Characterization of antinociceptive potency of endomorphin-2 derivatives with unnatural amino acids in rats

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This study reports on the in vivo effects of four endomorphin-2 (EM-2) derivatives (EMD1-4) containing unnatural amino acids, i.e. 2-aminocyclohexanecarboxylic acid (Achc), para-fluorophenylalanine (pFPhe), β-methyl-phenylalanine (βMePhe) and/or 2',6'-dimethyltyrosine (Dmt). After induction of osteoarthritis by monosodium iodoacetate into the ankle joint of male Wistar rats, a chronic intrathecal catheter was inserted for spinal drug delivery. The mechanical threshold was assessed by a dynamic aesthesiometer. Intrathecal injection of the original EM-2 and the ligands (0.3–10 µg) caused dose-dependent antiallodynic effects. The comparison of the different substances revealed that EMD3 and EMD4 showed more prolonged antinociception than EM-2, and the effects of the highest dose of EMD4 were comparable to morphine, while EMD3 caused paralysis at this dose. The potency of the different ligands did not differ from EM-2. The results show that the derivatives of EM-2 have similar in vivo potency to the original ligand, but their effects were more prolonged suggesting that these structural modifications may play a role in the development of novel endomorphin analogues with increased therapeutic potential.

Keywords: opioid peptide, endomorphin, pain, spinal, intrathecal, osteoarthritis

Morphine and related compounds that are clinically valuable for pain relief, act primarily at the μ-opioid receptor (MOR), a member of the G-protein-coupled receptor superfamily (39). A major goal in opioid peptide research is the development of novel analgesics that could substitute for morphine without its well-known side effects of dependence, tolerance, respiratory depression and reward-seeking behavior (25). The study of naturally occurring peptides provides a rational and powerful approach in the design of peptide medications. Endomorphin-1 (EM-1, Tyr-Pro-Trp-Phe-NH₂) and endomorphin-2 (EM-2, Tyr-Pro-Phe-Phe-NH₂) are high-affinity, MOR-selective endogenous opioids which also inhibit nociception similarly to opiates of plant origin in both acute and chronic pain models (10, 31, 44). The exogenous application of EMs encounters serious limitations, including a short duration of action, a lack of activity after oral administration and poor metabolic stability (23, 34, 37). Aminopeptidases play a key role in the biodegradation of EMs, during which the main cleavage occurs at the Pro₂-Trp³ and Pro²-Phe³ peptide bonds. For their consideration as actual therapeutic drugs it is essential to enhance their resistance to enzymatic degradation (12).
Such objectives may possibly be achieved through systematic modification of the peptide sequence (9, 11, 15).

The solution structures of the EMs and their analogues have been investigated in detail in relation to their bioactivity (15). In a recent paper, the synthesis and structure-activity study of new analogues with unnatural amino acids were reported (22). The systematic replacement of natural amino acids by 2',6'-dimethyltyrosine (Dmt¹), 2-amino-cyclohexanecarboxylic acid [cis-(1S,2R)Achc²/cis-(1R,2S)Achc²], β-methylphenylalanine [(2R,3R)βMePhe⁴/(2S,3S)βMePhe⁴] and para-fluorophenylalanine (pFPhe⁴) in different positions resulted in proteolytically stable compounds with high MOR affinity in some cases (22). Thus, it was found that the analogues carrying Dmt¹ and Achc² residues displayed the highest MOR affinities, depending upon the configuration of the incorporated Achc². Combination of such derivatives with pFPhe⁴ or βMePhe⁴ yielded compounds with high binding potency, while their efficacy did not differ from the parent ligand. Several earlier studies investigated the in vivo activities of different endomorphin derivatives in acute heat or chemical pain models (2, 17, 24, 27, 45). The goal of this study was to investigate the antiallodynic effects of the recently synthetized and most potent EM-2 derivatives (EMD1-4, Table I) at spinal level, in a chronic joint pain model.

