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Potentiation of morphine-induced antinociception and locomotion by citalopram is accompanied by anxiolytic effects

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Key words: morphine, citalopram, antinociception, anxiety, mice, tolerance.

Abstract

Morphine and related opioids are the mainstay of analgesic treatment, especially in patients suffering chronic pain. Besides their antinociceptive effects they may also exhibit anxiolytic-like properties that could contribute to pain relief. The pharmacological manipulation of the serotonergic system may not only modulate pain transmission and processing but also other behavioral effects of opioids. The present study aimed to analyze the effect of the concurrent treatment with citalopram, a selective serotonin reuptake inhibitor, on the antinociceptive, locomotor and anxiety-related effects induced by acute and subchronic administration of morphine in mice. Citalopram (15 mg/Kg) enhanced the acute antinociceptive effects of morphine when concurrently administered as evidenced by a two-fold increase in the ED_{50} for the antinociceptive effect of morphine in the hot-plate test. Chronic studies also revealed that concurrent citalopram treatment (15 mg/Kg) delayed the development of tolerance to the thermal antinociceptive effects of morphine. Additionally, morphine-induced hyperlocomotion was potentiated by citalopram as assessed in the open-field test and in the spontaneous activity recording in the home cage, a behavioural outcome to which tolerance or desensitization was not developed. Interestingly, chronic administration of both drugs promoted an anxiolytic effect as evidenced by the increased central activity in the open field test. Future investigations on this pharmacological interaction, such as the possible translational research in clinics, might have consequences in future strategies for the therapeutic management of pain.

1. Introduction

Pain modulation by opioids is intricately regulated by other neurotransmitter systems, essentially monoamines such as noradrenalin and serotonin. Both monoamines are generated in specific neurons allocated in discrete nuclei from the midbrain and brain stem which descending axonal projections reach those areas of the spinal cord involved in the transmission and processing of pain signals through different ascending nociception pathways (Millan, 2002; Ossipov *et al*., 2010). In this sense, the potentiation of monoamine neurotransmission by non-selective reuptake inhibitors such as tricyclic antidepressants (TCA) has been described as an effective therapeutic indication for the treatment of chronic pain either when used alone or in combination with other opioid analgesic drugs (Dharmshaktu et *al.,* 2012; Knotkova and Pappagallo, 2007; Patetsos and Horjales-Araujo, 2016). Nociception studies conducted with experimentation animals have also described an antinociceptive effect of either non-selective monoamine as serotonin reuptake inhibitors, such as citalopram, on its own (Fasmer *et al.* , 1989, Gatch *et al.* , 1998), or as effective adjuvants to enhance the analgesic properties of some opioids compounds (Gatch *et al.*, 1998, Larsen and Christensen, 1982, Larsen and Hyttel, 1985, Larson and Takemori, 1977, Sugrue, 1979). In addition, previous investigations carried out with rats assessed for thermal nociception described an increase of the analgesic effect together with a delay of the expression of morphine tolerance when this drug was co-administered with either amitriptyline or venlafaxine, both of them noradrenaline/serotonin reuptake inhibitors (Ozdemir *et al.* , 2012) and also with fluoxetine, a serotonin selective reuptake inhibitor (Ozdemir *et al.* , 2011). These results further confirmed previous observations that pointed out serotonin as an

essential element in the prevention of morphine tolerance upon sustained treatment. Moreover, it has been described that administration of 5-hydroxytryptophan, a precursor of serotonin, prior to morphine decreases the occurrence of tolerance to this opiate in mice (Contreras *et al.*, 1973). Similarly, a more recent report demonstrated that the combination of morphine with fenfluramine attenuates the development of tolerance in rats chronically treated with morphine (Arends *et al.*, 1998). On the other hand, preclinical and clinical studies have reported the modulatory role of opioidergic system in anxiety (Colasanti *et al.*, 2011), a behavioral feature dependent on the serotonergic tone. In this regard, opioid agonists, especially morphine, have been shown to exhibit anxiolytic-like actions (Glover and Davis, 2008) that may also contribute to pain relief. Interestingly, the effect of the concomitant administration of serotonergic drugs upon these anxiolytic actions of morphine has not been addressed yet.

Therefore, the aim of the present work is to further examine and characterize the effect of citalopram on morphine-induced antinociception in C57BL6 mice submitted to the hot-plate thermal test when acutely administered in terms of potentiation of the opiate response together with the modulation of the development of morphine tolerance after chronic treatment. Moreover, changes in other behavioral responses such as locomotion as well as exploratory and anxiety-related behaviors evoked by morphine alone and combined with citalopram were also evaluated by the open field test.

2. Materials and Methods

2.1. Drugs

Morphine sulphate was supplied by Alcaliber S.A. (Madrid, Spain) and citalopram hydrobromide was generously gifted by H. Lundbeck A/S (Copenhagen, Denmark).

2.2. Animals and experimental groups

Experiments were conducted with 2–3-month old male C57BL/6 mice weighing 25–30 g. All procedures were approved by the Animal Care Committee of the University of Cantabria and according to the Spanish legislation (RD 53/2013) and the European Communities Council Directive (2010/63/UE) on "Protection of Animals Used in Experimental and Other Scientific Purposes". Food and water were given *ad libitum*. Development of tolerance experiments were conducted with four experimental groups according to the drug treatment, i.e., vehicle, morphine, citalopram and morphine plus citalopram, each of them comprised by 10 mice. Three independent experiments were completed (120 mice in total). Dose-response curves to determine the ED_{50} of morphine and morphine plus citalopram were carried out with seven dose groups including 3-4 animals per group. These experiments were independently performed five times (125 mice in total).

