CHARACTERIZATION OF NEW ANTINOCICEPTIVE LIGANDS IN A RAT MODEL: PRECLINICAL STUDIES

Ph.D. Thesis Summery

Gyula Kovács, M.D.

University of Szeged

Faculty of Medicine

Department of Physiology

Szeged

2017

CHARACTERIZATION OF NEW ANTINOCICEPTIVE LIGANDS IN A RAT MODEL: PRECLINICAL STUDIES

Ph.D. Thesis Summery

Gyula Kovács, M. D.

University of Szeged

Faculty of Medicine

Department of Physiology

Szeged

2017

Introduction

Pain is a crucial part of our life, a vital signal about the effects which may damage our body. The International Association for Study of Pain (IASP) defines pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage" (Merskey and Bogduk 1994). Furthermore, the IASP declared in 2010 that adequate pain management is a fundamental human right, mentioned chronic pain as a separate entity, and determined pain management as an independent specialty in medical science, which requires adequate training and resources (Declaration of Montreal, 2013). Pain management, especially chronic pain, is a major public health problem, which is associated with devastating consequences to patients and families, a high rate of health care utilization, and huge society costs related to loss in work productivity.

Opioids have been regarded for millennia to be among the most effective drugs for the treatment of pain. Their use in the management of acute and chronic pain related to advanced medical illness is considered to be the standard of care in most of the world. However, long-term opioid treatment in chronic non-cancer pain continues to be controversial, but the new guidelines suggest their administration in these cases, too (Trescot et al., 2008). The incidence and severity of side effects of opioids may play an important role in the success or failure of pain management in patients. Thus, the development of new ligands with fewer side effects may increase the safety of treatments.

Three major opioid receptor families, the μ (MOR), -κ (KOR), and δ (DOR)-opioid receptors, were cloned in the early 1990s (Law and Loh 1999; Pasternak 2004) and a fourth member of the opioid receptor family, nociceptin or orphanin FQ receptor (NOP) or the opioid receptor-like orphan receptor (ORL) was added to the list in 1994 (Mollereau et al., 1994; Meunier et al., 1995). Opioid receptors are members of the superfamily of seven helix transmembrane (TM) proteins known as G-protein coupled receptors (GPCRs). Besides morphine, several ligands including endogenous peptides can activate these receptors (e.g. endorphins, enkephalins and dynorphins). The structures of traditional opioid peptides contain Tyr-Gly-Gly-Phe sequence at the N terminus. Zadina's group discovered and identified a new biologically sequence, Tyr-Pro-Trp-Phe-NH_2, in bovine brain (Zadina et al.,1977) and human

cortex (Hackler et al.,1977) (named endomorphin-1) which, showed remarkable affinity for the μ-opioid receptor and selectivity for the μ-opioid receptor over the δ- and κ-opioid receptors. The other peptide, endomorphin-2, which differs by one amino acid from endomorphin-1 (Tyr-Pro-Phe-Phe-NH_2), was also isolated. Endomorphin-2 was shown to be almost as potent as endomorphin-1 (Hackler et al., 1997; Zadina et al., 1997). A major goal in opioid peptide research is the development of novel analgesics that could substitute morphine without its well-known side effects (Olson et al., 1998). However, they have short half life time due to fast methabolization. The systematic replacement of natural amino acids by 2',6'-dimethyltyrosine (Dmt^1), 2-aminocyclohexanecarboxylic acid [cis-(1S,2R)$Achc^2$/cis-(1R,2S)$Achc^2$], β-methylphenylalanine [(2R,3R)β$MePhe^4$/(2S,3S)β$MePhe^4$] and para-fluorophenylalanine ($pFPhe^4$) in different positions resulted in proteolytically stable compounds with high MOR affinity in some cases (Mallareddy et al., 2011). Thus, it was found that the analogues carrying Dmt^1 and $Achc^2$ residues displayed the highest MOR affinities, depending upon the configuration of the incorporated $Achc^2$. Combination of such derivatives with $pFPhe^4$ or β$MePhe^4$ yielded compounds with high binding potency, while their efficacy did not differ from the parent ligand.

Cannabis are also widely used to treat pain for many centuries, and interest in cannabis–based medicines has also emerged, recently. After the discovery of cannabinoid receptors (CB1 and CB2), which are members of the superfamily of G protein-coupled receptors (GPCRs) (Howlett et al., 2002), the next step was the detection of endogenous ligands. Arachidonoyl ethanolamide (anandamide, AEA) was the first brain metabolite shown to act as a ligand of CB1 cannabinoid receptors (Devane et al., 1992) and then, it was followed by 2-arachidonoylgycerol (2-AG) extracted from the canine gut and later from the brain (Mechoulam et al., 1995; Stella et al., 1997).

AEA binds to the CB1 receptor and evokes agonist activity. There is evidence that AEA also binds to the CB2 receptor, although it does not evoke CB2 receptor-mediated effects to a biologically significant degree (Devane et al., 1992; Felder et al., 1995; DiMarzo and Deutsch 1998). However, anandamide activates other receptors as well, including the capsaicin-sensitive transient receptor potential vanilloid 1 channels (TRPV1), and some of its effects (like antinociception) may be at least partially due to TRPV1 activation (Zygmunt et al., 1999; Di Marzo et al., 2002; Horvath et al., 2008). It is known that 2-AG is a full agonist at the CB1 receptor, albeit it has a relatively low binding affinity, and it has been suggested

that it is the optimal known candidate as the natural ligand at the CB2 receptor (Sugiura et al., 2000), but there is no direct binding to the TRPV1 receptor (Mechoulam et al.,1995).

The antinociceptive action of plant originated and synthetic cannabinoids has been investigated widely in inflammatory pain after peripheral (Richardson et al., 1998; Hargraeves et al., 1998), spinal (Hohmann et al., 1998; Drew et al., 2000) and intracerebral administration (Lichtman et al., 1996; Martin et al., 1999). The antinociceptive doses of cannabinoids have frequently been accompanied by side effects which limited the use for treatment of chronic pain states. One alternative strategy might be to develop selective CB1 receptor agonists that do not penetrate the blood–brain barrier, thereby decrease the side effects. Another option is to develop peripherally acting selective inhibitors of endocannabinoids metabolism to elevate the level of endocannabinoids, and so would increase the activation of both CB1 and CB2 receptors (Kathuria et al., 2003).