Materials and Methods

Animals

After institutional ethical approval had been obtained (Institutional Animal Care Committee of the Faculty of Medicine at the University of Szeged), male Wistar rats (Charles River strain, Bioplan, Budapest, Hungary; 334 ± 3.8 g; n = 7–14/group) were housed in groups of 5–6 per cage, with free access to food and water, and with a natural light/dark cycle.

Drugs

The following drugs were purchased: ketamine hydrochloride (Calypsol, Richter Gedeon RT, Budapest, Hungary), xylazine hydrochloride (Rompun, Bayer, Leverkusen, Germany), Gentamycin (Sanofi-Aventis, Budapest, Hungary), monosodium iodoacetate (MIA; Sigma-Aldrich Ltd. Budapest, Hungary) and morphine hydrochloride (Hungaropharma, Budapest, Hungary). EM-2 and the derivatives (Table I) were synthesized as was described earlier (22). All substances were dissolved in saline. Intrathecally (i.t.) administered drugs were injected over 120 s in a volume of 10 µl, followed by a 10 µl flush of physiological saline.

Abbreviations

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Name</th>
</tr>
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<tbody>
<tr>
<td>Achc</td>
<td>2-aminocyclohexanecarboxylic acid</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AUC</td>
<td>area under the curve</td>
</tr>
<tr>
<td>βMePhe</td>
<td>β-methylphenylalanine</td>
</tr>
<tr>
<td>CI</td>
<td>95% confidence intervals</td>
</tr>
<tr>
<td>Dmt</td>
<td>2’,6’-dimethyltyrosine</td>
</tr>
<tr>
<td>ED₂₅</td>
<td>25% effective dose</td>
</tr>
<tr>
<td>EM-1</td>
<td>endomorphin-1 (Tyr-Pro-Trp-Phe-NH₂)</td>
</tr>
<tr>
<td>EM-2</td>
<td>endomorphin-2 (Tyr-Pro-Phe-Phe-NH₂)</td>
</tr>
<tr>
<td>EMD1</td>
<td>Tyr-(1S,2R)Achc-Phe-pFPhe-NH₂</td>
</tr>
<tr>
<td>EMD2</td>
<td>Tyr-(1S,2R)Achc-Phe-(2S,3S)βMePhe-NH₂</td>
</tr>
<tr>
<td>EMD3</td>
<td>Dmt-(1S,2R)Achc-Phe-pFPhe-NH₂</td>
</tr>
<tr>
<td>EMD4</td>
<td>Dmt-(1S,2R)Achc-Phe-(2S,3S)βMePhe-NH₂</td>
</tr>
<tr>
<td>HP</td>
<td>hot-plate</td>
</tr>
<tr>
<td>i.t</td>
<td>intrathecally</td>
</tr>
<tr>
<td>i.c.v.</td>
<td>intracerebroventrical</td>
</tr>
<tr>
<td>MOR</td>
<td>μ-opioid receptor</td>
</tr>
<tr>
<td>%MPE</td>
<td>% maximum possible effect</td>
</tr>
<tr>
<td>pFPhe</td>
<td>para-fluorophenylalanine</td>
</tr>
<tr>
<td>TF</td>
<td>tail-flick</td>
</tr>
</tbody>
</table>
Antinociception by endomorphin-2 derivatives

Monosodium iodoacetate-induced inflammation

Osteoarthritis was induced by injecting MIA (1 mg/30 µl) into the tibiotarsal joint of the right hind leg on two consecutive days. All treatments were given to gently restrained conscious animals, using a 27-gauge needle, without anaesthesia so as to exclude any drug interaction. These injections did not elicit signs of major distress. Animals were allowed to recover for 14 days, which has consistently been shown to cause severe end-stage cartilage destruction resulting in osteoarthritis-like joint pain (3, 13).