2.3. Nociception assays

Hot plate nociception test was carried out with a BIO-CHP apparatus (Bioseb, France). Animals were placed on a surface at 55 °C and the latency time for reaction, defined as paw-licking or jumping, was counted. After reaction, mice were removed immediately from the hot surface. A cut-off time of 30 sec was considered throughout all the assays. Each animal was submitted to two consecutive tests 2 min apart and the mean of both determinations was considered as the final result which was expressed

either as hot-plate latency in seconds or as percentage of maximum possible effect (%MPE) and according to the following formula:

$$\%\text{MPE} = \frac{\text{drug response} - \text{predrug control}}{\text{cut-off time} - \text{predrug control}} \text{ X } 100$$

All drugs were diluted in a saline solution (0.15 M NaCl) and administered via the intraperitoneal route. For development of tolerance assays, drugs were administered at 9:00 a.m. and 5:30 p.m during 7 days at the following doses per injection: morphine 30 mg/kg, citalopram 15 mg/kg, morphine plus citalopram were co-administered at their respective doses and vehicle group was injected with an equivalent volume of saline solution. Nociception test was conducted on a daily basis 45 min after the first injection (9:00 a.m.). The day before starting this experimental schedule, all the animals were subjected to the tests in absence of drug administration in order to evaluate their basal response. Dose response experiments to discern the ED_{50} of morphine administered alone or concomitantly with citalopram (15 mg/kg) were performed by acute injections of morphine at the following doses: 5, 10, 15, 20, 25, 30 and 50 mg/kg. ED_{50} calculation was done by non-lineal regression using the GraphPad Prism Software (GraphPad, San Diego, CA, USA) according to the following equation $Y = 100/(1+10^{(\log EC50-X)})$.

2.4. Actimetry

Mice spontaneous home-cage activity was evaluated with the Acti-System II device (Panlab, Spain), which detects changes produced in a magnetic field generated by the

animal movement. Animal activity was recorded during 1 hour.

2.5. Open field test

The open field apparatus was a brightly lit (350 lx) white wooden box (50 cm × 50 cm × 30 cm) with white floor and luminescent walls. Mice were released in the center of the apparatus for 5 min, and behavior was video-tracked by a computerized system (Any-maze Video-Tracking software, Stoelting Co., U.S.A.). Total distance travelled and time spent in the center area (30 cm × 30 cm) were considered for analysis. The test was conducted with those animals submitted to the development of tolerance schedule 2 h and 30 min after the first injection (9:00 a.m.) on day 1 and 7.

2.6. Statistical analysis

The statistical analyses were performed using Student's t-test, one-way or two-way ANOVA where appropriate. When effects of independent variables (dose, time), or interactions were significant, two-way ANOVA analysis was followed by Tukey's multiple comparisons post-hoc test. The type of statistical analysis is indicated in the results/discussion section and in the figure legends. The level of significance was set at $p < 0.05$. Graph editing and statistical analyses were performed using the GraphPad Prism Software (GraphPad, San Diego, CA, USA).

3. Results

3.1. Antinociceptive potentiation

In order to determine the degree of morphine analgesic effect on thermal nociception,

mice were administered with different doses of this opioid agonist, ranging from 10 to 50 mg/kg, and subsequently submitted to hot-plate test evaluation. The hot plate test is a thermal nociception assay that contemplates two behavioral components, i.e. mouse paw licking and jumping, resulting from the supraspinal integration of different neural processes (Le Bars *et al.*, 2001). Response measurements, considered as the reaction time of the first of any of those behavioral elements, were taken at 15 min intervals during 2 hours. The graphical plot of these results (Figure 1A) reveals an evident dose response effect of morphine on thermal nociception that was confirmed by statistical analysis (two-way ANOVA for dose variable $F_{(4,15)} = 8.832$, $p < 0.005$); in this sense, morphine administered at 10 mg/kg resulted completely ineffective, compared to vehicle, in terms of thermal antinociception whereas the maximal response, arbitrarily established at 30 sec to avoid animal tissue damage, was achieved at the highest doses, i.e. 40 and 50 mg/kg. Regarding the time course of morphine antinociceptive effect (two-way ANOVA for time variable $F_{(8,120)} = 16.93$, $p < 0.0001$), all the effective doses reached their maximal response between 45 and 60 min after injection (Tukey´s multiple comparison tests $P < 0.001$) (Figure 1A). Equivalent experiments were conducted, combining various doses of morphine with citalopram administered at 15 mg/kg (Figure 1B). In this case we decided to use a lower range of morphine doses, from 5 to 20 mg/kg, because it was observed in previous pilot tests a substantial potentiation of the morphine antinociceptive effect when injected in combination with citalopram. The confirmation of this potentiation is evident in data shown in Figure 1B, where the lowest morphine dose (5 mg/kg) evoked a robust antinociceptive reaction compared to mice only treated with citalopram, whereas doses ranging from 10 to 20 mg/kg already achieved 30 sec of

response latency. When comparing the time course of the antinociceptive effect after morphine injection, responses observed with the combination of both drugs appears to be less sustained than the equivalent obtained when morphine was administered alone (Figure 1A and 1B), with the exception of the morphine highest doses in combination with citalopram, 15 and 20 mg/kg, where responses are more stable across the time after reaching the maximal value between 45 and 60 min (two-way ANOVA for time variable $F_{(8,120)} = 21.20$, $p < 0.0001$). This fluctuation observed in the antinociceptive response throughout time is reflected by the absence of statistical significance when comparing the different doses (two-way ANOVA for dose variable $F_{(4,15)} = 1.368$, $p = 0.2917$). According to these results, we designated 45 min as the time interval after drug administration before nociception evaluation in the following experiments.

