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Experiences and reflections about behavioral pain assays in laboratory animals☆

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ABSTRACT

Pharmacological assays based on the measurement of nociceptive responses in laboratory animals are a fundamental tool to assess analgesic strategies. During our experience with this type of experiments, we have been repeatedly challenged by different concerns related to their interpretation or relevance. Although these subjects are frequently discussed in our lab, they do not usually find a place in research articles with original data, in which the focus on results seems mandatory. In the present manuscript we try to discuss as central issues some of these aspects that often cross transversally our research.

We have gathered them in five topics inspired by the results obtained in our laboratory. The two initial sections are devoted to the influence of the behavioral method used to assess nociception on the results achieved, as well as to the possibility that data may be more easily accepted when obtained with standard methods than with alternative ones. The third topic is related to the difficulties encountered when working with a molecule that may evoke dual effects, acting as pronociceptive or antinociceptive depending on the dose. The fourth point deals with the situation in which a particular hyperalgesic reaction is related to several molecules but the single inhibition of only one of them can completely prevent it. Finally, the last issue is addressed to comment the impact in the progress of pain research of experiments performed in animal models of pathological settings.

1. Introduction

We are engaged in experimental pain research with laboratory animals from more than three decades and, with the essential participation of different collaborators, we have published several tens of original research articles dealing with the pharmacological modulation of nociception. Some of our initial publications only included behavioral experiments but, as our lab was being able to incorporate different techniques, in vivo experiments were progressively accompanied by complementary in vitro assays. Although the majority cannot be considered as first level publications, some of our manuscripts appear in journals of the first quartile group of their respective category and, globally, they have been cited by about two thousand papers. As it would be expected, we have always intended that the results shown in our publications were reliable, offering the most relevant information to facilitate their understanding and we have tried to discuss appropriately the main topics. In spite of that, we are aware that laboratory results are often complex, can be analysed from different points of view and their interpretation can sometimes be nuanced with time. During these years, when discussing about behavioral results obtained in our lab, it has not been infrequent to focus on concerns or doubts related to aspects that are not usually associated to a certain set of experiments but rather to methodological or conceptual dilemmas that pass through our global work. Due to their transversal nature, we feel that several of these topics have not been specifically exposed or discussed with the calm required and this has been the main reason to elaborate the present manuscript. Thus, our primary aim is to share a part of the intramural discussions maintained during these years in our lab in relation to behavioral assays of pain, discussing several particular cases and trying to offer some interpretations. Although we have attempted to maintain the spirit of a scientific publication, the style is perhaps closer to an essay and in no case we mean to establish tenets but, instead, to open subjects for

☆ We would like to dedicate this manuscript to our friend, mentor and colleague, Agustín Hidalgo Balsera. Dr. Hidalgo was founder, at the end of the seventies, of our laboratory at the Universidad de Oviedo, where he was Professor of Pharmacology until, unfortunately, he passed away on August 2022. Agustín has exerted a fundamental influence in our professional and personal trajectories and he will always be present in our memory.

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reflection and discussion. The manuscript considers five different topics separated in the corresponding sections that, in turn, are subdivided in three subsections addressed to initially state the problem itself, next to describe some related experiences from our lab and, finally, to offer some general comments.

2. Topic 1: Results obtained in behavioral nociceptive assays are strongly determined by the test used

Trying to measure nociception is not so simple as to quantify many other more objective body parameters and, as widely experienced by pain researchers, slight changes in the procedure used or in the nociceptive stimulus applied can strongly modify the result obtained. Several physiological reasons, such as the activation of different nociceptive receptor channels or the recruitment of a particular population of peripheral nociceptive neurons can justify these discrepancies but, in any case, these features provoke that an effect on nociception might show dissimilar pharmacological properties when measured by different methods and also that a certain response might be measured by a particular method but not by another one (Ebbinghaus et al., 2012; Mohammadi et al., 2014; DuBreuil et al., 2021). Since the obtaining of contradictory results can considerably complicate data interpretation or its contextualization, we initially refer to some examples coming from our laboratory related to this problem.

2.1. Our experience about topic 1

The first case is related to our work on stress-induced analgesia (SIA) during the late eighties and early nineties. At that moment, we observed that mice showed opioid-induced analgesia after receiving 80 electrical footshocks of particular characteristics. This effect was very reliable when measured by the tail-flick test and we determined that it was dependent on the activation of spinal kappa-opioid receptors (Menendez et al., 1993). Later, we also remarked that, following this stress procedure, if the nociceptive response was measured by using an acetic acid-based writhing test, analgesia was also detected, but it was no longer dependent on endogenous opioids (Menendez et al., 1994). Although we initially deduced that the opioid nature of the response could be related to the application of a thermal stimulus and the non-opioid to a chemical one, we further learned that this was an oversimplification. As schematized in Fig. 1, when using a tail-immersion test in which the stimulus was also thermal but evoked shorter withdrawal latencies than the tail-flick test, analgesia was naloxone-insensitive, despite the thermal nature of the noxious stimulus applied. In consequence, we considered that the opioid component of this stress could perhaps be only detected when measured by radiant heat, but not by other modalities of noxious heat. Further experiments showed more nuances, since the increase in the intensity of the radiant stimulus used in tail-flick test also led to the disappearance of the opioid response and the measurement of naloxone-insensitive analgesia.

At that time, stressful stimuli were usually labeled as able to produce opioid or non-opioid analgesia and it was considered that its opioid or non-opioid nature could be determined by different methodological variables (application of escapable vs inescapable stress or continuous vs intermittent shocks, for example) (Lewis et al., 1980; Maier et al., 1983). Certainly, we were not ready to find that a single stress procedure could produce an analgesic response that could only be classified as opioid when assessed by a particular stimulus at a precise intensity.

As commented above, in some cases the method not only conditions qualitative differences in the responses obtained, but even makes possible or not their detection. For instance, when we were studying the involvement of endothelins in inflammatory nociception in mice by assessing the effect of selective antagonists for endothelin type A (ETA) or type B (ETB) receptors, we observed that the administration of an ETA receptor antagonist relieved hyperalgesia measured by the application of either a thermal or a mechanical nociceptive stimulus. However, the blockade of ETB receptors inhibited inflammatory hyperalgesia only when measured by using a mechanical stimulus but not a thermal one (Menéndez et al., 2003a). It might be initially assumed that this fact could derive from a different involvement of ETA and ETB receptors in the processing of thermal and mechanical nociceptive stimuli but, considering the influence of methodological variables, we would like to remark that the attribution of analgesic properties to these drugs would have been different if only one of these methods were used. Thus, ETA receptor antagonist would have been considered as antihyperalgesic in both cases, whereas the consideration of the ETB antagonist as analgesic would be completely dependent on the test chosen.

We faced a similar situation when characterizing the modulation of murine experimental bone cancer pain by peripheral opioid receptor stimulation. In these experiments, the stimulation of peripheral opioid receptors counteracted thermal hyperalgesia measured in mice intratibially inoculated with NCTC 2472 tumoral cells (Menéndez et al., 2003b) but results were more complex when using mechanical stimuli. As occurred with thermal hyperalgesia, mechanical hyperalgesia was also prevented by the stimulation of peripheral opioid receptors (Menéndez et al., 2005) but the nocifensive allodynic response evoked by an innocuous von Frey filament was completely unaffected by this pharmacological strategy (Baamonde et al., 2006), as depicted in Fig. 4a. Thus, although the different results obtained may be a consequence of the different mechanisms involved in hyperalgesia and allodynia, the fact that not all the tests used yield positive results can raise doubts relative to the possible interest of this pharmacological strategy, as further commented in topic 5.

