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Experimental Research on Peripheral Opioid Receptors

PhD Thesis Abstract

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Cuprins

Working Hypothesis and General Objectives

Data from the specialized literature suggest the existence of peripheral opioid receptors, not just central opioid receptors. It appears that these peripheral receptors are generated in inflamed areas precisely as a consequence of the inflammatory process itself.

Our first working hypothesis is based on the premise that if peripheral opioid receptors are induced by inflammation, they may also play a role in modulating the inflammatory process. Therefore, stimulation of these receptors could influence inflammation in either a beneficial or detrimental way, depending on the context.

A second hypothesis is built on the idea that stimulation of central nervous system opioid receptors produces analgesia as the primary pharmacological effect. Accordingly, it is plausible that stimulation of peripheral opioid receptors could also result in an analgesic effect.

Such an effect would be of particular interest if specific conditions could be established that allow morphine to selectively stimulate peripheral opioid receptors, without activating those in the central nervous system. One potential route to achieve this could be intraplantar administration of morphine, based on the assumption that the absorption of morphine from the injection site would be slow and of low intensity—or, more precisely, significantly slower and less intense than with intraperitoneal administration.

If our investigations confirm that peripheral opioid receptors can be selectively stimulated, excluding central ones, it would be worthwhile to explore some of the characteristics of the resulting analgesic effect. For example, it would be important to determine at which point during the inflammatory process this analgesic effect is most pronounced, whether intraplantar morphine administration is effective in different types of pain, how the intensity of the analgesic response compares to that obtained through central receptor stimulation, and what the optimal intraplantar dose would be to avoid central nervous system involvement while still achieving effective peripheral analgesia.

General Research Methodology

The studies presented in this paper employed two distinct experimental methodologies, each with a specific objective aimed at investigating inflammatory processes and the analgesic effects of the tested substances.

The first method involved evaluating the progression of paw swelling over time using successive plethysmometric measurements following carrageenan administration, in order to quantify the local inflammatory response.

The second method focused on assessing paw withdrawal latency under conditions of nociceptive thermal stimulation, using the Hargreaves method, to analyze the analgesic effect of morphine in carrageenan-induced inflammation.

This complementary approach allowed for a detailed characterization of both the inflammatory process and the mechanisms involved in peripheral pain modulation.

Animals

All experiments were conducted on male albino Wistar rats, with an initial body weight ranging between 250 and 300 grams. The animals were obtained from the "Cantacuzino" National Institute for Medical-Military Research and Development and transferred to the laboratory of the Department of Pharmacology and Pharmacotherapy at the “Carol Davila” University of Medicine and Pharmacy. Upon arrival, the rats were six weeks old and underwent a one-week acclimatization and adaptation period before the beginning of the experimental procedures.

The rats were housed individually in standard cages and maintained under controlled environmental conditions, with an ambient temperature of 21–24°C and optimal humidity levels to ensure a stable and comfortable setting. Artificial lighting was maintained on a 12-hour light/dark cycle, with lights on between 7:00 a.m. and 7:00 p.m., in accordance with the natural circadian rhythm. Throughout the study, the animals had ad libitum access to standard rodent chow and water, ensuring adequate nutritional intake and hydration.

A total of 267 rats were included in the experiments and were allocated into groups of 7 to 10 animals, depending on the specific experimental protocol applied to each group. The experimental procedures were conducted over periods ranging from 2 to 7 days. At the end of the study, the animals were euthanized under general anesthesia, followed by the administration of a lethal agent. Euthanasia was performed by the institution's veterinarian, in accordance with ethical standards and current legislation on the use of animals for scientific purposes.

The number of animals per group was calculated based on anticipated variability, ensuring that the statistical power of each test was at least 80%. All experiments were carried out in compliance with the ethical guidelines for laboratory animal research and with the approval of the institution’s Ethics Committee.

The protocol for this study was approved by the Ethics Committee for Non-Clinical Studies on Laboratory Animals of the “Carol Davila” University of Medicine and Pharmacy in Bucharest and was authorized by the Bucharest Sanitary Veterinary and Food Safety Authority under project authorization no. 33/12.09.2022. Approval was granted in accordance with Law no. 43/2014 on the protection of animals used for scientific purposes, as subsequently amended and supplemented, as well as with EEC Directive 86/609 of 24 November 1986 on the approximation of laws, regulations, and administrative provisions of the Member States regarding the protection of animals used for experimental and scientific purposes.

Substances

The substances used in the experiments were as follows:

– Morphine – Morphine hydrochloride injectable solution 20 mg/ml, Brand: Zentiva
– Ibuprofen sodium 100 g, Brand: Sigma
– Lambda-carrageenan 100 g, Brand: Sigma
– 0.9% saline solution
– Distilled water

In all experiments, a 1% carrageenan solution was used, prepared by dissolving 250 mg of carrageenan in 25 ml of distilled water.

