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Melanocortin receptor agonists and antagonists modulate nociceptive sensitivity in the mouse formalin test

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Abstract

A number of studies suggest the involvement of melanocortins in nociception, and although the mechanism through which this occurs is still unknown, experimental evidence would suggest an involvement of melanocortin MC_4 receptors. We investigated the effect of melanocortin receptor agonist and antagonists on nociceptive behaviour induced by formalin in the mouse. The intrathecal injection of the melanocortin receptor agonist MTII ([Ac-Nle^4,Asp^5,D-Phe^7,Lys^{10}]cyclo-α-MSH-(4–10) amide) (5 nmol; $P<0.05$) significantly increased nociception in both phases of the formalin test, whereas the synthetic melanocortin receptor antagonists, SHU9119 ([Ac-Nle^4,Asp^5,D-2-Nal^7,Lys^{10}]cyclo-α-MSH-(4–10) amide) (5 nmol), HS014 ([Ac-Cys^{11},D-2-Nal^{14},Cys^{18}]β-MSH-(11–22)amide) (5 nmol), and JKC-363 (cyclic [Mpr^{11},D-Nal^{14},Cys^{18},Asp^{22}-NH$_2$]β-MSH-11–22)) (5 nmol), and the endogenous receptor antagonist Agouti-related protein (AgRP) (1.5 nmol) were effective in reducing nociception in the late phase of the formalin test (50–60% of reduction in licking/flinching response; $P<0.05$). The present findings further support the involvement of the melanocortin system in the control of nociception. Moreover, considering that melanocortin MC_4 receptors are the only melanocortin subtype receptors present in the spinal cord, we can assume that the activity of the peptides in the formalin model is mediated through melanocortin MC_4 receptors.

Keywords: Chronic pain; Formalin test; Melanocortin MC_4 receptor

1. Introduction

Melanocortins are a family of endogenous peptides generated by enzymatic cleavage of a common precursor molecule, proopiomelanocortin (POMC). Main members of the melanocortin family are α, β, γ-melanocyte-stimulating hormone (MSH) and adrenocorticotropic hormone (ACTH). Melanocortins exert their actions through activation of at least five subtypes of receptors (MC_1 to MC_5). Melanocortin MC_1, MC_2 and MC_5 receptors are predominantly expressed in peripheral tissues, whereas melanocortin MC_3 and MC_4 receptors are mainly restricted to the central nervous system. A further peculiarity of the system is that, in addition to endogenous agonists, there are also endogenous antagonists such as the Agouti protein, which binds preferentially to melanocortin MC_1 receptors and is expressed mainly in the skin, and the Agouti-related protein (AgRP), which is a competitive antagonist of both melanocortin MC_3 and MC_4 receptors and is mainly expressed in the brain (Dinulescu and Cone, 2000). Other evidence has demonstrated that AgRP is an inverse agonist at the human melanocortin MC_4 receptor (Nijenhuis et al., 2001).

As a consequence of their widespread expression, melanocortins have been implicated in the regulation of several physiological functions both at peripheral and central levels. Briefly, a large body of evidence links melanocortins to skin pigmentation, grooming behaviour, food intake and energy consumption, inflammation, temperature control, blood pressure, and sexual function (Adan and Gispen, 2000).

A possible link between melanocortins and nociception was first postulated by pioneering studies in late 1970s–early 1980s, showing that central administration of α-MSH and ACTH causes hyperalgesia in various nociceptive models (Bertolini et al., 1979; Sandman and Kastin, 1981; Williams et al., 1986) and reverses the analgesic effects of morphine and β-endorphin (Gispen et al., 1976; Wiegant et al., 1977; Smock and Fields, 1981).

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More recently, Vrinten et al. (2000, 2001) confirmed this initial suggestion by showing that administration of melanocortin receptor antagonists could modulate allodynia in rats with neuropathic pain, i.e., the melanocortin MC_3/MC_4 receptor antagonist SHU9119 ([Ac-Nle^4,Asp^5, D-2-Nal^7,Lys^{10}]cyclo-α-MSH-(4–10) amide) had anti-allodynic effects, while melanocortin MC_3/MC_4 receptor agonists MTII ([Ac-Nle^4,Asp^5,D-Phe^7,Lys^{10}]cyclo-α-MSH-(4–10) amide) and D-Tyr-MTII (Acetyl-[Nle^4,Asp^5,D-Tyr^7, Lys^{10}]cyclo-α-MSH-(4–10) amide) increased nociceptive sensitivity. These findings confirmed the initial observations and expanded the role of melanocortins from an acute to a more chronic nociceptive condition. These observations received recent support by Starowicz et al. (2002).

