

**STUDY OF  $\kappa$ -OPIOID RECEPTOR EXPRESSION  
IN CELL CULTURES AND IN HUMAN BRAIN**

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**Summary of Ph.D. thesis**

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## LIST OF FULL PAPERS RELATED TO THE THESIS

- I. Péter, A., Tóth, G., **Cserpán, E.**, Tourwé, D., (1994) Monitoring of optical isomers of  $\beta$ -methylphenylalanine in opioid peptides, *J. Chromatography A*, **660**: 283-291.
- II. Wevers, A., Schmidt, P., **Cserpán, E.**, Cserpán, I., Maderspach, K., Staak, M., and Schröder, H., (1995) Cellular distribution of the mRNA for the  $\kappa$ -opioid receptor in the human neocortex: a non-isotopic *in situ* hybridization study, *Neurosci. Lett.*, **195**: 125-128.
- III. **Cserpán, E.**, Tryoen-Tóth P., Bajenaru, L., Benyhe, S., and Maderspach, K., (1997) Differential expression of the  $\kappa_2$ -opioid receptor protein under the effect of opioid agonists in embryonic chick neurons *in vitro*, *Neurobiology* **5**: 233-248.

## INTRODUCTION

Opioid receptors have been the focus of intensive research since their discovery in 1973 with the hopes of elucidating their roles in development of the nervous system and in the adult plastic function. Development of new, selective agonists and antagonists made possible identification of  $\kappa$ -,  $\mu$ - and  $\delta$ -opioid receptor types and their subtypes  $\kappa_1$ ,  $\kappa_2$  and  $\kappa_3$ ,  $\mu_1$ ,  $\mu_2$ ,  $\delta_1$ ,  $\delta_2$  by radioligand binding studies. Localization of the opioid receptors has been carried out for a long time by autoradiography studies using specific opioid ligands. Application of this method was limited because of its low resolution. Design of more and more selective opioid ligands is still an actual goal in science. To construct these new ligands, unusual amino acids are often used. After development of opioid receptor specific antibodies, cellular and subcellular localization of receptors has become also possible. Later it was revealed that each type of opioid receptors has unique pharmacological properties and are differentially distributed in the nervous system. They are implicated in a broad range of functions including release of neurotransmitters, neuroendocrine modulation and regulation of pain. Furthermore, they play a role in mediation of the effects of chronic opioid drug addiction, resulting in tolerance and dependence. Opioid peptides and receptors are present in the early stages of ontogeny and influence the proliferation and differentiation of neurons and glial cells.

Molecular cloning of the opioid receptors confirmed the suggestion that they are members of the family of G-protein-coupled receptors with

seven transmembrane domains The  $\kappa$ -,  $\mu$ - and  $\delta$ -receptors, which are encoded by different genes, are highly homologous in their nucleic acid and amino acid sequences. The knowledge of the cDNA and amino acid sequences of these receptors allowed the synthesis of peptide fragments on the basis of the cloned structures. Using these fragments as antigens, preparation of specific antibodies was possible.

The cloning of opioid receptors also resulted in developing of immortalized cell lines, expressing the opioid receptor proteins for further analysis of the pharmacological and biochemical properties of the opioid receptors. On the basis of the cloned sequences, construction of specific hybridization probes became possible to localization of opioid receptor mRNA expression in the nervous system by *in situ* hybridization.

#### AIM OF THE STUDIES

The aim of the present work was to develop new, opioid receptor specific peptide ligand and to investigate the  $\kappa$ -opioid receptor expression in the nervous system. The experiments were directed in four main subjects.

I. *Developing new, stereoselective, opioid receptor specific ligands.* Synthesis of an unusual amino acid,  $\beta$ -methylphenylalanine ( $\beta$ -MePhe), separation its enantiomers and incorporation into the original peptide, deltorphin C. Finally, separation, identification and purification of the  $\beta$ -MePhe-deltorphin C diastereomers was carried out.

