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

PhD Thesis

by

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ABBRAVIATIONS

Ac	Acetyl
BSA	Bovine serum albumin
cAMP	Adenosine-3,5'-cyclic monophosphate
cDNA	Complementary DNA copied from an mRNA coding for a protein; it is inserted into surrogate host cells to cause them to express the protein
CHO	Chinese hamster ovary
CTAP	D-Phe-Cys-Thr-D-Trp-Arg-Thr-Pen-Thr-NH_2
DAMGO	Tyr-D-Ala-Gly-nMe-Phe-Gly-ol
DIDI	Tyr-D-Ala-Phe-Glu-Ile-Ile-Gly-NH_2
DOP	*delta*-opioid peptide receptor
DPDPE	Tyr-D-Pen-Gly-Phe-D-Pen
EC_{50}	Potency - the agonist molar concentration that produces 50% of the maximal possible effect of that agonist.
EDTA	Ethylenediamine-tetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
EKC	Ethylketocyclazocine
EL	Exracellular loop
E_{max}	Efficacy - the maximal effect that an agonist can elicit in a given tissue/preparation
GDP	Guanosin diphosphate
G-protein	Guanine nucleotide binding protein
GPCR	G-protein coupled receptor
GPI	Guinea pig ileum
GTP	Guanosin 5'-triphosphate
GTPγS	Guanosine-5'-O-(3-thiotriphosphate)
[^{35}S]GTPγS	Guanosine-5'-[γ-35S]thiophosphate
IL	Intracellular loop
IUPHAR	International Union of Pharmacology
J-113397	(±)trans-1-[1-cyclooctylmethyl-3-hydroxymethyl-4-piperidyl]-3-ethyl-1,3- dihydro-2H-benzimidazol-2-one
K_d	Equilibrium dissociation constant of a radioligand-receptor complex
K_i	equilibrium inhibition constant
KOP	*kappa*-opioid peptide receptor
MOP	*mu*-opioid peptide receptor
mRNA	Messenger ribonucleic acid
mVD	Mouse *vas deferens*
N/OFQ	Nociceptin/Orphanin FQ (Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-Leu-Ala-Asn-Gln)
NOP receptor	Nociceptin/Orphanin FQ peptide receptor
ORL-1 receptor	Opioid receptor-like 1
pA_2	Antagonist potency - the negative logarithm to the base 10 of the antagonist molar concentration that makes it necessary to double the agonist concentration to elicit the original submaximal response.
PBS	Phosphate-buffered saline
PDYN	Pro-dynorphin

pEC_{50}	Agonist potency - the negative logarithm to base 10 of the agonist molar concentration that produces 50% of the maximal possible effect of that agonist.
PEI	Polyethyleneimine
PENK	Pro-enkephalin
pK_B	Antagonist potency - when one single concentration of antagonist is used.
pK_i	Antilogarithm of the equilibrium inhibition constant
PMSF	Phenylmethylsulfonyl fluoride
PNOC	Pro-nociceptin
POMC	Pro-opiomelanocortin
Ro-65-6570	(8-acenaphthen-1-yl-1-phenyl-1,3,8-triaza spiro [4,5] decan-4-one)
SEM	Standard error of the mean
TM	Transmembrane
Tris	Tris-(hydroxymethyl)-aminomethane
U-50488	2-(3,4-dichlorophenyl)-N-methyl-N-[(1R,2R)-2-pyrrolidin-1-ylcyclohexyl]acetamide
U-69593	(+)-(5α,7α,8β)-N-Methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro-[4,5] dec-8-yl]
UFP-101	[$Nphe^{1}$,Arg^{14},Lys^{15}]N/OFQ-NH_2
UFP-102	[(pF)Phe^{4},Arg^{14},Lys^{15}]N/OFQ-NH_2
ZP-120	Ac-Arg-Tyr-Tyr-Arg-Trp-Lys-Lys-Lys-Lys-Lys-Lys-Lys-NH_2

INTRODUCTION

1. Historical overview of poppy plant and opium

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; after all, opium is prepared from the juice of the poppy plant. The name of the plant means, the "sleep-bringing poppy", referring to its narcotic properties.

The medical historic writings of Theophrastus (3rd century B.C.) were the first known written source mentioning opium. The Egyptians cultivated opium thebaicum in famous poppy fields around 1300 B.C. Opium was used as a poison to let people to death without pain, but it was also employed in medicine. In the ancient time, poppy plant has been applied for anaesthesia, analgesia and for treating diarrhoea as well as sleeping disorders. During the Middle Ages, many of the uses of opium were appreciated. In 1680, Sydenham wrote: "*Among the cures which it has given to man to relieve his sufferings, none is as efficacious as opium*" *(Wong, 2003)*

Fig. 1. *Papaver somniferum L., Opium poppy.*

Opioids have been the basis of pain treatment for thousands of years, and they remain so today. Opioids are the collective names for alkaloid compounds directly derived from opium and for many synthetic derivatives that have been created which imitate the effects of those drugs, as well as the endogenous peptides that interact with the opioid receptors. The poppy plant itself produces at least 20 different alkaloids. The most important ones are the analgesic and narcotic morphine, the antitussive codeine, the smooth muscle relaxant papaverine and the convulsant thebaine. The pain

relieving effect of opium and later of pure morphine has been successfully used against severe pain. The term 'opiate' includes all natural and synthetic drugs with morphine like action. The old term opiates is now more and more replaced by the term opioids which applies to any substance, whether endogenous or synthetic, peptidic or non-peptidic, that produces morphine-like effects through an action on opioid receptors *(Trescot et al., 2008)*.

2. Opioid and nociceptin system

Opioids, like many other bioactive compounds, are acting through specific cellular elements called receptors. Consistent with the 'receptor theory' established by Paul Ehrlich: '*Corpora non agunt nisi fixata'* ('substances do not act unless bound'), opioid binding cellular structures are opioid receptors. The discovery of opioid receptors in 1973 by demonstrating the existence of specific binding sites for opioid radioligands in neural cell membranes turned out to be a breakthrough in biochemical pharmacology *(Terenius, 1973; Pert and Snyder, 1973; Simon et al., 1973)*.

Receptor theory suggested also that opioid receptors in physiological conditions are activated by endogeneous molecules within the body. These natural compounds were designated '**endorphins**' by fusing the terms 'endogenous' and 'morphine'. The intensive search for endogenous opioids resulted in the discovery of two related pentapeptides methionine (Met-) and leucine (Leu-) enkephalin in 1975 *(Hughes et al., 1975)*. Endogenous opioid peptides and their G-protein-coupled receptors are located in the central nervous system and peripheral tissues. The opioid system has been studied to determine the intrinsic mechanism of modulation of pain and many other physiological processes and to develop uniquely effective pain-control substances with minimal abuse potential and fewer side effects *(Prüll et al., 2003)*. Opioids produce analgesia by binding to opioid receptors both within and outside the central nervous system.