The intraplantar injection of formalin in mice provides a quick, reproducible and robust nociceptive model to test potential anti-nociceptive targets in an experimental setting mimicking simultaneously both acute and chronic nociceptive conditions. In fact, after formalin injection two distinct behavioural phases can be observed. An early phase (lasting for the initial 5–10 min) due to the activation of peripheral nociceptive mechanisms and mimicking acute nociception, followed by a later phase starting 15 min after formalin and lasting for an additional 20–30 min, reflecting central sensitization similar to that occurring in neuropathic pain (Tjølsen et al., 1992).

To further investigate the involvement of melanocortins in the modulation of nociception, we decided to take a systematic approach and investigated the effects of different melanocortin ligands (both agonist and antagonists) in a mouse model of nociception, the formalin test.

2. Materials and methods

2.1. Animals

Male CD1 mice (Charles River, Calco, Italy) weighing 25–30 g were used in the experiments. They were housed 10 per cage and kept under a 12-h day/night cycle at a constant room temperature of 22 ± 1 °C. Food and water were available ad libitum. All behavioural tests were conducted in the morning.

Procedures involving animals and their care were conducted in conformity with institutional guidelines, in compliance with the European Community Council Directive 86/609 (OJ L 358, 1, December 12, 1987).

2.2. Formalin test

Mice were gently restrained and 30 μl of formalin solution (1.5% in saline) was injected subcutaneously into the plantar surface of the right hind paw, using a microsyringe with a 27-gauge needle. After the formalin injection, the mouse was immediately put back into the Plexiglas observation chamber (30 × 20 × 20 cm) and the nociceptive response of the animal to formalin injection was observed for 60 min. The duration of licking and flinching of the injected paw was recorded and quantified every 5 min for the total observation period. The recording of the early phase (first phase) started immediately and lasted for 5 min. The late phase (second phase) started about 10–15 min after formalin injection.

2.3. Injection procedures

All peptidic compounds were administered by the intrathecal (i.t.) route, whereas SHU9119 and HS014 ([Ac-Cys^{11},D-2-Nal^{14},Cys^{18}]β-MSH-(11–22)amide) were also administered by the intracerebroventricular (i.c.v.) route. Both injections were performed according to the method described by Bertorelli et al. (2002).

Briefly, the mouse was manually restrained and a 27-gauge needle connected to a 10-μl Hamilton syringe was inserted between vertebrae L5 and L6 of the mouse spinal column for i.t. injection. Tail flick behaviour confirmed the correct penetration of the subarachnoid space. A volume of 5 μl was injected as bolus injection in about 5 s. The i.c.v. administration was done free-hand. According to the stereotaxic coordinates for the lateral ventricle in the mouse (2 mm lateral and caudal from bregma and a depth of 3 mm), a metal cannula 1–2 mm of diameter and 1.5 mm in length directly connected with a 10-μl Hamilton syringe by a PE10 tube, was used to penetrate the skull and reached the lateral ventricle. A volume of 5 μl was injected over about 10 s.

2.4. Drugs

Formalin was prepared by diluting a formaldehyde solution (37%, Scharlau, Barcelona, Spain) with saline to obtain the concentration of 1.5%.

MTII, SHU9119, HS014, AgRP (Human, 86–132) (Bachem, Bubendorf, Switzerland) and JKC-363 (cyclic [Mpr^{11},D-Nal^{14},Cys^{18},Asp^{22}-NH_2]β-MSH-(11–22)) (Phoenix Pharmaceuticals, Belmont, CA) were dissolved in saline and stored at −20 °C as stock solutions. Just before use, all peptides were diluted with saline to the appropriate concentration.

2.5. Statistical analysis

Data from the formalin test are expressed as mean nociceptive response (licking and flinching) time (s) ± S.E.M. in an interval of 5 min. Only in the experiment with SHU9119 and HS014, data are expressed as the area under the curve (AUC) for the interval between 15 and 30 min (second phase).