II. *Study of the  $\kappa$ -opioid receptor expression in the course of*

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mAb; in parallel with  $\kappa_1$ -OR mRNA by *in situ* hybridization with the aid a specific oligonucleotide probe complementary to the  $\kappa_1$ -opioid receptor transcript. Our work was the first localization of  $\kappa_1$ -opioid receptor mRNA in the human frontal cortex area 10. Specific hybridisation signal was detected in pyramidal-like neurons, in layers II., III. and V., and some of the ovoid neurons were labelled in layer VI. This result is in good agreement with the distribution pattern of immunoreactive cells detected previously using the K8 mAb. A similar parallel localisation was also performed on rat granule cell cultures. Specific hybridisation and immunolabelling was seen in type II astrocytes. The level of specific signals increased simultaneously during the *in vitro* differentiation. Based on these results, we concluded that 1. / The  $\kappa_1$ - and  $\kappa_2$ -opioid receptors could be expressed by the same cells; 2. / The  $\kappa_1$ -receptor-specific oligonucleotide probe recognized both the  $\kappa_1$ - and  $\kappa_2$ -opioid receptor mRNAs; 3. / Statements 1. / and 2. / are simultaneously true. This indicated that the sequences of the  $\kappa_1$ - and  $\kappa_2$ -opioid receptor mRNAs could be highly homologous or even identical in the region recognized by the oligonucleotide probe.

*development.* The developmental changes of the  $\kappa$ -opioid receptor protein expression were examined in neuronal and glial cell cultures, which were derived from embryonic chick forebrain and postnatal rat cerebellum.

III. *Investigation the possible regulation of the  $\kappa$ -receptor expression, exerted by chronic opioid signals in cultured neurons.* Chronic treatment of cultured neurons by opioid ligands served as the *in vitro* model of drug addiction.

IV. *Comparative cellular localization of the  $\kappa$ -opioid receptor protein and the encoding mRNA in human brain cortical sections and in rat cerebellar granule cell cultures.*

## METHODS

**Synthesis of  $\beta$ -Me-Phe · HCl:** The synthesis was carried out according to modified method of Kataoka *et al.* (1976). Structures were confirmed by  $^1\text{H}$  NMR spectroscopy.

**Synthesis of [ $\beta$ -MePhe $^3$ ] Deltorphin C (Tyr-D-Ala- $\beta$ -Me-Phe-Asp-Val-Val-Glu-NH $_2$ ) analogues:**  $\beta$ -MePhe isomers were incorporated into the deltorphin C peptide in position 3. Peptides were synthesized by solid-phase peptide synthesis (SPPS), using BOC-protected amino acids.

**Separation of amino acid isomers and peptide diastereomers:** HPLC separation was carried out with variety of columns and eluents. To achieve better separation, different derivatives of  $\beta$ -MePhe were analysed on Nucleosil columns. Gradient elution of N-acetyl form was

investigated in a range 5-80% MeOH content. Other derivatives, o-phthaldialde-hyde-mercaptopropionic acid (OPA) and 2,4-dinitrophenyl-5-L-alanin-amide (FDAA) were chromatographed in isocratic solvent systems. N-trifluoroacetylated isobutyl ester (TAB) derivative was analysed by GC-MS as reported in Peter *et al.*, (1994). HPLC separation of deltorphin C peptide was carried out with acetonitrile-phosphate buffer (20:80) on Vydac semipreparative column. TLC analysis of  $\beta$ -MePhe isomers and peptides containing them were done on Kieselgel- and Chiral plates with various of mobil phase system.

**Primary chick neuronal cell cultures:** they were prepared from forebrains of seven-day old chick embryos according to the method described by Maderspach and Solomonia (1988). Cells were plated in a density of  $6 \times 10^6$  cells/cm<sup>2</sup> onto coverslips or Petri dishes coated previously with 0.01% poly-L-lysine.

**Rat primary granule cell cultures:** they were prepared from the cerebellum of 8-day-old Wistar rats similarly as described above. Cells were plated in a density of  $6.4 \times 10^5$  cells/cm<sup>2</sup> onto coverslips or Petri dishes previously coated with 0.01 % poly-L-lysine.

**Chronic drug treatments of primary chick neuronal cultures:**The applied opioid drugs and the final concentrations were as follows. Agonists, morphine (MORPH):  $10^{-6}$  M; bremazocine (BREM):  $10^{-7}$ ,  $10^{-8}$  M; dinorphine (DYN):  $10^{-6}$ ,  $10^{-7}$  M; U-50, 488 H:  $10^{-6}$  M. Antagonists, naloxone(NAL):  $10^{-5}$  M; NBI:  $10^{-7}$ ,  $10^{-8}$  M.