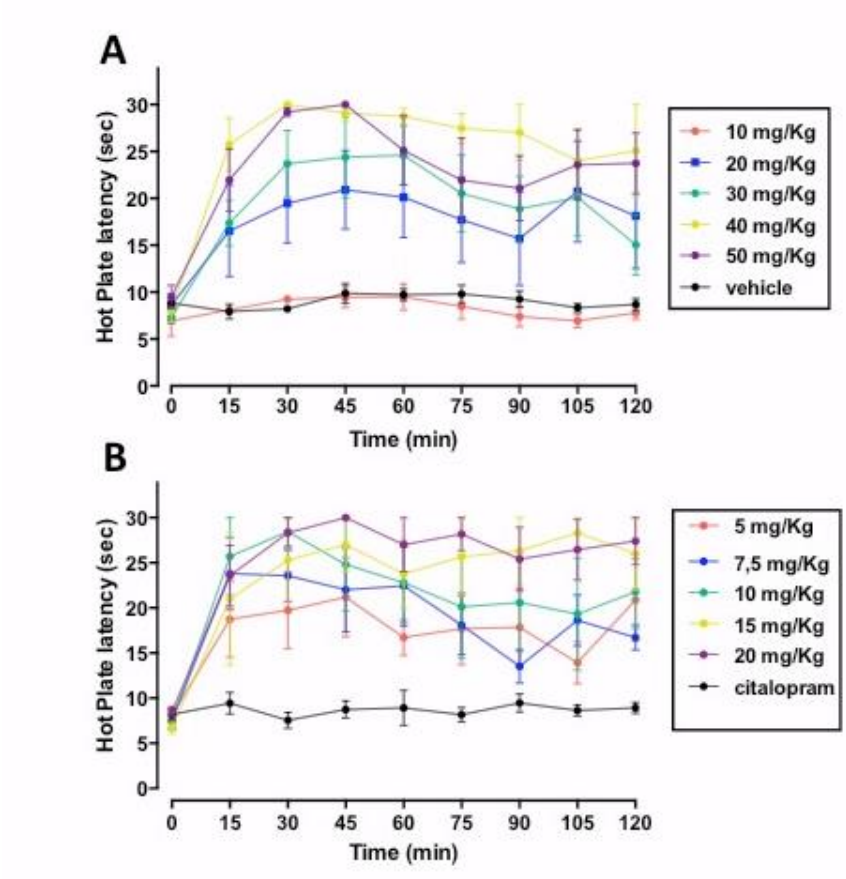

Figure 1. Time course of morphine antinociceptive effect evaluated by hot-plate test.
Data in graphs represent different responses measured as the latency to appear thermal nociceptive reaction in mice treated either with different doses of morphine alone **(A)** or in combination with citalopram at 15 mg/kg **(B).** Insets indicate the different doses of morphine used in each treatment. Each point represents mean ± SEM (n = 4 mice).

Next, morphine dose-response experiments to determine the extent of citalopram enhancement in thermal antinociception assays were carried out by acutely treating animals with a range of increasing doses of morphine from 5 to 50 mg/kg, either solely or in combination with citalopram at 15 mg/kg. Comparison of curves corresponding to both experimental conditions in a single graph (Figure 2) reveals a robust leftward shift of the curve obtained with animals treated with the combination of morphine plus citalopram, indicating the gain in potency of morphine when co-administered with citalopram. Non-lineal regression analysis of sigmoid curves from independent experiments resulted in a ED_{50} = 24.50 ± 1.31 (mean ± SEM; n = 5 experiments) for morphine that was significantly larger (*t-student* $_{(8)}$ = 4.329, p = 0.0025) than the equivalent value obtained in morphine plus citalopram experiments, i.e. ED_{50} = 13.49 ± 2.18 (mean ± SEM; n = 5 experiments).

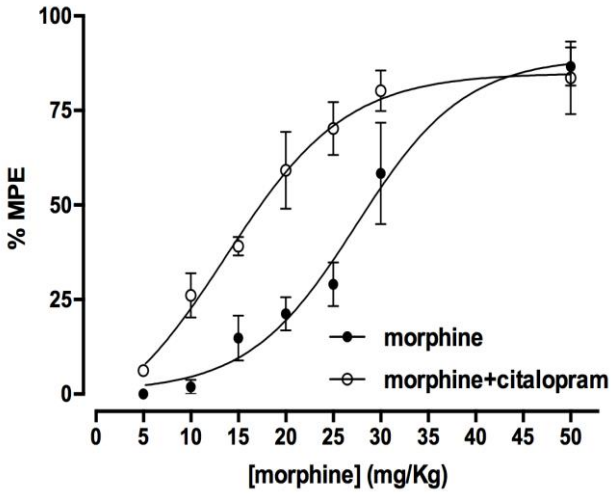

Figure 2. Evaluation of morphine dose-response effect on thermal nociception.
Data represented as % MPE were obtained from mice acutely treated with different doses of morphine alone (filled symbols) or in combination with citalopram at 15 mg/kg (opened symbols) and submitted to hot-plate test. Each point represents mean ± SEM (n = 3-4 mice)

3.2. Development of tolerance.

In order to induce morphine tolerance to antinociception in the hot plate test, mice were chronically treated with morphine (30 mg/kg), citalopram (15 mg/kg) or concurrently with morphine plus citalopram by injecting them twice daily during seven days as described in the Methods section. Thermal nociception was evaluated every day 45 min after the first drug injection and results from one representative experiment expressed as the percentage of maximum possible effect (%MPE) are shown in Figure 3.

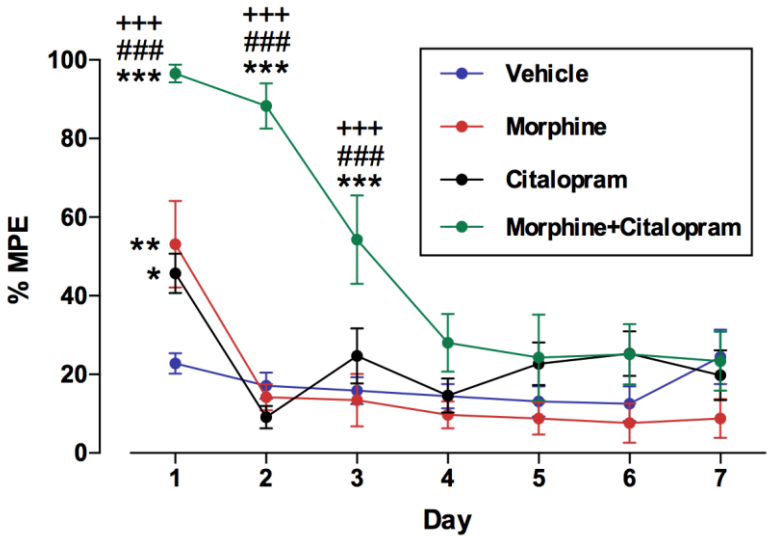

Figure 3. Induction of morphine tolerance to thermal antinociception evaluated by hot-plate test.
Data expressed as % MPE were obtained from mice treated chronically during 7 days with morphine (30 mg/Kg), citalopram (15mg/Kg), morphine plus citalopram and vehicle. Nociception was assessed in a daily basis during the treatment period. * $p < 0.01$, ** $p < 0.001$ and *** $p < 0.0001$ vs vehicle group. ### $p < 0.0001$ vs morphine group. +++ $p < 0.0001$ vs citalopram group (Tukey's multiple comparisons post-hoc test). Each point represents mean ± SEM (n = 10 mice).