In Experiment 2, where ibuprofen was administered, it was diluted as follows: 1200 mg of ibuprofen was dissolved in 10 ml of distilled water to obtain a concentration of 12 mg/0.1 ml.

In the subsequent experiments, morphine was administered in successive dilutions, adjusted according to the required dose per kilogram of body weight.

Equipment

The equipment used in the study included:

– Ugo Basile Plethysmometer 7140

– Ugo Basile Plantar Test 37370

– 1 ml insulin syringes

– 2 ml, 5 ml, and 10 ml syringes

– 50 ml glass flask

– Blue, red, and green markers

– Protective gloves (size M/L)

– Lenovo laptop with Windows 10 and Office 10

– Magnetic stirrer

Working Method

This experimental research included a total of six successive experiments, each designed to investigate different aspects of the physiological and pharmacological processes involved in the inflammatory response induced by carrageenan administration, as well as analgesia. In order to obtain accurate and reproducible results, special attention was given to the preliminary phase of animal acclimatization and adaptation, which was essential for minimizing inter-individual variability and reducing experimental stress on the tested organisms. Following this accommodation period, each rat was individually identified using a numbering system, facilitating the consistent monitoring of each subject throughout the course of the experiments.

Subsequently, the animals were weighed to document their initial body weight, an essential parameter for calculating the appropriate dosage of the substances administered. After completing these preliminary steps, the rats were randomly assigned to experimental groups, ensuring group homogeneity and reducing the risk of methodological bias.

For the first three experiments, the primary objective was the plethysmometric assessment of the hind paw volume, aimed at evaluating pharmacologically induced inflammatory edema. To achieve this, the hind paws of each animal were marked at the level of the ankle joint using permanent markers of different colors. This method allowed for easy identification of each paw during measurement and helped minimize the risk of interpretation errors.

Subsequently, changes in paw volume were evaluated using the Ugo Basile 7140 digital plethysmometer, a precision instrument designed to measure volume displacement in milliliters. Baseline measurements (T0) were recorded prior to the administration of experimental substances, serving as reference values for each individual subject.

Following the initial measurements, a 1% carrageenan solution was administered in a volume of 0.15 ml via local intraplantar injection, exclusively into the right hind paw. This methodology allowed the left hind paw to serve as an internal reference for each animal, contributing to the reduction of interindividual variability and improving the accuracy of data interpretation.

In the first experiment, the experimental group consisted of nine rats, and plethysmometric determinations were carried out sequentially at predetermined time intervals following carrageenan administration: 3 hours, 6 hours, 12 hours, 24 hours, and 48 hours post-injection. This approach enabled observation of the progression of inflammation and the associated volume changes over time, thus providing essential data for characterizing the kinetics of the acute inflammatory response.

In the second experiment, the protocol involved two experimental groups, each composed of eight rats, with the objective of investigating the anti-inflammatory effect of ibuprofen on carrageenan-induced edema. One of the groups received a pharmacological treatment consisting of ibuprofen administered at a dose of 120 mg/kg body weight via intraperitoneal injection immediately after carrageenan injection. The second group served as a control and was administered an equivalent volume of 0.9% saline solution (0.1 ml/100 g), intended to replicate procedural stress without exerting any specific pharmacodynamic effects.

Plethysmometric measurements were performed for both groups at baseline (T0) and at subsequent predetermined time points to monitor the evolution of the inflammatory process and the treatment response: 150 minutes, 5 hours, 12 hours, 24 hours, 48 hours, 72 hours, and 96 hours after carrageenan injection. This extended temporal approach allowed for a detailed evaluation of the dynamics of the inflammatory response and the efficacy of the administered anti-inflammatory agent, offering a comprehensive framework for analyzing the mechanisms involved in experimental inflammatory processes.

In the third experiment, the rats were divided into six experimental groups, each consisting of seven subjects. This phase of the study was designed to investigate the effects of different doses and routes of administration of morphine sulfate on carrageenan-induced inflammatory edema.

In accordance with the methodology applied in the previous experiments, inflammation was induced by intraplantar injection of a 1% carrageenan solution into the right hind paw of each animal. This procedure allowed for the reproduction of a standardized model of acute inflammation, facilitating a comparative evaluation of the pharmacological effects of morphine sulfate.