Statistical analysis was carried out to compare control responses to test responses, both in the first and the second phases, using one-way analysis of variance (ANOVA)

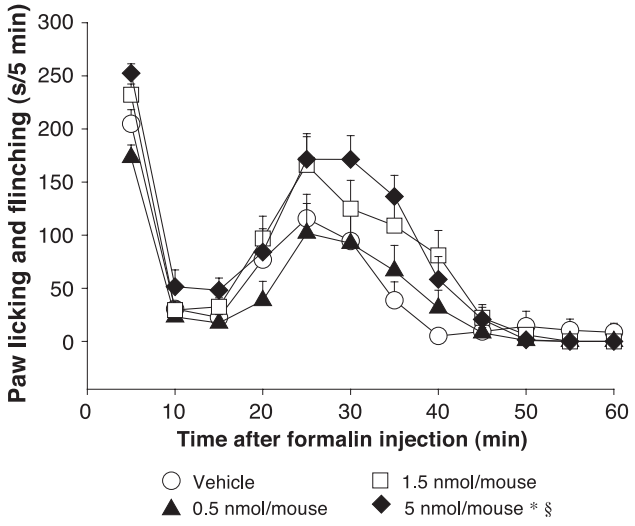

Fig. 1. Pro-hyperalgesic effects of the melanocortin receptor agonist MTII in the formalin test in mice. MTII (0.5–1.5–5 nmol/mouse, i.t.) was injected immediately before intraplantar formalin injection. Formalin (1.5%; 30 μl) was injected s.c. into the plantar surface of the right hindpaw of the mouse. Licking and flinching of the injected paw were recorded as the total time (s) per 5-min observation period, for a total duration of 60 min. Data are means ± S.E.M. for 9–13 animals per group. §$P<0.05$ vs. vehicle-treated group (first phase). *$P<0.05$ vs. vehicle-treated group (second phase). ANOVA followed by post hoc Dunnett test.

followed by a post hoc Dunnett test, where $P<0.05$ was considered significant. To define significance, the early phase was considered to be the first observation period of 5 min, and the second phase was considered to be the interval from 10 to 45 min.

3. Results

3.1. The effect of melanocortin receptor agonist MTII on the nociceptive behavioural response elicited by intraplantar injection of formalin

As expected, the intraplantar injection of 1.5% formalin solution into the hindpaw caused an acute, immediate nociceptive response, i.e., licking and flinching of the injected paw, which lasted for 5 min (early phase), followed by a later phase starting 15–20 min after formalin administration and lasting for an additional 20–30 min (Fig. 1).

As shown in the same figure, the i.t. administration of the nonselective melanocortin MC_3/MC_4 receptor agonist MTII at doses of 0.5–1.5–5 nmol/mouse, immediately before formalin injection, caused a decrease in the nociceptive threshold. The effect was detectable at the dose of 1.5 nmol/mouse and was significant at 5 nmol/mouse ($P<0.05$). Interestingly, the effect of MTII was present in both phases although apparently more pronounced in the second phase.

3.2. The effect of melanocortin receptor antagonists SHU9119, HS014, JKC-363 and AgRP on the nociceptive behavioural response elicited by intraplantar injection of formalin

As shown in Fig. 2, both i.t. and i.c.v. administration of SHU9119 and HS014 after intraplantar formalin injection caused a reduction of nociceptive behaviour over the range of doses tested (0.5–5 nmol/mouse). Results are expressed