To determine the changes in the size of the inflamed joint, we measured the anteroposterior and mediolateral diameter of the paw at the level of ankle joint with a digital caliper. The cross-section area was calculated with the formula $a \times b \times \pi$, where $a$ and $b$ are the radii in the two aspects.

Intrathecal catheterization

Two weeks after MIA administration rats were anaesthetized with a mixture of ketamine hydrochloride and xylazine (72 and 8 mg/kg intraperitoneally: i.p., respectively). An i.t. catheter (PE-10 tubing; Intramedic, Clay Adams; Becton Dickinson; Parsippany, NJ; I.D. 0.28 mm; O.D. 0.61 mm) was inserted via the cisterna magna and passed 8.5 cm caudally into the subarachnoid space (42), which served to place the catheter tip between Th12 and L2 vertebrae, corresponding to the spinal segments that innervate the hindpaws (6). After surgery, rats were housed individually and had free access to food and water. Animals exhibiting postoperative neurologic deficits (about 10%), and also those that did not show paralysis of one of the hindpaws after the administration of 100 µg lidocaine (about 0.5%) were excluded (6). After the surgery, animals were administered antibiotics (13 mg/kg gentamicin, subcutaneously) to prevent infection. The rats were allowed to recover for at least four days before testing, and were assigned randomly to the treatment groups. The observer was blind to the treatment administered. Repeated intrathecal injections in the same animals were separated by 5–7 days.

Behavioral nociceptive testing

Mechanical allodynia was determined using a dynamic plantar aesthesiometer (Ugo Basile, Comerio, Italy). Prior to baseline testing, each rat was habituated to a testing box with a wire-mesh grid floor for at least 20 min. Measurements were done with a straight metal filament that exerts an increasing upward force at a constant rate (4.25 g/s) with a maximum cut-off force of 50 g. The filament was placed under the plantar surface of the hind paw. Measurement was stopped when the paw was withdrawn, and results were expressed as paw withdrawal thresholds in grams.

Experimental protocol

After baseline determination of tibiotarsal joint diameter and mechanical paw withdrawal threshold (pre-MIA baseline values at 1st day), MIA was injected. These measurements were repeated 7 and 14 days later, and then the intrathecal catheterization was performed. One week later the non-paralysed rats were selected and after the post-MIA baseline value determination, the different ligands were administered (Table I). The control group received physiological saline. In the positive control group, animals were treated with 10 µg morphine. The pain thresholds were registered 10, 20, 30, 45, 60, 75, 90, 105 and 120 min after the intrathecal injection, and the mean of the values obtained between 10–30, 45–75 and 90–120 min were analyzed.
At the end of the experiment, the joint diameters were measured again. We did not examine the motor behavior systematically, nor did we quantify it, but the animals’ behaviors were observed, and in most animals there were no signs of altered behavior (immobility, flaccidity, excitation or motor weakness), except for the administration of 10 µg EMD3 which produced paralysis. Animal suffering and the number of animals per group were kept at a minimum.

Statistical analysis
Data are presented as means ± SEM. Paw withdrawal thresholds on the inflamed side were transformed to % maximum possible effect (%MPE) by the following formula:

\[
%\text{MPE} = \frac{\text{observed threshold} - \text{post-MIA baseline threshold}}{50 - \text{post-MIA baseline threshold}} \times 100.
\]

Therefore, 100% MPE means perfect relief of allodynia (equivalent to cut-off value [50 g] for all measurements), while 0% MPE means that the observed threshold is equivalent to the post-MIA baseline value. The time-course data sets were examined by two-way analysis of variance. The significance of differences between the different groups was calculated using the Fisher LSD test for post hoc comparison (p value < 0.05 was considered significant).