Hemopressin (HP), a nonapeptide (H-Pro-Val-Asn-Phe-Lys-Leu-Leu-Ser-His-OH) is a product of the hemoglobin α chain, discovered in rat brain, and so named because it can cause small decreases in blood pressure (Rioli et. al., 2003; Lippton et al., 2006). A number of in vitro studies show that HP acts as a CB1 receptor inverse agonist, and it can act on both peripheral and central pain pathways in vivo (Heimann et al. 2007; Dodd et al., 2010). These studies showed that HP pretreatment caused antinociceptive effects at systemic, local, spinal and cerebral levels.

Aims of our experiments were

- To investigate the antiallodynic effects of new EM-2 derivatives (EMD1: Tyr-(1S,2R)Ache-Phe-pFPhe-NH2, EMD2: Tyr-(1S,2R)Ache-Phe-(2S,3S)βMePhe-NH2, EMD3: Dmt-(1S,2R)Ache-Phe-pFPhe-NH2, EMD4: Dmt-(1S,2R)Ache-Phe-(2S,3S)βMePhe-NH2) at spinal level, in a chronic pain model.

- To compare the dose-depend effect the EM2 and different EM2 derivates with morphine as a gold standard in joint inflammation model at spinal level.

- To characterize the antinociceptive potency of anandamide and 2 AG, applied intrathecally in acute joint inflammation model.

- To determine the effects of hemopressin on the mechanical pain threshold in acute joint inflammation model at spinal level.

- To describe the influence of the intrathecal administration of synthetic CB1 and CB2 antagonists and hemopressin on the effects of 2-AG in acute joint inflammation model.

Materials and Methods

Animals and drugs

After institutional ethical approval had been obtained (Institutional Animal Care Committee of the Faculty of Medicine at the University of Szeged), male Wistar rats were used.
The following drugs were used: ketamine hydrochloride, xylazine hydrochloride, Gentamycin, monosodium iodoacetate (MIA), λ-carrageenan, morphine hydrochloride, 2-arachidoylglycerol (2-AG) and AM 251 (CB1 receptor antagonist), SSR144528-2 (CB_2 receptor antagonist), EM-2 and its derivatives (EMD1: Tyr-(1S,2R)Ache-Phe-pFPhe-NH2, EMD2: Tyr-(1S,2R)Ache-Phe-(2S,3S)βMePhe-NH2, EMD3: Dmt-(1S,2R)Ache-Phe-pFPhe-NH2, EMD4: Dmt-(1S,2R)Ache-Phe-(2S,3S)βPhe-NH2), dimethylsulfoxide (DMSO), ethanol and hemopressin.

Experiments

Intrathecal catheterization

Rats were anesthetized with a mixture of ketamine hydrochloride and xylazine 72 and 8 mg/kg intraperitoneally (i.p.) respectively. An intrathecal (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 (Yaks and Rudy 1976), which served to place the catheter tip between the T12 and L2 vertebrae, corresponding to the spinal segments that innervate the hindpaws (Dobos et al., 2003).

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 (Dobos et al., 2003). 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.

Induction of inflammation

Series 1

Intra-articular injection of MIA in the joint of rats disrupts chondrocyte glycolysis through the inhibition of glyceraldehyde-3-phosphate dehydrogenase, resulting in cartilage degeneration and subsequent nociceptive behavior that has been described as a model of osteoarthritis (OA) pain (Bove, 2010). Osteoarthritis was induced by injecting MIA (1 mg/30μL) into the tibiotarsal joint of the right hindleg on two consecutive days.

Series 2

The inflammation was elicited by injecting λ-carrageenan (carrageenan is a family of linear sulphated polysaccharides that are extracted from red edible seaweeds) (300 μg/30μL) into one of the tibiotarsal joints (on the paralyzed side during lidocaine administration) (Dobos et al., 2003; Mecs et al., 2009). In both experiments, to determine the changes in the size of the inflamed joint, the anteroposterior and mediolateral diameters of the paw were measured at the level of the ankle joint with a digital caliper.

Behavioral nociceptive testing

The threshold for withdrawal from mechanical stimulation to the plantar aspect of the hindpaws was assessed using a dynamic plantar aesthesiometer (Ugo Basile, Comerio, Italy), which consists of an elevated wire mesh platform to allow access to the ventral surface of the hindpaws. The maximum cut-off force was 50 g over an 8 s period.

Experimental protocols

Series 1

After baseline determination of the tibiotarsal joint diameter and mechanical paw withdrawal (PWD) threshold (pre-MIA baseline values on Day 1), MIA was injected. These measurements were repeated 7 and 14 days later, and then, the i.t. catheterization was performed. One week later, the post-MIA baseline values were determined, the EM2 and the different analogs (0.3, 1, 3 and 10 μg) were administered.

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, 70, 90 and 120 min after the i.t. injection. The mean of the values obtained between 10–30, 45–70 and 90–120 min were analyzed.

Series 2

After baseline determination of the joint diameter and mechanical PWD threshold (pre-carrageenan baseline value at -180 min), carrageenan was injected. These measurements were carried out again 3 hours after carrageenan injection (post-carrageenan baseline values at 0 min), and then HP (0.3–30 μg), 2-AG (1–200 μg) or anandamide (10–200 μg) was given i.t. and mechanical sensitivity was defined at 10, 20, 30, 45, 60, 75, 90 and 105 min post-administration. The control group received physiological saline (vehicle of HP) or vehicle of 2-AG/anandamide.

Since vehicle-treated groups did not differ from the saline-treated one, we merged the data of these animals. To determine the involvement of CB_1 and CB_2 receptors in the effects of 2-AG, separate groups of animals were pretreated with AM 251 (antagonist of CB_1 receptors, 10 μg) or SSR144528-2 (antagonist of CB_2 receptors, 15 μg) 20 min before 200 μg 2-AG injection. The control group was injected with vehicles of 2-AG and CB-antagonists. To investigate the potential antagonistic effects of HP on the 2-AG induced antinociception, we co-administered 3 or 30 μg HP with 200 μg 2-AG.

Statistical analysis

Data are presented as means ± SEM. Data sets were examined by one way or repeated measures of ANOVA. *Post hoc* comparisons were carried out with the Fisher LSD test. A *p* value lower than 0.05 was considered significant. Data analyses were performed with the STATISTICA (Statistica Inc., Tulsa, Oklahoma, USA) software.

Results

Joint edema

MIA injection caused permanent but moderate increase in joint cross-section area compared with the contralateral side (48.4±0.37 mm^2 vs 38.3±0.15 mm^2, $p<0.01$).
3 hours after the injection of carrageenan into the ankle, there was significant increase in joint cross-section area compared with pre-injection control levels from 36 mm^2±0.1 to 73±0.5 mm^2, ($p<0.01$)).
None of the treatments influenced the degree of oedema (series1 and series 2).