Following the elegant biochemical presentation of opioid receptors *(Terenius, 1973; Pert and Snyder, 1973; Simon et al., 1973)*, the first evidence for their heterogeneity was achieved in the mid seventies and based upon detailed and comprehensive pharmacological analyses of various opioid drugs *(Martin et al., 1976; Gilbert and Martin, 1976)*. Receptor-binding and cloning studies confirmed the existence of three main receptor types: mu- (μ), delta- (δ), kappa- (κ). A fourth member of the opioid peptide receptor family, the nociceptin/orphanin FQ (N/OFQ) receptor, was cloned in

1994, which does not interact with any of the classical opiate ligands *(Meunier et al., 1995)*, but it is part of the opioid family based on extensive sequence homology. First, the N-terminus of N/OFQ, FGGF, is highly reminiscent of the canonical YGGF of the opioid peptides. Furthermore, the N/OFQ precursor protein exhibits several analogous structures as compared to the opioid precursors: the active peptides are located in the C-terminal part and seven Cys residues are found conserved at the N-terminus of pronociceptin, prodynorphin, and proenkephalin *(Nothacker et al., 1996)*. Then, the NOP receptor shares more than 60% identity with the three opioid receptors *(Bunzow et al., 1994; Mollereau et al., 1994)*.Altogether, these data support the view that the receptors, as well as the neuropeptide precursors of both the opioid and the OFQ/N systems, have evolved from common ancestral genes.

In 2000, the Committee on Receptor Nomenclature and Drug Classification of the International Union of Pharmacology (NC-IUPHAR, http:/www.iuphar.org) adopted the terms MOP, DOP, KOP and NOP to indicate *mu-*, *delta-*, *kappa-* opioid and nociceptin/orphanin FQ (N/OFQ) peptide receptors, respectively.

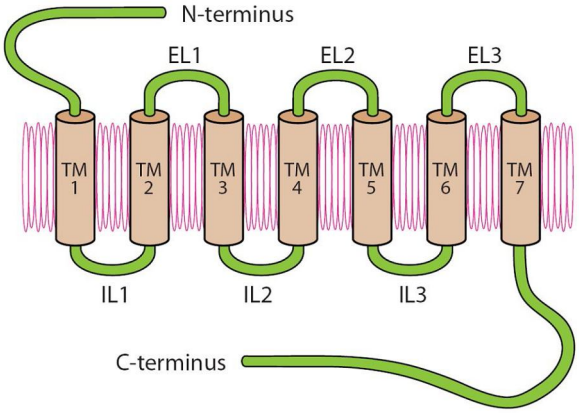

Fig. 2 *Opioid receptor structure.* EL -*Extracellular loop;* TM - *Transmembrane helix;* IL - *Intracellular loop*

Opioid receptors are cell surface glycoproteins and they are tightly integrated into the cell membranes. The human MOP, DOP, KOP and NOP genes encodes a heptahelical protein comprised of 400, 372, 380 and 370 amino acids respectively. Several splice variants of MOP (formerly MOR-1) have been cloned *(Cadet et al., 2004)*. Only one DOP gene has been cloned to date. Pharmacological experiments in rodents indicate the subdivision into δ_1 and δ_2 receptors. From the binding characteristics of the prototypical ligand ethylketocyclazocine, evidence for the subdivisions in κ_1, κ_2 and κ_3 has been provided *(Paternak et al., 2004)*. Splice variants have been found in the

human and mouse NOP receptor *(Xie et al., 1999)*. These receptor variants differ in their distribution in the central nervous system and in the rate of internalization and desensitization upon agonist exposure but have similar binding and coupling properties. MOP, DOP, KOP and NOP genes are located on paralogous (gene from the same homologous superfamily found in another part of the genome of a particular taxon, paralogous are the result of a gene duplication, not a lineage splitting event) regions of human chromosomes which is evidence for gene duplication for opioid receptors *(Fredriksson et al., 2003)*. Human MOP and DOP genes are mapped to chromosome 6 and 1, respectively, while KOP and NOP genes map to chromosomes 8 and 20.

All of the cloned opioid receptor types belong to the G_i/G_o-coupled superfamily of receptors (GPCRs). Such receptors are composed of a single polypeptide chain. The main feature of these proteins is the presence of seven membrane-spanning sequences containing hydrophobic amino acid side-chains permitting the integration of the macromolecule into the lipid bilayer of the cell membrane by hydrophobic lipid-protein interactions *(Corbett et al., 1993)*. Covalent modifications, e.g. palmitoylation of the C-terminal, and composition of disulphide bridge(s) between the extracellular loops can help to maintain the properly folded active receptor conformation. All of the opioid receptor types possess the same general structure of an extracellular N-terminal region containing N-glycosylation recognition sites, the seven transmembrane domains taking part in the formation of the binding pocket, and intracellular C-terminal tail structure with phosphorylation- and G-protein interacting sites (Fig. 2.). The water soluble connecting portions of the polypeptide chain form three extracellular (facing outside) and three intracellular (located whithin the cytoplasma) loops *(Devi et al., 2001)*.

3. Effector mechanisms

Upon agonist ligand binding (morphine, enkephalin, N/OFQ etc.), receptor proteins become activated due to conformational changes that results in the transduction of the information signal. The major downstream element of opioid signaling whithin the membrane is the activation of the G_α subunit of regulatory G-proteins (G_i/G_q and G_o types mainly). Activation of any type of opioid receptor consecutively inhibits the enzyme adenylate cyclase via inhibitory G-proteins, resulting in a fall in the intracellular level of the key second-messenger molecule cAMP and diminishes action

potential firing *(Chevlen, 2003)*. Conversely, opioid addicts undergoing withdrawal suffer elevated cAMP levels and enhanced protein kinase A activity, resulting in increased neurotransmitter release *(Corbett et al., 2006)*.

4. Compounds targeting opioid receptors

The MOP is the classical target for morphine and mediates the analgesic and addictive affects of the drug. Therefore, in MOP-deficient mice morphine does not exhibit analgesic and positive reinforcing properties *(Matthes et al., 1996)*. Endomorphin-1 and 2 are two pentapeptides that show the highest selectivity for this receptor *(Zadina et al., 1997)*. Another selective agonist is the synthetic peptide DAMGO (D-Ala^2, nMe-Phe^4, Gly^5-ol) enkephalin) *(Handa et al., 1981)*. The action of MOP agonists are competitively blocked by the antagonist naloxone, which is not absolutely specific for MOPs. The somatostatin analogue CTAP (D-Phe-Cys-Thr-D-Trp-Arg-Thr-Pen-Thr-NH2) has been found to be a more selective MOP antagonist *(Pelton et al., 1986)*.

The DOP receptor is the primary target for met- and leu-enkephalin which also exhibits affinities for MOP and KOP receptors. DIDI (Ile^{5,6}deltorphin-2) and DPDPE (D-Pen^2, D-Pen^5) enkephalin *(Mosberg et al., 1983)* are selective agonists, and naltrindole is a selective antagonist on DOP.