Area under the curve (AUC) values were obtained by calculating the area between 0 and 120 min to construct dose response curves for different doses of ligands. AUC 5500 (AUC\text{max} value) would mean the complete relief of hyperalgesia (50 g) during the whole period. We observed almost no effects regarding the AUC values after saline treatment (AUC\text{min} = 2813 ± 227). The mean AUC values were used for linear regression analysis (least square method) to determine the ED\text{25} values with 95% confidence intervals (CI). The 25% effective dose (ED\text{25}) means the dose that yielded 25 % increase in the PWD latency for the whole period (32).

The AUC data sets were examined by one-way analysis of variance (ANOVA), and the time-course curves were analyzed by repeated measurement of ANOVA. Post hoc comparisons were carried out with the Fisher’s LSD test. Statistical analysis was performed with the STATISTICA for Windows (Statistica Inc., Tulsa, Oklahoma, USA) and GraphPad Prism 4.0 (GraphPad Software Inc. La Jolla, CA, US) softwares.

Results

Joint edema
The MIA injection caused a permanent increase in joint cross-section area compared with the contralateral side (48.4 ± 0.37 vs. 38.3 ± 0.15 mm\(^2\), p < 0.01). This conspicuous increase in joint size was a result of edema formation, confirming that the MIA treatment resulted in an inflammatory reaction (1). None of the treatments influenced the degree of edema; the cross section of the ankle was 49.5 ± 0.53 mm\(^2\) at the end of the experiments, which did not differ from the post-MIA baseline value.

Mechanosensitivity
Basal mechanical withdrawal threshold was 41 ± 0.6 g, and MIA caused a significant decrease in paw withdrawal threshold on the inflamed side. This threshold was lowest after 1 week of MIA (15 ± 0.6 g), and later it stabilized at 24 ± 0.5 g. There was no significant difference between before and after intrathecal catheterization (p = 0.62), suggesting that the catheterization did not change the inflammatory pain sensitivity. MIA did not have a
significant influence on the non-inflamed side (43 ± 0.5 g). None of the treatments changed the mechanosensitivity on the non-inflamed side; therefore, results were analyzed only on the inflamed paws.

All the drugs had antinoallodynic potency therefore, we compared the effects of different doses of the analogues with EM-2. As for the lowest dose (0.3 µg), ANOVA with repeated measurements showed significant effects of time ($F_{2,104} = 9.6, \ p < 0.001$) and interaction ($F_{10,104} = 2.4, \ p < 0.05$). The post hoc comparison revealed that EMD3 and EMD4 produced significant antinociception, while EM-2 and the other two ligands were ineffective in this dose (Fig. 1).

At 3 µg, all of the ligands produced antiallodynia. ANOVA with repeated measurements showed significant effects of treatment ($F_{5,55} = 5.4, \ p < 0.001$) and time ($F_{2,110} = 5.6, \ p < 0.01$). Post hoc comparison showed that EMD3 was more effective than EM-2 during the last investigated interval (75–120 min, Fig. 2).

Regarding the highest dose, 10 µg of EMD3 caused prolonged paralysis of the animals, therefore, we could not analyze their data on the pain test. ANOVA with repeated measurements showed significant effects of treatment ($F_{5,50} = 8.4, \ p < 0.001$) and time ($F_{2,100} = 19.8, \ p < 0.001$). Post hoc comparison showed that all the drugs were effective compared to the control group at the first and second investigation period, however, only EMD4 was effective during the whole period compared to both control and EM-2 treated groups (Fig. 3). Morphine, as a positive control, produced long-lasting and highly effective antinociception. EM-2, EMD1 and EMD2 were effective as morphine only at the first investigated period (10–30 min), while the effect of EMD4 did not differ significantly from morphine during the whole period.

Fig. 1. Time-course effects of EM2 and the derivatives at 0.3 µg dose.
Each point signifies the mean ± SEM of the results. * indicates a significant ($p < 0.05$) difference as compared with the saline-treated group. # denotes a significant difference as compared with the EM-2-treated group.