Mechanosensitivity

Series 1

Basal PWD threshold was 41±0.6 g, and MIA caused significant decrease in PWD threshold on the injected side. This threshold was lowest 1 week after MIA (15±0.6 g), and later it stabilized at 24±0.5 g.
MIA did not have 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 applied drugs had antinociceptive potency; therefore, we compared the effects of different doses of the analogues with parent 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. 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). Regarding the highest dose (10 μg), 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 periods; however, EMD4 was effective during the whole period compared to both control and EM-2 treated groups. Morphine, as a positive control, produced long-lasting and highly effective antinociception. EM-2, EMD1 and EMD2 were as effective as morphine only in the first investigated phase (10–30 min), while the effect of EMD4 did not differ significantly from morphine during the whole session.

Series 2

The basal mechanical PWD threshold was 45±0.4 g, and carrageenan caused significant decrease in PWD threshold on the inflamed side (10±0.3 g), but it did not have a significant influence on the non-inflamed side. None of the treatments changed the mechanosensitivity on the normal side; therefore, results were analyzed only on the inflamed paws.

Neither did HP cause significant antiallodynic effect compared to the control group, nor were any motor impairments observed in this wide dose-range (0.3–30 μg).

2-AG by itself produced a dose-dependent antiallodynic effect, which developed gradually, and it reached a maximum between 45 and 60 min. ANOVA with repeated measures showed significant effects of treatment ($F_{4,48}$=4.7, p<0.005) and time ($F_{9,432}$=94.3, p<0.001). Thus, 1 μg 2-AG was ineffective, while 200 μg caused a prolonged antinociceptive effect.

Anandamide elicited a dose-dependent antinociceptive effect, which reached a maximum approximately at 20 min post-administration. ANOVA with repeated measures showed significant effects of treatment ($F_{4,47}$=5.2, p<0.005), time ($F_{9,423}$=68.5, p<0.001), and interaction ($F_{36,423}$=1.9, p<0.005). Thus, 10 μg anandamide was ineffective, while 200 μg caused a prolonged effect. Regarding the effects of antagonists AM 251 and SSR144528-2 at CB_1 and CB_2 receptors, respectively, none of the substances influenced the pain threshold in themselves. AM 251 pretreatment antagonized the antiallodynic effect of 2-AG (200 μg), while SSR144528-2 did not influence it.

Co-treatment of 3 μg or 30 μg HP with 200 μg 2-AG significantly decreased the antinociceptive effect of 2-AG.

Discussion

Series 1

Regarding the in vivo antinociceptive potency of different endomorphin derivatives, several studies have investigated the effects of the ligands 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 have been 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 (Kruszynsk et al., 2005; Perlikowska et al., 2009; and 2010). 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 (Perlikowska et al., 2010). 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 (Gao et al., 2006; Yu et al., 2007; Wang, et al., 2011). A number of studies proved that in contrast to the parent ligands, some analogues can produce antinociception after peripheral administration, too, which suggests that these substances can pass through the blood–brain barrier (Hau et al., 2002; Kruszynski et al., 2005; Shi et al., 2007; Bedini et al., 2010; Perlikowska et al., 2010; Wang, et al., 2011). A few studies have found that analogues of EMs can antagonize opioid-induced antinociception after i.t. or i.c.v. administration in Hp or TF tests in mice (Sakurada et al., 2002; Fichna et al., 2005; Kruszynski et al., 2005; Mizoguchi et al., 2006).

EM-2 analogs containing N-methylated amino acids consecutively in each position showed the strongest analgesic effect when administered centrally in the Hp test in mice (Kruszynski et al., 2005). An earlier study showed that a dimethyl-analogue of EM-2 (Dmt^1-EM-2) produced antinociception after i.t. injection in formalin test (rats) (Labuz et al., 2003). The effect evoked by Dmt^1-EM-2 was similar to 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 EMD1 and EMD2 had similar effects as EM-2, and this is in agreement with their K_i values for MOR, too (Mallaredy et al., 2011). EMD3 and EMD4 showed high potency to the MOR in vitro,

and these ligands had also long half-life in a crude rat brain membrane homogenate (Mallaredy et al., 2011). 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 patients in the world accompanied by chronic pain. An earlier study showed that the systemic administration of morphine reversed the hindlimb weight bearing decrease in this model (Pomonis et al., 2005). A recent study proved that MIA-induced joint pain is associated with significant changes in the spinal cord, too (Le et al., 2011). Our study showed that i.t. 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.

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.

To our knowledge, our results have been the first to demonstrate that complex modification of endomorphins by the 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. This structural modification 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.

Series 2

Spinally administered anandamide and 2-AG significantly decreased the mechanical inflammatory pain sensitivity. The use of cannabinoids for the management of a wide range of painful disorders has been well documented at spinal, supraspinal, and peripheral levels (Hohmann et al., 2002; Guindon et al., 2007), while data about the endogenous ligands are scarce, especially at spinal level. Earlier studies have shown that intrathecal anandamide decreases the acute heat pain sensitivity (in Hp and TF tests) and the carrageenan-induced thermal hyperalgesia in rodents, and that both the CB_1 and TRPV1 receptors play a role in these effects (Yaksh et al., 2006; Horvath et al., 2008; Tuboly et al., 2009). To our knowledge, we have been the first to offer evidence to suggest that anandamide inhibits mechanical allodynia at the spinal level as well. Since several systems may be influenced by

anandamide (e.g., CB-, TRPV1- glycine and serotonin-3 receptors), their net effect may be observed under these circumstances (Hajos et al., 2001; Oz et al., 2002, 2004; Kim et al., 2005; Lozovaya et al., 2005; Hejazi et al., 2006). As the high dose of anandamide caused temporary pain, the desensitization of TRPV1 receptors can also be involved in its antinociceptive effect, as suggested earlier (van der Stelt and Di Marzo, 2005; Horvath et al., 2008). Therefore, it is possible that alterations in the release of excitatory and inhibitory transmitters can modify the activation of projection neurons, either pre-synaptically from primary sensory neurons or post-synaptically from interneurons, or both.