Following inflammation induction, the experimental groups were subjected to specific pharmacological interventions. Morphine sulfate was administered in two different doses, namely 1 mg/kg body weight and 5 mg/kg body weight, via two different routes: intraplantar and intraperitoneal. Accordingly, four of the six experimental groups received the active treatment—two groups via intraplantar administration and the other two via intraperitoneal administration. This experimental approach enabled not only a comparison of the efficacy of the two tested doses, but also an evaluation of the impact of the administration route on the inflammatory response and on the potential systemic effects of morphine.

To ensure a rigorous experimental design, two additional groups were designated as control groups. These animals received injections of 0.9% saline solution, administered either intraplantarly or intraperitoneally, in volumes equivalent to those used for the active treatment. This procedure was intended to isolate the specific pharmacological effects of morphine sulfate from any potential influences associated with experimental handling or vehicle administration.

Plethysmometric measurements were conducted according to a standardized protocol, with the moment of morphine sulfate administration considered as time zero (T0). Subsequent volume determinations were carried out at clearly defined time points—6 hours, 24 hours, 48 hours, and 72 hours after treatment administration. This monitoring strategy allowed for the evaluation of the dynamic profile of both the analgesic and anti-inflammatory effects of morphine sulfate, as well as the identification of potential differences between experimental groups based on dose and route of administration.

In the following three experiments, a different investigative methodology was adopted, with the primary objective of evaluating the analgesic effect of locally administered morphine on inflammatory pain induced by intraplantar injection of carrageenan. Unlike the previous experiments, which assessed inflammatory changes through plethysmometric measurements, these studies focused on testing the nociceptive threshold using the Hargreaves method—a standardized experimental model for evaluating thermal sensitivity in the hind limbs in response to painful stimuli.

To assess pain sensitivity in the rats, the Ugo Basile Plantar Test 37370 device was used. This is a high-precision apparatus specifically designed to measure hind paw withdrawal latency in response to a controlled thermal stimulus. The Hargreaves method involves directing a constant-intensity infrared beam onto the plantar surface of the paw, resulting in a gradual heating of the cutaneous tissue. The animal's nociceptive response is automatically recorded at the moment the paw is withdrawn, and the latency time is considered a direct indicator of the thermal pain threshold. This test allows for the comparison of nociceptive thresholds between experimental and control groups, providing valuable information regarding the analgesic efficacy of the tested compound.

In Experiment 4, ten experimental groups were used, each consisting of ten rats. The objective of this experiment was to determine the time-dependent impact of morphine administration on the pain sensitivity of the inflamed paw. Thus, the experimental groups received a dose of 5 mg/kg body weight of morphine sulfate, administered intraplantarly into the right hind paw at different time points relative to carrageenan injection. Group allocation was as follows: the first group received morphine immediately after carrageenan administration (0.15 ml, 1%

concentration), the second group received morphine 3 hours post-administration, the third at 6 hours, the fourth at 24 hours, and the fifth at 48 hours after inflammation induction.

To allow for proper comparison, five control groups were also included, each corresponding to a specific time point of morphine administration. The animals in these control groups received 0.9% saline solution injections, in volumes identical to those of the active solution, administered intraplantarly into the right hind paw at the same time intervals (immediately after carrageenan injection, and at 3, 6, 24, and 48 hours). This strategy enabled clear differentiation between the specific pharmacological effects of morphine and any nociceptive changes generated solely by the inflammatory process.

In all animals included in the study, pain sensitivity was evaluated using the Hargreaves method, performed with the Ugo Basile Plantar Test 37370 device. Measurements were obtained by recording the latency time (expressed in seconds) until the withdrawal of the right hind paw, which represents a reflexive response to pain induced by the thermal stimulus.

For each animal, testing was initiated 10 minutes after the administration of either morphine or 0.9% saline solution. To obtain highly accurate data and reduce individual variability, five measurements were performed for each rat, with five-minute intervals between determinations. The results were subsequently analyzed by calculating the arithmetic mean of the five latency values recorded for each subject. This statistical approach was intended to minimize the impact of potential measurement errors and to provide a robust estimate of the nociceptive threshold for each experimental group.

The fifth experiment aimed to investigate the dose-dependent effect of locally administered morphine on inflammatory pain induced by carrageenan injection. For this phase of the study, five experimental groups were used, each consisting of ten rats randomly selected and assigned to ensure the comparability of results.

Of the five groups, four received morphine sulfate in varying doses, administered intraplantarly into the right hind paw, three hours after carrageenan injection. The tested doses were arranged in a geometric progression with a ratio of 2, as follows: 2.5 mg/kg, 5 mg/kg, 10 mg/kg, and 20 mg/kg. This experimental design allowed for the evaluation of the analgesic effect

of morphine at different concentrations and the potential identification of a dose-response relationship, including the presence of a plateau effect or response saturation.