Fig. 2. Time-course effects of EM2 and the derivatives at 3 µg dose. Each point signifies the mean ± SEM of the results. * indicates a significant ($p < 0.05$) difference as compared with the saline-treated group. # denotes a significant difference as compared with the EM-2-treated group.

Fig. 3. Time-course effects of EM2, morphine and the derivatives at 10 µg dose. Each point signifies the mean ± SEM of the results. * indicates a significant ($p < 0.05$) difference as compared with the saline-treated group. # denotes a significant difference as compared with the EM-2-treated group.
As for the dose-response curves of the ligands, we observed that in the case of EMD3 the steepness of the curve is higher, and the curve of EMD4 is also slightly left-shifted (Fig. 4). However, the ED25 values did not reveal significant difference between these ligands (Table I).

*Table I.* The applied drugs and their dosage, the number of animals in each group and *in vivo* potency

<table>
<thead>
<tr>
<th>Doses (µg) and number of animals</th>
<th>0</th>
<th>0.3</th>
<th>1</th>
<th>3</th>
<th>10</th>
<th>ED25 (CI) (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EM-2: Tyr-Pro-Phe-Phe-NH2</td>
<td>8</td>
<td>8</td>
<td>9</td>
<td></td>
<td></td>
<td>6.3 [1.30–10.00]</td>
</tr>
<tr>
<td>EMD1: Tyr-(1S,2R)Achc-Phe-pFPhe-NH2</td>
<td>10</td>
<td>9</td>
<td>10</td>
<td>7</td>
<td></td>
<td>5.3 [1.73–8.85]</td>
</tr>
<tr>
<td>EMD2: Tyr-(1S,2R)Achc-Phe-(2S,3S)βMePhe-NH2</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>7</td>
<td></td>
<td>7.2 [2.95–10.00]</td>
</tr>
<tr>
<td>EMD3: Dmt-(1S,2R)Achc-Phe-pFPhe-NH2</td>
<td>10</td>
<td>9</td>
<td>14</td>
<td></td>
<td></td>
<td>1.6 [1.02–2.11]</td>
</tr>
<tr>
<td>EMD4: Dmt-(1S,2R)Achc-Phe-(2S,3S)βMePhe-NH2</td>
<td>10</td>
<td>10</td>
<td>8</td>
<td></td>
<td></td>
<td>2.6 [0.31–4.81]</td>
</tr>
<tr>
<td>Morphine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>9</td>
</tr>
</tbody>
</table>

Abbreviations: ED25: 25% effective dose after intrathecal administration. CI: confidence interval.

*Fig. 4.* The dose-dependent effects of EM and derivatives (AUC values between 0–120 min)
**Discussion**

We found that new EM-2 analogues with unnatural amino acids produced dose-dependent antinociception. In agreement with the *in vitro* results, the ligands with high potency at MOR and long half-life (EMD3 and EMD4) were the most effective in the *in vivo* tests (22). To our knowledge, we are also the first to prove the antinociceptive effects of intrathecally administered morphine and EM-2 in a MIA-induced osteoarthritis model. These data are in agreement with some earlier studies showing the antinociceptive effect of systemically administered morphine in this model (5, 40).

The modification of different opioid ligands is a well-known method for the enhancement of their antinociceptive potency. An earlier study showed that replacement of Tyr\(^1\) by Dmt resulted in marked increases in receptor-binding affinity and bioactivity in numerous opioid peptide agonists and antagonists (4). Replacement of Pro\(^2\) by alicyclic β-amino acids, pseudoprolines or piperidine-2-, -3- and -4-carboxylic acids resulted in increased affinity for the MOR and enhanced proteolytic stability (7, 14, 16, 35). The insertion of pFPhe in place of the Phe\(^4\) in enkephalin or endomorphins resulted in increased potency in functional assays (22, 38). Regarding the *in vivo* antinociceptive potency of endomorphin derivatives, several studies investigated the effects of different analogues after systemic or intracerebroventricular (i.c.v.) administration in acute pain tests, while only a few studies are available on the effects of derivatives at spinal level. Furthermore, no data were available about their effects in chronic pain models. Thus, different cyclic analogues of EM-2 induced more potent and/or prolonged antinociception in the hot-plate (HP) test after i.c.v. administration in mice compared to the parent ligand (17, 27, 29). EM analogues containing D-amino acids also induced effective antinociception in mice assessed in HP or tail-flick (TF) test after i.c.v. administration (21, 28). EM analogues containing other natural (e.g. arginine) or non-natural aminoacids (e.g. phenylglycine or homophenylalanine) had more prolonged and/or more potent antinociception in acute heat pain tests after i.c.v. administration in mice (8, 41, 43).