2-AG, similarly to anandamide, reduced allodynia in the carrageenan-induced arthritis model, and its antinociceptive effect was inhibited by a CB1 antagonist, while it was not influenced by a CB2 antagonist. This is the most abundant endogenous cannabinoid, and its concentration in the brain is 50–500 times as high as that of anandamide. It has also been identified peripherally (Kondo et al., 1998; Agarwal et al, 2007,). 2-AG is a full agonist for CB1 and CB2 receptors with no direct binding to the TRPV1 receptor (Mechoulam et al., 1995). There is only little evidence to support the antinociceptive potency of 2-AG. Endogenous 2-AG has been implicated as a major transmitter involved in endocannabinoid-mediated stress-induced analgesia (Hohmann, 2005; Suplita, 2006). Thus, 2-AG, but not anandamide, is mobilized in the lumbar spinal cord following exposure to footshock stress, and spinal 2-AG levels show marked correlation with stress-induced antinociception (Suplita et al., 2006; Hohmann and Suplita 2008). Additionally, i.t. administration of an inhibitor of the 2-AG hydrolyzing enzyme, monoacylglycerol lipase, enhances stress-induced antinociception in a CB1-dependent manner (Suplita et al., 2006). In systemic administration to mice, 2-AG (ED_{50}=12.5 mg/kg) has caused antinociception in acute pain tests, immobility, reduction of spontaneous activity, and lowering of rectal temperature (Mechoulam et al., 1995; Ben Shabat et al., 1998). Topical administration of 2-AG has also decreased the nocifensive behavior in a formalin test, decreased mechanical allodynia and thermal hyperalgesia in a neuropathic pain model, and it has also been effective in the alleviation of inflammatory joint pain (Guindon et al., 2007; Desroches et al., 2008; Mecs et al, 2010). The local antinociceptive effects of 2-AG have been prevented by CB1 and/or CB2 antagonists (Guindon et al., 2007; Desroches et al., 2008; Mecs et al., 2010). As far as the spinal level is concerned, we have been the first to show its antinociceptive potency, and that the effect is reversed by a CB1 antagonist drug (but not by a CB2 antagonist), suggesting that the antiallodynic effect of 2-AG is mainly due to the activation of CB1 receptors at spinal level.

CB1 receptors, the molecular targets of 2-AG, are located on primary afferent fiber endings and/or on intrinsic interneurons in the dorsal horn of the spinal cord (Nyilas et al., 2009; Hegyi et al., 2009); therefore, their activation could lead to the observed antinociception.
It is important to consider that these ligands can influence the activity of neurons in the dorsal root ganglia (DRG), too, since the cannabinoid receptors can be found on DRG neurons (Bridges et al., 2003; Sagar et al., 2005), and it has been shown that i.t. injection of sodium fluorescein results in massive staining in the DRG both in the cellular and fiber portions (Abram, 2006). As for the ineffectivity of CB1 and CB2 antagonists on inflamed and on the non-inflamed sides in themselves, a number of scenarios may be suggested. First, it might be supposed that the mechanical pain threshold after carrageenan administration (~10-15 g) is a very low value, which could not be further decreased by an antagonist. However, the threshold on the normal side did not change either; therefore, this is not likely. Another possibility is that the endogenously released cannabinoids have no significant inhibitory effect on the mechanical threshold in inflammatory circumstances, either on the normal, or on the inflamed side. Similar results have been found in a bone cancer-induced pain model (Curto-Reyes et al., 2010); however, other studies have shown that i.t. injection of CB1 receptor antagonists can evoke nociceptive responses (Chapman, 1999; Lever and Malcangio, 2002). It is assumed that the differences in the pain models can lead to these controversial findings. However, the level of the released endogenous cannabinoids was not determined in our study; therefore, it cannot be decided whether this is due to the lack of production or the lack of effect of endogenous cannabinoid agonists.

So far only a few studies have investigated the in vivo and in vitro characteristics of HP. Conformation-state sensitive antibodies have been used for the investigation of binding characteristics of HP to different opioid, cannabinoid, adrenergic, bradykinin and angiotensin receptors in cell-lines and striatum (Heimann et al., 2007). It has been found that HP is an inverse agonist of CB_1 receptors; thus, HP is able to block the constitutive activity of CB_1 but not CB_2 receptors (Heimann et al., 2007). Regarding the antinociceptive potency of HP, Dale et al. have found that intraplantarly administered HP (0.1–20 μg) did not affect the paw pressure threshold in the non-inflamed paws, but co-treatment with carrageenan or bradykinin significantly decreased the development of mechanical allodynia, as measured with the paw pressure test, and the effect was not inhibited by an opioid antagonist (Dale et al.,2005). Since the contralaterally administered HP was also effective in this respect, the data suggest systemic effects of the ligand. Orally (50 or 100 μg/kg) or i.t. (0.5 or 5 μg) administered HP

pretreatments were also effective in the same test (Heimann et al., 2007). Intraperitoneally administered HP (50 or 500 μg/kg) exhibited marked antinociceptive potency in the acetic acid-induced visceral nociception model. This high dose of HP did not impair motor activity or alter pentobarbital-induced sleeping time, indicating the absence of unwanted sedative or motor side-effects. Unfortunately, we did not observe similar antinociceptive effects in our model. It is possible that the controversial results might be due to differences in the timing of the administration. That is, we applied HP after that mechanical allodynia had been established (post-treatment), while earlier studies prevented the development of the hyperalgesia (pre-treatment). Furthermore, there were differences either regarding the applied pain test (paw pressure vs. von-Frey) or the site of administration of carrageenan (intraplantar vs intra-joint administration). In agreement with our results, the latest evidence suggests inefficacy of HP at spinal level in an acute heat pain test and in a neuropathic pain model (Hama and Sagen, 2011; Hama and Segan 2011). The authors have observed the inefficacy of HP as an antagonist after CB1 receptor activation. This is in contrast with our results, since HP, similarly to the synthetic CB_1 antagonist, antagonized the antinociceptive effect of 2-AG in our study. We suppose that the differences in the pain models and the applied cannabinoid ligand (WIN 55,212-2 vs 2-AG) might be the explanation of the different results.

In conclusion, we found that HP was not capable of influencing the established mechanical allodynia in a model of arthritic pain, but it inhibited the antinociceptive effects of 2-AG at spinal level. Furthermore, these findings are the first to demonstrate the antinociceptive potency of 2-AG at spinal level, and to report on the effect of anandamide on mechanical allodynia in an arthritic pain model.

General conclusions

- It has been verified that four different derivatives of EM2 have similar in vivo potency to the original ligand in the osteoarthritic pain model.

- Effects of EMD3 and EMD4 were more prolonged suggesting long-lasting stability and high affinity to MOR, in vivo.

- Compared to morphine, which is a long-lasting pain analgetic, the EM2, EMD1 and EMD3 were efficient only in the first period (10–30 min), while the EMD4 was effective all along the period.

- Spinally administered anandamide and 2-AG significantly decreased mechanical inflammatory pain sensitivity.

- We proved that the antinociceptive potency of 2-AG was inhibited by a CB1 antagonist drug (but not by CB2 antagonist ligand), suggesting that the antiallodynic effect of 2-AG is mainly due to the activation of CB1 receptors at spinal level.