2.2. Some comments about topic 1

Multiple reports illustrate the idea that an analgesic or hyperalgesic response can be detected by using particular nociceptive stimuli

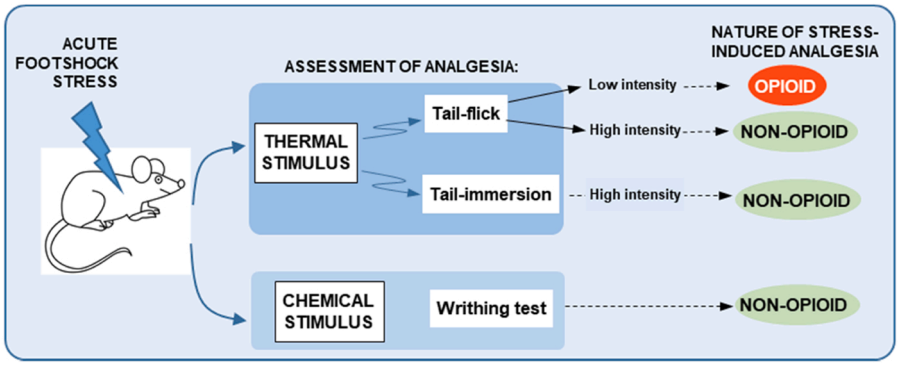

Fig. 1. Schematic representation of the different results obtained after the application of the same footshock stress protocol to mice depending on the method used to assess analgesia (Data extracted from Menéndez et al., 1993; Menendez et al., 1994).

whereas it might not be measured when different noxious stimuli are applied. For instance, the administration of the IL-1R1 antagonist anakinra was able to markedly reduce thermal hyperalgesia in a model of murine antigen-induced arthritis but did not modify the severity of mechanical hyperalgesia evoked in the same situation (Ebbinghaus et al., 2012). Some experimental approaches focused at molecular level also lead to rather similar conclusions. Thus, a significant reduction of mechanical hyperalgesia in response to inflammation and nerve injury appears after the selective deletion of α9-nAChR in mice, while the development of cold and mechanical allodynia remained unaffected (Mohammadi et al., 2014). Besides, it has been recently demonstrated that peripheral Cav2.2 channels are essential for heat hypersensitivity following capsaicin exposure, but they are not involved in mechanical hypersensitivity induced by this TRPV1 agonist (DuBreuil et al., 2021). The interpretation of such heterogeneous data is, undoubtedly, complicated and highlights the enormous intricacy of nociceptive transmission. Probably, these results showing the involvement of different mechanisms in each process offers valuable information useful to understand the complex puzzle of pain modulation. However, in the context of a basic laboratory of pharmacology trying to assess the possible relevance of experimental analgesic strategies, this remarkable variability related to the algesimeter behavioral method used constitutes a considerable complication for data interpretation.

It is usually accepted that, when the efficacy of an analgesic treatment is observed in a method and a positive result is further obtained in a second test, the assayed strategy is solid and interesting. In contrast, a non-confirmative result could suggest that the treatment is probably not promising. Although common sense could initially support this rationale, it might be partially fallacious, since the obtaining of positive results with the second method can be more or less casual, depending on the ability of the strategy studied to specifically inhibit particular modalities of nociception and not others. In fact, we do not generally use tens of tests but, at most, three or four different assays and, considering the chance of obtaining variable responses, it is possible that the introduction of a third or fourth method could probably offer a different pattern. For this reason, to consider that a pharmacological treatment could be potentially less interesting if one of the tests used does not give the expected result is not so obvious. It seems likely that universal, potent and effective analgesics, as opiate drugs, will accomplish the requirement that a second measurement should confirm the result obtained in the first one, since these drugs are efficacious in almost all tests. However, many of the above mentioned results in which some assays offered negative results, do not necessarily mean that the respective strategies are useless, but probably suggest that they could be useful in particular situations but not others. The effects induced by aspirin are remarkable when assessed in inflammatory pain, but the destiny of this molecule could have been not so brilliant if tested in a hot plate or other standard thermal behavioral test, considering that it could produce no effect even at doses as high as 100 mg/kg (Eschalier et al., 1983). In spite of this, its efficacy in many clinical settings is indisputable.

Finally, a possible undesirable consequence of this method-dependency could be that, in order to construct solid, linear, messages, the lack of effect in a test could act as an invitation to only describe positive results. It seems clear that this strategy would help to avoid contradictory results, but it also could lead to the elaboration of unrealistic or simplified messages. In our opinion, the inability to adequately understand results obtained with methods offering opposite results cannot justify such a bias and results should be communicated, even if they could considerably complicate the panorama.

3. Topic 2: Could some algesimetric methods be more accurate than others?

As we have just described, results obtained with different methods, or even when using different stimulus intensities in the same method, can be considerably dissimilar. In addition, it is well known that there are particular methods for nociceptive testing more established and more frequently used than others. However, it is not clear if we have consistent reasons to consider results obtained with usual methods as more interesting, reliable or predictive that those related to alternative ones. Effectively, it should be accepted that more standardized methods can make easier comparisons among results obtained in different laboratories. However, if some methods can be more useful to detect a particular nociceptive response, perhaps undetected by others, maybe we should be prone to accept the use of different or modified algesimetric assays.

3.1. Our experience about topic 2

About 20 years ago, we started to work with a modified, non-standard, variation of the classical hot plate test method, that we named unilateral hot plate test (Menéndez et al., 2002). We adopted it when we started to perform experiments focused on the pharmacology of neoplastic pain. Our first approach was based on the intraplantar (i.pl.) inoculation of XC Rous sarcoma-virus-transformed rat fibroblasts cells (XC), a procedure that showed the advantage of evoking tumoral growth in CD-1 mice (Wlodarski et al., 1987), the standard strain of laboratory mice. The inoculation of these cells evoked a relatively rapid tumefaction in the injected paws, but they did not generate a true tumoral process and we abandoned soon this strategy. Although we were initially interested in measuring if the tumefaction in response to XC cells was accompanied by enhanced nociceptive responses, measurements performed with standard tests such as the classical hot plate or the Hargreaves test did not reveal differences between inoculated and not inoculated mice. Thus, we decided to measure withdrawal latencies in the hot plate by restraining mice and separately maintaining the plantar surface of each individual hind paw in contact with the plate. After having worked with other behavioral procedures, such as the tail immersion test, we were trained to restrain mice and we observed that the unilateral hot plate allowed the detection of remarkable thermal hyperalgesia in paws injected with tumoral XC cells (Baamonde et al., 2004a). However, since this behavioral protocol had not been previously reported, we tried to standardize the technique and to describe its main characteristics in a methodological manuscript, in which we depicted that this procedure was easy, useful and sensitive (Menéndez et al., 2002).

Once we handled accurately this method, we further studied the peripheral local effect of opiates in a model of tumoral hyperalgesia based on the inoculation of NCTC 2472 cells and described the anti-hyperalgesic effect of opiates acting on peripheral receptors (Menéndez et al., 2003b). However, when submitting these results for publication, we found certain resistance to the acceptation of unilateral hot plate assays because it was not a usual method. In fact, we needed to give a great deal of arguments explaining that our results could be valid. Sometime later, we assessed mechanical hyperalgesia in tumor-bearing mice by using a mouse paw pressure test (Menéndez et al., 2005) that drew attention by the fact that this test had been designed for rats but not mice. A common criticism on both methods, unilateral hot plate and paw pressure tests, was the possibility that mice restraining could activate analgesic responses due to stress. This putative bias related to unilateral hot plate method has also been reflected in a more recent review (Deuis et al., 2017). However, although this risk seems completely feasible, we never detected any remarkable effect in the experimental group treated with solvent. Actually, similar restraint procedures are applied when using for example the tail-immersion test in mice or the paw-pressure tests in rats, two standard methods with which many experiments have been performed for a long time (Deuis et al., 2017).

Another example related to the use of an atypical method was also included in our first publication describing tumoral nociception evoked by intratibial NCTC 2472 cells in mice (Menéndez et al., 2003b). The pioneer team working with intrafemoral administration of NCTC 2472

cells described that the quantification of spontaneous flinches could be used to measure ongoing pain as an indication of spontaneous nociception (Luger et al., 2001). Since it seemed a reasonable method, probably rather related to pain symptoms in patients, we tried to use it as well. However, we were unable to observe this flinching behavior in our mice inoculated with the same tumoral cells. It is not easy to know what was the reason, although it could perhaps be related to the fact that NCTC 2472 tumoral cells were inoculated into the tibia, instead of the femur. Once more, the attempt to use a method previously described was not possible for us and we were obliged to use a different strategy, that in this case was based on the formalin test scores. Formalin test is based in the assessment of nocifensive behaviors observed in a hind paw after receiving the administration of the intraplantar injection of formalin, and the application of these criteria allowed to us to quantify the difficulty in weight bearing of the affected limb suffered by mice during tumor development.