The two-way ANOVA of this data resulted significant when considering either the

time variable ($F_{(6,120)}$ = 42.34, p < 0.0001), the group treatment variable ($F_{(3,35)}$ = 13.98, p < 0.0001) or the interaction between both variables ($F_{(18,120)}$ = 14.67, p < 0.0001). Similarly as observed in prior acute treatments, animals treated with morphine at this dose and evaluated on the day 1 presented an increase in the reaction time of some 50 % of the maximal response and significantly different from the reaction evoked by the group treated with vehicle (Post-hoc test p < 0.001). This effect was completely absent on the second day of treatment and not significantly different to the response observed for the vehicle group during the following 5 days demonstrating, therefore, a rapid development of morphine tolerance in mice to this type of thermal nociceptive stimulus. In relation to citalopram treatment, the time course curve presented a profile comparable to that observed with morphine (Figure 3), i.e., the acute administration on day 1 resulted in an antinociceptive response of some 50 % of the maximal response that was followed by values not different from the vehicle group during the next 6 days. Further actimetry experiments were conducted to evaluate the spontaneous activity of mice in their home cages 1 hour after drug administration in order to verify that the increment in locomotion observed in our experiments was due to the animal exposition to a novel environment. Actimetry tests (Supplemental Figure 1) resulted in a significant hyperlocomotion effect in animals treated either with morphine alone or in combination with citalopram (two-way ANOVA for treatment variable $F_{(3,48)}$ = 225.5, p < 0.0001). In contrast, treatment with only citalopram resulted in values significantly lower to those ones observed in groups treated with morphine (Post-hoc test for curve comparison, p < 0.0001) and not different to the vehicle control group.

As expected, the combination of morphine plus citalopram evoked the highest

antinociceptive reaction on the first day of treatment (Figure 3). In fact, this response was about two fold larger than the one observed with each of these drugs individually administered (Figure 3, day 1) suggesting the summation of their effects in the final response. Conversely, morphine plus citalopram reached an antinociceptive response of some 90% on day 2 regardless the lack of effect of each drug when separately used at this time point (Figure 3, day 2) ruling out any assumption that considers a final accumulative effect upon concurrent administration. This difference, although at a lesser but significant extent, was still detected on the third day delaying the manifestation of tolerance to the analgesic effect of morphine co-administered with citalopram until the day 4 of treatment (Figure 3).

3.3. Actimetry evaluation.

Further actimetry experiments were conducted to evaluate the spontaneous activity of mice in their home cages 1 hour after drug administration in order to verify that the increment in locomotion observed in our experiments was due to the animal exposition to a novel environment. Actimetry tests (Figure 4) resulted in a significant hyperlocomotion effect in animals treated either with morphine alone or in combination with citalopram (two-way ANOVA for treatment variable $F_{(3,48)} = 225.5$, $p < 0.0001$). In contrast, treatment with only citalopram resulted in values significantly lower to those ones observed in groups treated with morphine (Post-hoc test for curve comparison, $p < 0.0001$) and not different to the vehicle control group.

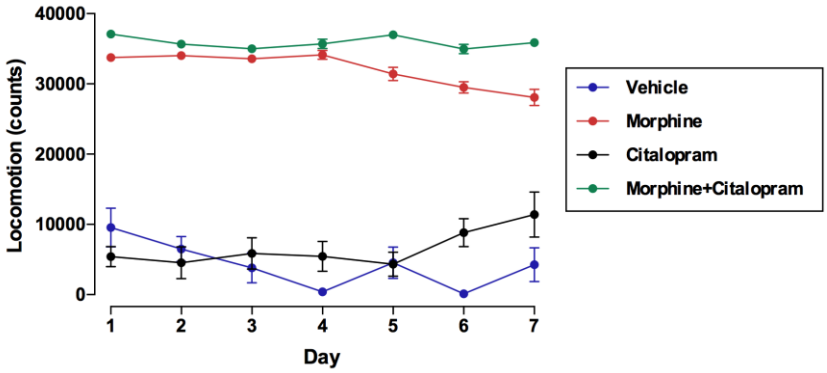

Figure4. Evaluation of mice spontaneous activity. Mice actimetry measurements were conducted in a daily basis in their habitual home cages 1 hour after treatment with morphine (30 mg/Kg), citalopram (15 mg/Kg), morphine plus citalopram and vehicle.

3.4. Open field test assessment.

Mice assessed for thermal nociception in experiments of morphine development of tolerance were also evaluated in the open field test on days 1 and 7 by recording their behavior within the apparatus during 5 min. Two parameters were taken into account when analyzing the final results, i.e., locomotor activity as total distance and central activity as time spent in central area. In good agreement with actimetry results, locomotor hyperactivity was also detected on the open field test in mice administered with morphine alone or in combination with citalopram (two-way ANOVA for treatment variable $F_{(3,58)} = 38.94$, $p < 0.0001$). Moreover, posthoc analysis revealed a potentiation of morphine-induced hyperlocomotion by citalopram that persisted after 7 days of treatment ($p < 0.0001$ morphine plus citalopram *versus* morphine alone on days 1 and 7) (Figure 5A). Regarding central activity, the combined treatment of morphine plus citalopram induced a significant anxiolytic effect as evidenced by an increase in this parameter, particularly on day 7 (two-way ANOVA for interaction $F_{(3,49)} = 3.377$, $p < 0.0255$ and time variable $F_{(3,49)} = 6.456$, $p < 0.0143$) (Figure 4B).