- Intrathecal administration of hemopressin was not capable of influencing the mechanical allodynia in a wide dose–range in the model of arthritic pain, but it inhibited the antinociceptive effects of 2-AG at spinal level.

Acknowledgement

I would like to express my special gratitude to my advisor, Prof. Dr. Gyongyi Horvath, who has always supported my work and put her faith in me and urged my to do better. I am thankful to her for giving the right advice at the right time, and for being a source of motivation. She encouraged and taught me throughout my Ph.D.

I would also like to thanks to Prof. Gabor Jancso for making it possible to work in the neuroscience program.

I am also hugely appreciative to co-workers of the Department of Physiology, especially to Zita Petrovszki, Gabrialla Kekes, Agnes Abraham-Tandari for being so generous with their time and energy, and for sharing their suggestions and experiences, and for being ensured me the scientific backgroud.

I owe my special thanks to my previous teacher, Prof. Dr. Kalman Toth who has raised my attention and introduced me into the world of science. He was always willing to help and give his best suggestions to my thesis and life as a good friend.

I am grateful to my friends and co-workers at the Department of Orthopedics for their help and encouragement.

Finally, but by no means least, tanks to my parents and my family (my wife and children) for the solid and peaceful background in private life. They are the most important people in my world and I dedicate this thesis to them.

Publications

Original publications and presentations the present work is based on:

I. Kovacs G, Petrovszki Z, Toth G, Mallareddy JR, Benedek G, Horvath G.

Characterization of antinociceptive potencies of endomorphin – 2 derivatives.

Acta Physiologica Hungarica 2012;99: 353-63. **IF: 0.821, citation 1**

II. Petrovszki Z, Kovacs G, Tomboly C, Benedek G, Horvath G.

The effects of peptide and lipid endocannabionids on arthritic pain at spinal level.

Anasthesia and Analgesia 2012;114: 1346-52. **IF: 3,274, citation 4**

Other publications

I. Kovács Gy, Tóth K.
Hátfájás – ami mindenkinek van I. rész. Praxis 1999;8: 37-44.

II. Kovács Gy, Tóth K.
Hátfájás – ami mindenkinek van II. rész. Praxis 1999;8: 43-6.

III. Kovács Gy, Tóth K.
Hátfájás - ami mindenkinek van. Nővérpraxis 1999;2: 23-9.

IV. Kovács Gy, Tóth K.
Csigolyaközti fúzió hengeres titán cage alkalmazásával.
Magyar Traumatológia Ortopédia Kézsebészet és Plasztikai Sebészet
2003;46: 204-9. **citation 1**

V. Nagy E, Tóth K, Janositz G, Kiss A, Kovács Gy, Horváth Gy.
Az ironmen triatlon hatása a testtartás kontrollra. Magyar Sporttudományi Szemle 2004; 2-3: 43-6.

VI. Nagy E, Toth K, Janositz, G, Kovacs G, Kiss A, Horvath Gy, Angyan L.
Postural controll in athletes participating in an ironmen triatlon. European Journal of Applied Physiology 2004;92: 407-13. **IF: 1. 332, citation 58**

VII. Tóth K, Janositz G, Kovács Gy.
Cement nélküli vápával végzett revíziók középtávú tapasztalatai. Magyar Traumatológia Ortopédia Kézsebészet Plasztikai Sebészet 2009;52: 241-8.

VIII. Toth K, Janositz G, Kovacs Gy, Sisak K.
Midterm results of up revisions using uncemneted acetabular components. Revista De Ortopedie Si Traumatologie 2010;1: 1-7.

IX. Toth K, Janositz G, Kovacs Gy, Sisak K, Rudner E.
Successful treatment of late Salmonella infections into total hip replacement-report of two cases. BMC Infections Diseases 2010;10: 160. **IF: 2.825, citation 10**

X. Klára T, Janositz G, Kovács Gy, Csönge L, Csernátony Z, Lacza Zs.
Humán albuminnal kezelt liofilizált strukturális allograftokkal szerzett sebészi tapasztalatok. Magyar Traumatológia Ortopédia Kézsebészet Plasztikai Sebészet 2012;55: 251-58.

XI. Klara T, Janositz G, Kovacs G, Csonge L, Csernatony Z, Lacza Zs.
Albumin coated structural lyophilized bone graft: A clinical report of 10 cases. Cell and Tissue Banking 2014:15: 89–97 **IF: 0,965, citation 8**

XII. Kovács Gyula
Felnöttek könyökfájdalmának kivizsgálása. Orvostovábbképző Szemle. 2015;11: 68-72.

References

Abram SE, Yi J, Fuchs A, Hogan QH. Permeability of injured and intact peripheral nerves and dorsal root ganglia. Anesthesiology 2006;105: 146-53.

Agarwal N, Pacher P, Tegeder I, Amaya F, Constantin CE, Brenner GJ, et al. Cannabinoids mediate analgesia largely via peripheral type 1 cannabinoid receptors in nociceptors. Nature Neuroscience 2007;10: 870-9.

Bedini A, Baiula M, Gentilucci L, Tolomelli A, De Marco R, Spampinato S. Peripheral antinociceptive effects of the cyclic endomorphin-1 analog c[YpwFG] in a mouse visceral pain model. Peptides 2010;31: 2135-40.

Ben Shabat S, Fride E, Sheskin T, Tamiri T, Rhee MH, Vogel Z, et al. An entourage effect: Inactive endogenous fatty acid glycerol esters enhance 2-arachidonoyl-glycerol cannabinoid activity. Nature Neuroscience 1998;353: 23-31.

Blais PA, Cote J, Morin J, Larouche A, Gendron G, Fortier A, et al. Hypotensive effects of hemopressin and bradykinin in rabbits, rats and mice: A comparative study. Peptides 2005;26: 1317-22.

Bove SE, Calcaterra SL, Brooker RM, Huber CM, Guzman RE, Juneau PL, et al. Weight bearing as a measure of disease progression and efficacy of anti-inflammatory compounds in a model of monosodium iodoacetate-induced osteoarthritis. Osteoarthritis Cartilage 2003;11: 821-80.

Bridges D, Rice ASC, Egertova M, Elphick MR, Winter J, Michael GJ. Localisation of cannabinoid receptor 1 in rat dorsal root ganglion using in situ hybridisation and immunohistochemistry. Neuroscience 2003;119: 803-12.

Bryant SD, Jinsmaa Y, Salvadori S, Okada Y, Lazarus LH. Dmt and opioid peptides: A potent alliance. Biopolymers 2003;71: 86-102.

