

Summary of the Ph.D. thesis

**ONE STEP CLOSER TO THE ROLE OF THE
BRAIN CB₂ CANNABINOID RECEPTORS:
INTERACTION WITH THE μ -OPIOID SYSTEM**

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INTRODUCTION

Natural defense mechanisms allow organisms to survive under threatening environmental conditions. However, extreme levels of pain can be redundant and could disrupt performance at inopportune times. The organism's ability to produce analgesia has been exploited by scientists to develop pharmaceuticals that can be used to eliminate aversive sensation of pain. The discovery of analgesic drugs dates back to more than 3000 years ago, when it was found that opium could be obtained from the poppy plant (*Papaver somniferum*). Also the anesthetic and analgesic properties of the cannabinoids derived from *Cannabis sativa* have been described in the first medicinal documents of the traditional Chinese and Indian culture.

Opioids and cannabinoids share a number of common features, although in the cell they activate different receptors (μ -, δ - and κ -opioid, and CB₁ és CB₂ cannabinoid receptors). They belong to the G-protein-coupled receptor (GPCR) superfamily that consist of a single polypeptide chain with an extracellular N-terminal domain, seven transmembrane (7TM) helical domains and an intracellular C-terminal tail. They transduce signals through activation of pertussis-toxin sensitive G_{i/o} proteins. Both receptor families are targets of drugs of abuse and are implicated in pain processes, therefore an understanding of the mechanisms modulating their activities is of significant clinical interest.

CB₂ cannabinoid receptors

The first studies that explored the tissue and cell distribution of CB₂ cannabinoid receptors indicated that this receptor type was exclusively present in tissues and cells of the immune system, being absent from the central nervous system (CNS). However, recent evidences have shown that CB₂ receptors can be found in different brain structures like in the cerebral cortex, thalamic nuclei, cerebellum or brainstem. This allowed to relate the CB₂ receptor to neurobiological processes located in the regions described, like in the control of pain, brain reward and others. Because the μ -opioid and CB₂ cannabinoid receptors overlap in these parts of the brain, the co-localization of these receptors can not be excluded.

AIMS OF THE STUDY

It is well known that opioid and cannabinoid receptors are able to heterodimerize with distantly related GPCRs resulting in novel characteristics and altered pharmacological properties. Interactions between the opioid and CB₁ cannabinoid systems have been proposed in many publications. However, there is no such data that links the possible effects of cannabinoids on the opioid system mediated through the brain CB₂ cannabinoid receptor.

In our work we aim to study the interactions between brain cannabinoid and opioid systems, searching for the possible

involvement of the CB₂ receptors. For this the following experiments were designed:

1. To acutely treat (intraperitoneally) wild-type and CB₁ cannabinoid receptor knockout mice with endocannabinoid noladin ether alone, in combination with the CB₂ receptor antagonist SR144528 and with SR144528 alone.

2. To characterize and compare the changes of the μ -opioid receptor (MOR) gene expression level in forebrain and brainstem, after the *in vivo* cannabinoid treatments by Quantitative Real-time PCR.

3. To examine the changes of the μ -opioid receptor G-protein activation in CB₁ wild-type and CB₁ knockout mice forebrain and brainstem membranes, after the *in vivo* cannabinoid treatments by using [³⁵S]GTP γ S functional binding assay.

4. To investigate the binding properties of noladin ether and SR144528 to the μ -opioid receptor in CB₁ wild-type and CB₁ knockout mice forebrain and brainstem membranes in competition binding studies.

5. To test the *in vitro* effects of the cannabinoids on MOR G-protein activation in either CB₁ or CB₂ cannabinoid receptor knockout mice brain membranes in [³⁵S]GTP γ S binding experiments.

METHODS

Animal treatments

CB₁ wild-type and CB₁ receptor knockout mice were generated in Dr. Ledent's lab (Brussels, Belgium) and was bred and treated at the Department of Human Morphology and Developmental Biology, SOTE, Budapest. The animals were acutely treated (intraperitoneally, i.p.) with endocannabinoid noladin ether (NE) (1mg/kg) or with CB₂ antagonist SR144528 (0.1 mg/kg). When used in combined treatments SR144528 was delivered 30 min prior to the agonist treatment. The CB₂ wild-type and CB₂ receptor knockout mice were generated in Dr. Zimmer's lab (Bonn, Germany).