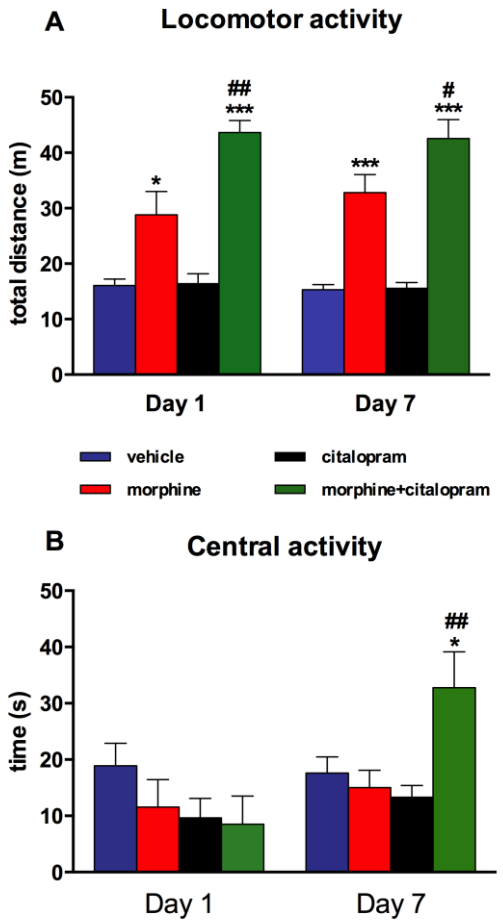

Figure 5. Behavioural effects of morphine, citalopram and their coadministration in the open-field test.
Mice were administered with morphine (30 mg/kg), citalopram (15 mg/kg), morphine plus citalopram and vehicle. Locomotor activity (total distance) **(A)** and central activity (time in the center area) **(B)** were evaluated after drug administration during 5 min at days 1 and 7. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs respective vehicle group; # $p\ 0.05 <$ and ## $p < 0.01$ vs respective morphine group (Tukey's multiple comparisons post-hoc test). Each bar represents mean ± SEM (n = 10 mice).

Discussion

Early reports already described the augmentation of morphine antinociceptive effects in experimentation animals when citalopram was simultaneously administered. In this regard, hot plate nociception assays conducted with rats determined 10 mg/kg as the minimum dose of citalopram required for a significant increase in reaction times of morphine analgesia (Sugrue, 1979) and, interestingly, this potentiation was selective for morphine since no equivalent effects were observed with methadone and pethidine. Nevertheless, this was contradicted by later results also obtained with rats evaluated for thermal nociception by hot plate test (Larsen and Hyttel, 1985). The

potentiation of morphine analgesia by citalopram observed in the present work confirmed previous results in rats assessed in hot plate test and in mice evaluated in grid shock assays (Larsen and Christensen, 1982) although a precise analgesic potency of morphine in terms of ED_{50} was not previously resolved. On the other hand, a more recent publication reported results in complete disagreement with those described herein and by others, since the combination of citalopram and morphine in acute and chronic treatments performed in mice not only resulted in a potentiation of morphine, but also caused a decrease of its analgesic effect in both tail-flick and hot plate tests (Pakulska and Czarnecka, 2001).

It has been extensively described that chronic treatments with morphine, and other opioid compounds, leads to the development of tolerance to the analgesic effects of these drugs. The pharmacological basis of this manifestation is related with the functionality of mu opioid receptors, the main site of action of morphine, and the desensitization processes that take place upon sustained receptor activation (Williams *et al.* , 2013). Furthermore, other adaptive changes within the CNS resulting from the continued receptor stimulation by morphine concern alterations in the expression of different proteins at the cellular level or modifications in the connectivity of neurons involved in the nociception transmission and/or supraespinal integration (Christie, 2008). The antinociceptive-like effect detected after the acute administration of citalopram observed in development of tolerance experiments (Figure 3) has not been consistently described in previous investigations regardless its possible participation as potentiator of morphine analgesia (Fasmer *et al.*, 1989, Larsen and Christensen, 1982, Larsen and Hyttel, 1985, Lee *et al.* , 2012, Sugrue, 1979). The reason for this discrepancy probably resides in the nature of the hot-plate nociception assay where

experimentation animals are subjected to a novel environment in order to generate paw thermal stimulation introducing, consequently, an additional element that might interfere the behavioral outcome considered as response, i.e. paw licking and/or jumping, and influencing therefore the final interpretation of experimental observations. In this sense, it has been previously described in the case of citalopram, along with other SSRIs, a specific increase of spontaneous locomotor activity mediated by 5-HT_{1B} and 5-HT_{2A} serotonin receptors associated with the exposition of animals to a novel environment only observed in mice and not when tested in rats (Brocco *et al.*, 2002, Millan *et al.* , 2003). Interestingly, this induced hyperlocomotion completely disappeared when mice treated with a similar dose of citalopram were pre-exposed to the activity chamber consisting in a white plexiglass cage different to the one where the animals were habitually housed. In our case, the hot plate apparatus containing plexiglass walls to impede animal evasion could be considered as the new environment responsible of the increase in the locomotion activity only observed in animals treated with citalopram the first day of treatment (see Supplemental Video 1). This generalized hyperactivity would delay the appearance of behavioral signs associated to thermal nociception (paw licking and/or jumping) and would augment, consequently, the latency of the response. As far as we know, the only previous investigation that used the hot-plate test to evaluate antinociceptive properties of citalopram in mice also described an 86% increase of the response latency when administered at 40 mg/kg while it was ineffective at 10 mg/kg (Fasmer *et al.*, 1989). This dose-response effect of citalopram observed in nociception assays was similarly described by Brocco *et al.* in experiments conducted to investigate the hyperlocomotion induced by this drug in animals subjected to a novel environment