Receptor Subtype	MOP	DOP	KOP	NOP
Prototypic ligands	Morphine	Met-enkephalin Leu-enkephalin	Ethylketocyclacozine	N/OFQ
Endogenous ligand	Endomorphin-1 Endomorphin-2	Met-enkephalin Leu-enkephalin	Dynorphyn A Dynorphyn B	N/OFQ
Selective agonist	DAMGO Dermorphin	DIDI DPDPE	U-69593 U-50488	UFP-102 Ro 65-6570
Selective antagonist	Naloxone CTAP	Naloxone Naltrindole	Naloxone Nor-binaltorphine	J-113397 UFP-101

Table 1. *Opioid receptors and their selective ligands*

The KOP receptor is the natural target for prodynorphin-derived peptides, such as dynorphin A, dynorphin B, α-neoendorphin etc. The prototypical, although non-selective ligand is ethylketocyclazocine (EKC). U-69593 and U-50488 are selective agonists and norbinaltorphimine is an irreversible selective antagonist *(Takemori et al., 1988)*.

None of the endogenous opioid peptides or the opiate drugs shows high affinity for the NOP receptor. An endogenous ligand that binds to NOP with high affinity has been identified and termed nociceptin FQ *(Meunier et al, 1995) or orphanin FQ*

(Reinscheid et al., 1995), currently also referred as N/OFQ.. Pseudopeptide bond containing UFP-102 *(Carrà et al., 2005)* and non-peptide Ro-656570 *(Rizzi et al., 2001)* are one of the most potent NOP agonists. Recently, peptide UFP-101 *(Calo et al., 2005, Arduin et al., 2007)* and non peptide J-113397 *(Kawamoto et al., 1999)*, a drug with potent and selective antagonist activity at NOP receptors, has been characterized.

Hexapeptides Ac-RYYRIK-NH_2, Ac-RYYRWK-NH_2, Ac-RYYRWR-NH_2 etc. with high affinity and selectivity to the NOP receptor were identified from a combinatorial peptide library containing about 52 millions of compounds and reported to have partial agonist properties at the NOP receptors (*Dooley et al., 1996; Dooley et al., 1997).* These structures have also become templates for other peptide analogues with additional chemical modifications, such as ZP-120: Ac-RYYRWKKKKKKK-NH_2 *(Rizzi et al., 2002),* Ac-Arg-D-Cha-Qaa-D-Arg-D-pClPhe-NH_2 (*van Cauwenberghe et al., 2004*) or Ac-RYYRIR-NH_2 (*Ambo et al., 2007*).

5. Endogenous opioid peptides

Natural opioid peptides, defined as endogenous peptides with opioid-like pharmacological effects, are derived from distinct precursor polypeptides and have a characteristic anatomical distribution. Precursor polypeptides are encoded in the genome and are synthesized by protein translation using an mRNA template with the help of the ribosomal machinery *(Costa et al., 1987).* Each precursor is subjected to complex enzymatic cleavages, transport and other posttranslational modifications resulting in the accomplished active peptides. Many non-neuronal cells, including endocrine- and exocrine glands, cells of the immune system and heart, produce opioid peptides *(Cabot, 2001).* These peptides play a regulatory role in many different physiological systems.

Endogenous opioid peptides were first found and identified chemically in porcine brain and pituitary (hypophysis) extracts by Scottish researchers *(Hughes at al., 1975),* they reported the existence of an endogenous morphine-like substance that interacted with morphine (MOP) and enkephalin (DOP) receptors. Later, they purified these substances to homogeneity and named them enkephalin (‘in the head’). Enkephalins are closely related pentapeptides with the structure of Tyr-Gly-Gly-Phe-**Met/Leu** (or in the one-letter code: YGGP**M/L**).

β-endorphin, a endogenous opioid peptide derived from the Pro-opiomelanocortin which was discovered from camel pituitary extracts *(Li et al.,, 1976)* is equiactive at MOP and DOP receptors with much lower affinity for KOP receptors *(Corbett et al., 2006)*. Met- and Leu-enkephalin have high affinities for DOP receptors, ten-fold lower affinities for MOP receptors and negligible affinity for KOP receptors. In addition, two endogenous tetrapeptides have been isolated that exhibit a high selectivity for MOP receptors and have been called endomorphin-1 and endomorphin-2 with the general structure of Tyr-Pro-**Trp/Phe**-Phe-NH_2 *(Zadina et al., 1997)*. Opioid peptides and their receptors have subsequently been studied through the use of immunohistochemistry *(Covenas et al., 2004)*, and have been found both at a central and at a peripheral levels.

Proopiomelanocortin (POMC)	Proenkephalin (PENK)	Prodynorphin (PDYN)	Pronociceptin (PNOC)	(Proendomorphin)*
β-endorphin	Met-enkephalin	Dynorphin A	Nociceptin/OFQ	Endomorphin-1
	Leu-enkephalin	Dynorphin B		Endomorphin-2
	Met-enk-Arg-Phe	α-neoendorphin		
	Met-enk-Arg-Gly-Leu	β-neoendorphin		

Table 2. *Precursor proteins and endogenous opioid peptides. *Presently unknown.*

6. Precursor polypeptides

In mammals the endogenous opioid peptides are derived from four precursors: pro-opiomelanocortin (POMC), pro-enkephalin (PENK), pro-dynorphin (PDYN) and pro-nociceptin/orphanin FQ (PNOC) (Table 2) *(Nakanishi et al., 1979; Noda et al., 1982; Kakidani et al., 1982)*.

The opioid peptide precursor genes are located in different chromosomes in the species investigated. Genetic structures are closely related, all opioid precursor genes have only one introne within a region encoding the N-terminus of the full-length precursor protein *(Nikoshkov et al., 2005)*. Each prepro-polypeptide precursor, as product of the source gene, undergoes post-translational modifications (processing) that result in multiple bioactive peptides. These peptides share (with the exception of nociceptin) the common N-terminal sequence of Tyr-Gly-Gly-Phe-(Met or Leu), which has been termed either 'opioid motif' or 'message sequence'; this pattern is followed by various C-terminal extensions yielding peptides ranging from 5 to 31 residues in length. Each precursor polypeptides have N-terminal signal peptid sequence for transport and release, cysteine rich N-terminal region, and differing

numbers of opioid core sequences encompassed by specific recognition sites for enzymatic cleavage. Pairs of basic amino acids, such as Lys-Arg (KR) or Lys-Lys (KK) or Arg-Arg (RR) surround enkephalin (opioid) units in the precursor. This, highly protonated positively charged dipeptide repeats determine recognition sites for the processing endopeptidase enzymes, e.g., prohormone convertase.

POMC is a multifunctional precursor giving rise to adrenocorticotropin (ACTH), α-, γ- and β-melanocyte stimulating hormone (MSH) but apparently only one opioid peptide, β-endorphin *(Li et al., 1976)*. Besides the pentapeptides Met-enkephalin (**ME**) and Leu-enkephalins (**LE**), proenkephalin is processed in the heptapeptide Met-enkephalin-Arg^6-Phe^7 (**MERF**) and the octapeptide Met-enkephalin-Arg^6-Gly^7-Leu^8 (**MERGL**) (Table 2). PDYN is processed into dynorphin A, dynorphin A (1–8), dynorphin B, α-, β-neoendorphin *(Goldstein et al., 1981)*. The bioactive fragments of PDYN, particularly dynorphin A and dynorphin B, have high affinity for KOP receptors but also have significant affinity for MOP and DOP receptors *(Danielson et al., 1999)*. Other important feature of the mammalian PDYN is the lack of any Met-enkephalin sequence in the precursor. N/OFQ was discovered by the first successful application of the 'reverse pharmacology approach', *i.e.*, the use of an orphan receptor to identify its endogenous ligand and its processed from its precursor polypeptide PNOC *(Meunier et al., 1995; Reinscheid et al., 1995)*.