Chapman V. The cannabinoid CB1 receptor antagonist, SR141716A, selectively facilitates nociceptive responses of dorsal horn neurones in the rat. British Journal of Pharmacology 1999;127:1 765-7.

Curto-Reyes V, Llames S, Hidalgo A, Menendez L, Baamonde A. Spinal and peripheral analgesic effects of the CB2 cannabinoid receptor agonist AM1241 in two models of bone cancer-induced pain. British Journal of Pharmacology 2010;160: 561-73.

Dale CS, Pagano RdL, Rioli V, Hyslop S, Giorgi R, Ferro ES. Antinociceptive action of hemopressin in experimental hyperalgesia. Peptides 2005;26: 431-6.

Decleration of Montreal 2013
www.iasp-pain.org/Content/NavigationMenu/Advocacy/DeclarationofMontr233al/default.htm

Desroches J, Guindon J, Lambert C, Beaulieu P. Modulation of the anti-nociceptive effects of 2-arachidonoyl glycerol by peripherally administered FAAH and MGL inhibitors in a neuropathic pain model. British Journal of Pharmacology 2008;155: 913-24.

Devane WA, Hanus L, Breuer A, Pertwee RG, Stevenson LA, Griffin G, et al. Isolation and structure of a brain constituent that binds to the cannabinoid receptor. Science 1992;258: 1946-19.

Di Marzo V, Deutsch DG. Biochemistry of the endogenous ligands of cannabinoid receptors. Neurobiology of Disease 1998;5: 386-404 .

Di Marzo V, Blumberg PM, Szallasi A. Endovanilloid signaling in pain. Current Opinion in Neurobiology 2002;12: 372-9.

Dobos I, Toth K, Kekesi G, Joo G, Csullog E, Klimscha W, et al.. The significance of intrathecal catheter location in rats. Anesthesia and Analgesia 2003;96: 487-92.

Dodd GT, Mancini G, Lutz B, Luckman SM. The peptide hemopressin acts through CB1 cannabinoid receptors to reduce food intake in rats and mice. Journal of Neuroscience 2010;30: 7369-76.

Drew LJ, Harris J, Millns PJ, Kendall DA, Chapman V. Activation of spinal cannabinoid 1 receptors inhibits C-fibre driven hyperexcitable neuronal responses and increases GTPγS binding in the dorsal horn of the spinal cord of non-inflamed and inflamed rats. European Journal of Neuroscience 2000; 12: 2079-86.

Felder CC, Joyce KE, Briley EM. Comparison of the pharmacology and signal transduction of the human cannabinoid CB1 and CB2 receptors. Molecular Pharmacology 1995;48: 443-50.

Fichna J, do-Rego JC, Kosson P, Costentin J, Janecka A. Characterization of antinociceptive activity of novel endomorphin-2 and morphiceptin analogs modified in the third position. Biochemical Pharmacology 2005;69: 179-15.

Fichna J, Gach K, Perlikowska R, Cravezic A, Bonnet JJ, et al. Novel endomorphin analogues with antagonist activity at the mu-opioid receptor in the gastrointestinal tract. Regulatory Peptides 2010;162: 109-14.

Gao Y, Liu X, Liu W, Qi Y, Liu X, Zhou Y, Wang R. Opioid receptor binding and antinociceptive activity of the analogues of endomorphin-2 and morphiceptin with phenylalanine mimics in the position 3 or 4. Bioorganic and Medical Chemistry Letters 2016;16: 3688-92.

Guindon J, Desroches J, Beaulieu P. The antinociceptive effects of intraplantar injections of 2-arachidonoyl glycerol are mediated by cannabinoid CB2 receptors. British Journal of Pharmacology 2007;150: 693-01.

Guindon J, Hohmann AG. Cannabinoid CB2 receptors: A therapeutic target for the treatment of inflammatory and neuropathic pain. British Journal of Pharmacology 2007;153: 319-34.

Hackler L, Zadina JE, Ge LJ, Kastin AJ. Isolation of relatively large amounts of endomorphin-1 and endomorphin-2 from human brain cortex. Peptides 1997;18**:** 1635-9.

Hama A, Segan J. Activation of spinal and supraspinal cannabinoid-1 receptors leads to antinociception in a rat model of neuropathic spinal cord injury pain. Brain Research 2011;1412: 44-54.

Hama A, Sagen J. Centrally mediated antinociceptive effects of cannabinoid receptor ligands in rat models of nociception. Pharmacology Biochemistry and Behavior 2011;100: 340-6.

Hajos N, Ledent C, Freund T. Novel cannabinoid-sensitive receptor mediates inhibition of glutamatergic synaptic transmission in the hippocampus. Neuroscience 2001;106: 1-4.

Hargreaves KM, Dubner R, Brown F, Flores C, Joris J. A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. Pain 1988;32: 77-88.

Hau VS, Huber JD, Campos CR, Lipkowski AW, Misicka A, Davis TP. Effect of guanidino modification and proline substitution on the in vitro stability and blood-brain barrier permeability of endomorphin II. Journal of Pharmacology 2002;91: 2140-9.

Heimann AS, Gomes L, Dale CS, Pagano RL, Gupta A, de Souza LL, et al. Hemopressin is an inverse agonist of CB1 cannabinoid receptors. Proceedings of the National of Sciences of the USA 2007;104: 20588-93.

Hejazi N, Zhou C, Oz M, Sun H, Ye JH, Zhang L. Delta9-tetrahydrocannabinol and endogenous cannabinoid anandamide directly potentiate the function of glycine receptors. Molecular Pharmacology 2006;69: 991-7.

Hegyi Z, Kis G, Hollo K, Ledent C, Antal M. Neuronal and glial localization of the cannabinoid-1 receptor in the superficial spinal dorsal horn of the rodent spinal cord. European Journal of Neuroscience 2009;30: 251-62.

Hohmann AG, Tsou K, Walker JM. Cannabinoid modulation of wide dynamic range neurons in the lumbar dorsal horn of the rat by spinally administered WIN55,212-2. Neuroscience Letters 1998;257: 119-22.

Hohmann AG. Spinal and peripheral mechanisms of cannabinoid antinociception: behavioral, neurophysiological and neuroanatomical perspectives. Chemistry and Physics Lipids 2002;121: 173-90.

Hohmann AG, Suplita RL. Endocannabinoid mechanisms of pain modulation. American Association of Pharmaceutical Scientist Journal 2006;8: 693-08.

Horvath G, Kekesi G, Nagy E, Benedek G. The role of TRPV1 receptors in the antinociceptive effect of anandamide at spinal level. Pain 2008;134: 277-84.