A number of studies proved that in contrast to the parent ligands, some analogues could produce antinociception after peripheral administration, too, which suggests that these substances can pass through the blood-brain barrier (27). A few studies found that analogues of EMs could antagonise opioid-induced antinociception after i.t. or i.c.v. administration in HP or TF tests in mice (33).

EM-2 analogues containing N-methylated amino acids consecutively in each position showed the strongest analgesic effect when administered centrally in the HP test in mice (18). An earlier study showed that a dimethyl-analogue of EM-2 (Dmt\(^1\)-EM-2) produced antinociception after i.t. injection in formalin test (rat) (19). The effect evoked by Dmt\(^1\)-EM-2 was similar to the antinociceptive effect of EM-2 in the first phase but it was much stronger in the second phase. As for our results, we found that following the modification of the parent ligand at the 1\(^{\text{st}}\), 2\(^{\text{nd}}\) and 4\(^{\text{th}}\) positions by unnatural aminoacids the antinociceptive potency of these analogues remained. EMD1 and EMD2 which was modified in the 2\(^{\text{nd}}\) and 4\(^{\text{th}}\) positions had similar effects as EM-2, and this is in agreement with their \(K_i\) values for MOR, too (22). The insertion of 3 amino acids at the 1\(^{\text{st}}\), 2\(^{\text{nd}}\) and 4\(^{\text{th}}\) positions (EMD3 and EMD4) caused a considerable potency increase at MOR *in vitro*, and these ligands had also long half-life in a crude rat brain membrane homogenate (22). Therefore, the activation of the MOR and their high metabolic stability could have led to prolonged antinociception in our model.

All of the above-mentioned studies applied acute heat or chemical pain models. However, osteoarthritis, a widespread condition, affects several million in the World accompanied by chronic pain. Intra-articular injection of MIA in the joint of rats disrupts...
chondrocyte metabolism resulting in cartilage degeneration and subsequent nociceptive behavior that has been described as a model of osteoarthritis pain (3). An earlier study showed that systemic administration of morphine reversed the hind limb weight bearing decrease in this model (30). A recent study proved that MIA-induced joint pain was associated with significant changes in the spinal cord, too, that is associated with increased phosphorylation of mitogen activated protein kinases, and it was suggested that these changes were involved in nociceptive behaviors (20). Our study showed that intrathecally applied morphine, EM-2 and derivatives can decrease the MIA-induced mechanical allodynia, supporting the role of the opioid receptors in the spinal cord in this type of pain as well.

To our knowledge, our results are the first to demonstrate that complex modification of endomorphins by introduction of Dmt, alicyclic β-amino acids, βMePhe, and pFPhe in the EM-2 can induce effective and prolonged antinociception in a chronic arthritis model. It is very important that the antinociceptive effects are in agreement with the binding experiments, that is, the ligands with high potency at MOR with long half-life (EMD3 and EMD4) were the most effective in the in vivo tests, too. These structural modifications of EM-2 might be a promising strategy to enhance bioavailability of peptides and may serve a role in the development of novel endomorphin analogues with increased therapeutic potential. Further studies are required to clarify the possible side-effects of these ligands.

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