The organization of the PENK gene and the three other opioid precursor genes, PDYN, POMC, and PNOC, suggests that these four genes share a common evolutionary origin. From a phylogenetic point of view, PENK is believed to be the ancestral form, the present POMC, PNOC and especially PDYN forms appeared at later stages of evolution *(Danielson and Dores, 1999; Dores et al., 2002)*. To date, complete PENK, PDYN, POMC and PNOC cDNA sequences have been obtained for multiple species of mammals, amphibians and fish. Attempts to clone the genes for the endomorphin-1 and endomorphin-2 corresponding precursor molecules have not been successful.

7. Pharmacology of the opioid and nociceptin system

For decades of synthetic efforts in the opioid field has been the search for an alternative to morphine, which would induce powerful analgesia without the attendant effects of respiratory depression and, above all, liability for abuse. Due to their relatively rapid enzymatic decomposition and the limited transport across the blood

brain barrier the pharmacological effects of the enkephalins are generally less than that of morphine, at least in systemic administration.

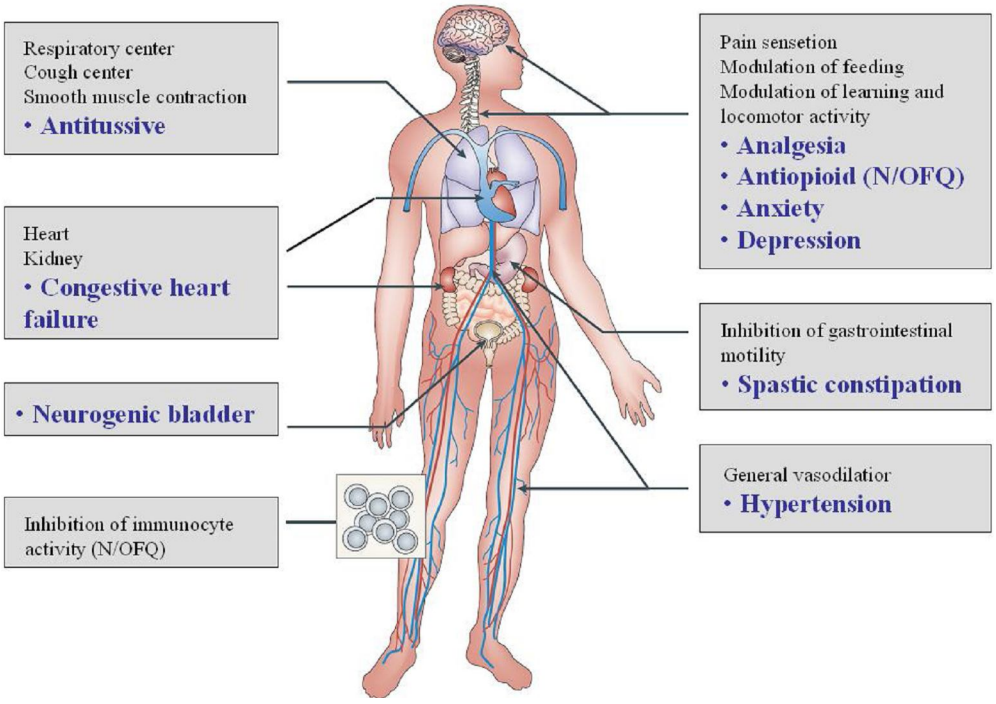

Figure 3. *Effects of the opioids and N/OFQ on major organ systems. Clinical indications are noted in bold.*

In humans, morphine-like drugs produce analgesia, drowsiness, changes in mood, and mental clouding. Opioids have long been used to treat acute pain (such as post-operative pain). They have also been found to be effective in severe, chronic, disabling pain of terminal conditions such as cancer, and degenerative conditions such as rheumatoid arthritis (Figure 3). A significant feature of the analgesia is that it occurs without loss of consciousness *(McQuay, 1999)*. Opioids are classified as central nervous system depressants, i.e., they are cellular inhibitors of the neuronal activity *(Chevley, 2003)*. Other synthetic opioids (e.g., fentanyl) are also effective as an analgesic *(McQuay, 1999)* or act as cough suppressants (e.g., codeine) *(Mogil and Paternak et al., 2001)*. Semi-synthetic opioids also have valuable medical uses either as analgesics (e.g., oxycodone) or antitussive compounds (e.g., hydrocodone)*(Mogil and Paternak et al., 2001)*. At higher doses, effects include increased sedation or euphoria, impaired concentration, reduced respiration and blood pressure, contraction of pupils, constipation *(Hargreaves and Joris, 1993)* and, in some cases, rapid and irregular heart rate *(Raehal et al., 2005)*. Tolerance for opiates, but typically not for

endogenous opioid peptides, develops fairly rapidly, making higher doses necessary to maintain the desired intensity of effects *(Stein et al., 1993)*. Most synthetic opioid narcotics and heroin, are quite addictive, and regular use of them may result in severe physical dependence, a serious and life-threatening health problem.

The N/OFQ is involved in a wide range of physiological responses with effects noted in the nervous system (central and peripheral), the cardiovascular system, the airways, the gastrointestinal tract, the urogenital tract and the immune system *(Lambert, 2008)* (Figure 3). The effects in the nervous system are complex and have received much attention. It is generally accepted that spinal N/OFQ is antinociceptive with many features that are common to the classical members of the opioid family *(Zeilhofer et al., 2003)*. Nevertheless, when given supraspinally, N/OFQ reverses the effects of opioids (anti-opioid action) with a whole-animal response that manifests as hyperalgesia *(Zeilhofer et al., 2003)*. In the brain, N/OFQ is affects the response to stress, anxiety and locomotion *(Chiou et al., 2007)*. In the cardiovascular system N/OFQ produces bradycardia and hypotension; this response is similar to that produced by classical opioids and more specifically to morphine used in the clinic.

8. Opioid system in lower vertebrates

The opioid/orphanin gene family provides a model system for analyzing the genome duplication events. Studies done with proenkephalin gene provided a support for the organizational arrangement which has been conserved throughout the extensive radiation of the vertebrates *(Dores et al., 2002, Dores et al., 2004)*. It has been proposed that the evolution of the opioid/orphanin precursor gene family resulted from successive duplication of the proenkephalin gene (‘Proenkephalin hypotheses`). These duplications gave rise first, to the proopiomelanocortin gene, then to the pronociceptin gene and most recently to the prodynorphin gene *(Khalap et al., 2005)*.