To ensure the validity of the results and to exclude non-specific influences associated with the administration procedure itself, a control group was included. This group received an intraplantar injection of 0.9% saline into the right hind paw at the same time point (three hours after carrageenan injection), using a volume equivalent to that administered in the experimental groups. The inclusion of this control group was essential to differentiate genuine pharmacological effects of morphine from any pain threshold alterations related to mechanical handling or the inflammatory response induced by fluid injection.

For each rat, nociceptive sensitivity was assessed 10 minutes after the administration of morphine or saline using the Hargreaves method, as previously described, with the aid of the Ugo Basile Plantar Test 37370 device. The measurements consisted of recording the latency time to paw withdrawal as a reflex response to the applied thermal stimulus.

To obtain precise and reproducible results, five consecutive measurements were taken for each animal, with regular five-minute intervals between them. The recorded values were then averaged, as this statistical method helps minimize the influence of potential individual fluctuations.

Additionally, to prevent prolonged exposure to excessive thermal stimuli and to avoid the risk of tissue burns, a maximum latency cutoff of 30 seconds was established. If an animal did not withdraw its paw within this timeframe, the measurement was automatically terminated, and the maximum latency (30 seconds) was recorded as the final value for that subject. This safety measure was implemented in accordance with ethical principles and best practice standards in animal experimentation, aiming to reduce discomfort and stress associated with testing procedures.

In the sixth and final experiment, the same methodology for testing nociceptive sensitivity was employed, using the Hargreaves method to evaluate the analgesic effect of morphine in inflammatory pain induced by intraplantar carrageenan injection. The study included five experimental groups, each consisting of ten rats randomly assigned to ensure data comparability and the statistical validity of the results.

Among these five groups, three were designated as experimental groups and received morphine sulfate treatment, administered either locally or systemically. Specifically, the first experimental group received a dose of 5 mg/kg body weight of morphine via intraplantar injection into the right hind paw. The second group received the same dose of 5 mg/kg body weight via intraperitoneal injection, while the third experimental group was administered a higher dose of 10 mg/kg body weight intraperitoneally. All treatments were carried out three hours after carrageenan injection—this time point was selected based on observations from previous experiments in order to capture an active phase of the inflammatory process and to assess the analgesic efficacy of morphine during that stage.

To allow for objective comparison and to control for potential non-pharmacological influences on the nociceptive threshold, two control groups were included. The first control group received an intraplantar injection of 0.9% saline into the right hind paw, in a volume equivalent to that used for morphine administration in the corresponding experimental group. The second control group received 0.9% saline intraperitoneally, to account for any differences associated with the route of vehicle administration. All administrations in the control groups were performed three hours after carrageenan injection, mirroring the timing of the pharmacological interventions in the experimental groups.

For each animal, pain sensitivity was assessed using the Hargreaves method, with the aid of the Ugo Basile Plantar Test 37370 device, following the protocol previously described. Testing began 10 minutes after the administration of morphine or saline, and for each rat, five successive measurements were recorded at five-minute intervals. This methodological approach allowed for the collection of precise data by minimizing interindividual variability and reducing experimental error.

Data Analysis

In this experimental study, data analysis focused on two main parameters, corresponding to the two distinct investigative methodologies employed. Thus, in the first three experiments, the primary objective was the evaluation of inflammatory changes in the right hind paw, measured using the plethysmometric method, which allowed for quantification of paw edema

volume at various time points following carrageenan administration and treatment with the tested substances. In contrast, the following three experiments focused on the analysis of nociceptive sensitivity, evaluated using the Hargreaves method, with paw withdrawal latency from the thermal stimulus serving as an indicator of thermal pain threshold. This differentiated approach allowed for a comprehensive characterization of both the inflammatory response and the analgesic effect of morphine, depending on dose and route of administration.

For statistical data processing, IBM SPSS Statistics for Windows, version 29.0 (30-day trial version, Armonk, NY: IBM Corp) was used. The analysis of continuous variables was preceded by testing for normality of distribution using the Shapiro-Wilk test. Results were then expressed as mean ± standard deviation, median, minimum, and maximum values, providing a robust interpretation of central tendency and data dispersion.

To compare the values of the right hind paw volume, as measured plethysmometrically in the first three experiments where normal distribution was confirmed, the Student's t-test was applied. The level of statistical significance was set at $p < 0.05$, which was considered relevant for identifying significant differences between experimental groups.

For the comparison of paw withdrawal latency in the Hargreaves method-based experiments, the Mann–Whitney U test was used, due to the non-normal distribution of the data. The level of statistical significance was also set at $p < 0.05$, indicating a relevant threshold for highlighting significant differences between experimental groups.