3.2. Some comments about topic 2

We think that alternative nociceptive testing methods are sometimes not easily admitted by the scientific community, even if slight methodological variations can be useful to obtain consistent information. As explained above, our initial motivation to introduce variations on the hot plate test was not an arbitrary decision, but the consequence of searching a method that offered the possibility of studying a particular experimental situation that was not detected by other methods. During the following years, we observed that results obtained with the unilateral hot plate test by trained researchers showed slight variability and were consistent and reliable over time, although the process of training a person to work with this method was sometimes long and hard, taking several weeks or even months. Thus, new methods can be welcomed if they meet needs and if their consistency is systematically demonstrated.

Although our studies have been more frequently related to evoked nociceptive responses, it seems possible that the introduction of innovative measures of spontaneous pain-related behaviors such as dynamic weight bearing or conditioned place preference might be interesting alternatives for the future. These methods offer the theoretical advantages of being performed in freely moving animals and addressing behavioral parameters perhaps more related to pain suffered in clinical settings. For these reasons, it could be claimed that they could offer more translational results than older methods although this consideration remains to be proven. In our opinion, the generalized use of these new algesimeter methods is happening at a slow rate. We cannot know if it could be due to the mentioned tendency of working with more traditional methods and it will be interesting to observe whether this trend progressively changes for the next years. At present, some publications that compare results obtained by dynamic weight bearing with more standard approaches confirm the mentioned divergences showing that data obtained with this method are relatively similar to those achieved when measuring thermal hyperalgesia (Sheehan et al., 2021) but not mechanical allodynia (Sheehan et al., 2021; Lu et al., 2022).

4. Topic 3: What to think when the same molecule can act as hyperalgesic or analgesic?

The initial analysis related to the nociceptive responses evoked by molecules whose properties are not well characterized usually aims to classify them as hyperalgesic, analgesic or inactive. Certainly, this classification can be easily adopted for molecules that show a clear analgesic or hyperalgesic profile as opioids or prostaglandins, respectively. However, things can become more complex when working with molecules that can show a dual nature, a characteristic that hinders their easy labeling. A paradigmatic case may be nociceptin, that received this name due to its pronociceptive properties (Reinscheid et al., 1995) although further studies demonstrated its ability to evoke analgesia in some circumstances (Xu et al., 1996). Really, this is not a so exceptional situation since several other molecules, such as capsaicin, dynorphin, nitric oxide or endothelin have been claimed to be hyperalgesic or analgesic depending on the testing condition. However, when these type of opposite results are found in experiments with a mediator scarcely characterized, the interpretation about its relevance becomes complicated.

4.1. Our experience about topic 3

A part of our research activity has dealt with the participation of chemokines in pain, especially in neoplastic and inflammatory settings and, since this was not a widely developed field of research, we also needed to characterize the effects of some members of this family in healthy mice. Initially, we were able to measure the hyperalgesic effects of some chemokines as chemokine (C-C motif) ligand 2 (CCL2) (Baamonde et al., 2011) or (C-C motif) ligand 5 CCL5 (Pevida et al., 2014) whose hypernociceptive role had been previously established. However, things became more difficult when focusing on the chemokine (C-C motif) ligand 5 (CCL5) or ligand 4 (CCL4). Thus, i.pl administration of 30 ng of CCL5 produced thermal hyperalgesia due to prostaglandin release and the subsequent sensitization of TRP channels. However, a slight increase in the dose of CCL5 up to 100 ng was able to evoke the release of dynorphin A from neutrophils and the subsequent stimulation of kappa-opioid receptors completely neutralized the hyperalgesic action of this molecule (Fig. 2a). In fact, this was the first description that a chemokine with C-C structure was able to trigger analgesic mechanisms (González-Rodríguez et al., 2017).

As summarized in Fig. 2b, the complexity was greater for CCL4. Thus, although its hypernociceptive properties had been reported (Saika et al., 2012), we observed that minimal i.pl. doses, in the pg order, evoked analgesia and that, in spite of its local administration, we had the impression that this effect was bilateral instead of unilateral. Our initial feeling was that it could be an artifact, because the detection of analgesia

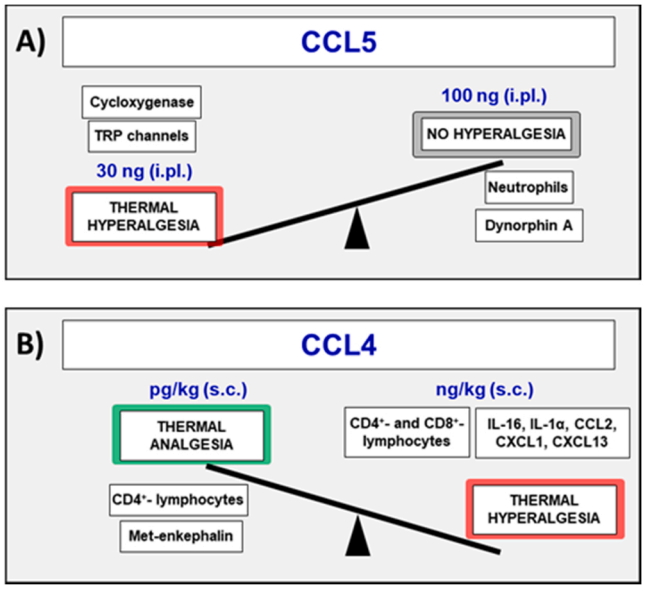

Fig. 2. Schematic representation of the dual effects induced after the administration of either i.pl. CCL5 or s.c. CCL4 in mice. a) The local injection of 30 ng of CCL5 evoked thermal hyperalgesia due to cyclooxygenase activation and TRP channels opening, whereas a x3 increase of this dose led to the neutralization of this effect following the chemoattraction of neutrophils able to release dynorphin. b) The systemic administration of CCL4 in the order of pg/kg produced thermal analgesia through the release of met-enk. In contrast, doses up to 1000 times higher lead to hyperalgesia by promoting increased levels of several interleukins and chemokines. (Data extracted from González-Rodríguez et al., 2017; García-Domínguez et al., 2019a; Aguirre et al., 2020).

in the non-injected, contralateral, paw seemed meaningless. However, we further observed that this bilateral response could also be evoked when these ultra-small doses (pg/kg) were administered under the fur of the neck. Finally, conscious of being in the antipodes of the current literature related to CCL4, we tried to explore with caution this topic and we were able to find some mechanisms involved in this analgesic response that, once again, could be consistently measured with the unilateral hot plate test and by the paw-pressure test, but undetected by other methods, including the conventional unrestrained hot plate assay (unpublished data). After a first manuscript describing this response (García-Domínguez et al., 2019a), we aimed to explore whether CCL4-induced analgesia could be detected in other tests by administering higher doses. We used the tail-flick, the tail immersion test and the licking behavior evoked by capsaicin, and we observed no analgesia but, if any, some tendency to hyperalgesia (unpublished data). At that moment of difficulties in data interpretation, results obtained in another experiment considerably complicated the situation. Trying to observe if a maintained analgesic effect could be evoked by CCL4, we designed a plasmid containing the sequence of the CCL4 gene and we administered it by using the hydrodynamic gene delivery technique (Liu et al., 1999), in order to induce the sustained CCL4 expression in mice. The procedure worked fine and, after the administration of the plasmid, we could detect the increased presence of endogenous CCL4 but, instead, of analgesia, we observed a maintained hyperalgesic reaction. Only some days later, we detected that this hyperalgesia switched to an analgesic response that lasted for 3–4 days, before recovering normal basal withdrawal latencies, when CCL4 endogenous production ended (Aguirre et al., 2020). Since these data pointed the possibility that CCL4 could evoke a hyperalgesic response at high concentrations and an analgesic one at lower ones, we focused on the detection of the possible hyperalgesic effect after its exogenous administration by exploring the effect evoked by doses much higher than the analgesic ones and we found it, but not increasing doses in a usual 3x or 10x factor related to the analgesic ones but in a completely unexpected 1000–3000 factor (from 30 pg/kg up to 100 ng/kg) (Aguirre et al., 2020). Really, we had never observed such an enormous difference between two active doses of a drug.