Among the Gnathostomes (animals that have developed the trait of a jaw), the organizational plan for the PENK precursor is remarkably conserved. For example in lineages as diverse as mammals, cartilaginous fish and bony fish there are usually seven opioid sequences present in this precursor *(Khalap et al., 2005)*. Yet, within vertebrate lineages there can be distinct sets of pentapeptide opioids (YGGFM or YGGFL). In the Sarcopterygii, the sixth opioid position in lungfishes and anuran amphibian proenkephalin genes encodes a Met-enkephalin (YGGFM) sequence *(Lecaude et al., 2000)*. However, in mammalian proenkephalin there is a Leu-

enkephalin (YGGFL) sequence at this position. These data are consistent with the conclusion that the transition from a Met-enkephalin sequence to a Leu-enkephalin sequence at the sixth opioid position in tetrapod proenkephalins occurred in the ancestral proto-reptiles *(Roberts et al., 2007)*.

There were a number of radioligand binding studies in brain homogenates from non-mammalian species including fish *(Gonzalez-Nunez et al., 2006)*, reptiles *(Xia et al., 2001)* and birds *(Martin et al 1992)*. However, the largest effort came from the study of opioid binding sites in amphibian brain membranes such as *Rana esculenta (Benyhe et al., 1990; Benyhe et al., 1994; Benyhe et al., 1999; Wolleman et al., 1999).*

8.1. Precursor polypeptides and opioid sequences

African clawed frog, *Xenopus laevis*, PDYN precursor contains a novel opioid pentapeptide, an N-terminally positioned Ile^5-enkephalin (IE) pentapeptide motif *(Pattee et al., 2003)*; this oligopeptide unit is absent in all mammalian PDYNs, but it is also found in some fish PDYNs, such as the ones cloned from eel (*Anguilla rostrata, Alrubaian et al., 2006)* and Nile tilapia (*Oreochromis niloticus, Alrubaian et al., 2006)*. Another unique enkephalin structure has been deduced from a partial cDNA sequence of the African lungfish (*Protopterus annectens*) prodynorhin *(Dores et al., 2000)*. African and Australian lungfish are well known as 'living fossils' among evolutionary biologists. Lungfish were dominant and widespread during the Paleozoic era, and the earliest fossils belong to the Devonian period; they are the closest living relatives of land Vertebrates *(Joss et al., 2006; Lee et al., 2006)*. Lungfish prodynorphin encodes for Phe^5-enkephalin (YGGF<u>F</u>) that is the second example of a different non-mammalian enkephalin structure.

In the case of PENK derived octapeptides, ubiquitous YGGFMRGL sequence is present in 26 mammals including the human. Several other mammalian octapeptides exist, e.g., YGGFMRG<u>Y</u> (*Xenopus laevis*, *Ornithorhyncus anatinus)*, YGGFMR<u>SY</u> (rainforest opossum, *Monodelphis domestica*), YGGFMR<u>A</u>L (cat and dog), YGGFMR<u>S</u>L (mouse), YGGFMRG<u>V</u> (European rabbit, *Oryctolagus cuniculus* and American pika, *Ochotona princeps*). Among other vertebrate animals, including fish, amphibians, reptiles and birds, variability in the structure of the octapeptide is present within the PENK polypeptide. Mouse PENK contains YGGFMR<u>S</u>L which is unique among mammals, but is also present in the Australian lungfish (*Neoceratodus forsteri*) and African lungfish (*Protopterus annectens*) proenkephalins *(Sollars et al.,*

2000; Dores et al., 2000). The double substituted YGGFMNGF sequence is present in a cartilaginous fish species *(Komorowski et al., 2004 direct submission)*, the bullhead shark *(Heterodontus portusjacksoni)*. While YGGFMRGY sequence occurs in the *Xenopus laevis* and *Xenopus tropicalis (Martens and Herbert, 1994)*. YGGFMRSV octapeptide is included in the chicken and florida gar proenkephalin *(Khalap et al., 2005)*.

Mutations of nucleotide bases in the coding regions of the DNA lead to changes in amino acid sequence, thus explain the differences observed at the amino acid level of certain neuropeptides in various animal species. The more distantly related two species are, the more substitutions can be found in one and the same neuropeptide. Difference in the amino acid sequence may cause difference in biological function, but usually, the orthologous family members seem to produce the same effect *(Holmgren and Jensen, 2001)*. The region carrying the biological activity is generally the most conserved segment in neuropeptides.

8.2. Nociception

There is a wealth of information regarding nociception in mammalian systems, however, recently non-mammalian vertebrates, amphibian and avian models, have been extensively studied *(Gentle, 1992; Stewens, 2003)*.

Nociceptors (sensory receptors that react to potentially damaging stimuli, such as pain) are associated with free nerve endings and are usually of two fibre types, small myelinated A-delta fibres and smaller unmyelinated C fibres *(Lynn, 1994)*. Yet in the the oldest living ancestor of the fishes, the lamprey, *Petromyzon marinus*, has only unmyelinated fibres, yet electrophysiological recordings indicated slowly adapting receptors that responded to noxious heat, therefore, possibly nociceptive *(Matthews and Wickelgren, 1978)*. Initial studies of the analgesic or antinociceptive effects of opioids in amphibians (*Rana pipiens*) were conducted using non-selective opioid agonists, endogenous opioid peptides, and antagonists *(Pezalla et al., 1984a)*. Tolerance to the analgesic effects of daily morphine administration was documented *(Stevens et al., 1993)* and stress-induced release of endogenous opioids produced analgesia which was potentiated by enkephalinase inhibitors and blocked by naltrexone *(Pezalla et al., 1984b)*. Those studies showed that both exogenous opioid agonists and endogenous opioid peptides could raise the nociceptive threshold in amphibians by an action at opioid receptors.

AIM OF THE STUDIES

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)*. Our analysis of upto 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.

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.

Present studies were devoted to characterize naturally occurring opioid penta- and octapeptides as well as the synthetic NOP receptor selective hexapeptide analogue Ac-RYYRIR-ol using biochemical, functional and pharmacological approach. *In-vitro* biochemical assays were performed in rat brain membranes. Receptor-type selectivity patterns were determined in radioreceptor binding experiments, while G-protein activating properties of the peptides were investigated in [^{35}S]GTPγS functional tests.