**Equilibrium binding was measured by adding 1 nM [<sup>3</sup>H]-naloxone or 4 nM [<sup>3</sup>H]-EKC (ethylketocyclazocine) into the medium of intact**

was suitable to achieve full separation and identification of the  $\beta$ -M-Phe amino acid- and  $\beta$ -Me-Phe deltorphin C peptide enantiomers.

### **Studies on the expression of $\kappa$ -opioid receptors**

Expression of the  $\kappa$ -opioid receptor was studied on chick neuronal cultures, rat granule cell cultures and on the human frontal cortex using radioligand binding, immunocytochemistry and *in situ* hybridization. Our results confirmed the presence of  $\kappa$ -opioid receptors in chick neuronal cells. Bremazocine and nor-binaltorphimine yielded biphasic curves in the heterologous displacement experiments, indicating a high-affinity interaction of these ligands with the binding sites. Binding kinetics showed positive cooperativity.

The chick neuronal cultures were used as a model system to study the effect of drug addiction. Chronic treatment with opioid agonists bremazocine and dynorphin<sub>1-13</sub> resulted in significant down-regulation of  $\kappa$ -binding sites of intact chick neurons, what could be antagonized by the non-selective antagonist naloxone and the  $\kappa$ -selective antagonist nor-binaltorphimine. This indicated that the down-regulation was indeed opioid-receptor mediated. Chronic treatment with the KA8 mAb also down-regulated the  $\kappa$ -opioid binding sites, pointing to an agonist character of the  $\kappa_2$ -receptor specific mAb. In chronically treated chick neuronal cultures, immunocytochemistry failed to confirm the down-regulation of the receptors.

Expression of  $\kappa$ -OR protein was studied using  $\kappa_2$ -OR specific

and better  $\alpha$  and  $R_s$  values, but it was still insufficient to achieve complete separation of the D-threo and D-erithro diastereomer pairs.

To overcome on this problem thin layer chromatography (TLC) and gas chromatography-mass spectrometry (GC-MS) analysis of  $\beta$ -Me-Phe was performed. Chiral TLC resolution of the four isomers was carried out. The elution sequence was the L-erithro and the L-threo form appeared as one spot, followed by D-threo and finally D-erithro  $\beta$ -Me-Phe. These values are in good agreement with the  $R_f$  values of standard amino acids. This method is suitable for resolution of the D-threo and D-erithro form as well. To confirm the expected structure of the four isomers, GC-MS analysis of TAB- $\beta$ -Me-Phe derivative was carried out. The elution sequence was D-erithro, L-erithro with overlapping peaks, followed by D-threo and L-threo as independent peaks.

HPLC analysis of the  $\beta$ -Me-Phe-deltorphine C peptide gave complete separation of the four isomers. The elution sequence was D-threo, D-erithro, L-threo and L-erithro. The  $\alpha$  and  $R_s$  values were also good.

Identification of the peaks was performed by TLC. Applying Kieselgel F<sub>254</sub> plate resulted in partial separation of the L-threo form, while the other three diastereomers were not resolved in system A. On the Chiral plate with the same mobile phase system (acetonitrile-methanol-water, 4:1:1), resolution of L-threo D,L-erithro as one spot and D-threo was achieved. Application of more polar mobile phase systems resulted in an increase of the  $R_f$  values and decrease of the quality of separation. Combination of the above-discussed procedures

cultures and by incubated for 4 min. at 37°C. Non-specific binding was determined in the simultaneous presence of 1 mM unlabelled naloxone or EKC. Heterologous displacement curves of BREM and nor-binaltorphimine (NBI) were measured at 0.8 nM [<sup>3</sup>H]-naloxone concentration, in a range 10<sup>-12</sup> M-10<sup>-5</sup> M of non-labelled ligand.

**Immunostaining for  $\kappa$ -opioid receptor with mAb KA8:** The cultures were fixed with ice-cold methanol and incubated with the KA8 mAb, 1:1000, 37°, 90 min. As a secondary antibody, a horseradish peroxidase conjugated goat-anti mouse Ig was applied, in a dilution 1:500. For detection 3,3'-diaminobenzidine tetrahydrochloride (DAB) and hydrogen peroxide were added.

