Summary of the Ph.D. Thesis

Biochemical and pharmacological characterizations of the novel endogenous opioid peptide motifs and synthetic nociceptin hexapeptide sequences

Ph.D. Thesis

by

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“We are all naturally dependent on opioids for our emotional health.”

INRODUCTION

The milky fluid extracted from the *Papaver somniferum* plant's seed-capsule (‘*poppyhead’*) is highly narcotic after drying. This product is referred as opium. The word ‘opium’ derives from the Greek word for juice of a plant. Opioids have been the mainstay of pain treatment for thousands of years, and they remain so today. Opioid receptors are cell surface glycoproteins and they are tightly integrated into the cell membranes. Opioids are the most effective analgesic drugs for acute and chronic pain. Opioid system consists of mu- (MOP) delta- (DOP) kappa (KOP) nociceptin (NOP) opioid receptors and endogenous opioid peptides (enkephalins, β-endorphin, dynorphins and nociceptin or N/OFQ) (*Corbett et al 2006*). All the endogenous opioid peptides are derived from four precursor polypeptides named as proenkephalin (PENK), prodynorphin (PDYN), proopiomelanocortin (POMC) and pronociceptin (PNOC). While PENK is source for the two enkephalin pentapeptides (Met- and Leu-enkephalin, YGGF**M** and YGGF**L** respectively), PDYN is source for dynorphins. Beside the enkephalins, PENK is also precursor for the Met-enkephalin extended heptapeptide, Met-enkephalin-Arg^6-Phe^7 (YGGFM**RF**) and octapeptide, Met-enkephalin-arg^6-gly^7-leu^8 (YGGFM**RGL**) sequences *(Patey and Rossier, 1986).*

Artificial hexapeptides isolated from peptide combinatorial chemical libraries are amongst the most selective ligands for the NOP receptor. One leading compound in this series is Ac-RYYRIK-NH_2, developed by Dooley *(Dooley et al 1996).* Recently a close derivative with a reduced C-terminus, Ac-RYYRIK-ol has been described by our group as a partial agonist at the NOP receptors using various *in vitro* and *in vivo* studies *(Gündüz et al., 2006).*

Being a partial agonist with intrinsic antagonist potency, this hexapeptide alcohol is capable of inhibiting competitively some effects mediated by full agonists at the NOP receptor. A further analogue, Ac-RYYRIR-ol, representing the $Lys^6 \rightarrow Arg^6$ replacement of its parent compound Ac-RYYRIK-ol, has been synthesized aiming even better potency.

AIM OF THE STUDIES

Main objective of the present studies were;

✓ *To collect all available PENK and PDYN sequences from the public database (PubMed, Ensmbl etc.) and align them to characterize* ***phylogenetic variability*** *of the opiod peptides within the precursors,*

✓ *To chemically synthesize and* ***to characterize the novel opioid sequences*** *using comparative biochemical methods such as* ***receptor binding*** *and* ***G-protein activation*** *assays,*

✓ *To investigate the* ***pharmacological properties of Ac-RYYRIR-ol*** *using various in vitro assays, such as receptor binding, G-protein activation, mouse vas deferens, guinea pig ileum, and mouse colon bioassays, and calcium ion mobilization experiments.*

✓ *To study the effective radiolabeling and detailed receptor binding properties of the* ***newly developed radioligand [^{3}H]Ac-RYYRIK-ol*** *to native and recombinant NOP receptors.*

METHODS

Inbred Wistar rats, guinea pigs (R9strain) and CHO cells stably expressing the wild type human nociceptin ($_{h}$NOP) receptor protein (Dr Jean-Claude Meunier, Toulouse, France) were used for the studies. Recombinant HEK293 cells expressing the fusion construct $_{h}$NOP-GFP (Dr. Maithe Corbani Montpellier, France) were used for the receptor internalization experiments. All the peptides were prepared by Dr. Magyar Anna in Research Group for Peptide Chemistry, Budapest, Hungary.

- **Receptor binding assays**

Radioligand binding assays are extremely powerful tools for studying receptors. They allow an analysis of the interactions of related drugs with the receptors, studies of second messenger systems, and characterization of changes in receptor number and physiological function. These assays were widely used by investigators in a variety of disciplines, including pharmacology, biochemistry, and cell biology.

- **[^{35}S]GTPγS binding assays**

[^{35}S]GTPγS binding assays represent a functional test determinig agonist-induced and receptor-mediated G-protein activation which is based on the increase in guanine nucleotide exchange at G-proteins upon agonist stimulation.

- **Isolated tissue bioassay**

The longitudinal muscle of the guinea pig ileum and the smooth muscle of the mouse and rat vas deferens were used for in vitro pharmacological

characterization (efficacy, potency and selectivity) of the NOP receptor ligand, Ac-RYYRIR-ol. The mouse vas deferens, the rat vas deferens and the guinea pig ileum was prepared according to Bigoni et al., (1999).