Main objective of the present studies were;

✓ *To collect all available PENK and PDYN sequences and aligning them to characterize variability of the opiod peptides within the precursors,*

✓ *To chemically synthesize and to characterize the novel opioid sequences (Table 3) 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 deferens, guinea pig ileum, and mouse colon bioassays, and calcium 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.*

Opioid Pentapeptides	Opioid Octapeptides	Synthetic Hexapeptides
YGGFM YGGFL *Mammalian* 	YGGFMRGL *Mammalian* 	Ac-RYYRIK-NH_2 *Dooley peptide*
YGGFI *Frog* 	YGGFMRGY YGGFM$_{(O)}$RGY *Frog* 	Ac-RYYRIR-ol
YGGFF *Lungfish* 	YGGFMRSV YGGFM$_{(O)}$RSV *Chicken* 	[^{3}H]Ac-RYYRIK-ol *New Radioprobe*
	YGGFMNGF YGGFM$_{(O)}$NGF *Shark* 	
	YGGFMRSL YGGFM$_{(O)}$RSL *Mouse & Fish* 	

Table 3. All the novel sequences studied in this thesis which includes, two non-mammalian enkephalin, four octapeptides together with its oxidized forms, one modified and one radiolabelled hexapeptide which selectively acts towards the NOP receptors

MATERIALS AND METHODS

1. Radiochemicals

[^{3}H]Naloxone (28 Ci/mmol), [^{3}H]DAMGO ([D-Ala^2,NMePhe^4,Gly^5-ol]enkephalin; 41 Ci/mmol), [^{3}H]Tyr^1,Ile^{5,6}deltorphin-2 (48 Ci/mmol), *(Nevin et al.,1994)* [^{3}H]D-Ala^3-dynorphin-[1-11]amide (40 Ci/mmol) (Lung et al., 1995), [^{3}H]Ac-RYYRIK-ol (94 Ci/mmol) (*Bojnik et al., 2009b*), [Phenylalanyl-^{3}H]nociceptin-amide (25 Ci/mmol) *(Wollemann et al., 2008)* were radiolabeled in the Isotope Laboratory of BRC, Szeged. [^{3}H]U-69,593 (55 Ci/mmol) and unlabelled U-69,593H that is (+)-(5α,7α,8β)-N-Methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro-[4,5]dec-8-yl] were purchased from Amersham Biosciences and the Upjohn Co., respectively, [Leucyl-^{3}H]nociceptin (TRK1047; specific activity 162 Ci/mmol were purchased from Amersham, U.K. Guanosine-5-[γ-^{35}S]-triphosphate (1204 Ci/mmol) was purchased from the Isotope Institute Ltd. (Budapest, Hungary).

2. Opioid and NOP receptor ligands

All peptides used in these studies were chemically synthesized and not extracted/purified from biological sources. Mammalian Leu- and Met-enkephalin (YGGFL/M) and methionine extended octapeptide (YGGFMRGL) and N/OFQ used in these studies were purchased from Bachem Feinbiochimica, Bubendorf, Switzerland. Lungfish Phe-enkephalin (YGGF**F)**,; non mammalian octapeptides, YGGFM**RGY** (African clawed frog), YGGFM**RSV** (Chicken), YGGFM**NGF** (shark) and YGGFM**RSL** (African lungfish) and NOP receptor selective hexapeptide alcohols, Ac-RYYRIR-ol and Ac-RYYRIK-ol and other analogues *(Kocsis et al., 2004)* were prepared at the Research Group for Peptide Chemistry of the Hungarian Academy of Sciences, Budapest, Hungary, by liquid phase peptide synthesis. Frog Ile-enkephalin (YGGF**I**) was prepared by automated solid phase peptide synthesis at the Isotope Laboratory of BRC, Szeged, Hungary. Naloxone hydrochloride was kindly provided by Du Pont de Nemours and buprenorphine was obtained from ICN-Alkaloida Inc., (Tiszavasvari, Hungary). The NOP receptor antagonists [Nphe^1,Arg^{14},Lys^{15}]nociceptin-NH_2 (UFP-101) and (1-[(3R,4R)-1-cyclooctylmethyl-3-hydroxy-methyl-4-piperidyl]-3-ethyl-1,3-dihydro-2H-benzimidazol-2-one; J-113397) were kindly provided by Dr. Girolamo Calo' (Ferrara, Italy). All the

compounds used in these studies were purified by analytical HPLC methods, and the structures were confirmed by mass spectrometry.

3. Other chemicals

$MgCl_2$, KCl, EDTA, polyethylenimine (PEI), Tris(hydroxymethyl)aminomethane (Tris), protease-free bovine serum albumin (protease-free BSA, fraction V), guanosine 5'-diphosphate (GDP), guanosine-5'-O-(3-thiotriphosphate), GTPγS were purchased from Sigma-Aldrich (St. Louis, MO, USA). Morphine was obtained from ICN Alkaloida, Tiszavasvári, Hungary. All the other chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

4. Animals

Inbred Wistar rats (250–300 g body weight) and guinea pigs (R9strain) were housed in the local animal house of the Biological Research Center (BRC, Szeged, Hungary). Rats were kept in groups of four, allowed free access to standard food and tap water and maintained on a 12-h light/dark cycle until the time of sacrifice. Male Swiss mice (25–30 g), guinea pigs (300–350 g), and Sprague Dawley rats (300–350 g) were used throughout the in vitro isolated tissue assays of Ac-RYYRIR-ol (Morini, Reggio Emilia, Italy). Mice were housed in 425 x 266 x 17 155-mm cages (Techniplast, Milan, Italy), eight animals/cage, under standard conditions (22ºC, 55 % humidity, 12-h light/dark cycle, light on at 7:00 am) with food (MIL, standard diet) and water ad libitium for at least 3 days before experiments began. Animals were handled according to the European Communities Council Directives (86/609/ECC) and the Hungarian Act for the Protection of Animals in Research (XXVIII.tv. 32.§) and Italian national regulations (D.L. 116/92). Accordingly, the number of animals and their suffering were minimized.

5. Rat brain membrane preparations

Crude membrane fractions were prepared from Wistar rat brains according to the method of Pasternak with small modification *(Simon et al., 1986)*. Rats were decapitated and the brains without cerebellum were quickly removed and washed several times to remove any unwanted blood or tissue particles with chilled 50 mM Tris–HCl (pH 7.4) buffer. The brains were blotted dry, weighed and suspended in five

volumes/weight of the original brain tissue with ice-cold 50 mM Tris–HCl buffer. The brains were than homogenized at 1000 rpm with an electrically driven Braun Teflon-glass rota-homogenizer at 4 °C, using 10–15 strokes of the homogenizer. The final volume of the homogenate was made up to 30 volumes/weight of the brain and filtered through four layers of gauze to remove any larger aggregates. After centrifugation with a Sorvall RC5C centrifuge at 4000xg (18,000 rpm) for 20 min at 4°C, the resulting pellet was resuspended in fresh buffer (30 volumes/weight) by using a vortex. The suspension was incubated for 30 min at 37 °C to remove any endogenous opioids. Centrifugation was repeated under the same conditions as described above, and final pellet was resuspended in five volumes of 50 mM Tris–HCl (pH 7.4) buffer containing 0.32 M sucrose to give a final concentration of 3–4 mg/ml protein. The presence of sucrose is necessary for stabilization of proteins for storage. The membranes were kept in 5 ml aliquots at -70 °C until use. The binding activity of the protein remained stable for at least 2 months. Membranes were thawed and resuspended in 50 mM Tris–HCl (pH 7.4) buffer and centrifuged at 40,000xg (18,000 rpm) for 20 min at 4 °C to remove the sucrose. The resulting pellets were taken up in appropriate fresh buffer and immediately used in binding assays. The protein concentration was determined according to the method of Bradford (1976), using bovine serum albumin as a standard.