Howlett AC, Barth F, Bonner TI, Cabral G, Casellas P, Devane WA. International union of pharmacology. XXVII. Classification of cannabinoid receptors. Pharmacological Reviews 2002;54: 161-02.

Hruby VJ, Li GG, Haskell-Luevano C, Shenderovich M. Design of peptides, proteins, and peptidomimetics in chi space. Biopolymers 1997;43: 219-66.

Janecka A, Fichna J, Janecki T. Opioid receptors and their ligand. Current Topics in Medical Chemistry 2004;4: 1-17.

Kathuria S, Gaetani S, Fegley D. Modulation of anxiety through blockade of anandamide hydrolysis. Nature Medicine 2003;9: 76-81.

Keller M, Boissard C, Patiny L, Chung NN, Lemieux C, Mutter M, Schiller PW. Pseudoproline-containing analogues of morphiceptin and endomorphin-2: Evidence for a cis Tyr-Pro amide bond in the bioactive conformation. Journal of Medical Chemistry 2001;44: 3896-03.

Keresztes A, Szucs M, Borics A, Kover KE, Forro E, Fulop F, et al. New endomorphin analogues containing alicyclic beta-amino acids: Influence on bioactive conformation and pharmacological profile. Journal of Medical Chemistry 2008;51: 4270-9.

Kim HI, Kim TH, Shin YK, Lee CS, Park M, Song JH. Anandamide suppression of Na^+ currents in rat dorsal root ganglion neurons. Brain Research 2005;062: 39-47.

Kondo S, Kondo H, Nakane S, Kodaka T, Tokumura A, Waku K, et al. 2-arachidonoylglycerol, an endogenous cannabinoid receptor agonist: Identification as one of the major species of monoacylglycerols in various rat tissues, and evidence for its generation through Ca^{2+}-dependent and -independent mechanisms. FEBS Letters 1998;429: 152-6.

Kozak KR, Rowlinson SW, Marnett LJ. Oxygenation of the endocannabinoid, 2-arachidonylglycerol, to glyceryl prostaglandins by cyclooxygenase-2. Journal of Biological Chemistry 2000;275: 33744-9.

Kruszynski R, Fichna J, Do-Rego JC, Chung NN, Schiller PW, Kosson P, et al. Novel endomorphin-2 analogs with mu-opioid receptor antagonist activity. Journal of Peptide Research 2005;66: 125-31.

Labuz D, Chocyk A, Wedzony K, Toth G, Przewlocka B. Endomorphin-2, deltorphin II and their analogs supress formaline-induced nociception and c-Fos expression in the rat spinal cord. Life Sciences 2003;73: 403-12.

Law PY, Loh HH. Regulation of opioid receptor activities. Journal of Pharmacology Experimental Therapeutics 1999;289: 607–24.

Lee Y, Pai M, Brederson JD, Wilcox D, Hsieh G, Jarvis M, et al. Monosodium iodoacetate-induced joint pain is associated with increased phosphorylation of mitogen activated protein kinases in the rat spinal cord. Molecular Pain 2011;7: 39.

Lever IJ, Malcangio M. CB1 receptor antagonist SR141716A increases capsaicin-evoked release of substance P from the adult mouse spinal cord. British Journal of Pharmacology 2002;135: 21-4.

Lichtman AH, Cook SA, Martin BJ. Investigation of brain sites mediating cannabinoid-induced antinociception in rats: Evidence supporting periaqueductal gray involvement. Journal of Pharmacology and Experimental Therapeutics 1996;276: 585-93.

Lippton H, Lin B, Gumusel B, Witriol N, Wasserman A, Knight M. Hemopressin, a hemoglobin fragment, dilates the rat systemic vascular bed through release of nitric oxide. Peptides 2006;27: 2284-8.

Lozovaya N, Yatsenko N, Beketov A, Tsintsadze T, Burnashev N. Glycine receptors in CNS neurons as a target for nonretrograde action of cannabinoids. Journal of Neuroscience 2005;25: 7499-06.

Mallareddy JR, Borics A, Keresztes A, Kover KE, Tourwe D, Toth G. Design, synthesis, pharmacological evaluation, and structure-activity study of novel endomorphin analogues with multiple structural modifications. Journal of Medical Chemistry 2011;54: 1462-72.

Martin WJ, Coffin PO, Attias E, Balinsky M, Tsou K, Walker JM. Anatomical basis for cannabinoid-induced antinociception as revealed by intracerebral microinjections. Brain Ressearch 1999;822: 237-42.

Mechoulam R, Ben Shabat S, Hanus L, Ligumsky M, Kaminski NE, Schatz AR, et al. Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. Biochemical Pharmacology 1995;50: 83-90.

Mecs L, Tuboly G, Toth K, Nagy E, Nyari T, Benedek G, et al. Peripheral antinociceptive effect of 2-arachidonoyl-glycerol and its interaction with endomorphin-1 in arthritic rat ankle joints. Clinical and Experimental Pharmacology and Physiology 2010;37: 544-50.

Merskey H, Bogduk N. Classification of chronic pain descriptions of chronic pain syndromes and definitions of pain terms. IASP Press International Association for the Study of Pain 1994 909 NE 43rd St. Suite 306

Meunier JC, Mollereau C, Toll L. Isolation and structure of the endogenous agonist of opioid receptor-like ORL1 receptor. Nature 1995;377: 532-5.

Mizoguchi H, Nakayama D, Watanabe H, Ito K, Sakurada W, Sawai T, et al. Involvement of spinal mu1-opioid receptors on the Tyr-d-Arg-Phe-sarcosine-induced antinociception. European Journal of Pharmacology 2006;540: 67-72.

Mollereau C, Parmentier M, Mailleux P. ORL1, a novel member of the opioid receptor family: Cloning, functional expression and localization. FEBS Lett. 1994;341: 33-8.

Nyilas R, Gregg LC, Mackie K, Watanabe M, Zimmer A, Hohmann AG, et al. Molecular architecture of endocannabinoid signaling at nociceptive synapses mediating analgesia. European Journal of Neuroscience 2009;29: 1964-78.

Oz M, Zhang L, Morales M. Endogenous cannabinoid, anandamide, acts as a noncompetitive inhibitor on 5-HT_3 receptor mediated responses in oocytes. Synapse 2002;46: 150-6.

Oz M. Receptor-independent actions of cannabinoids on cell membranes: focus on endocannabinoids. Pharmacology and Therapeutics 2006;111: 114-44.

Pasternak GW. Multiple opiate receptors: Déjà vu all over again. Neuropharmacology 2004;47: 312-23.