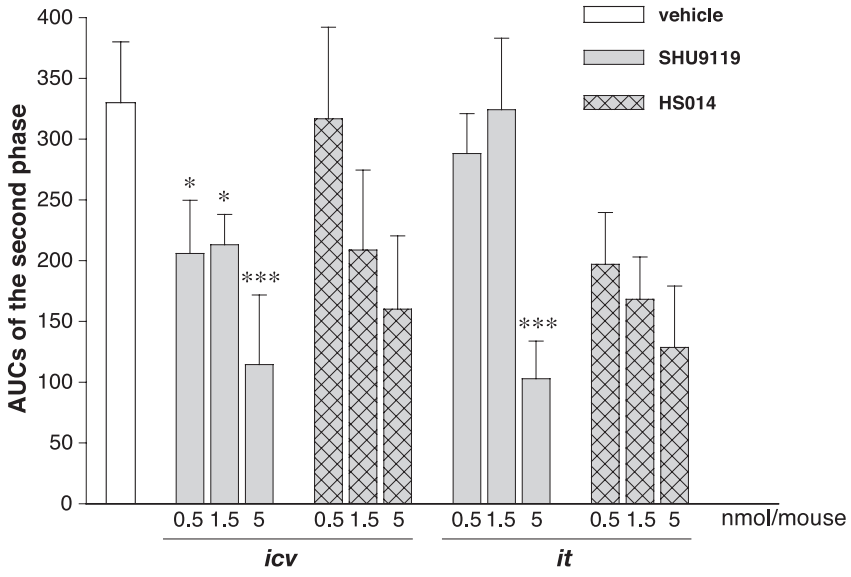

Fig. 2. Anti-hyperalgesic effect of melanocortin receptor antagonists in the formalin test in mice. SHU9119 and HS014 (0.5–1.5–5 nmol/mouse, i.t. and i.c.v.) were injected just before formalin injection. Formalin (1.5%; 30 μl) was injected s.c. into the plantar surface of the right hindpaw of the mouse. Time spent licking and flinching the paw was considered as a measure of nociceptive intensity. Area Under Curves (AUCs) of the second phase were calculated from 15 to 30 min after formalin injection. Data for the vehicle bar were obtained from the mean of data for the vehicle from the four experiments. Bars represent means ± S.E.M. for 8–12 animals per group. *$P<0.05$; ***$P<0.001$ vs. vehicle-treated group. ANOVA followed by post hoc Dunnett test.

as AUC in the second phase since, in contrast to agonist, no effect was seen in the first phase. Regardless of the route of administration, SHU9119 had a statistically significant effect at the three doses tested (0.5–1.5 nmol: $P<0.05$; 5 nmol: $P<0.001$). Conversely, significance was not achieved with HS014, although the magnitude of the effects was similar, i.e., at 5 nmol/mouse the reduction was 70% vs. 52% by i.c.v. route and 70% vs. 53% by i.t. route for SHU9119 and HS014, respectively. In Fig. 3, the effect of i.t. injection of JKC-363 on formalin-induced behaviour is shown. JKC-363 had a similar affinity for melanocortin MC_4 receptors as SHU9119 and HS014, but had better selectivity for melanocortin MC_4 receptors than for melanocortin MC_3 receptors (90-fold, Kim et al., 2002, compared to 3- and 17-fold, respectively, Schiöth et al., 1998). Nonetheless, as shown in Fig. 3, when tested at the doses of 1.5–5 nmol/mouse, JKC-363 retained the ability to reduce formalin-induced pain and a significant effect was seen at 5 nmol/mouse ($P<0.05$). Also in this case, the effect of the melanocortin receptor antagonist was selective for the second phase and the magnitude of inhibition was 63% compared to that of saline.

In Fig. 4, the effect of i.t. administration of the endogenous antagonist AgRP at the doses of 0.5 and 1.5 nmol/mouse is shown. In Fig. 4A, the compound was given immediately before, while in Fig. 4B the compound was given 10 min after, formalin (i.e., after the early phase but before the late phase).

Regardless of the time of administration, AgRP was always effective in reducing formalin-induced pain. Significance was achieved at 1.5 nmol/mouse in both protocols, but an increased efficacy and potency were noticed when AgRP administration was switched from before to 10 min after formalin. In fact, when AgRP was given before formalin, it reduced the nociceptive threshold by 60% measured as AUC, whereas when it was given 10 min later it completely blocked the hyperalgesic effects of formalin.