6. Preparation of $_{h}$NOP-CHO cells

Chinese hamster ovary (CHO) cells stably expressing the wild type human nociceptin ($_{h}$NOP) receptor protein were kindly provided by Dr Jean-Claude Meunier, Toulouse, France. Cells were cultured in a medium containing Nut Mix F-12 with l-glutamine (GIBCOInvitrogen) and 25 mM Hepes, 10 % FCS, 100 UI/ml penicillin, 100 µg/ml streptomycin and 0.4 mg/ml G418 at 37ºC in a humidified atmosphere consisting of 5 % CO2 and 95 % air. Cells were sub-cultured twice a week. Cells were harvested with trypsin solution (0.05 % trypsin, 0.02 % EDTA) in ice cold PBS by washing two times, frozen at -70°C for 2 hours to facilitate cell disruption by water crystallization and homogenized in 50 mM Tris-HCl (pH 7.4) buffer with a glass/glass hand homogenizer (Wheaton USA). Then centrifuged at 1000 x g for 10 min at 4ºC and the collected pellets were homogenized in 50 mM Tris-HCl (pH 7.4) buffer with a glass / glass hand homogenizer. Homogenates were centrifuged two times at 40,000 × g for 20 min at 4ºC. Pellets were suspended in Tris-HCl (pH 7.4) buffer and following the

protein determination the membrane preparation was aliquoted (0.3-0.4 mg/ml protein per series) and stored at -70ºC until use. Membranes for the GTPγS binding assays were prepared by the same procedure, but the pellets were suspended in TEM (50 mM Tris, 1 mM EGTA, 5 mM MgCl2) pH 7.4 buffer. Membrane fractions were aliquoted (~ 240 μg protein per series = ~ 10 μg protein/sample) and stored at -70ºC until use.

7. Receptor binding assays

All binding assays were performed at 25°C for 30 minutes in 50 mM Tris–HCl buffer (pH 7.4) in a final volume of 1 ml, containing 1 mg BSA and 0.2–0.4 mg/ml membrane protein. Samples were made in disposable plastic assay tubes (Sarstedt Co., Nümbrecht, Germany). Rat and Guinea brain membranes were incubated with the general opioid agonist radioligand [^{3}H]naloxone (0.8–1.2 nM), or with type-specific radioprobes, such as the selective MOP receptor agonist [^{3}H]DAMGO (0.9–1.2 nM), the DOP receptor selective agonist [^{3}H]Tyr^1,Ile^{5,6}deltorphin-2 *(Nevin et al., 1994)* (Tyr-D-Ala-Phe-Glu-Ile-Ile-Gly-NH_2; 0.8–1.3 nM), or with 0.4–0.7 nM of the KOP receptor-specific agonist [^{3}H]D-Ala^3-dynorphin-[1-11]amide and [^{3}H]U-69,593 (with guinea pig brain membranes) and NOP receptor radioprobes [^{3}H]Ac-RYYRIK-ol and [Leucyl-^{3}H]nociceptin in the presence of unlabeled test ligands (their concentrations ranged from 10^{-5} to 10^{-11} M). Conditions for incubations are given in the figure legends. Non-specific binding was determined in the presence of 10 μM naloxone for opioid and 10 μM N/OFQ for the NOP receptor studies. Reaction was terminated and bound and free radioligands were separated by rapid filtration under vacuum through Whatman GF/C (radiolabeled peptides) or GF/B (radiolabeled alkaloids) glass fiber filters by using Brandel M24R Cell Harvester. Filters were washed three times with 5 ml ice-cold 50 mM Tris–HCl buffer. After filtration and separation procedure had been completed, fiber-disks were dried under an infrared lamp, and then removed from the filter-sheet by tweezers. Each disk was inserted into UltimaGold™ environment friendly, non-volatile, toluene-free scintillation cocktail, and placed into individual sample vials (transparent glass, Packard). Bound radioactivity was determined in Packard Tricarb 2300TR liquid scintillation analyzer. Receptor binding experiments were performed in duplicate and repeated at least three times.

8. [^{35}S]GTPγS binding assays

Rat brain membrane fractions (~10 μg of protein/sample) were incubated at 30°C for 60 min in Tris–EGTA buffer (50 mM Tris–HCl, 1 mM EGTA, 3 mM $MgCl_2$, 100 mM NaCl, pH 7.4) containing [^{35}S]GTPγS (0.05 nM) and increasing concentrations (10^{-9} to 10^{-5} M) of the compounds tested in the presence of 30 μM GDP in a final volume of 1 ml. Total binding was measured in the absence of test compound, non-specific binding was determined in the presence of 10 μM unlabeled GTPγS and subtracted from total binding to calculate the specific binding. The reaction was started by addition of [^{35}S]GTPγS and terminated by filtrating the samples through Whatman GF/B glass fiber filters. Filters were washed three times with ice-cold 50 mM Tris–HCl buffer (pH 7.4) using Brandel M24R Cell Harvester, then dried, and bound radioactivity was detected in UltimaGold™ scintillation cocktail (Packard). Agonist-induced receptor-mediated G-protein stimulation is given as percentage over the specific [^{35}S]GTPγS binding observed in the absence of receptor ligands (basal activity).

9. Isolated tissue bioassay

Tissues for in-vitro studies were taken from male Swiss mice (25–30 g), guinea pigs (300–350 g), and Sprague Dawley rats (300–350 g). The mouse vas deferens, the rat vas deferens and the guinea pig ileum was prepared according to Bigoni et al., (1999). The tissues were suspended in 5-ml organ baths containing Krebs solution oxygenated with 95% O_2 and 5% CO_2. The temperature was set at 33°C for the mouse vas deferens and at 37°C for the other tissues. A resting tension of 0.3 g was applied to the mouse vas deferens, 1 g to the guinea pig ileum and rat vas deferens. For the experiments on the mouse vas deferens, an Mg^{2+}-free and a 2.5 mM $CaCl_2$, rat vas deferens 1.2 mM $MgSO_4$ and a 1.8 mM $CaCl_2$ Krebs solution were used, respectively. All tissues were continuously stimulated through two platinum ring electrodes with supramaximal voltage rectangular pulses of 1 ms duration and 0.1 Hz frequency. The electrically evoked contractions (twitches) were measured isotonically with a strain gauge transducer (Basile 7006) and recorded with the PC-based acquisition system Power Lab (ADInstrument, USA). After an equilibration period of about 60 min, the contractions induced by electrical field stimulation were stable; at this time, cumulative concentration-response curves to agonists were performed (0.5 log-unit

step) in the absence or presence (15 min preincubation time) of antagonists. In these preparations, the effects of agonists were expressed as percent inhibition of the control twitch.

For the mouse colon bioassay, approximately 1 cm length segments of mouse colon were prepared to record the isometric smooth muscle contraction as described previously *(Rizzi et al., 1999)*. Briefly, the preparations were mounted vertically under 1 g tension in an organ bath (10 ml) containing Krebs buffer at 37°C and continuously gassed with 5% CO_2 and 95% O_2. Tissues were equilibrated for 60 min with washing every 10 min. For recording the maximal contractile response of the tissues 100 µM carbachol (carbamylcholine chloride) was used. In the mouse colon, N/OFQ and Ac-RYYRIR-ol elicit concentration-dependent contractions; concentration-response curves to these peptides were performed non-cumulatively, adding to the bath different concentrations of peptide at 20-min intervals. Contractile effects of N/OFQ or Ac-RYYRIR-ol, were expressed as % of the contraction induced by carbachol 100 µM.