4.2. Some comments about topic 3

In our lab, we have worked with several drugs that induced effects different to those initially expected. Thus, in accordance with previous reports, we have detected analgesic responses evoked by nociceptin (Menéndez et al., 2003c) or nitric oxide (Menéndez et al., 2007), hypernociceptive reactions triggered by morphine (Alvarez-Vega et al., 1998) and even a hypoalgesic reaction evoked by spinal NMDA (Alvarez-Vega et al., 2000). However, besides these apparently conflicting results, all these molecules have been widely characterized and there is no doubt that morphine is a very valuable analgesic or that spinal NMDA activation leads to hyperalgesia. In contrast, the situation is not so easy when studying a molecule as CCL4, that begins to be tested almost without precedent studies. Besides, although in the case of CCL4 and CCL5, dual effects were obtained in our own lab, data related to another chemokine, CCL1, showed a contradiction among our results and those previously published by other laboratories. Although CCL1 is generally considered an hyperalgesic molecule (Akimoto et al., 2013), we have reported that this chemokine can also evoke analgesic responses, either after systemic or spinal administration (García-Domínguez et al., 2019b, 2021). Even if results are more reassuring when they support or complement those offered by previous publications, we think that the finding of data that contradict the more general view may simply be the consequence of the role played by some molecules in different mechanisms involved in nociceptive modulation. In our opinion, the obtaining of two apparently opposed results must not be considered as an unlucky contradiction that should imply to take a definite position related to the action of the molecule. On the contrary, the acceptance of both possibilities and their further detailed characterization might help to shed some light when these type of conflicting results appear.

A historical example of such a dilemma may be the case of the ORL-1 agonist nociceptin. This atypical endogenous opioid peptide was described in 1995, with a considerable delay compared to other endogenous opioids as enkephalins or endorphins, already identified in the seventies. In the former publication of 1995, it was described that mice became hyperreactive to nociceptive stimulation after its intracerebroventricular administration (Meunier et al., 1995) and this property was confirmed in another pioneer study published only one month later (Reinscheid et al., 1995). In fact, based on these data, it seemed appropriate to adopt the name of nociceptin for this new molecule, able to amplify nociceptive transmission. However, shortly afterwards, some reports described that this peptide could also evoke analgesic properties. Thus, a relevant manuscript published by the team of Dr. Pasternak, showed that orphanin FQ (nociceptin) elicited, apart from a rapid hyperalgesic response, a more delayed analgesia (Rossi et al., 1996). Hence, at these initial stages, the design of experimental strategies addressed to explore the putative usefulness of nociceptin in the modulation of pain was surely complicated, since it was not possible to anticipate whether the more practical approach to evoke analgesia could be to prevent its hyperalgesic effects or to potentiate its analgesic mechanisms. Two decades later, cebranopadol, an ORL-1 receptor agonist able to reproduce the analgesic properties of nociceptin also showing a certain affinity for MOR is being tested as a putative new type of analgesic drug in humans (Ziemichod et al., 2022). Thus, the current perspective obtained after all these years of research on nociceptin pharmacology was completely unexpected when its initial hyperalgesic effects were communicated in 1995. Coming back to the particular case of our studies with CCL4, subsequent experiments drove us to deepen in its hyperalgesic properties and oriented our experiments towards IL-16, a new putatively interesting molecule that is responsible for the hyperalgesic effect of CCL4 (González-Rodríguez et al., 2022) that has been poorly studied so far in the field of pain. However, we must recognize that we remain subject to the doubt whether we were following the right clue.

5. Topic 4: Why a part can sometimes become the whole?

An essential field of pharmacological studies on nociception is devoted to explore the mechanisms by which a molecule or a pathological situation can lead to hyperalgesic responses. Due to the complexity of pathological processes associated to hyperalgesia, generally involving a great number of mediators, it is often difficult to determine the role played by each one of them. Also, in many experiments performed after the exogenous administration of a hyperalgesic mediator, it is frequent that the administered molecule leads to the release of several other mediators probably contributing to its final effect. This situation often occurs after the administration of cytokines, that can promote the synthesis of other molecules, as prostaglandins, further acting on nociceptors leading to hyperalgesia. Thus, considering the usual involvement of several molecules in the instauration of hypernociceptive states, the determination of the role played by each one of them in behavioral experiments can become a laborious and subtle task. We would like to consider the fact that, when several mediators participate in a hyperalgesic process, the selective inhibition of only one of them does not generally lead to a partial prevention of hypernociception but to its complete suppression, as if this factor were the only variable involved (Fig. 3). Although this situation is, in our opinion, more common than expected, it raises the question about how a part can act as the whole.

5.1. Our experience about topic 4

During these years, we have had the opportunity of testing molecules able to prevent the action of particular endogenous hyperalgesic

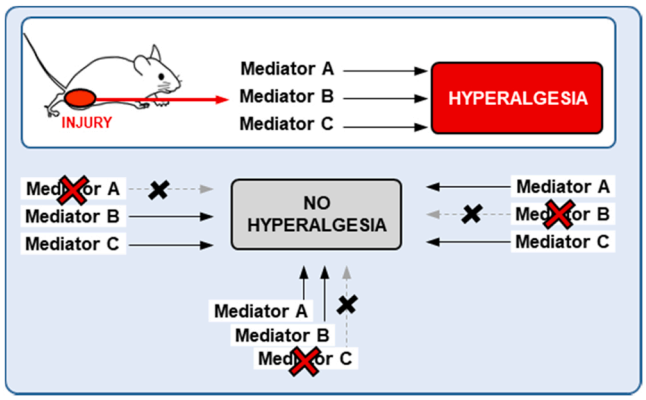

Fig. 3. Schematic representation of the experimental situation in which several mediators participate in a hyperalgesic response but the selective antagonism of only one of them prevents it.

mediators involved in the amplification of nociception in different settings of inflammatory or neoplastic pain. As previously commented, it would be rather expected that the selective inhibition of just one of these molecules would only relieve hyperalgesic responses partially, but very often it is not the case. For instance, we have described in different publications the complete suppression of thermal inflammatory hyperalgesia measured by the same method after preventing the effect of CCL2 on CCR2 (Llorián-Salvador et al., 2016a), CCL3 on CCR1 (Llorián-Salvador et al., 2016b) or endothelin-1 on ETA or in ETB receptors (Baamonde et al., 2004b) or by impeding TRPV1 activation (Baamonde et al., 2005). Related to NCTC 2472 fibrosarcoma cells, we have observed the total inhibition of tumoral hyperalgesia by blocking the activation of P2×3 receptors (González-Rodríguez et al., 2009) or α2δ-1 units of calcium channels (Menéndez et al., 2008) or by antagonizing CCR2 (target of CCL2) (Pevida et al., 2012), CCR1 (target of CCL5) (Pevida et al., 2014) or IL-1R receptors (target of IL-1) (Baamonde et al., 2007). Whereas these effects are coherent with the well-established hyperalgesic action of all the mediators mentioned, why the inhibition of only one of them leads to a complete disappearance of the hyperalgesia remains, in our opinion, poorly understood.

All the referred results come from data obtained in different studies performed in the same models, but a similar situation has also been observed in our lab when characterizing the hyperalgesia evoked by a particular exogenous mediator in the same set of experiments. For instance, trying to characterize thermal hyperalgesia triggered by CCL5, we observed that the administration of a COX-1 inhibitor completely prevented it and that a similar effect can be attained by selectively acting on COX-2, or by blocking either TRPV1 or TRPA1 channels (González-Rodríguez et al., 2017). In the same way, the hyperalgesic effect produced by the administration of CCL4 in mice is related to increased levels of different hyperalgesic molecules released from lymphocytes, such as IL-16, CCL2, IL-1α, CXCL1 or CXCL13 and again, the exclusive neutralization of a single mediator did not lead to a partial inhibition of the hyperalgesic effect but to its complete suppression (Aguirre et al., 2020).

5.2. Some comments about topic 4

Although this is a topic often discussed in our lab, we cannot offer any rational explanation. We think that, although not usually commented, similar findings are obtained in the majority of labs, as frequently reflected in publications. For example, it has been described that the administration of the natural flavone vitexin to mice with postoperative pain induces antinociceptive effects mediated by GABAergic and opioid mechanisms, being this effect completely reverted by the selective blockade of either $GABA_A$ or opioid receptors (Zhu et al., 2016). In the same way, neuropathic hypernociception evoked by paclitaxel in mice is related to both $TRPV_1$ and $TRPV_4$ channel activation and the selective blockade of each one of them did not evoke a partial blockade but the complete disappearance of this hyperalgesic response (Chen et al., 2011).