(Brocco *et al.*, 2002). In relation to the effect on the development of morphine tolerance when administered in combination with citalopram, the significance found in the ANOVA when considering the interaction between both variables, i.e. treatment and time, indicates that the slope of the tolerance curve corresponding to morphine plus citalopram treatment is distinct from the equivalent one obtained when using morphine alone. This result suggests that citalopram, in addition of delaying the appearance of tolerance to morphine thermal antinociceptive effect, promotes a different mechanism of morphine tolerance development when concurrently used. Equivalent results were reported in thermal nociception experiments conducted with rats concomitantly treated with morphine plus fenfluramine and evaluated by tail flick test (Arends *et al.*, 1998). With this respect, simultaneous acute administration of morphine and fenfluramine significantly enhanced the antinociceptive effect of the opiate by shifting morphine dose-response curves to the left. Additionally, in chronic treatments a delay of tolerance development was also observed when combining both drugs in a similar manner as described herein in mice exposed to hot plate test and treated with morphine plus citalopram. Pharmacokinetics determinations in this same study ruled out the possibility that the attenuation of morphine tolerance development facilitated by fenfluramine were due to a higher concentration accompanied by a more sustained presence of morphine and/or its active metabolites during the chronic treatment suggesting, therefore, that inhibition of morphine tolerance would occur mainly due to an interference with the pharmacological mechanism underlying the development of tolerance to this opiate (Arends *et al.*, 1998). More recent reports have also confirmed this delay of tolerance development to the morphine thermal antinociceptive effects in rats co-treated either with amitriptyline, venlafaxine or

fluoxetine and assessed with tail flick and hot plate tests (Ozdemir *et al.*, 2011, Ozdemir *et al.*, 2012). The biological basis underlying this enhancement of morphine antinociceptive potency along with the delay of tolerance development by serotonin reuptake inhibition is an issue that remains to be elucidated. Previous results excluded any direct effect promoted by SSRIs that may influence the affinity of morphine for opioid receptors (Hynes *et al.*, 1985) or the functional properties of mu opioid receptors evaluated by [^{35}S]GTPγS binding autoradiography upon DAMGO stimulation in rat brain (Hesketh *et al.* , 2008). Other investigations consisting in the inhibition of the enhancement of morphine effects by using antagonist compounds, i.e. mianserin and methysergide, suggested the participation of 5-HT_2 receptor subtypes in these processes (Gatch *et al.*, 1998, Lee *et al.*, 2012). At this respect, we have previously described an augmentation of morphine potency in [^{35}S]GTPγS binding assays using membranes from cells heterologously co-expressing human mu opioid (MOP) and 5-HT_{2A} receptors when cells were pretreated with serotonin (Lopez-Gimenez *et al.* , 2008); intriguingly, this enhancement of morphine EC_{50} was not paralleled by DAMGO in equivalent experiments. Morphine is not capable to induce MOP receptor internalization upon its activation in several heterologous and native tissues at difference of what is observed with other agonist compounds (for a review on this topic see (Lopez-Gimenez and Milligan, 2010)). It has been largely hypothesized that morphine functional deficiency in terms of MOP receptor endocytosis might be involved in the molecular basis of the development of tolerance after sustained treatments (Berger and Whistler, 2010, Whistler, 2012). Moreover, we also described in these same cells that co-activation of 5-HT_{2A} receptors facilitated the endocytosis of MOP receptors upon morphine treatment similarly as we later

described in an analogous experimental model expressing 5-HT_{2C} receptors instead (Campa *et al.*, 2015, Lopez-Gimenez *et al.*, 2008). Although rather speculative in terms of translating *in vitro* results obtained from heterologous systems to native or physiological models, these results may suggest future approaches to further explore the biological mechanisms implicated in the improvement of morphine analgesia by enhancing serotonergic neurotransmission. Furthermore and according to a recent publication (Brenchat *et al.*, 2011), additional possibilities should be taken into account in terms of considering other serotonin receptor subtypes, such as 5-HT_7, that could mediate the action of the serotonin remaining in the synaptic cleft resulting from SSRI treatment.

The open field test is a behavioral paradigm widely used to evaluate locomotor activity and anxiety levels in rodents. When mice are exposed to this new and challenging environment they are naturally inclined to thigmotaxis which is evidenced as the movement of the animal away from the center and towards the peripheral zone of the open field and closer to the limiting walls. In this sense, such behavior has been considered as an index of timidity (Walsh and Cummins, 1976), and it is assumed to be an indicator of animal fear/anxiety state. Contrarily, those animals that spent more time in the central region of the field are considered as less fearful or anxious than those ones that prefer the perimeter area (Stanford, 2007). Results obtained with mice treated with SSRI in anxiety behavioral tests present a dual component, i.e., acute administration of some SSRI induces anxiogenic effects (Birkett *et al.*, 2011, Mombereau *et al.*, 2010) whereas repeated treatment leads to an anxiolytic response dependent on CREB function (Mombereau *et al.*, 2010). In the present study, conducted with C57BL/6J mice and regarding central activity in the

open field tests, we do not observe any of these effects either in acute as in subchronic citalopram treatments. However, we detected an anxiolytic response upon sustained citalopram and morphine co-treatment that may be explained due to an additive effect of both drugs in terms of augmentation of serotonergic transmission. In this sense, previous neurochemical studies demonstrated an increase of extracellular serotonin in rodent brain after morphine administration (Tao and Auerbach, 1994). These anxiolytic-like properties observed after sustained citalopram and morphine co-treatment may hold clinical significance since chronic pain is usually associated with depression and anxiety disorders (Huyser and Parker, 1999, McWilliams *et al.*, 2003). Early investigations already described an excitatory effect elicited by morphine in rats that was not affected by tolerance development after chronic treatment (Babbini and Davis, 1972). Further studies conducted with mice characterized other behavioral traits in response to morphine such as Straub sign, i.e. contraction of the sacro-coccygeus dorsalis muscle with protrusion of the perineum and elevation of the tail, extension rigidity of the hind legs accompanied by increased motor activity and animal running in circles in their cages (Shuster *et al.*, 1975). An equivalent sterotypia was detected in mice from our study submitted to morphine treatment (See Supplemental Video). In particular, mouse strain C57BL/6 presents a considerably higher running response when compared to other strains (Oliverio and Castellano, 1974, Shuster *et al.*, 1975) and, at difference to what happens in relation to tolerance to the analgesic effects of morphine, this locomotion response presented sensitization after sustained treatment. Although the negative correlation between running and analgesia has been reproduced in different laboratories and mouse strains, some discrepancies appeared in terms of development of sensitization or tolerance to the

