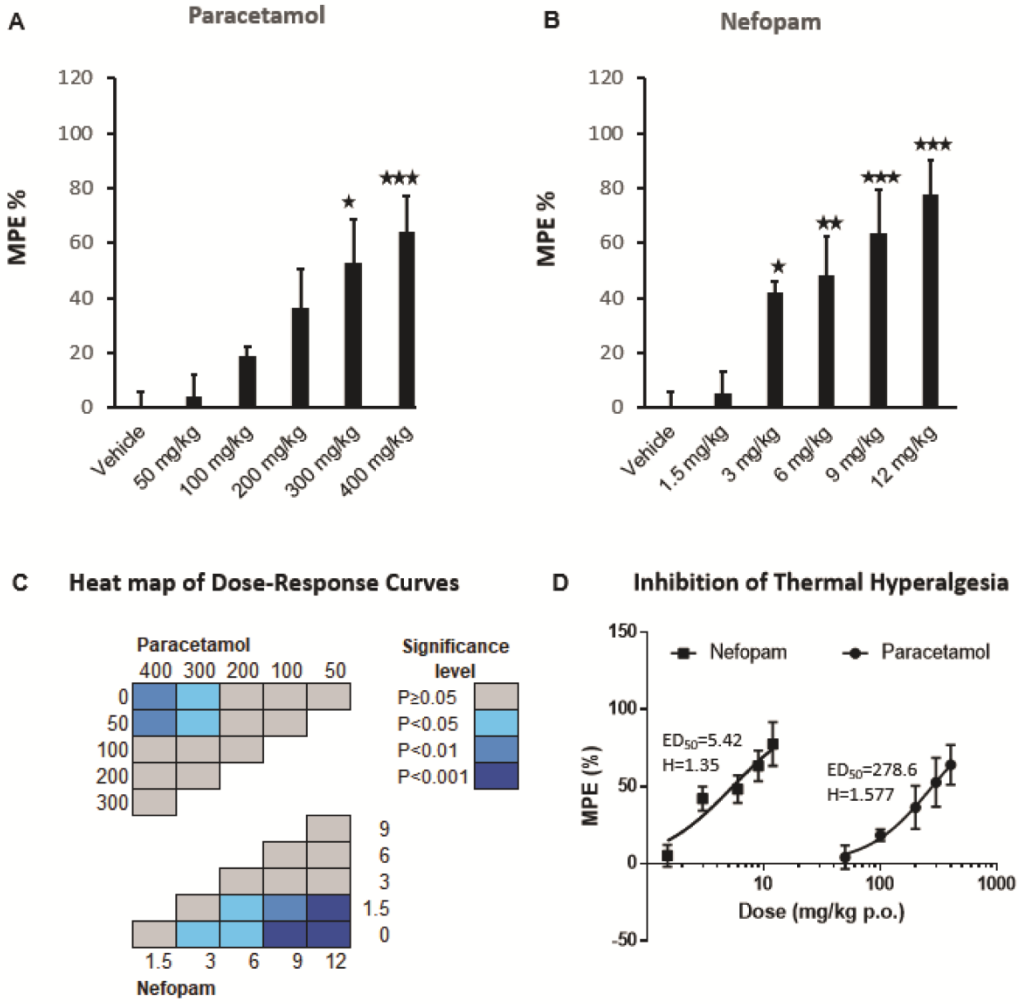

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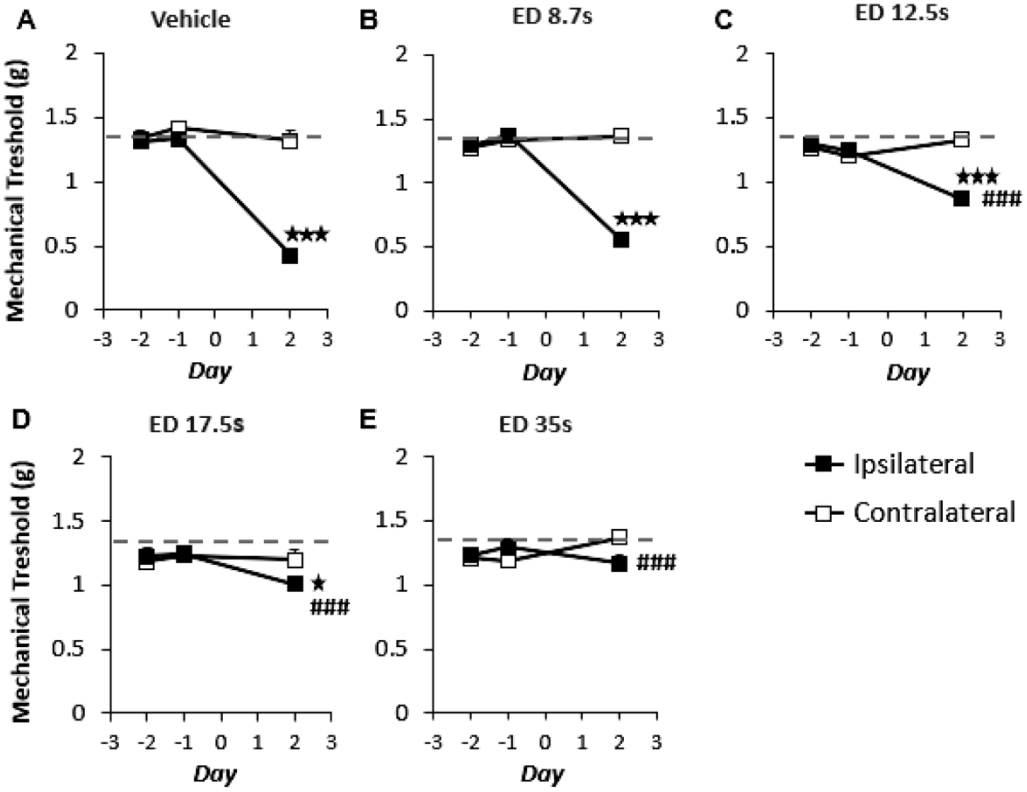