Whereas it could be expected that drugs able to hyperpolarize nociceptors or spinal nociceptive cells, as opioids or cannabinoids, lead to the suppression of a hyperalgesic response triggered by several molecules, its complete inhibition after the selective inhibition of only one particular excitatory mediator does not seem easily understandable. For example, when COX inhibitors are used to counteract inflammatory hyperalgesia it is frequent to achieve a complete inhibition as if prostaglandins were the unique mediators responsible for hyperalgesia and no other hypernociceptive molecules would participate. We consider that the most evident way to understand that a drug able to selectively prevent the effect of a molecule (or a family of molecules in the case of prostaglandins) can avoid a hyperalgesic response mediated by different mediators is a setting in which the target acts as the final effector of a pathway where the action of the others converge. Following with the NSAID example, their effect could be interpreted considering prostaglandins as the ending point for several hyperalgesic cascades initiated by different cytokines or chemokines during inflammation. However, assuming this hypothetical model, it could not be explained why, apart from prostaglandin synthesis inhibition, the selective antagonism of several of these cytokines can also lead to a total antihyperalgesic effect, as often occurs in laboratory assays.

Trying to find an alternative interpretation for the total suppression of the hyperalgesia obtained after the selective inhibition of one of the several signals involved, it could be proposed that neural sensitization that underlies hyperalgesia could require the combined participation of all these mediators in a synergic manner. This hypothetic interaction should occur in a multiplicative, non-additive, way so that the sole inhibition of one of these components would lead to the total suppression of the effect. Under this consideration, hyperalgesia could be looked as a sensitized state in which the selective inhibition of only one of these pathways could be enough to completely revert the process. Thinking about possible parallelisms with other systems in which different mediators collaborate to evoke a common response by acting in a synergic way, a classical example could be the regulation of acid secretion in the parietal cells of the stomach. This process is controlled basically by histamine, acetylcholine and gastrin that act on different receptors to stimulate acid secretion. However, in this system, the sole blockade of histamine type 2 receptors produces a marked inhibition of hydrochloric acid production not only due to the blockade of histaminergic secretion, but to an additional reduction of the secretion evoked by the two other systems (Bertaccini and Coruzzi, 1989). The possibility that a similar synergic phenomenon could occur on nociceptive sensitization might help to explain why the inhibition of a unique mediator can lead to the total suppression of hyperalgesia. Although we recognize that this is a speculative view without a true experimental demonstration, we mainly would like to raise a theoretical concern related to a rather common experimental finding that, in our opinion, has not merited great discussion so far.

Altogether, the results commented in this section show that, in laboratory assays, it is not unusual to observe that a hyperalgesic response is completely prevented by the sole administration of an inhibitor of the synthesis of a mediator or an antagonist of its receptors. However, this result should not be interpreted as a demonstration of the exclusive involvement in this process of the studied mediator but, instead, as an evidence of its participation, perhaps in cooperation with other mediators.

6. Topic 5: Difficulties in the interpretation of results obtained in pathological models of pain

Experiments performed in laboratory models designed to reproduce clinical situations offer a very attractive scenario for basic research. The

expectancy of obtaining results that anticipate the efficacy of drugs on pathological settings has served as an important stimulus to develop different models of, for example, inflammatory, neuropathic or neoplastic pain. However, a constant concern when designing experiments using these protocols of pathological pain, is whether they are actually predictive, that is, if they do properly mimic the clinical setting. Obviously, by no means we question the relevance of these approaches but, in fact, there are so many results obtained in these different models being the information gathered often discrepant, that it can be difficult to predict whether a particular treatment could be really useful. We would like to offer some reflections about this aspect related to experiments performed in our lab.

6.1. Our experience about topic 5

We have worked for a long time in the assessment of antinociceptive effects induced by drugs in inflammatory (Baamonde et al., 2005, 2007; Llorián-Salvador et al., 2016a,b) and neuropathic (Folgueras et al., 2009; Pevida et al., 2013) models of pain, although our focus was mainly addressed towards murine bone cancer pain (Menéndez et al., 2003b, 2005, 2007; Baamonde et al., 2006, 2007; Curto-Reyes et al., 2008; González-Rodríguez et al., 2009; Pevida et al., 2012, 2014). In the model most frequently used by us, based on the intratibial inoculation of fibrosarcoma derived NCTC 2472 cells we were able to measure thermal and mechanical hyperalgesia as well as allodynia in mice 4 weeks after inoculation (Menéndez et al., 2003b). In contrast, earlier, 2 weeks after cell inoculation, thermal withdrawal latencies assessed by the unilateral hot plate were increased, being this transient thermal hypoalgesia due to the action of local beta-endorphin (Baamonde et al., 2006).

The detection of endogenous analgesia during tumoral development seemed us particularly interesting because the absence of pain at initial stages of many cancer injuries can contribute to a delay in diagnosis. Furthermore, an early phase of endogenous analgesia had not been often described in other reports dealing with experimental nociceptive responses when tumoral cells were affecting bone and a window of pain-free period due to the release of endogenous opioids was only shown in a pancreatic pain cancer model almost at the same time (Sevcik et al., 2006). However, the most conflicting result obtained was that this initial analgesic effect was only detected when applying a thermal stimulus, since it coexisted with remarkable mechanical allodynia (Fig. 4a). This dual response was explained considering that, as commented, the stimulation of peripheral opioid receptors was able to counteract thermal hyperalgesia (Menéndez et al., 2003b) but not allodynia (Baamonde et al., 2006) in this model. In any case, our dilemma at that moment was to decide whether the study of these analgesic mechanisms could be interesting or a rather irrelevant laboratory finding considering its coexistence with mechanical allodynia.

In relation to the hypernociceptive response obtained at week 4 (Fig. 4a, bottom), already commented in topic 1, we addressed our initial efforts towards the characterization of the effect of potential drugs with analgesic properties and we assessed the effect of opioids acting at peripheral, local, receptors. As occurred in the study of the hypoalgesic phase, the results obtained were also complex. In this case, our main conflict was related to the fact that this strategy was effective to inhibit tumoral hyperalgesia but completely ineffective to suppress allodynia.

Trying to shed some light on the complex panorama related to both the early hypoalgesia and the late hyperalgesia, we considered that working with a single tumoral model could be a limitation and reasoned that, if we were able to assess our hypotheses in at least two different bone cancer settings, our results could be more easily generalized. With this aim in mind, we developed another bone cancer model based on the intratibial injection of melanoma B16-F10 cells (Curto-Reyes et al., 2008). However, the balance of introducing a new bone tumor to complement our studies did not clarify very much our concerns. Thus, related to the detection of initial hypoalgesia, we simply found that it was absent in the new model, showing an important difference that impeded to deepen into its characterization. In relation to tumoral hyperalgesia, results obtained with peripheral opiates in the second model were rather similar to those obtained in the first one. Although this reassured the consistency of our data, it also reinforced our doubts related to the possible interest of a strategy able to avoid the tumoral hyperalgesia but ineffective against allodynia (Fig. 4b).

In further experiments, we obtained also more discrepant results between both models. For instance, when testing CCR2 antagonists, we observed that their blockade was effective to relieve hyperalgesia and allodynia evoked by the intratibial inoculation of NCTC 2472 cells but not B16-F10 cells (Pevida et al., 2012). Whereas these differences are understandable taking into account the histopathological differences between tumors, this was also a practical demonstration that, although both experimental settings try to reproduce bone cancer pain, the conclusion we can obtain from them are completely different. Whether one of these models could be more helpful to make an initial assessment of the putative usefulness of different analgesic strategies in this setting is a question that remains to be answered.