Paterlini MG, Avitabile F, Ostrowski BG, Ferguson DM, Portoghese PS. Stereochemical requirements for receptor recognition of the μ-opioid peptide endomorphin-1. Biophysical Journal 2000;78: 590-9.

Perlikowska R, Gach K, Fichna J, Toth G, Walkowiak B, do-Rego JC, et al. Biological activity of endomorphin and [Dmt1]endomorphin analogs with six-membered proline surrogates in position 2. Bioorganic and Medical Chemistry Letters 2009;17:3 789-94.

Perlikowska R, do-Rego JC, Cravezic A, Fichna J, Wyrebska A, Toth G, et al. Synthesis and biological evaluation of cyclic endomorphin-2 analogs. Peptides 2010;31: 339-45.

Podlogar BL, Paterlini MG, Ferguson DM, Leo GC, Demeter DA, Brown FK, et al. Conformational analysis of the endogenous mu-opioid agonist endomorphin-1 using NMR spectroscopy and molecular modeling. FEBS Lett 1998;439**:** 13-20.

Pomonis JD, Boulet JM, Gottshall SL, Phillips S, Sellers R, Bunton T, et al. Development and pharmacological characterization of a rat model of osteoarthritis pain. Pain 2005;114; 339-346.

Richardson JD, Kilo S, Hargreaves KM. Cannabinoids reduce hyperalgesia and inflammation via interaction with peripheral CB1 receptors. Pain 1998;75: 111-9.

Rioli V, Gozzo FC, Heimann AS, Linardi A, Krieger JE, Shida CS, et al. Novel natural peptide substrates for endopeptidase 24.15, neurolysin, and angiotensin-converting enzyme. Journal of Biological Chemistry 2003;278: 8547-55.

Sagar DR, Kelly S, Millns PJ, O'Shaughnessey CT, Kendall DA, Chapman V. Inhibitory effects of CB1 and CB2 receptor agonists on responses of DRG neurons and dorsal horn neurons in neuropathic rats. European Journal of Neuroscience 2005;22: 371-9.

Sakurada S, Watanabe H, Hayashi T, Yuhki M, Fujimura T, Murayama K, et al. Endomorphin analogues containing D-Pro2 discriminate different mu-opioid receptor mediated antinociception in mice. British Journal of Pharmacology 2002;137: 1143-6.

Shi Z-H, Wei Y-Y, Wang C-J, Yu L. Synthesis and analgesic activities of endomorphin-2 and its analogues. Chemistry and Biodiversity 2007;4: 458-67.

Staniszewska R, Fichna J, Gach K, Toth G, Poels J, Broeck JV, et al. Synthesis and biological activity of endomorphin-2 analogs incorporating piperidine-2-, 3- or 4-carboxylic acids instead of proline in position 2. Chemical Biology and Drug Design 2008;72: 91-4.

Stella N, Schweizter P, Piomelli D. Characterisation of a second endogenous cannabinoid ligand that modulates long term potentiation (LTP) in hippocampus. Society for Neuroscience 1997;23: 264-9.

Sugiura T, Kondo S, Kishimoto S, Miyashita T, Nakane S, Kodaka T, et al. Evidence that 2-arachidonoylglycerol but not N-palmitoylethanolamine or anandamide is the physiological ligand for the cannabinoid CB2 receptor. Comparison of the agonistic activities of various cannabinoid receptor ligands in HL-60 cells. Journal of Biological Chemistry 2000;275: 605-12.

Suplita II RL, Gutierrez T, Fegley D, Piomelli D, Hohmann AG. Endocannabinoids at the spinal level regulate, but do not mediate, nonopioid stress-induced analgesia. Neuropharmacology 2006;50: 372-9.

Tomboly C, Kover KE, Peter A, Tourwe D, Biyashev D, Benyhe S, et al. Structure-activity study on the Phe side chain arrangement of endomorphins using conformationally constrained analogues. Journal of Medical Chemistry 2004;47:7 35-43.

Toth G, Kramer TH, Knapp R, Lui G, Davis P, Burks TF, et al. [D-Pen2,D-Pen5] Enkephalin analogs with increased affinity and selectivity for delta-opioid receptors. Journal of Medical Chemistry 1990;33: 249-53.

Trescot AM, Helm S, Hansen H, BenyaminR, Glaser SE, Adlaka R, et al. Opioids in the management of chronic non-cancer pain: An update of American Society of the Interventional Pain Physicians' (ASIPP) Guidelines Pain Physician 2008: Opioids Special Issue: 11: 5-62.

Tuboly G, Mecs L, Benedek G, Horvath G. Antinociceptive interactions between anandamide and endomorphin-1 at the spinal level. Clinical and Experimental Pharmacology and Physiology 2009;36: 400-5.

van der Stelt M, Di Marzo V. Endovanilloids - putative endogenous ligands of transient receptor potential vanilloid 1 channels. European Journal of Biochemistry 2004;271: 1827-34.

Wang Cl, Guo C, Wang YQ, Zhou Y, Li Q, Ni JM, et al. Synthesis and antinociceptive effects of endomorphin-1 analogs with C-terminal linked by oligoarginine. Peptides 2011;32: 293-9.

Yaks TL, Rudy TA. Analgesia mediated by a direct spinal action of narcotics. Science 1976;192: 1357-8.

Yaksh TL, Kokotos G, Svensson CI, Stephens D, Kokotos CG, Fitzsimmons B, et al. Systemic and intrathecal effects of a novel series of phospholipase A2 inhibitors on hyperalgesia and spinal prostaglandin E2 release. Journal of Pharmacology and Experimental Therapeutics 2006;316: 466-75.

Yamazaki T, Ro S, Goodman M, Chung NN, Schiller PWA. Topochemical approach to explain morphiceptin bioactivity. Journal of Medical Chemistry1993;36: 708-19.

Yu Y, Shao X, Wang Cl, Liu HM, Cui Y, Fan YZ, et al. In vitro and in vivo characterization of opioid activities of endomorphins analogs with novel constrained C-terminus: Evidence for the important role of proper spatial disposition of the third aromatic ring. Peptides 2007;2: 859-70.

Zadina JE, Hackler L, Ge LJ, Kastin AJ. A potent and selective endogenous agonist for the μ-opioid receptor. Nature1997;386: 499-02.

Zhang J, Chen C. Endocannabinoid 2-arachidonoylglycerol protects neurons by limiting COX-2 elevation. Journal of Biological Chemistry 2008;283: 22601-11.

Zygmunt PM, Petersson J, Andersson DA, Chuang H, Sorgard M, Di Marzo V, et al. Vanilloid receptors on sensory nerves mediate the va