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

Ph.D. Thesis Summary

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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 (Tresco 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-NH₂, 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)βMePhe$^4$//(2S,3S)βMePhe$^4$] 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 βMePhe$^4$ 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-arachidonoylglycerol (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), \( \lambda \)-carrageenan, morphine hydrochloride, 2-arachidoylglycerol (2-AG) and AM 251 (CB1 receptor antagonist), SSR144528-2 (CB2 receptor antagonist), EM-2 and its derivatives (EMD1: Tyr-(1S,2R)Ache-Phe-pFPhe-NH2, EMD2: Tyr-(1S,2R)Ache-Phe-(2S,3S)\( \beta \)MePhe-NH2, EMD3: Dmt-(1S,2R)Ache-Phe-pFPhe-NH2, EMD4: Dmt-(1S,2R)Ache-Phe-(2S,3S)\( \beta \)Phe-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 tibiotalar 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 tibiotalar 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 CB₁ and CB₂ receptors in the effects of 2-AG, separate groups of animals were pretreated with AM 251 (antagonist of CB₁ receptors, 10 µg) or SSR144528-2 (antagonist of CB₂ 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 (series 1 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 CB1 and CB2 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 (Kruszynski 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 (Dmt1-EM-2) produced antinociception after i.t. injection in formalin test (rats) (Labuz et al., 2003). The effect evoked by Dmt1-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 CB1 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 alldynia 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 (ED50=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 alldynia 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 CB1 receptors; thus, HP is able to block the constitutive activity of CB1 but not CB2 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 CB1 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.

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