6.2. Some comments about topic 5

The difficulties in reaching an appropriate translation from basic science data to useful and commercially available analgesic therapies has been previously analyzed (Abboud et al., 2021) and, from a more subjective point of view related to researchers, it has even argued that

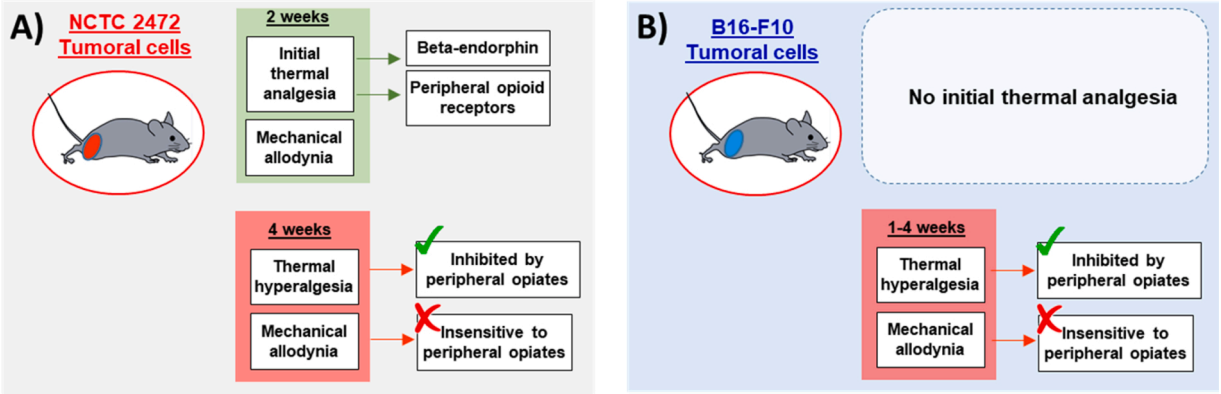

Fig. 4. a) Schematic representation of the detection of thermal analgesia accompanied by mechanical allodynia 2 weeks after the intratibial inoculation of fibrosarcoma NCTC 2472 cells to C3H/He mice, followed by thermal hyperalgesia and mechanical allodynia at week 4. b) In contrast, the intratibial inoculation of melanoma B16-F10 to C57BL/6 mice evoked early thermal hyperalgesia and mechanical allodynia not preceded by a period of thermal analgesia (Data extracted from Menéndez et al., 2003b; Curto-Reyes et al., 2008).

scientific community feels an increasing frustration related to the limited success in translating basic scientific data into new, effective and safe clinical analgesics (Mogil, 2009).

Obviously, the effort of working in experimental pathological models should not be underestimated since its contribution to generate a great body of knowledge in this field is unquestionable. However, the diverse pathophysiology of each painful experimental situation (type of inflammation, neuropathy or tumor), the different mediators involved in each setting, or the heterogeneous methods used to evaluate their consequences on nociception make difficult to elucidate if an experimental treatment assayed in a particular model might really be useful to define new strategies to counteract clinical pain. Thus, although the predictability of laboratory models is the clue for their validity, we must be conscious that to dispose of experimental settings that exactly match with human pathologies is a very difficult task and, in fact, there are several examples showing that strategies able to alleviate nociception in animal models can be completely ineffective in clinics. One of the most notorious predictive failures in the field was the unexpected lack of NK1 receptor antagonists to produce analgesia in patients. The promising effects of these drugs in laboratory animals gave support to the idea that they could become a new class of effective analgesics but, in spite of the large investments of the pharmaceutical industry, clinical studies showed that these drugs were ineffective in humans (Hill, 2000). Apart from the economic consequences for the pharmaceutical companies involved, this negative outcome contributed to generate a certain loss of confidence on the usefulness of basic pain research to find new pain killers. However, this type of discouraging situations can also be an opportunity to remember the indisputable fact that the accumulation of positive evidence obtained from experiments with animals is not enough to guarantee positive results in humans. Also, it should be recognized that not all animal assays gave insights into the wrong direction since particular reports also described that this strategy did not evoke significant antinociception in rats (Garces et al., 1993), as finally occurred in humans.

On the other hand, conflicting situations as the just described emphasize in the obvious assertion that only prospective experiments in humans can give a valuable estimate related to the translational potential of experimental analgesic strategies. From our point of view, a more fluid feed-back between laboratory experiments and the initial assessment of their efficacy in humans could be the theoretical desirable frame to obtain more solid and useful information, avoiding the generation of false expectations as the one described and also preventing the risk that laboratory animal assays might generate lots of dispersed, inconsistent and, most probably, forgotten results. Ourselves could serve as a modest example of this last case, since we have worked for many years trying to assess the effect of tens of drugs on neoplastic pain in mice and, although we have generated some knowledge, it is very unlikely that it will contribute to solve the problem focused. It might be expected that the availability of at least a glance of clinical information related to some of these topics could have helped to more precisely decide which of the targets, if any, should merit preferential attention in the design of further experiments. It seems clear that the difficulty in obtaining early clinical data able to guide experimental research makes difficult this ideal feedback between basic and clinical information. In any case, as it has been recently proposed (Mogil, 2022), the possible development in the next few years of more sophisticated or alternative methods or the possible use of biomarkers of pain in clinics could perhaps help to fulfill this gap between basic and clinical studies improving the predictability of new analgesic strategies.

Overall, we describe here some concerns or, more properly, doubts acquired during our work related to nociceptive behavioral assays in laboratory animals focussing on difficulties of interpretation or contextualization of the experiments. Behavioral results rarely follow a straightforward pathway where all different data fit perfectly and, although our analysis can be influenced by the temporal or conceptual frame, this set of doubts depict a part of the multiple limitations associated to the assessment of nociception in experimental animals.

Declarations of interest

None.

Data availability

This manuscript discusses about data previously published in the referenced cites.

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References

Abboud, C., Duveau, A., Bouali-Benazzouz, R., Massé, K., Mattar, J., Brochoire, L., Fossat, P., Boué-Grabot, E., Hleihel, W., Landry, M., 2021. Animal models of pain: diversity and benefits. J. Neurosci. Methods 348, 108997. https://doi.org/10.1016/j.jneumeth.2020.

Aguirre, A., González-Rodríguez, S., García-Domínguez, M., Lastra, A., Gutiérrez-Fernández, A., Hidalgo, A.,1, Menéndez, L., Baamonde, A., 2020. Dual dose-related effects evoked by CCL4 on thermal nociception after gene delivery or exogenous administration in mice. Biochem. Pharmacol. 175, 113903 https://doi.org/10.1016/j.bcp.2020.113903.

Akimoto, N., Honda, K., Uta, D., Beppu, K., Ushijima, Y., Matsuzaki, Y., Nakashima, S., Kido, M.A., Imoto, K., Takano, Y., Noda, M., 2013. CCL-1 in the spinal cord contributes to neuropathic pain induced by nerve injury. Cell Death Dis. 4, e679 https://doi.org/10.1038/cddis.2013.198.

Alvarez-Vega, M., Baamonde, A., Gutiérrez, M., Hidalgo, A., Menéndez, L., 1998. Comparison of the effects of calmidazolium, morphine and bupivacaine on N-methyl-D-aspartate- and septide-induced nociceptive behaviour. Naunyn Schmiede Arch. Pharmacol. 358, 628–634. https://doi.org/10.1007/pl00005304.

Alvarez-Vega, M., Baamonde, A., Gutiérrez, M., Hidalgo, A., Menéndez, L., 2000. Intrathecal N-methyl-D-aspartate (NMDA) induces paradoxical analgesia in the tail-flick test in rats. Pharmacol. Biochem. Behav. 65, 621–625. https://doi.org/10.1016/s0091-3057(99)00231-2.

Baamonde, A., Lastra, A., Fresno, M.F., Llames, S., Meana, A., Hidalgo, A., Menéndez, L., 2004a. Implantation of tumoral XC cells induces chronic, endothelin-dependent, thermal hyperalgesia in mice. Cell Mol. Neurobiol. 24, 269–281. https://doi.org/10.1023/b:cemn.0000018621.58328.ea.

Baamonde, A., Lastra, A., Villazón, M., Bordallo, J., Hidalgo, A., Menéndez, L., 2004b. Involvement of endogenous endothelins in thermal and mechanical inflammatory hyperalgesia in mice. Naunyn Schmiede Arch. Pharmacol. 369, 245–251. https://doi.org/10.1007/s00210-003-0841-1.

Baamonde, A., Lastra, A., Juarez, L., Hidalgo, A., Menéndez, L., 2005. TRPV1 desensitisation and endogenous vanilloid involvement in the enhanced analgesia induced by capsaicin in inflamed tissues. Brain Res. Bull. 67, 476–481. https://doi.org/10.1016/j.brainresbull.2005.07.001.

Baamonde, A., Lastra, A., Juárez, L., García-Suárez, O., Meana, A., Hidalgo, A., Menéndez, L., 2006. Endogenous beta-endorphin induces thermal analgesia at the initial stages of a murine osteosarcoma. Peptides 27, 2778–2785. https://doi.org/10.1016/j.peptides.2006.07.004.