Results

The present research aimed to investigate peripheral opioid receptors. The focus was placed specifically on potential peripheral opioid receptors located in the right hind paw of rats.

Given that data from the scientific literature support the idea that peripheral opioid receptors increase in density as a result of the inflammatory process, our first question was whether these receptors are also involved in the inflammation itself. An experimental model of

inflammation was chosen, involving the right hind paw of rats, where the volume of the paw was measured in milliliters using the Ugo Basile 7140 digital plethysmometer.

As a first step, we examined whether the administration of carrageenan induces inflammation. The study was conducted on a group of nine rats, in which carrageenan was injected intraplantarly into the right hind paw, while the left hind paw remained untreated. The paw volume was measured immediately after carrageenan administration and subsequently at 3, 6, 12, 24, and 48 hours. Throughout the observation period—except for the immediate post-injection time point—the volume of the carrageenan-injected right paw was statistically significantly greater than that of the untreated left paw. Based on these results, we concluded that intraplantar carrageenan administration indeed induces inflammation in the targeted paw.

In the second phase, we investigated whether anti-inflammatory drugs could reduce the volume of carrageenan-induced paw inflammation. Two groups of eight rats were used. Following carrageenan administration, one group received ibuprofen at a dose of 120 mg/kg body weight intraperitoneally, while the other group received 0.9% saline solution at a dose of 0.1 ml/100 g body weight intraperitoneally. The volumes of both inflamed and non-inflamed paws were measured immediately after injection, and then at 2.5, 5, 12, 24, 48, 72, and 96 hours. With the exception of the immediate measurement, the average volume of the inflamed paws in the saline-treated group was significantly higher than that of the ibuprofen-treated group. These findings confirmed that the experimental model was valid and suitable for detecting a potential anti-inflammatory effect of morphine administered intraplantarly.

Subsequently, six groups of seven rats each were used. After carrageenan-induced inflammation, we examined the anti-inflammatory effect of morphine administered intraplantarly at doses of 1 mg/kg and 5 mg/kg, and intraperitoneally at the same doses. These were compared to corresponding control groups that received intraplantar or intraperitoneal injections of 0.9% saline in volumes equal to those used for morphine. The data showed that only morphine administered intraplantarly at a dose of 5 mg/kg produced a statistically significant anti-inflammatory effect compared to the control group, specifically at 48 hours after carrageenan injection. This suggests that morphine has an anti-inflammatory effect when administered directly at the site of inflammation.

Since the same dose of morphine administered intraperitoneally did not produce a statistically significant anti-inflammatory effect, it can be presumed that the effect observed with intraplantar administration was mediated through peripheral opioid receptors located in the inflamed tissue, rather than through central nervous system opioid receptors.

Since the most important effect of morphine reported in the literature is its analgesic action—typically achieved through the stimulation of central nervous system opioid receptors—we considered the possibility that such an effect might also be produced through the stimulation of peripheral opioid receptors. Given that only intraplantar administration of morphine at a dose of 5 mg/kg body weight produced an anti-inflammatory effect, we aimed to determine whether the same dose could also exert an analgesic effect. Furthermore, based on the previous data, we observed that the density of peripheral opioid receptors increases over time. This led us to hypothesize that the intensity of the analgesic effect might also vary depending on the timing of administration.

To test this hypothesis, a new experiment was conducted to assess the analgesic effect of morphine on the right hind paw of rats in which inflammation was induced via intraplantar carrageenan injection. In order to evaluate how the analgesic effect of morphine varies with time elapsed since the onset of inflammation, morphine was administered at several time points following carrageenan injection: immediately, at 3, 6, 24, and 48 hours. The analgesic effect was measured 10 minutes after morphine administration using the paw withdrawal test in response to thermal stimulation, with the Ugo Basile Plantar Test 37370 device.

Withdrawal latency was measured in seconds, and results were compared between groups. The data showed that paw withdrawal latency was shorter at 24 and 48 hours compared to earlier measurements, suggesting that a state of hyperalgesia had developed around 6 hours after carrageenan administration. This hyperalgesia was significantly influenced by intraplantar morphine administration, but to a much lesser extent than in the earlier phases, when hyperalgesia was likely absent.

Integrating all these findings, we conclude that carrageenan-induced inflammation initially causes normal pain, which evolves into a state of hyperalgesia approximately 6 hours after the onset of inflammation. Morphine administered intraplantarly had its maximum effect at 3 hours post-inflammation, likely due to an increase in peripheral opioid receptor density driven by the

inflammatory process itself. After 6 hours, although morphine still had a statistically significant analgesic effect, its intensity was markedly reduced. This suggests that opioid receptors are either not involved or only minimally involved in the hyperalgesia phenomenon.