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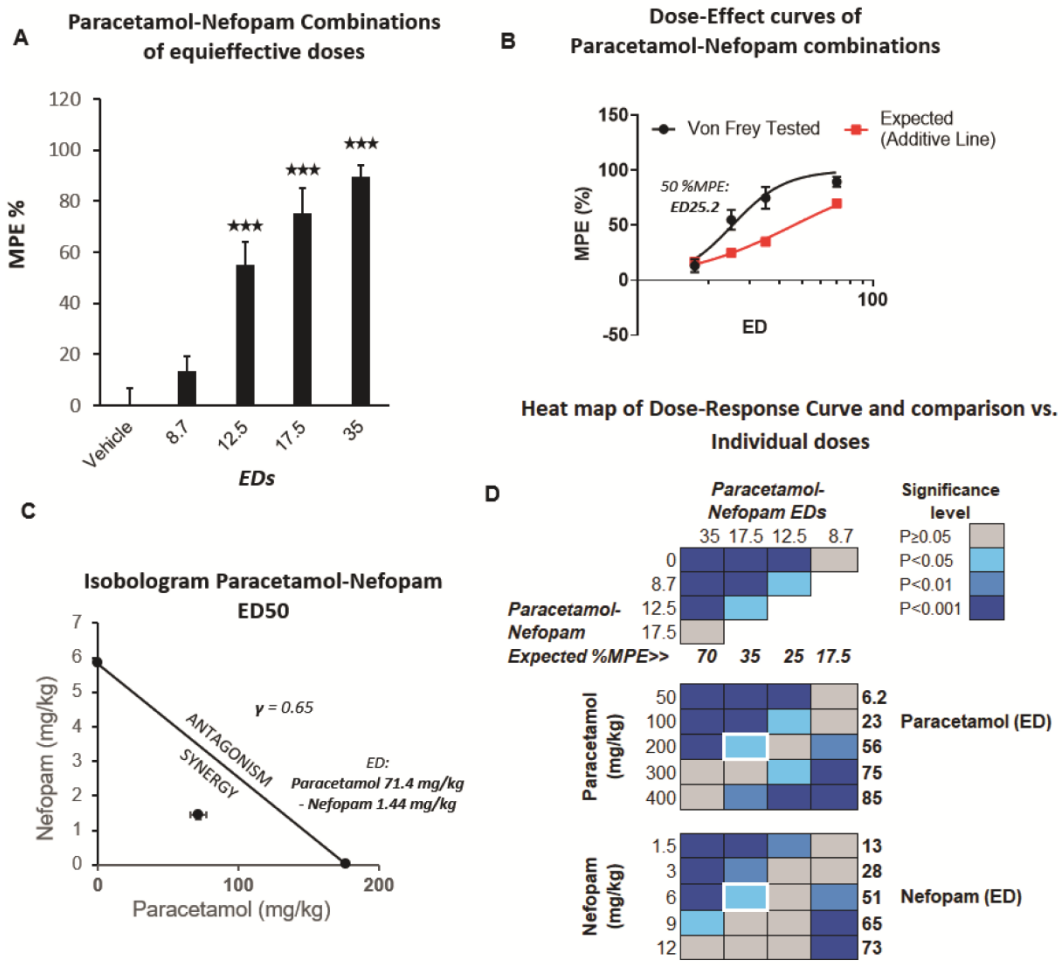