Quantitative Real-Time PCR

Total RNAs were extracted from forebrain and brainstem tissues of wild-type and CB₁- and CB₂ receptor knockout mice. First strand cDNA was synthesized by reverse transcription using MuLV reverse transcriptase and Revertaid H-Minus Kit. Quantitative Real-time PCR was performed with MOR primers. Expression ratios were calculated using the Pfaffl method. Results were analyzed using GraphPad InStat 3.06 software.

Competition binding assay

Forebrain and brainstem membrane fractions from CB₁ wild-type and CB₁ receptor knockout mice were prepared. In competition binding experiments μ -opioid receptor specific agonist

[³H]DAMGO was used with unlabelled ligands (DAMGO, noladin ether, SR144528). Total binding was measured in the presence of radioligand, non-specific binding was determined in the presence of naloxone. Experimental data were analyzed by GraphPad Prism 3.0 software program to determine the concentration of the drug that displaced 50% of [³H]DAMGO. For the statistical analysis GraphPad InStat 3.06 software was used.

[³⁵S]GTPγS binding assay

Forebrain and brainstem membrane fractions from wild-type and CB₁- and CB₂ receptor knockout mice were prepared. The membrane fractions were incubated at 30 °C for 60 min in Tris-EGTA buffer (pH 7,4) containing 20 MBq/0,05 cm³ [³⁵S]GTPγS (0,05 nM), 30 μM GDP and in the *in vivo* experiments DAMGO (10⁻¹⁰ – 10⁻⁵ M), in the *in vitro* experiments DAMGO (10⁻¹⁰ – 10⁻⁵ M), NE and/or SR144528 (10⁻⁶ M). Total binding was measured in the absence of test compounds, non-specific binding was determined in the presence of 10 μM unlabelled GTPγS and was subtracted from total binding. Experimental data were analyzed by GraphPad Prism 3.0 software program. For the statistical analysis GraphPad InStat 3.06 software was used.

SUMMARY OF THE RESULTS

Forebrain

1. Intraperitoneal administration of noladin ether to CB₁ wild-type and CB₁ receptor knockout mice decreased MOR gene expression level and MOR G-protein activation in the forebrain. This attenuation can be reversed by pretreatment of mice with the CB₂ cannabinoid antagonist SR144528.

2. *In vitro* studies with noladin ether in forebrain membranes of wild-type and CB₁ receptor knockout provided similar decrease in the efficacy of DAMGO in [³⁵S]GTPγS binding assays. The combined *in vitro* treatments with SR144528 and noladin ether resulted in an enhancement of the efficacy when compared to the noladin ether administration alone.

The observed attenuations caused by noladin ether are similar in both wild-type and CB₁ receptor knockout mice therefore we can exclude CB₁ receptor contribution in these effects.

3. In competition binding experiments noladin ether failed to displace [³H]DAMGO with high affinity from μ-receptors when compared to the affinity of the unlabelled DAMGO.

These results reach the conclusions that in mice forebrain the attenuation caused by noladin ether on MOR signaling is mediated *via* CB₂ cannabinoid receptors.

Brainstem

4. In mouse brainstem, no significant changes were observed in MORs mRNA expression and signaling after *in vivo* and *in vitro* noladin ether treatments.

It seems that the mechanisms involved in noladin ether induced effects on MOR signaling are different depending on the investigated area.

After combined treatment with noladin ether and SR144528 we observed an attenuation in MOR activity both *in vivo* and *in vitro*, suggesting the presence of interactions between SR144528 and MOR. To reveal this possibility additional animal treatments were done with SR144528 alone.

5. *In vivo* treatment with SR144528 caused a significant decrease in MOR mRNA level and MOR G-protein activation in wild-type and CB₁ receptor knockout mice brainstem.

6. Ligand stimulated [³⁵S]GTPγS binding assays on CB₂ knockout mice showed that SR144528 have a significant inhibitory effect on the efficacy of DAMGO in wild-type but not in CB₂ knockout mice brainstem.

7. In competition binding studies our data demonstrate that [³H]DAMGO binding was basically not affected by SR144528 when compared to the affinity of unlabelled DAMGO.

These results indicate that in mouse brainstem SR144528 has an inhibitory effect on MOR signaling and this attenuation is mediated *via* CB₂ cannabinoid receptors.

Our results extend the previous evidence that CB₂ receptors are present in different parts of the brain and that they play a role in putative neuronal functions. Although our data clearly show that there are functional interactions between the brain CB₂ and the μ -opioid receptors, the mechanisms involved in these interactions needs to be clarified.