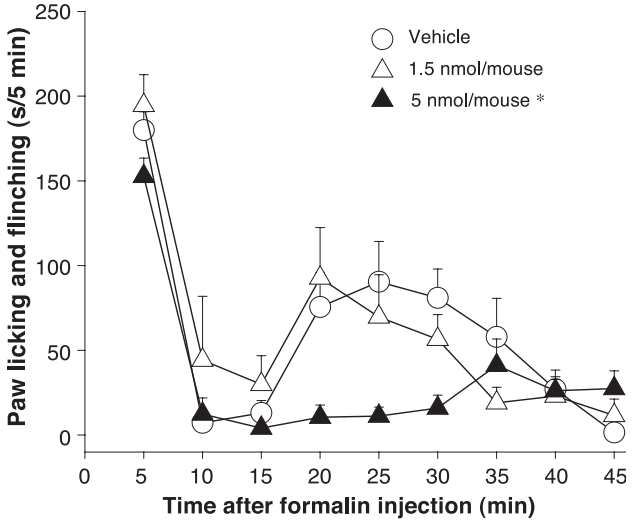

Fig. 3. Anti-hyperalgesic effects of selective melanocortin MC_4 receptor antagonist, JKC-363, in the formalin test in mice. JKC-363 (1.5–5 nmol/mouse, i.t.) was injected immediately before intraplantar formalin injection. Formalin (1.5%; 30 μl) was injected s.c. into the plantar surface of the right hindpaw of the mouse. Licking and flinching of the injected paw were recorded as the total time (s) per 5-min observation period, for a total duration of 45 min. Data are means ± S.E.M. for 6–10 animals per group. *$P<0.05$ vs. vehicle-treated group. ANOVA followed by post hoc Dunnett test.

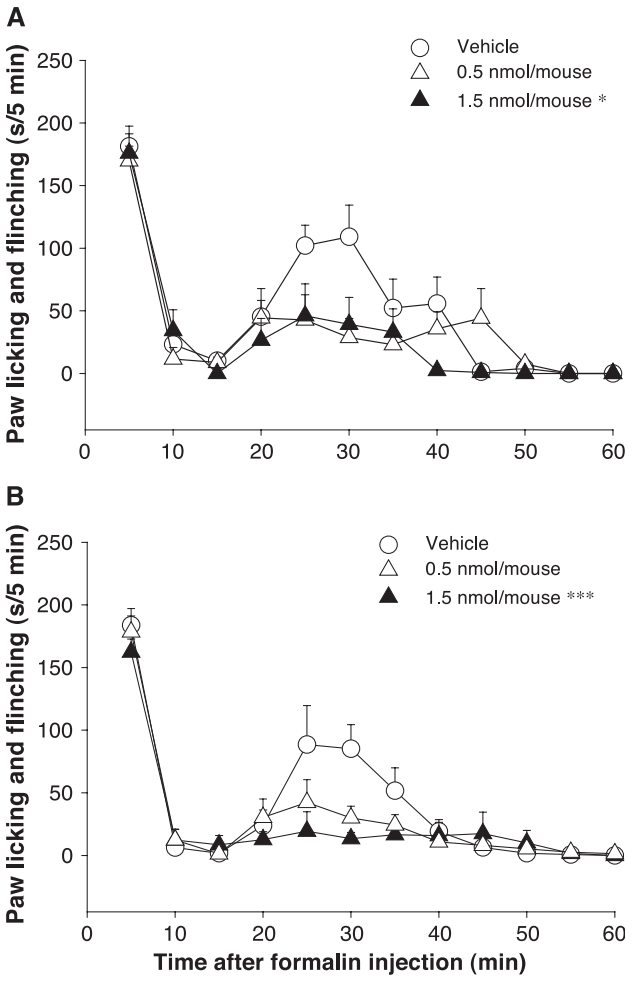

Fig. 4. Anti-hyperalgesic effects of the endogenous melanocortin receptor antagonist, Agouti-related protein, in the formalin test in mice. AgRP (0.5–1.5 nmol/mouse, i.t.) was injected immediately before (A) and 10 min after intraplantar formalin injection (B). Formalin (1.5%; 30 μl) was injected s.c. into the plantar surface of the right hindpaw of the mouse. Licking and flinching of the injected paw were recorded as the total time (s) per 5-min observation period, for a total duration of 60 min. Data are means ± S.E.M. for 6–10 animals per group. *$P<0.05$; ***$P<0.001$ vs. vehicle-treated group. ANOVA followed by post hoc Dunnett test.

4. Discussion

The present study provides further evidence supporting a role of melanocortins in the regulation of nociceptive sensitivity. The intraplantar injection of formalin in mice was used as experimental nociceptive model. In this model, the melanocortin receptor agonist MTII was pro-nociceptive

whereas melanocortin receptor antagonists had the opposite effect. Our results support and expand previous observations that SHU9119, a melanocortin receptor antagonist, can alleviate allodynic behaviour in rats with neuropathic pain after either acute or chronic treatments (Vrinten et al., 2000, 2001; Starowicz et al., 2002). In addition, in our studies, the antihyperalgesic effects of melanocortin receptor antagonists were demonstrated using several compounds. Furthermore, our data confirm previous results showing that central administration of melanocortin receptor agonists such as ACTH and α-MSH can cause hyperalgesia in various nociceptive tests (Bertolini et al., 1979; Sandman and Kastin, 1981; Williams et al., 1986).