running response (Oliverio and Castellano, 1974). In the present study morphine administration the first day of treatment evoked a locomotor response significantly higher than animals treated either with vehicle or citalopram. That hyperlocomotion remained at equivalent levels after 7 days of treatment, excluding any episode of sensitization or development of tolerance to this response in our case. As previously described (Popik, 1999), citalopram presented no effects on locomotor activity in mice. However, a significant potentiation of the hyperlocomotion effect caused by morphine was observed when both drugs were concurrently administered either on day 1 as on day 7 of treatment. A possible explanation to the augmentation of locomotion upon morphine treatment, that considers a neurochemical mechanism mediated by the enhancement of dopamine neurotransmission, was proposed after microdialysis studies in rats (Di Chiara and Imperato, 1988). However, later investigations on the relationship between morphine induced changes in locomotor activity and mesolimbic dopamine release conducted with three different mouse strains, including C57BL/6, found no correlation between these two variables, i.e. locomotion and dopamine release, in any of the considered strains (Murphy *et al.*, 2001). Previous results concerning the effect of drugs facilitating serotonergic transmission on the locomotion enhancement promoted by morphine treatment are also in contradiction with our present observations. In this sense, chronic treatments combining fluoxetine and morphine in rats resulted in attenuation of the locomotor stimulating effects of morphine (Sills and Fletcher, 1997). Similarly, fluvoxamine reduced morphine-induced hyperlocomotion in mice in a dose dependent manner in parallel to the potentiation of its antinociceptive effects (Ise *et al.*, 2001). This disparity on locomotion effects could be due to the different chemical nature of the

SSRIs used in the different studies. Further investigations in the future should elucidate the reason for these discrepancies.

In conclusion, in the present work we have fully characterized the interaction between citalopram and morphine in terms of functional response in a thermal nociception test and on behavioral responses. Citalopram enhanced the antinociceptive effects of morphine when concurrently administered in mice in two ways, i.e. by increasing its pharmacological potency and by attenuating the development of tolerance in sustained treatments. Additionally, hyperlocomotion induced by morphine is also potentiated by citalopram although no signs of tolerance or sensitization were observed in relation to this behavior. Interestingly, we firstly described that the combination of both drugs promotes an anxiolytic effect that is clearly evidenced in central activity measurements in the open field test after subchronic treatments. Future investigations on this pharmacological interaction, such as the possible translational research in clinics, might have consequences in future strategies for the therapeutic management of pain.

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References

Arends RH, Hayashi TG, Luger TJ, Shen DD. Cotreatment with racemic fenfluramine inhibits the development of tolerance to morphine analgesia in rats. J Pharmacol Exp Ther. 1998;286:585-92.

Babbini M, Davis WM. Time-dose relationships for locomotor activity effects of morphine after acute or repeated treatment. Br J Pharmacol. 1972;46:213-24.

Berger AC, Whistler JL. How to design an opioid drug that causes reduced tolerance and dependence. Ann Neurol. 2010;67:559-69.

Birkett MA, Shinday NM, Kessler EJ, Meyer JS, Ritchie S, Rowlett JK. Acute anxiogenic-like effects of selective serotonin reuptake inhibitors are attenuated by the benzodiazepine diazepam in BALB/c mice. Pharmacol Biochem Behav. 2011;98:544-51.

Brenchat A, Ejarque M, Zamanillo D, Vela JM, Romero L. Potentiation of morphine analgesia by adjuvant activation of 5-HT7 receptors. J Pharmacol Sci. 2011;116:388-91.

Brocco M, Dekeyne A, Veiga S, Girardon S, Millan MJ. Induction of hyperlocomotion in mice exposed to a novel environment by inhibition of serotonin reuptake. A pharmacological characterization of diverse classes of antidepressant agents. Pharmacol Biochem Behav. 2002;71:667-80.

Campa VM, Capilla A, Varela MJ, de la Rocha AM, Fernandez-Troyano JC, Barreiro RB, et al. Endocytosis as a biological response in receptor pharmacology: evaluation by fluorescence microscopy. PLoS One. 2015;10:e0122604.

Christie MJ. Cellular neuroadaptations to chronic opioids: tolerance, withdrawal and addiction. Br J Pharmacol. 2008;154:384-96.

Colasanti A, Rabiner EA, Lingford-Hughes A, Nutt DJ. Opioids and anxiety. J Psychopharmacol. 2011;25:1415-33.

Contreras E, Tamayo L, Quijada L, Silva E. Decrease of tolerance development to morphine by 5-hydroxytryptophan and some related drugs. Eur J Pharmacol. 1973;22:339-43.

Dharmshaktu P, Tayal V, Kalra BS. Efficacy of antidepressants as analgesics: a review. J Clin Pharmacol. 2012;52:6-17.

Di Chiara G, Imperato A. Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. Proc Natl Acad Sci U S A. 1988;85:5274-8.

Esmailian M, Keshavarz M. Synergistic Effects of Citalopram and Morphine in the

Renal Colic Pain Relief; a Randomized Clinical Trial. Emergency. 2014;2:26-9.

Fasmer OB, Hunskaar S, Hole K. Antinociceptive effects of serotonergic reuptake inhibitors in mice. Neuropharmacology. 1989;28:1363-6.

Gatch MB, Negus SS, Mello NK. Antinociceptive effects of monoamine reuptake inhibitors administered alone or in combination with mu opioid agonists in rhesus monkeys. Psychopharmacology (Berl). 1998;135:99-106.

Glover EM, Davis M. Anxiolytic-like effects of morphine and buprenorphine in the rat model of fear-potentiated startle: tolerance, cross-tolerance, and blockade by naloxone. Psychopharmacology (Berl). 2008;198:167-80.