Baamonde, A., Curto-Reyes, V., Juárez, L., Meana, A., Hidalgo, A., Menéndez, L., 2007. Antihyperalgesic effects induced by the IL-1 receptor antagonist anakinra and increased IL-1beta levels in inflamed and osteosarcoma-bearing mice. Life Sci. 81, 673–682. https://doi.org/10.1016/j.lfs.2007.07.003.

Baamonde, A., Hidalgo, A., Menéndez, L., 2011. Involvement of glutamate NMDA and AMPA receptors, glial cells and IL-1β in the spinal hyperalgesia evoked by the chemokine CCL2 in mice. Neurosci. Lett. 502, 178–181. https://doi.org/10.1016/j.neulet.2011.07.038.

Bertaccini, G., Coruzzi, G., 1989. Control of gastric acid secretion by histamine H2 receptor antagonists and anticholinergics. Pharmacol. Res. 21, 339–352. https://doi.org/10.1016/1043-6618(89)90151-5.

Chen, Y., Yang, C., Wang, Z.J., 2011. Proteinase-activated receptor 2 sensitizes transient receptor potential vanilloid 1, transient receptor potential vanilloid 4, and transient receptor potential ankyrin 1 in paclitaxel-induced neuropathic pain. Neuroscience 13 (193), 440–451. https://doi.org/10.1016/j.neuroscience.

Curto-Reyes, V., Juárez, L., García-Pérez, E., Fresno, M.F., Hidalgo, A., Menéndez, L., Baamonde, A., 2008. Local loperamide inhibits thermal hyperalgesia but not mechanical allodynia induced by intratibial inoculation of melanoma cells in mice. Cell Mol. Neurobiol. 28, 981–990. https://doi.org/10.1007/s10571-008-9272-3.

Deuis, J.R., Dvorakova, L.S., Vetter, I., 2017. Methods used to evaluate pain behaviors in rodents. Front. Mol. Neurosci. 10, 284. https://doi.org/10.3389/fnmol.2017.00284.

DuBreuil, D.M., Lopez Soto, E.J., Daste, S., Meir, R., Li, D., Wainger, B., Fleischmann, A., Lipscombe, D., 2021. Heat but not mechanical hypersensitivity depends on voltage-gated CaV2.2 calcium channel activity in peripheral axon terminals innervating skin. J. Neurosci. 41, 7546–7560. https://doi.org/10.1523/JNEUROSCI.0195-21.

Ebbinghaus, M., Uhlig, B., Richter, F., von Banchet, G.S., Gajda, M., Bräuer, R., Schaible, H.G., 2012. The role of interleukin-1β in arthritic pain: main involvement in thermal, but not mechanical, hyperalgesia in rat antigen-induced arthritis. Arthritis Rheumatol. 64, 3897–3907. https://doi.org/10.1002/art.34675.

Eschalier, A., Aumaître, O., Decamps, A., Dordain, G., 1983. A comparison of the effects of vitamin B12 and aspirin in three experimental pain models in rats and mice. Psychopharmacology 81, 228–231. https://doi.org/10.1007/BF00427267.

Folgueras, A.R., Valdés-Sánchez, T., Llano, E., Menéndez, L., Baamonde, A., Denlinger, B. L., Belmonte, C., Juárez, L., Lastra, A., García-Suárez, O., Astudillo, A., Kirstein, M., Pendás, A.M., Fariñas, I., López-Otín, C., 2009. Metalloproteinase MT5-MMP is an essential modulator of neuro-immune interactions in thermal pain stimulation. Proc. Natl. Acad. Sci. USA 106, 16451–16456. https://doi.org/10.1073/pnas.0908507106.

Garces, Y.I., Rabito, S.F., Minshall, R.D., Sagen, J., 1993. Lack of potent antinociceptive activity by substance P antagonist CP-96,345 in the rat spinal cord. Life Sci. 52, 353–360. https://doi.org/10.1016/0024-3205(93)90148-v.

García-Domínguez, M., Lastra, A., Folgueras, A.R., Cernuda-Cernuda, R., Fernández-García, M.T., Hidalgo, A., Menéndez, L., Baamonde, A., 2019a. The chemokine CCL4 (MIP-1β) evokes antinociceptive effects in mice: a role for CD4+ lymphocytes and met-enkephalin. Mol. Neurobiol. 56, 1578–1595. https://doi.org/10.1007/s12035-018-1176-8.

García-Domínguez, M., Aguirre, A., Lastra, A., Hidalgo, A., Baamonde, A., Menéndez, L., 2019b. The systemic administration of the chemokine CCL1 evokes thermal analgesia in mice through the activation of the endocannabinoid system. Cell Mol. Neurobiol. 39, 1115–1124. https://doi.org/10.1007/s10571-019-00706-3.

García-Domínguez, M., González-Rodríguez, S., Hidalgo, A., Baamonde, A., Menéndez, L., 2021. Kappa-opioid receptor-mediated thermal analgesia evoked by the intrathecal administration of the chemokine CCL1 in mice. Fundam. Clin. Pharmacol. 35, 1109–1118. https://doi.org/10.1111/fcp.12685.

González-Rodríguez, S., Pevida, M., Roques, B.P., Fournié-Zaluski, M.C., Hidalgo, A., Menéndez, L., Baamonde, A., 2009. Involvement of enkephalins in the inhibition of osteosarcoma-induced thermal hyperalgesia evoked by the blockade of peripheral P2×3 receptors. Neurosci. Lett. 465, 285–289. https://doi.org/10.1016/j.neulet.2009.09.015.

González-Rodríguez, S., Álvarez, M.G., García-Domínguez, M., Lastra, A., Cernuda-Cernuda, R., Folgueras, A.R., Fernández-García, M.T., Hidalgo, A., Baamonde, A., Menéndez, L., 2017. Hyperalgesic and hypoalgesic mechanisms evoked by the acute administration of CCL5 in mice. Brain Behav. Immun. 62, 151–161. https://doi.org/10.1016/j.bbi.2017.01.014.

González-Rodríguez, S., Lorenzo-Herrero, S., Sordo-Bahamonde, C., Hidalgo, A., González, S., Menéndez, L., Baamonde, A., 2022. Involvement of CD4+ and CD8+ T-lymphocytes in the modulation of nociceptive processing evoked by CCL4 in mice. Life Sci. 291, 120302 https://doi.org/10.1016/j.lfs.2022.120302.

Hill, R., 2000. NK1 (substance P) receptor antagonists–why are they not analgesic in humans? Trends Pharmacol. Sci. 21, 244–246. https://doi.org/10.1016/s0165-6147(00)01502-9.

Lewis, J.W., Cannon, J.T., Liebeskind, J.C., 1980. Opioid and nonopioid mechanisms of stress analgesia. Science 208, 623–625. https://doi.org/10.1126/science.7367889.

Liu, F., Song, Y., Liu, D., 1999. Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. Gene Ther. 6, 1258–1266. https://doi.org/10.1038/sj.gt.3300947.

Llorián-Salvador, M., Pevida, M., González-Rodríguez, S., Lastra, A., Fernández-García, M.T., Hidalgo, A., Baamonde, A., Menéndez, L., 2016a. Analgesic effects evoked by a CCR2 antagonist or an anti-CCL2 antibody in inflamed mice. Fundam. Clin. Pharmacol. 30, 235–247. https://doi.org/10.1111/fcp.12182.

Llorián-Salvador, M., González-Rodríguez, S., Lastra, A., Fernández-García, M.T., Hidalgo, A., Menéndez, L., Baamonde, A., 2016b. Involvement of CC chemokine receptor 1 and CCL3 in acute and chronic inflammatory pain in mice. Basic Clin. Pharmacol. Toxicol. 119, 32–40. https://doi.org/10.1111/bcpt.12543.

Lu, F., Kato, J., Toramaru, T., Sugai, M., Zhang, M., Morisaki, H., 2022. Objective and quantitative evaluation of spontaneous pain-like behaviors using dynamic weight-bearing system in mouse models of postsurgical pain. J. Pain. Res. 15, 1601–1612. https://doi.org/10.2147/JPR.S359220.