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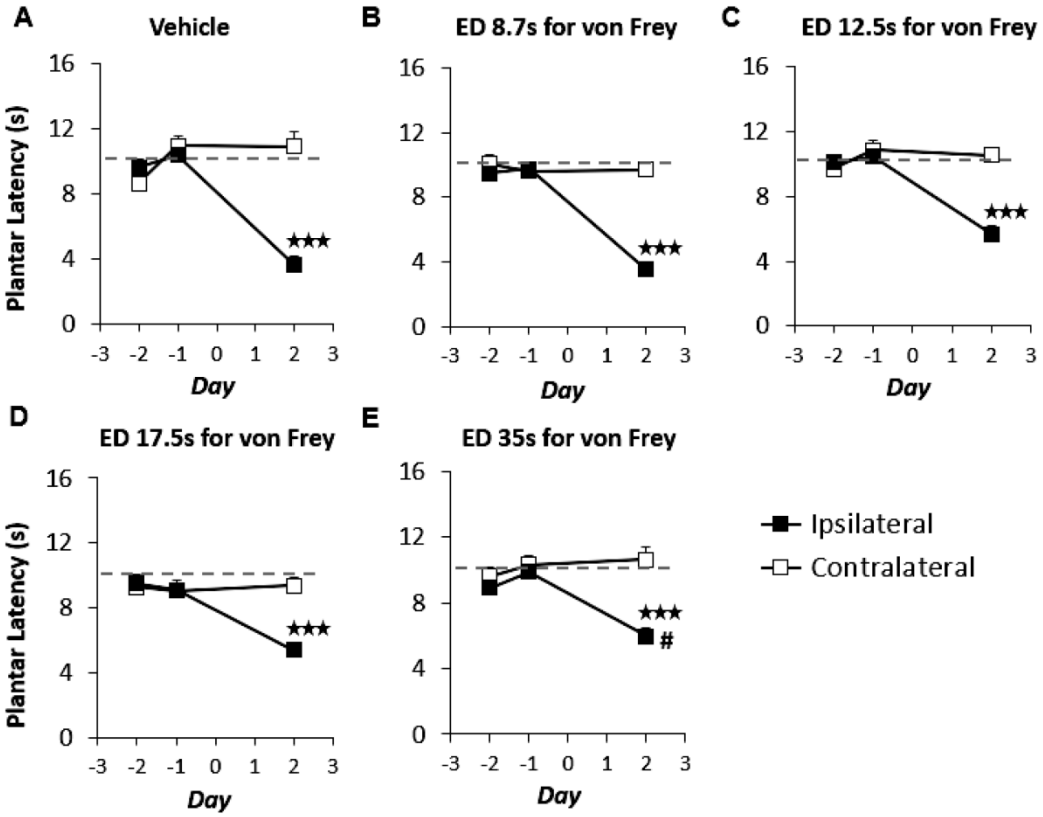

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