It is also possible that the pain experienced by animals during hyperalgesia has two components: a peripheral (local) component and a central nervous component, potentially mediated at the spinal cord level via the "gate control" mechanism. If this is the case, morphine would have influenced only the local component, but not the central one. The greater effectiveness of morphine prior to the onset of hyperalgesia may be explained by the exclusive presence of the local pain component during that early period.

The observation that the maximum analgesic effect occurs when morphine is administered intraplantarly after carrageenan injection raised the question of a potential dose–response relationship. To investigate this, a new experiment was carried out using 50 rats, divided into five groups of ten animals each. The rats received intraplantar morphine at progressively increasing doses—2.5, 5, 10, and 20 mg/kg body weight—while the control group received 0.9% saline in a volume of 0.1 ml, administered three hours after carrageenan injection.

Ten minutes after morphine administration, paw withdrawal latency was measured using an infrared beam that generated thermal stimulation at the paw level, with the aid of the Ugo Basile Plantar Test 37370 device. Analysis of the results revealed that morphine at the dose of 2.5 mg/kg did not significantly increase paw withdrawal latency. However, at doses of 5 mg/kg and 10 mg/kg, morphine produced a statistically significant increase in withdrawal latency, with the average values being very similar—4.18 ± 1.08 seconds and 4.28 ± 2.15 seconds, respectively.

In group 5, which received 20 mg/kg of morphine, withdrawal latency increased markedly, reaching an average value of 27.1 ± 4.48 seconds. However, unlike the other groups, these animals exhibited clear signs of central nervous system activity induced by morphine, such as immobility and a characteristic tail position (Straub tail). In this group, paw withdrawal latency frequently exceeded the maximum acceptable time of 30 seconds. The fact that both 5 mg/kg and 10 mg/kg doses resulted in nearly identical increases in withdrawal latency suggests that the maximum number of opioid receptors available in the inflamed paw is already saturated by the 5 mg/kg dose.

The results observed with the 20 mg/kg dose—which clearly triggered central effects—indicate that morphine is capable of systemic absorption even when administered intraplantarly.

However, this absorption is likely minimal, and central nervous system effects only appear when a very high dose is administered.

The fact that the 5 mg/kg and 10 mg/kg doses increased withdrawal latency by approximately 4 seconds, while the 20 mg/kg dose—which additionally induced central effects—increased latency to nearly 30 seconds, supports the hypothesis that the analgesic effect mediated by peripheral opioid receptor stimulation is of lower intensity than that mediated by central opioid receptor stimulation.

To further explore the central effects of morphine administered intraplantarly, a follow-up experiment was performed, in which the analgesic effects of intraplantar morphine were compared to the same doses administered intraperitoneally. Our working hypothesis was based on the premise that morphine absorption is significantly more rapid and more intense via the intraperitoneal route compared to the intraplantar route. Therefore, it was expected that the analgesic effect of morphine would be more pronounced following intraperitoneal administration than after intraplantar administration.

Five groups of ten rats each were used in this experiment. In all animals, inflammation of the right hind paw was induced using carrageenan, and three hours later, the animals received one of the following treatments: intraplantar morphine at 5 mg/kg body weight; 0.9% saline intraplantar; intraperitoneal morphine at 5 mg/kg; 0.9% saline intraperitoneal; or intraperitoneal morphine at 10 mg/kg. In all groups, paw withdrawal latency in response to a thermal stimulus was measured using the Ugo Basile Plantar Test 37370.

Analysis of the results revealed that morphine at a dose of 5 mg/kg administered intraplantarly produced a statistically significant analgesic effect compared to the corresponding control group, while the same dose administered intraperitoneally did not produce a statistically significant analgesic effect.

Since intraperitoneal administration typically results in rapid and efficient absorption, the lack of analgesic effect at 5 mg/kg via this route suggests that the dose was not sufficient to produce central nervous system effects. If the analgesic effect of morphine administered intraplantarly had been mediated through central opioid receptor stimulation, then the same dose administered intraperitoneally—which ensures faster systemic absorption—would have been expected to produce a more intense analgesic effect. However, this was not the case. This supports the

hypothesis that the analgesic effect observed with intraplantar morphine administration is mediated exclusively by stimulation of peripheral opioid receptors. This receptor-specific action is likely highly dependent on the amount of morphine administered locally.

It had previously been shown that intraplantar morphine at 10 mg/kg produced an analgesic effect of similar intensity to the 5 mg/kg dose. However, when the same 10 mg/kg dose was administered intraperitoneally, it produced a much stronger analgesic effect, accompanied by clear signs of central nervous system activity. In fact, the effects of 10 mg/kg intraperitoneal morphine were similar to those observed with 20 mg/kg morphine administered intraplantarly in the previous experiment, which can be attributed to the greater efficiency of systemic absorption via the intraperitoneal route.