Luger, N.M., Honore, P., Sabino, M.A., Schwei, M.J., Rogers, S.D., Mach, D.B., Clohisy, D. R., Mantyh, P.W., 2001. Osteoprotegerin diminishes advanced bone cancer pain. Cancer Res. 61, 4038–4047.

Maier, S.F., Sherman, J.E., Lewis, J.W., Terman, G.W., Liebeskind, J.C., 1983. The opioid/nonopioid nature of stress-induced analgesia and learned helplessness. J. Exp. Psychol.: Anim. Behav. Process. 9, 80–90. https://doi.org/10.1037/0097-7403.9.1.80.

Menendez, L., Andres-Trelles, F., Hidalgo, A., Baamonde, A., 1993. Involvement of spinal kappa opioid receptors in a type of footshock induced analgesia in mice. Brain Res. 611, 264–271. https://doi.org/10.1016/0006-8993(93)90512-l.

Menendez, L., Andrés-Trelles, F., Hidalgo, A., Baamonde, A., 1994. Gender and test dependence of a type of kappa mediated stress induced analgesia in mice. Gen. Pharmacol. 25, 903–908. https://doi.org/10.1016/0306-3623(94)90094-9.

Menéndez, L., Lastra, A., Hidalgo, A., Baamonde, A., 2002. Unilateral hot plate test: a simple and sensitive method for detecting central and peripheral hyperalgesia in mice. J. Neurosci. Methods 113, 91–97. https://doi.org/10.1016/s0165-0270(01)00483-6.

Menéndez, L., Lastra, A., Hidalgo, A., Baamonde, A., 2003a. Nociceptive reaction and thermal hyperalgesia induced by local ET-1 in mice: a behavioral and Fos study. Naunyn Schmiede Arch. Pharmacol. 367, 28–34. https://doi.org/10.1007/s00210-002-0655-6.

Menéndez, L., Lastra, A., Fresno, M.F., Llames, S., Meana, A., Hidalgo, A., Baamonde, A., 2003b. Initial thermal heat hypoalgesia and delayed hyperalgesia in a murine model of bone cancer pain. Brain Res. 969, 102–109. https://doi.org/10.1016/s0006-8993(03)02284-4.

Menéndez, L., Lastra, A., Villanueva, N., Hidalgo, A., Baamonde, A., 2003c. Spinal nociceptin inhibits AMPA-induced nociceptive behavior and Fos expression in rat spinal cord. Pharmacol. Biochem. Behav. 74, 657–661. https://doi.org/10.1016/s0091-3057(02)01042-0.

Menéndez, L., Lastra, A., Meana, A., Hidalgo, A., Baamonde, A., 2005. Analgesic effects of loperamide in bone cancer pain in mice. Pharm. Biochem. Behav. 81, 114–121. https://doi.org/10.1016/j.pbb.2005.02.007.

Menéndez, L., Juárez, L., García, V., Hidalgo, A., Baamonde, A., 2007. Involvement of nitric oxide in the inhibition of bone cancer-induced hyperalgesia through the activation of peripheral opioid receptors in mice. Neuropharmacology 53, 71–80. https://doi.org/10.1016/j.neuropharm.2007.04.011.

Menéndez, L., Hidalgo, A., Meana, A., Poras, H., Fournié-Zaluski, M.C., Roques, B.P., Baamonde, A., 2008. Inhibition of osteosarcoma-induced thermal hyperalgesia in mice by the orally active dual enkephalinase inhibitor PL37. Potentiation by gabapentin. Eur. J. Pharmacol. 596 (50–5) https://doi.org/10.1016/j.ejphar.2008.07.043.

Meunier, J.C., Mollereau, C., Toll, L., Suaudeau, C., Moisand, C., Alvinerie, P., Butour, J. L., Guillemot, J.C., Ferrara, P., Monsarrat, B., et al., 1995. Isolation and structure of the endogenous agonist of opioid receptor-like ORL1 receptor. Nature 377 (6549), 532–535. https://doi.org/10.1038/377532a0.

Mogil, J.S., 2009. Animal models of pain: progress and challenges. Nat. Rev. Neurosci. 10, 283–294. https://doi.org/10.1038/nrn2606.

Mogil, J.S., 2022. The history of pain measurement in humans and animals. Front Pain. Res. 3, 1031058 https://doi.org/10.3389/fpain.2022.

Mohammadi, S., Christie, M.J., 2014. α9-nicotinic acetylcholine receptors contribute to the maintenance of chronic mechanical hyperalgesia, but not thermal or mechanical allodynia. Mol. Pain 10, 64. https://doi.org/10.1186/1744-8069-10-64.

Pevida, M., González-Rodríguez, S., Lastra, A., Hidalgo, A., Menéndez, L., Baamonde, A., 2012. CCL2 released at tumoral level contributes to the hyperalgesia evoked by intratibial inoculation of NCTC 2472 but not B16-F10 cells in mice. Naunyn Schmiede Arch. Pharmacol. 385, 1053–1061. https://doi.org/10.1007/s00210-012-0787-2.

Pevida, M., Lastra, A., Hidalgo, A., Baamonde, A., Menéndez, L., 2013. Spinal CCL2 and microglial activation are involved in paclitaxel-evoked cold hyperalgesia. Brain Res. Bull. 95, 21–27. https://doi.org/10.1016/j.brainresbull.2013.03.005.

Pevida, M., Lastra, A., Meana, Á., Hidalgo, A., Baamonde, A., Menéndez, L., 2014. The chemokine CCL5 induces CCR1-mediated hyperalgesia in mice inoculated with NCTC 2472 tumoral cells. Neuroscience 259, 113–125. https://doi.org/10.1016/j.neuroscience.2013.11.055.

Reinscheid, R.K., Nothacker, H.P., Bourson, A., Ardati, A., Henningsen, R.A., Bunzow, J. R., Grandy, D.K., Langen, H., Monsma Jr, F.J., Civelli, O., 1995. Orphanin FQ: a neuropeptide that activates an opioidlike G protein-coupled receptor. Science 270, 792–794. https://doi.org/10.1126/science.270.5237.792.

Rossi, G.C., Leventhal, L., Pasternak, G.W., 1996. Naloxone sensitive orphanin FQ-induced analgesia in mice. Eur. J. Pharmacol. 311, R7–R8. https://doi.org/10.1016/0014-2999(96)00578-x.

Saika, F., Kiguchi, N., Kobayashi, Y., Fukazawa, Y., Kishioka, S., 2012. CC-chemokine ligand 4/macrophage inflammatory protein-1β participates in the induction of neuropathic pain after peripheral nerve injury. Eur. J. Pain 16, 1271–1280. https://doi.org/10.1002/j.1532-2149.2012.00146.x.

Sevcik, M.A., Jonas, B.M., Lindsay, T.H., Halvorson, K.G., Ghilardi, J.R., Kuskowski, M. A., Mukherjee, P., Maggio, J.E., Mantyh, P.W., 2006. Endogenous opioids inhibit early-stage pancreatic pain in a mouse model of pancreatic cancer. Gastroenterology 131, 900–910. https://doi.org/10.1053/j.gastro.2006.06.021.

Sheehan, G.D., Martin, M.K., Young, V.A., Powell, R., Bhattacharjee, A., 2021. Thermal hyperalgesia and dynamic weight bearing share similar recovery dynamics in a sciatic nerve entrapment injury model. Neurobiol. Pain 10, 100079. https://doi.org/10.1016/j.ynpai.2021.100079.

Wlodarski, K.H., Reddi, H.A., 1987. Tumor cells stimulate in vivo periosteal bone formation. Bone Min. 2, 185–192.

Xu, X.J., Hao, J.X., Wiesenfeld-Hallin, Z., 1996. Nociceptin or antinociceptin: potent spinal antinociceptive effect of orphanin FQ/nociceptin in the rat. Neuroreport 7, 2092–2094.

Zhu, Q., Mao, L.N., Liu, C.P., Sun, Y.H., Jiang, B., Zhang, W., Li, J.X., 2016. Antinociceptive effects of vitexin in a mouse model of postoperative pain. Sci. Rep. 6, 19266 https://doi.org/10.1038/srep19266.

Ziemichod, W., Kotlinska, J., Gibula-Tarlowska, E., Karkoszka, N., Kedzierska, E., 2022. Cebranopadol as a novel promising agent for the treatment of pain. Molecules 27, 3987. https://doi.org/10.3390/molecules27133987.