- **Calcium-ion mobilization experiments**

Calcium mobilization assays were performed in a fluorometric imaging plate reader FlexStation II using the CHO cells expressing the recombinant human NOP receptor and the chimeric protein $G\alpha_{qi5}$. Maximum change in fluorescence, expressed as percent over the baseline fluorescence, was used to determine agonist response.

- **Internalization of the GFP tagged $_{h}$NOP receptor**

Ac-RYYRIR-ol regulated internalization of the NOP receptors were studied using HEK293 cells expressing the $_{h}$NOP-GFP receptors. Olympus Cell-R fluorescence microscope was used with GFP filter set combination and 20 x objectives.

SUMMARY OF THE RESULTS

IDENTIFICATION OF THE NOVEL NATURALLY OCCURRING OPIOID PEPTIDES

Our analysis of up to 55 different PENK and 39 PDYN sequences available in major protein databases showed that the pentapeptide and octapeptide units are variable among the different species studied. Among the novel peptides **Ile-enkephalin** (Tyr-Gly-Gly-Phe-Ile) was primarily observed in the African clawed frog (*Xenopus laevis*) PDYNs, while the structure of **Phe-enkephalin** (Tyr-Gly-Gly-Phe-Phe) was predicted by analyzing brain cDNA sequences encoding a prodynorphin of the African lungfish (*Protopterus annectens*). Four of the orthologous Met-enkephalin octapeptide sequences have been identified by bioinformatic means which were; YGGFM**RGY** (three frog species, platypus), YGGFM**RSV** (chicken, one fish species), YGGFM**NGF** (shark) and YGGFM**RSL** (mouse, two lungfish species). All the novel sequences were chemically synthesized and studied and compared to those of the well known Met-enkephalin-Arg^{6}-Gly^{7}-Leu^{8} and the pentapeptide Met-enkephalin in receptor binding and G-protein activation assays performed on rat brain membranes.

Our experimental findings revealed that newly recognized PDYN and PENK derived opioid sequences have **good/moderate affinity** towards opioid receptors. Among pentapeptide enkephalin structures tested, Ile-enkephalin and Phe-enkephalins were found to be less effective than mammalian Met-enkephalin which exhibited the highest affinities in receptor binding assays and produced the most efficacious G-protein stimulation in brain membranes. Of the four octapeptide structures studied, the ‘chicken type’

peptide variant (YGGFMRSV) exhibited the highest affinities in receptor binding assays. These novel endogenously occurring sequences represent further examples of the **natural diversity** observed within the opioid peptide family. Importantly, these new endogenous peptides represent further illustration of the **chemical biodiversity** observed within the opioid peptide family.

IN-VITRO PHARMACOLOGICAL CHARACTERIZATION OF A NOVEL, NOP SELECTIVE HEXAPEPTIDE, Ac-RYYRIR-ol

The pharmacological profile of the **novel** hexapeptide **Ac-RYYRIR-ol** was investigated using various in vitro assays including receptor binding and G protein activation in rat brain membranes, mouse and rat vas deferens, guinea pig ileum, mouse colon and Ca^{2+} ion mobilization assay in chinese hamster ovary (CHO) cells co-expressing the human recombinant NOP receptor and the C-terminally modified $G_{\alpha qi5}$ protein. Ac-RYYRIR-ol displaced [^{3}H]Ac-RYYRIK-ol with high affinity and stimulated [^{35}S]GTPγS binding but showing lower maximal effects than N/OFQ. In antagonist type experiments Ac-RYYRIR-ol inhibited the stimulatory effects induced by N/OFQ. Meanwhile in mouse colon Ac-RYYRIR-ol produced concentration dependent contractile effects with similar potency and maximal effects as N/OFQ and finally, in the Ca^{2+} ion mobilization assay Ac-RYYRIR-ol displayed lower potency. NOP receptor selective hexapeptide Ac-RYYRIR-ol had **fine selectivity**, **high potency**, furthermore **agonist** and **antagonist** effects toward the NOP receptors which is likely due to its partial agonist pharmacological activity.

SELECTIVE AND HIGH AFFINITY LABELING OF THE NOP RECEPTORS WITH THE [^{3}H]Ac-RYYRIK-ol

Novel radiolabeled hexapeptide analogue **[^{3}H]Ac-RYYRIK-ol**, selective for the NOP receptor has been described. Heterologous competition assays with various nociceptin analogues and some opioid compounds revealed that [^{3}H]Ac-RYYRIK-ol specific binding was effectively inhibited by NOP receptor-selective ligands, whereas opioids displayed moderate to negligible affinities. The results revealed that the new tritiated peptide analogue [^{3}H]Ac-RYYRIK-ol **recognized and labeled NOP receptors** specifically in rat brain membrane preparations and in cellular system expressing the recombinant human NOP receptors.