The key feature of the formalin model is that two clearly distinguishable behavioural phases reflecting two different nociceptive states can be identified. Interestingly, the melanocortin receptor agonist MTII potentiated both phases, whereas all the antagonists tested affected only the second phase. The explanation for this finding could be that the effect of MTII in the first phase of the response may be mediated via other effects of activation of the melanocortin system. In fact, it is known that melanocortin receptor agonists stimulate grooming behaviour (Adan et al., 1999), which may explain the increased paw licking. An alternative explanation can be considered, namely, that once formalin is injected there is a tonic release of endogenous melanocortin peptides so that antagonists become effective. The appearance of melanocortin tone, which is sensitive to melanocortin receptor antagonists, seems to overlap the late phase, i.e., the sensitization phase, thus supporting the therapeutic use of melanocortin receptor antagonists in chronic nociceptive states. This hypothesis would explain the apparently increased potency of AgRP when the time of administration was moved to the beginning of the second phase, i.e., when presumably melanocortin tone is in its ascending phase. It remains to be established if this increased, melanocortin tone is a cause or a consequence of the central sensitization.

From a therapeutic perspective, a critical question is to define which melanocortin receptor is involved in this nociceptive modulation. Considering that the central nervous system has mainly melanocortin MC_3 and MC_4 receptors, we made the reasonable assumption of restricting our investigation to these two subtypes. The present study was done with the best tools available at presents, but none of them had a sufficient degree of selectivity to answer clearly the question. Moreover, all compounds tested exhibited activity in the same range of doses, in line with their comparable affinity for melanocortin MC_4 receptors rather than melanocortin MC_3 receptors, and, in this respect, a remarkable observation was that JKC-363 retained full activity despite its relative selectivity for melanocortin MC_4 receptors (90-fold higher affinity over melanocortin MC_3 receptors, Kim et al., 2002). Furthermore, considering that only melanocortin MC_4 receptors, but not melanocortin MC_3 receptors, are expressed in the spinal cord (van der Kraan et al., 1999; Vrinten et al., 2000) and that all compounds tested are also effective by the i.t. route, suggesting an involvement of spinal receptors, our findings support the primary involvement of melanocortin MC_4 receptors rather than melanocortin MC_3 receptors. Affinity data from binding assays showed that HS014 has a slightly lower affinity for both melanocortin MC_3 and MC_4 receptors than SHU9119 (40–10 times, Schiöth et al., 1998). We believe that this lack of relevance of HS014 is due to this slightly lower affinity, although the compounds may have different pharmacokinetic properties. Recently, Chaki et al. (2003) described antidepressant and anxiolytic-like activities of a novel, non-peptidic antagonist with a K_i value of 7.9 nM for melanocortin MC_4 receptors and no affinity for melanocortin MC_3 receptors up to 10 μM. This compound could be helpful in better understanding melanocortin MC_4 receptor involvement in the modulation of nociception.

Previous studies emphasized the importance of an altered regulation of AgRP in well-investigated physiological functions such as feeding behaviour (Dinulescu and Cone, 2000). For example, upregulation of AgRP production is induced by food intake restriction, and ectopic over-expression of AgRP in transgenic mice results in obesity. Whether a similar AgRP role is operant also in pain is not known but the present study demonstrates, for the first time, that AgRP reduces pain, suggesting that also in this case an endogenous balance between agonist and antagonist activities could regulate receptor function. Thus, alterations of this balance could promote the shift from a normal to a pathological state.

In summary, the main findings of the present study were that a melanocortin receptor agonist potentiates, while various melanocortin receptor antagonists (preferentially acting on melanocortin MC_4 receptors) attenuate, nociceptive behaviour after intraplantar formalin injection in mice, suggesting that targetting melanocortin MC_4 receptors could generate a novel treatment for chronic nociceptive states. Further studies are needed to clarify the mechanisms of action as well as the identification of selective, non-peptidic ligands to support target validation studies and allow clarification of the receptor subtype involved.

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