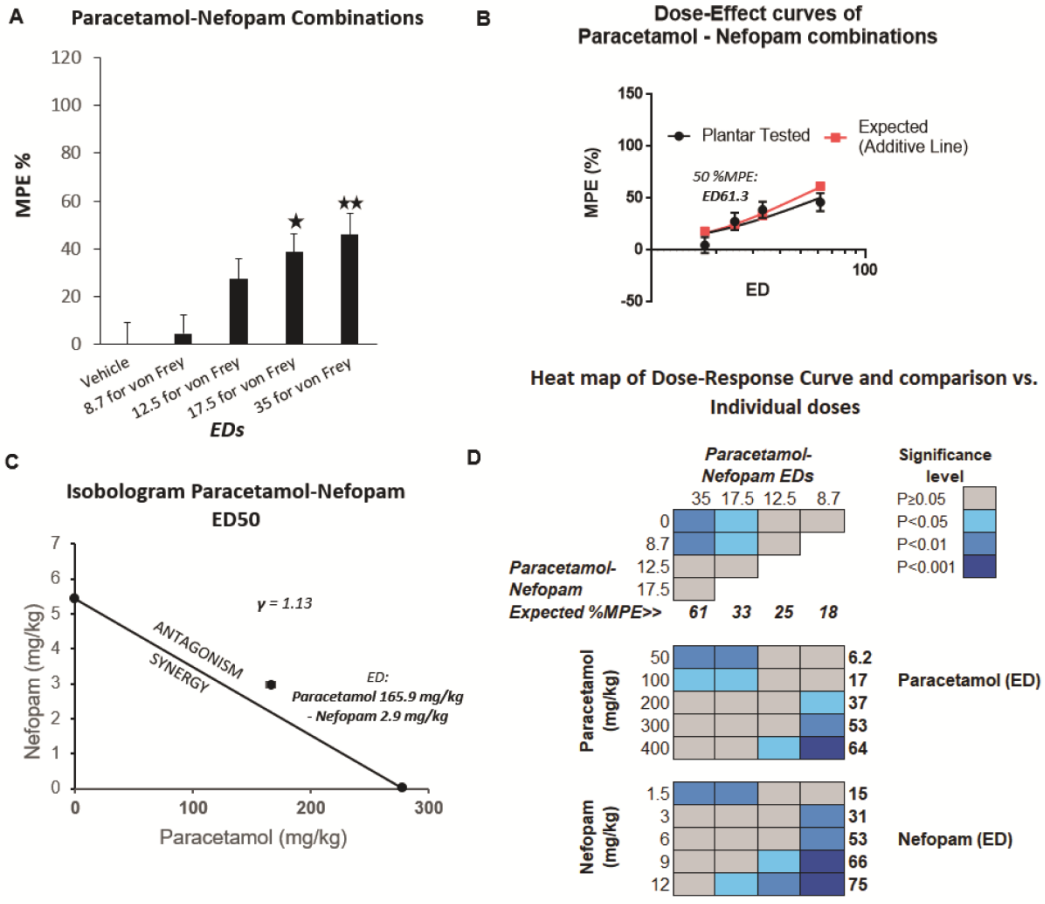
Figure 8



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