The pharmacology of cannabinoid-mediated actions is far from straightforward, indicated by reports of species and tissue differences in actions of ligands. Many of the ligands that are thought to be either CB₁ or CB₂ receptor specific have varying degrees of activity at other receptors, and this must be considered in interpreting experimental responses. Clearly, more research is required to illuminate further the nature of cannabinoid receptors agonist and antagonist action in various conditions. The development of conditional CB₂ receptor knockout mice and of ligands with greater selectivity will serve to further clarify the role of the CB₂ receptor in many physiological and pathological processes in which it appears to be implicated. It is clear that after some years where cannabinoid signaling seemed relatively straightforward, a new phase of research in this field lies ahead.

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LIST OF THE THESIS RELATED PUBLICATIONS

1. **Paldy, E.**, Bereczki, E., Santha, M., Wenger, T., Borsodi, A., Zimmer, A. & Benyhe, S. (2008). CB(2) cannabinoid receptor antagonist SR144528 decreases mu-opioid receptor expression and activation in mouse brainstem: role of CB(2) receptors in pain. *Neurochem Int* aug 29. ahead of print. (IF:2,975)
2. **Paldyova, E.**, Bereczki, E., Santha, M., Wenger, T., Borsodi, A. & Benyhe, S. (2008). Noladin ether, a putative endocannabinoid, inhibits μ -opioid receptor activity *via* CB2 cannabinoid receptors. *Neurochem Int* 52, 321-328. (IF: 2,975)

3. **Paldyova, E.**, Bereczki, E., Santha, M., Wenger, T., Borsodi, A. & Benyhe, S. (2007). Altered gene expression and functional activity of opioid receptors in the cerebellum of CB1 cannabinoid receptor knockout mice after acute treatments with cannabinoids. *Acta Biol Hun* 58, 113-129. (IF: 0,447)

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1. Kolarovszki-Sipiczki, Z., Gaspar, R., Ducza, E., **Paldy, E.**, Benyhe, S., Borsodi, A. & Falkay, G. (2007). Effect of alpha-adrenoceptor subtype-selective inverse agonists on non-pregnant and late-pregnant cervical resistance in vitro in the rat. *Clin Exp Pharmacol Physiol* 34, 42-7. (IF: 2,038)

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1. **E. Páldy**, A. Higuera-Matas, M. Miguéns, G. Tóth, T. Wenger, A. Borsodi, G.L. Montoya, O. Olías, C. García-Lecumberri & E. Ambrosio. Cerebral distribution of endomorphin-2 binding sites in CB1 knockout male and female mice: an autoradiography study. Spanish Society on Cannabinoid Research, Madrid, Spain, 2008. 27-29 November. (poszter)
2. A. Borsodi, **E. Páldyová**, E. Bereczki, M. Sántha, T. Wenger, L. Rocha & S. Benyhe. Altered mu-opioid receptor activity caused by brainstem CB₂ cannabinoid receptors. 13th NIDA International Forum, San Juan, Puerto Rico, 2008. 13-17 June. (poszter)
3. **E. Páldyová**, E. Bereczki, M. Sántha, T. Wenger, A. Borsodi & S. Benyhe. The CB2 cannabinoid receptor antagonist SR144528, inhibits MOR gene expression and DAMGO induced G-protein activation in mouse brainstem. European Opioid Conference, EOC-ENC Joint Meeting, Ferrara, Italy, 2008. 8-11 April. (poszter)
4. **E. Páldyová**, T. Wenger, A. Borsodi & S. Benyhe. Inhibition of μ -opioid receptor functional activity after *in vitro* noladin ether treatment in mice forebrain. Magyar Experimentális Farmakológia Tavaszi Szimpóziuma, Budapest, Magyarország, 2007. június 1-2. (poszter)
5. **E. Páldyová**, T. Wenger, S. Benyhe & A. Borsodi. Attenuation of opioid receptor G-protein activation after acute treatment by CB1 cannabinoid agonist noladin ether. C.I.N.P. Workshop, Addiction and Eating Disorders – Neurobiology and Comorbides, Brno, Czech Republic, 2007. 26-29 April. (poszter)

6. **E. Páldyová**, E. Bereczki, M. Sántha, T. Wenger, A. Borsodi & S. Benyhe. Altered gene expression and functional activity of μ -opioid receptors after acute nolidin ether treatment in the cerebellum of CB1 cannabis receptor knockout mice. MITT XI. Konferenciája, Szeged, Magyarország, 2007. január 25-27. (poszter)

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