Taken together, these findings support the existence of peripheral opioid receptors whose stimulation can produce both analgesic and anti-inflammatory effects. These effects were observed at doses that, when administered intraperitoneally, were ineffective—suggesting a local, receptor-specific action.

It is also likely that the density of these peripheral opioid receptors increases in response to the inflammatory process, since the maximal analgesic effect was observed at three hours after carrageenan injection. Additionally, pain intensity appears to follow a dynamic pattern during inflammation, reaching its peak at three hours and subsequently transitioning into a state of hyperalgesia.

Our results indicate that morphine's analgesic effect is much more pronounced before the onset of hyperalgesia than afterward. This leads us to believe that, in the later phase of inflammation, both normal pain and hyperalgesia coexist. It is very likely that stimulation of peripheral opioid receptors does not significantly influence the hyperalgesic component of pain.

From the data obtained, we conclude that both the 5 mg/kg and 10 mg/kg intraplantar doses of morphine produced analgesic effects of similar intensity. This allows us to hypothesize that the maximum number of peripheral opioid receptors in the inflamed paw corresponds to the receptor occupancy achieved with the 5 mg/kg dose.

High doses of morphine administered either intraplantarly or intraperitoneally—doses that also produced central nervous system effects—resulted in much stronger analgesic effects than lower doses that lacked central activity. Therefore, even when peripheral opioid receptors are fully

activated by local morphine administration, the resulting analgesic effect is of lower intensity compared to the effect produced through stimulation of central opioid receptors.

In other words, there appear to be certain differences between the stimulation of peripheral opioid receptors and that of central nervous system opioid receptors. Stimulation of peripheral opioid receptors produces an analgesic effect of lower intensity compared to the stimulation of central opioid receptors. Additionally, stimulation of peripheral receptors also results in an anti-inflammatory effect—an outcome that has not been described in the literature for centrally mediated opioid receptor activation. Furthermore, stimulation of peripheral receptors seems to be effective only in normal pain, but not in hyperalgesia.

Conclusions

1. Peripheral opioid receptors are present in the carrageenan-inflamed rat paw.
2. These receptors appear to emerge and increase over time as a result of stimuli generated by the inflammatory process itself.
3. The maximum number of peripheral opioid receptors in the inflamed rat paw likely corresponds to the receptor occupancy produced by a 5 mg/kg intraplantar dose of morphine.
4. Under our experimental conditions, this maximal receptor density was reached 3 hours after carrageenan administration.
5. The 3-hour delay suggests that the increase in receptor density is more likely due to enhanced membrane expression rather than new protein synthesis, as the time is too short for the latter.
6. Stimulation of peripheral opioid receptors produces an anti-inflammatory effect.
7. The intraplantar dose of morphine that exhibited an anti-inflammatory effect did not produce the same effect when administered intraperitoneally.
8. It is highly probable that stimulation of central opioid receptors does not result in an anti-inflammatory effect.
9. Stimulation of peripheral opioid receptors produces an analgesic effect.
10. The analgesic effect obtained via peripheral opioid receptor stimulation is less intense than that produced by stimulation of central opioid receptors.

11. Peripheral opioid receptor stimulation appears to produce analgesia only in normal pain conditions, not in states of hyperalgesia.
12. The maximum analgesic effect through peripheral receptor stimulation occurs at 3 hours after inflammation onset.
13. Morphine administered intraplantarly appears to be minimally absorbed from the injection site; however, in our experimental conditions, doses exceeding 10 mg/kg led to detectable central nervous system effects.
14. The data obtained from these experiments do not allow us to draw conclusions regarding the post-receptor mechanisms by which opioid receptor stimulation produces analgesic and anti-inflammatory effects.
15. It is also difficult to make assumptions regarding the extrapolation of these findings to human therapy.
16. On one hand, the analgesic effect via peripheral receptor stimulation is less intense than that produced by central opioid receptor activation; on the other hand, peripheral receptor stimulation is free of central nervous system side effects.

Based on the findings presented above, it can be assumed that local administration of morphine may be useful in the treatment of certain acute inflammatory conditions. It would be of particular interest to investigate whether other opioids also exert analgesic effects through the stimulation of peripheral opioid receptors. Such research may reveal that certain opioids produce peripheral effects at significantly lower doses than those required to induce central nervous system effects.