LIST OF THESIS RELATED PUBLICATIONS

Bojnik E., Magyar A., Toth G., Bajusz S., Borsodi A., Benyhe S. Binding studies of novel, non-mammalian enkephalins, structures predicted from frog and lungfish brain cDNA sequences. Neuroscience, (2009); 158, 867-874.

Bojnik E., Farkas J., Magyar A., Tömböly C., Güclü U., Gündüz O., Borsodi A., Corbani M., Benyhe S. Selective and high affinity labeling of neuronal and recombinant nociceptin receptors with the hexapeptide radioprobe [^{3}H]Ac-RYYRIK-ol. Neurochemistry International, (2009); 55, 458–466.

Bojnik E., Fischetti C., Babos F., Magyar A., Camarda V., Borsodi A., Bajusz S., Calo' G., Benyhe S. Comparative biochemical and pharmacological characterization of a novel, NOP receptor selective hexapeptide, Ac-RYYRIR-ol. Brain Research Bulletin. *Accepted for publication*

Bojnik E., Babos F., Magyar A., Borsodi A., Benyhe S., 2009. Bioinformatical and biochemical studies on the phylogenetic variability of proenkephalin-derived octapeptides. Neuroscience. *Accepted for publication*

LIST OF ORAL PRESENTATIONS

Bojnik E., Magyar A., Toth G., Borsodi A., Benyhe S. Receptor binding properties of novel, non-mammalian enkephalin pepntapeptides. Balatonszemes, Hungary, Peptide Chemistry Conference, May, 2006

Benyhe S., Bojnik E. Evolutionary opioid peptides. Balatonszemes, Hungary, Peptide Chemistry Conference, April, 2008

Benyhe S, Bojnik E, Babos F, Magyar A, Borsodi A. Synthetic hexapeptides targeting NOP receptors. Invited seminar lecture, Institute Genomique Fonctionelle (IGF), CNRS, Montpellier, France, December 2008

Benyhe S, Bojnik E. Phylogenetic diversity of opioid peptides. (Evolúciós diverzitás opioid peptidekben) Balatonszemes, Hungary, Peptide Chemistry Conference, May, 2009

Bojnik E., Babos F, Maygar A., Bajusz S., Borsodi A., Benyhe S. Biochemical diversity of various non-mammalian enkephalins: receptor binding and G-protein activation studies. Balatonszemes, Hungary, Peptide Chemistry Conference, May, 2009

LIST OF POSTER PRESENTATIONS

Bojnik E., Toth G., Magyar A., Borsodi A., Benyhe S. Receptor binding properties of novel, non-mammalian enkephalin pepntapeptides. ITC Alumni Meeting, Modern Trends in Biological Science, Szeged, Hungary, October, 2006

Bojnik E., Magyar A., Borsodi A., Benyhe S. Receptor binding and G-protein activation by novel non-mammalian enkephalin pentapeptides. Magyar Idegtudományi Társaság (Hungarian Neuroscience Society Meeting), Szeged, Hungary, January 2007

Bojnik E., Toth G., Maygar A., Borsodi A., Benyhe S. Biochemical Characterisation of novel non-mammalian enkephalin pentapeptides, deduced from *Xenopus laevis* and *Protopterus annectens* cDNA sequences. Magyar

Experimentális Farmakológia, (Hungarian Experimental Pharmacology Meeting), Budapest, Hungary, June 2007

Bojnik E., Toth G., Maygar A., Borsodi A., Benyhe S. Opioid receptor binding and G-Protein activation characteristics of novel non-mammalian enkephalin pentapeptides, deduced from *Xenopus laevis* and *Potopterus annectens* cDNA sequences. European Opioid Conference and European Neuropeptide Club, Ferrara, Italy, April 2008

Bojnik E., Babos F., Fischetti C., Magyar A., Borsodi A., Bajusz S., Calo' G., Benyhe S. Comparative biochemical and pharmacological characterization of a novel, NOP receptor selective hexapeptide, Ac-RYYRIR-ol. Stress, Drug Addiction and Eating Disorders, PENS–BLACKWELL SUMMER SCHOOL, Dubrovnik, Croatia, June, 2009

Bojnik E., Kanit L., Yalcin A., Bnenyhe S., Borsodi A. Changes in the Opioid/Nociceptin System in Kainic Acid Model of Epilepsy. Neuropeptide Festival, Joint Meeting of the European Neuropeptide Club and the Summer Neuropeptide Conference, Salzburg, Austria, July, 2009

Bojnik E., Babos F., Fischetti C., Magyar A., Borsodi A., Bajusz S., Calo' G., Benyhe S. Arginine (Arg^6) Replacement in the NOP Receptor Partial Agonist Ac-RYYRIK-ol Results In Equipotent Hexapeptide Ligand. Neuropeptide Festival, Joint Meeting of the European Neuropeptide Club and the Summer Neuropeptide Conference, Salzburg, Austria, July, 2009

*Dedicated to the **Srebrenica Victims***