#### **Immunostaining with anti-GFAP Ab**

Anti-GFAP Ab was diluted in blocking buffer in 1:500, and the cultures were incubated at 37°C for 90 min. As a second antibody, an anti-rabbit Ig, conjugated with alkaline phosphatase was used, in dilution 1:500. The samples were incubated for 90 min at 37°C. For 30 min, followed by immunostaining for GFAP done similarly as given above.

**Double immunostaining for the  $\kappa$ -OR and GFAP:** Kappa-opioid receptor immunolabelling was carried out as described above, except, that after the final washing step the samples were incubated again in blocking buffer at 37°C for 30 min, followed by immunostaining for GFAP done similarly as described above.

***In situ* hybridization probes:** The sequence of the oligonucleotide probe was designed on the basis of the rat  $\kappa$ -opioid receptor cDNA (Minami et al., 1993, GenBank database accession no.: D16829) The

antisense probe for  $\kappa$ -opioid receptor mRNA was a 48-mer oligonucleotide, corresponding to nt. -36 bp to +12. The sense and antisense oligonucleotide probes were 3'-end-labeled with digoxigenin-11-ddUTP.

**Preparation of cultures and sections for *in situ* hybridization:** One to four days-old primary granule cell cultures were fixed with methanol-acetic acid (3:1). Human frontal cortex (A10) autopsy samples were obtained from seven neurologically healthy patients (4 males, 3 females, age range 30-69 years, post mortem delay range 17-55h). Sections were prepared for *in situ* hybridisation as described by Wevers et al., (1994), fixed with ethanol/acetic acid (3:1; 1 week, 4°C), dehydrated, embedded in paraplast and 7- $\mu$ m thick sections were mounted onto adhesion slides. Before *in situ* hybridization, sections were deparaffinized in xylene, rehydrated in ethanol series.

***In situ* hybridization:** The procedure was carried out by a slightly modified method of Wevers *et al.*, 1994) The tissue sections and cultures were treated with 10 $\mu$ g / ml proteinase K for 10 min at 37°C. Prehybridisation was performed for 1 h at 42°C or 37°C. Hybridization was carried out with 75ng/100ng of DIG-labelled oligonucleotide probe in a moist chamber at 42°C (sections) or at 37°C (cultures) overnight. Specific hybridization was visualized by applying an alkaline phosphatase-conjugated anti-digoxigenin antibody in dilutions 1:100 for sections and 1:500 for cultures. To detect specific hybridisation signal, NBT/BCIP and levamisole was applied. *Control experiments: in situ* hybridisations were performed with the labelled sense oligonucleotide probe and with the omission of the probe.

## RESULTS AND DISCUSSION

Development of the new peptide ligand [ $\beta$ -Me-Phe<sup>3</sup>]deltorphan C peptide, Tyr-D-Ala- $\beta$ -Me-Phe-Asp-Val-Val-Glu-NH<sub>2</sub> was carried out using an unusual amino acid  $\beta$ -methylphenylalanine ( $\beta$ -MePhe). Since it has two chiral centers, four stereoisomers were possible. For separation of these isomers, various chromatographic methods were applied. To achieve better separation, different derivatives were analyzed by HPLC.

The capacity factor ( $k'$ ) and resolution ( $R_s$ ) values of the D,L-erithro and D,L-threo forms of N-acetyl- $\beta$ -MePhe were investigated at variety eluent composition and pH. With methanol-phosphate buffer, separation of the threo- (first) and erithro (second) forms started at 20-60% methanol concentration, depending on the pH. The  $k'$  and ( $R_s$ ) values increased with decreasing methanol concentration of mobile phase and with decreasing pH.

Analysis of OPA-derivative with the eluent, acetonitrile-sodium acetate resulted in elution of D,L-erithro form first and the D,L-threo second.

The effects of pH and eluent composition on separation of the FDAA- $\beta$ -MePhe were studied. At pH 2 and at pH 4 the L-erithro,threo- $\beta$ -Me-Phe and the D-erithro,threo- $\beta$ -MePhe could be separated, while at pH 3, the erithro- and threo forms were only partially separated. The influence of the eluent composition for  $k'$ ,  $\alpha$ , and  $R_s$  were also studied. Decreasing the organic content of the eluent increased the  $k'$  value and improved the separation. Adding a second organic modifier to the eluent, led to more effective separation of L-erithro and L-threo forms