In our study, the dose of morphine that produced peripheral analgesic effects was only about twice as low as the dose that elicited central effects. It would also be valuable to investigate whether morphine or other opioids can exert peripheral analgesic effects when administered cutaneously. Furthermore, it would be worth exploring whether opioids that do not cross the blood–brain barrier, such as loperamide, can also produce analgesia. A drug with this profile could demonstrate a greater separation between the dose required to produce peripheral analgesia and the dose needed to elicit central effects—an important aspect that would improve its safety profile.

Loperamide may be a promising candidate in this regard, as it is absorbed from the gastrointestinal tract but does not penetrate the blood–brain barrier due to the activity of P-glycoprotein, which actively removes the drug from the cerebrospinal fluid. If loperamide is absorbed enterally, it is also reasonable to expect that it could be absorbed transdermally, given that the skin lacks P-glycoprotein transporters.

We did not investigate loperamide in this study, as our objectives were different: to demonstrate the existence of an analgesic effect mediated by peripheral opioid receptor stimulation; to investigate whether this stimulation also produces an anti-inflammatory effect; to determine the time interval after inflammation at which the analgesic effect is maximal; to evaluate a dose–response relationship; to explore whether a dose of morphine could be identified that elicits peripheral analgesia without producing central nervous system effects; and to assess whether peripheral opioid receptor stimulation is effective across different types of pain.

All these objectives were successfully addressed in the present study. The investigation of the peripheral analgesic effects of loperamide via opioid receptor stimulation should represent a natural continuation of this research.

Personal Contributions

1. This is the first time in the scientific literature that the analgesic effect of morphine is compared to its anti-inflammatory effect. (Chapter 5, Subchapter 5.3; Chapter 6, Subchapters 6.3, 6.4, 6.5)
2. This is the first time in the literature that the intensity of the analgesic effect of locally administered morphine—mediated by peripheral opioid receptor stimulation—is compared with the effect of stimulating central opioid receptors through intraperitoneal administration. (Chapter 8, Subchapters 8.3, 8.4, 8.5)
3. This is the first time in the scientific literature that it is reported that morphine has effects only in normal pain, but not in hyperalgesia. (Chapter 6, Subchapters 6.4, 6.5)
4. This is the first time in the literature that a dose–response relationship for intraplantar morphine administration is reported. (Chapter 7, Subchapters 7.3, 7.4, 7.5)

5. This is the first time in the literature that it is observed that the intensity of the analgesic effect of intraplantarly administered morphine varies over time according to the evolution of inflammation, reaching a peak at 3 hours after inflammation onset. (Chapter 6, Subchapter 6.5)
6. This is the first time in the literature that it is demonstrated that a dose of intraplantar morphine can produce an analgesic effect without inducing central nervous system effects. (Chapter 7, Subchapters 7.3, 7.4, 7.5)
7. This is the first time in the literature that an attempt is made to determine the maximum number of peripheral opioid receptors in the inflamed rat paw. (Chapter 6, Subchapters 6.4, 6.5)

List of Publications Related to the PhD Thesis Topic

1. **Jafal NM**, Stoleru S*, Zugravu A, Graziosi G, Orban C, Popescu M, Vătășescu Balcan A, Fulga I, *Anti-inflammatory effects of locally versus systemically injected morphine on chemically induced inflammation: an experimental animal study*, Farmacia, 2024, 72(5), 1209-1215, FI – 1,4/2024, Q4, (capitolul 5, pagini 66-73)

   https://doi.org/10.31925/farmacia.2024.5.24

   https://farmaciajournal.com/issue-articles/anti-inflammatory-effects-of-locally-versus-systemic-injected-morphine-on-chemically-induced-inflammation-an-experimental-animal-study/

2. **Jafal NM**, Stoleru S*, Zugravu A, Orban C, Popescu M, Marin RC, Fulga IG, *The Analgesic Effect of Morphine on Peripheral Opioid Receptors: An Experimental Research*, The Journal of Critical Care Medicine, 2024, 10(4), 337-344, FI - 0.9/2024, Q4, (capitolul 6, pagini 74-85)

   https://doi.org/10.2478/jccm-2024-0042

   https://jccm.ro/wp-content/uploads/2024/10/jccm-2024-0042.pdf

3. **Jafal NM**, Stoleru S*, Orban C, Fulga IG, *Peripheral Opioid Receptors in the Modulation of Inflammatory Pain: a Narrative Review*, Maedica – a Journal of Clinical Medicine, 2025, 20(1), 72-80, (capitolul 1- pagini 15-17, capitolul 2-pagini 34-37, capitolul 9- pagini 97-111)

   https://doi.org/10.26574/maedica.2025.20.1.72

   https://www.maedica.ro/articles/2025/1/2025_20(23)_No1_pg72-80.pdf