Hesketh SA, Brennan AK, Jessop DS, Finn DP. Effects of chronic treatment with citalopram on cannabinoid and opioid receptor-mediated G-protein coupling in discrete rat brain regions. Psychopharmacology (Berl). 2008;198:29-36.

Huyser BA, Parker JC. Negative affect and pain in arthritis. Rheumatic diseases clinics of North America. 1999;25:105-21, vi.

Hynes MD, Lochner MA, Bemis KG, Hymson DL. Fluoxetine, a selective inhibitor of serotonin uptake, potentiates morphine analgesia without altering its discriminative stimulus properties or affinity for opioid receptors. Life Sci. 1985;36:2317-23.

Ise Y, Katayama S, Hirano M, Aoki T, Narita M, Suzuki T. Effects of fluvoxamine on morphine-induced inhibition of gastrointestinal transit, antinociception and hyperlocomotion in mice. Neurosci Lett. 2001;299:29-32.

Knotkova H, Pappagallo M. Adjuvant analgesics. Anesthesiology clinics. 2007;25:775-86, vi.

Larsen JJ, Christensen AV. Subarachnoidal administration of the 5-HT uptake inhibitor citalopram points to the spinal role of 5-HT in morphine antinociception. Pain. 1982;14:339-45.

Larsen JJ, Hyttel J. 5-HT-uptake inhibition potentiates antinociception induced by morphine, pethidine, methadone and ketobemidone in rats. Acta pharmacologica et toxicologica. 1985;57:214-8.

Larson AA, Takemori AE. Effect of fluoxetine hydrochloride (Lilly 110140), a specific inhibitor of serotonin uptake, on morphine analgesia and the development of tolerance. Life Sci. 1977;21:1807-11.

Le Bars D, Gozariu M, Cadden SW. Animal models of nociception. Pharmacol Rev. 2001;53:597-652.

Lee BS, Jun IG, Kim SH, Park JY. Interaction of morphine and selective serotonin receptor inhibitors in rats experiencing inflammatory pain. J Korean Med Sci.

2012;27:430-6.

Lopez-Gimenez JF, Milligan G. Opioid regulation of mu receptor internalisation: relevance to the development of tolerance and dependence. CNS Neurol Disord Drug Targets. 2010;9:616-26.

Lopez-Gimenez JF, Vilaro MT, Milligan G. Morphine desensitization, internalization, and down-regulation of the mu opioid receptor is facilitated by serotonin 5-hydroxytryptamine2A receptor coactivation. Mol Pharmacol. 2008;74:1278-91.

McWilliams LA, Cox BJ, Enns MW. Mood and anxiety disorders associated with chronic pain: an examination in a nationally representative sample. Pain. 2003;106:127-33.

Millan MJ. Descending control of pain. Prog Neurobiol. 2002;66:355-474.

Millan MJ, Veiga S, Girardon S, Brocco M. Blockade of serotonin 5-HT1B and 5-HT2A receptors suppresses the induction of locomotor activity by 5-HT reuptake inhibitors, citalopram and fluvoxamine, in NMRI mice exposed to a novel environment: a comparison to other 5-HT receptor subtypes. Psychopharmacology (Berl). 2003;168:397-409.

Mombereau C, Gur TL, Onksen J, Blendy JA. Differential effects of acute and repeated citalopram in mouse models of anxiety and depression. Int J Neuropsychopharmacol. 2010;13:321-34.

Murphy NP, Lam HA, Maidment NT. A comparison of morphine-induced locomotor activity and mesolimbic dopamine release in C57BL6, 129Sv and DBA2 mice. J Neurochem. 2001;79:626-35.

Oliverio A, Castellano C. Genotype-dependent sensitivity and tolerance to morphine and heroin: dissociation between opiate-induced running and analgesia in the mouse. Psychopharmacologia. 1974;39:13-22.

Ossipov MH, Dussor GO, Porreca F. Central modulation of pain.J Clin Invest. 2010;120:3779-87.

Ozdemir E, Bagcivan I, Gursoy S, Altun A, Durmus N. Effects of fluoxetine and LY 367265 on tolerance to the analgesic effect of morphine in rats. Acta Physiol Hung. 2011;98:205-13.

Ozdemir E, Gursoy S, Bagcivan I. The effects of serotonin/norepinephrine reuptake inhibitors and serotonin receptor agonist on morphine analgesia and tolerance in rats. J Physiol Sci. 2012;62:317-23.

Pakulska W, Czarnecka E. Effect of citalopram and buspirone on the antinociceptive action of analgesic drugs. Acta Pol Pharm. 2001;58:299-305.

Patetsos E, Horjales-Araujo E. Treating Chronic Pain with SSRIs: What Do We Know? Pain Res Manag. 2016; 2020915.

Popik P. Preclinical pharmacology of citalopram. J Clin Psychopharmacol. 1999;19:4S-22S.

Shuster L, Webster GW, Yu G. Increased running response to morphine in morphine-pretreated mice. J Pharmacol Exp Ther. 1975;192:64-7.

Sills TL, Fletcher PJ. Fluoxetine attenuates morphine-induced locomotion and blocks morphine-sensitization. Eur J Pharmacol. 1997;337:161-4.

Stanford SC. The Open Field Test: reinventing the wheel. J Psychopharmacol. 2007;21:134-5.

Sugrue MF. On the role of 5-hydroxytryptamine in drug-induced antinociception. Br J Pharmacol. 1979;65:677-81.

Tao R, Auerbach SB. Increased extracellular serotonin in rat brain after systemic or intraraphe administration of morphine. J Neurochem. 1994;63:517-24.

Walsh RN, Cummins RA. The Open-Field Test: a critical review. Psychological bulletin. 1976;83:482-504.

Whistler JL. Examining the role of mu opioid receptor endocytosis in the beneficial and side-effects of prolonged opioid use: from a symposium on new concepts in mu-opioid pharmacology. Drug Alcohol Depend. 2012;121:189-204.

Williams JT, Ingram SL, Henderson G, Chavkin C, von Zastrow M, Schulz S, et al. Regulation of mu-opioid receptors: desensitization, phosphorylation, internalization, and tolerance. Pharmacol Rev. 2013;65:223-54.