10. Calcium-ion mobilization experiments

Cultured chinese hamster ovary (CHO) cells stably co-expressing the human recombinant NOP receptor and the chimeric protein $G\alpha_{qi5}$ *(Camarda et al., 2009)* were maintained in Dulbecco's Medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 100 mg/L hygromycin and cultured at 37°C in 5% CO_2 humidified air. Cells were seeded at a density of 50,000 cells/well into poly D-lysine coated, 96-well, black, and clear-bottom plates. The following day, the cells were incubated with medium supplemented with 2.5 mM of probenecid, 3 µM of the calcium sensitive fluorescent dye Fluo-4 AM, and 0.01% pluronic acid for 30 min at 37°C. After that time, the loading solution was aspirated, and 100 µL/well of assay buffer (Hank's balanced salt solution; HBSS) supplemented with 20 mmol of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2.5 mM of probenecid, and 500 µM of Brilliant Black (Aldrich) were added. Concentrated solutions (1 mmol) of N/OFQ and Ac-RYYRIR-ol were made in bidistilled water and kept at -20 °C. Serial dilutions were carried out in HBSS/HEPES (20 mM) buffer (containing 0.02% BSA fraction V) in order to prepare a master plate at 3× concentration. After placing both plates (cell culture and master plate) into the fluorometric imaging plate reader

FlexStation II (Molecular Devices, Sunnyvale, CA), fluorescence changes were measured at room temperature (≈ 22°C). On-line additions were carried out in a volume of 50 µL/well. Maximum change in fluorescence, expressed as percent over the baseline fluorescence, was used to determine agonist response.

11. Internalization of the fluorescent receptor

The recombinant HEK293 cells (which were obtained from Dr. Maithe Corbani Montpellier, France) were harvested using trypsin solution (0.05 % trypsin, 0.02 % EDTA). Cells (numbers of 30,000 - 50,000) were seeded in glass bottom flask in a 2-ml volume. The next day, the cells were incubated in 1 ml of Nut Mix F-12 (HAM) with l-glutamine (GIBCOInvitrogen) culture medium containing 0.1 mg BSA, 25 mm HEPES (pH 7.4) and the ligand at the desired concentration. Olympus Cell-R fluorescence microscope was used with GFP filter set combination and 20 x objectives (37ºC).

12. Data analysis and terminology

All data are expressed as means ± standard error of the mean of n experiments. Curve fitting was performed using PRISM 4.0 (GraphPad Software Inc., San Diego, U.S.A.). Agonist potencies were expressed as pEC_{50}, which is the negative logarithm to base 10 of the agonist molar concentration that produces 50% of the maximal possible effect of that agonist. The E_{max} is the maximal effect that an agonist can elicit in a given tissue/preparation. All radioligand binding experiments were performed in duplicates and the [^{35}S]GTPγS binding assays were performed in triplicates. Displacement curves were fitted by non-linear regression using the one-site competition fitting option with no ligand depletion. G-protein stimulation data were analyzed by the sigmoid dose-response curve fit option of Prism. The equilibrium inhibition constant (K_i value) was calculated from the IC_{50} values according to the built-in Cheng-Prusoff (1973) equation module. Whereas the IC_{50} value for a compound may vary between experiments depending on radioligand concentration, the K_i is an absolute value:

$$K_i = \frac{IC_{50}}{1 + \frac{[L]}{K_d}}$$

where [L] is the concentration of free radioligand used in the assay and K_d is the dissociation constant of the radioligand for the receptor.

It is known that partial agonists can antagonize the stimulatory effect caused by the full agonists. In the [^{35}S]GTPγS binding inhibition experiments the antagonist potency, expressed as pK_B, was derived from the following equation:

$$pK_B = \frac{IC_{50}}{\left(\left[2+\left(\frac{[A]}{EC_{50}}\right)^{n}\right]^{\frac{1}{n-1}}\right)}$$

where IC_{50} is the concentration of antagonist that produces 50% inhibition of the agonist response, [A] is the concentration of agonist, EC_{50} is the concentration of agonist producing 50% of the maximal response, and n is the Hill coefficient of the concentration response curve to the agonist *(Kenakin, 2004)*.

In the assays of the electrically stimulated mouse vas deferens, rat vas deferens and guinea pig ileum, the E_{max} of agonists is expressed as % of inhibition of the control twitch, while for mouse colon data as percent of the contraction elicited by 100 μM carbachol. Antagonist potencies were expressed as pA_2 which is the negative logarithm to the base 10 of the antagonist molar concentration that makes it necessary to double the agonist concentration to elicit the original submaximal response (*Arunlakshana and Schild, 1959*) and were calculated by Schild's linear regression.

RESULTS

NATURALLY OCCURRING NOVEL OPIOID PEPTIDES

1. Bioinformatic examination of the opioid motifs in PDYN and PENK precursor polypeptides of various species

An analysis of public protein databases (NCBI Protein, Ensembl) has shown significant diversity in the amino acid sequences of prodynorphin and proenkephalin-derived opioid peptides. Altogether 39 PDYN sequences have been collected by database searching. Non-allelic PDYN gene duplicates have been identified in the tetraploid *Xenopus laevis (Pattee et al., 2003)*. Mammalian PDYN end-products are identical at the amino acid level with the exception of the C-terminal tail region of dynorphin B. Organization and peptide sequence variability appears more complex among PDYNs, since both the number and the length of the opioid units are rather different within this precursor. The C-terminal section of PDYNs that contains α-neo-endorphin, dynorphin A and dynorphin B (also termed as rimorphin) is notably conserved, while the N-terminal segment of the non-mammalian PDYN precursors is substantially flexible in terms of amino acid composition. A common feature of these PDYNs is the presence of at least one or even more opioid core motif located upstream relative to the position of the 'opioid triad' in mammalian PDYNs. Thus, four enkephalin units are present in the PDYN of the cane toad *Bufo marinus*, the zebrafish *Danio rerio*, and three other fish species. The PDYNs of the Australian lungfish (*Neoceratodus forsteri*), and two frog species including *Bombina orientalis* and *Xenopus laevis* contain five opioid repeats. The recently identified Ile-enkephalin lies in identical position amongst the PDYN orthologs. The unique Phe-enkephalin pentapeptide is located in the second place within the partially sequenced PDYN of the African lungfish (*Protopterus annectens*). Based on structural information, PDYNs might be biosynthetic sources for at least four kinds of enkephalins in some species. Frogs appear quite unusual animals in this context, because frog PENKs include only Met-enkephalin repeats, whereas PDYNs of certain frog species contain an atypical Met^5-dynorphin A, thus suggesting a further, non proenkephalin derived source of Met-enkephalin in these amphibians. Fish dynorphin A is seemingly a

Met^5-dynorphin in each species studied so far, although fish α-neo-endorphin and dynorphin B sequences contain the conventional Leu-enkephalin units.

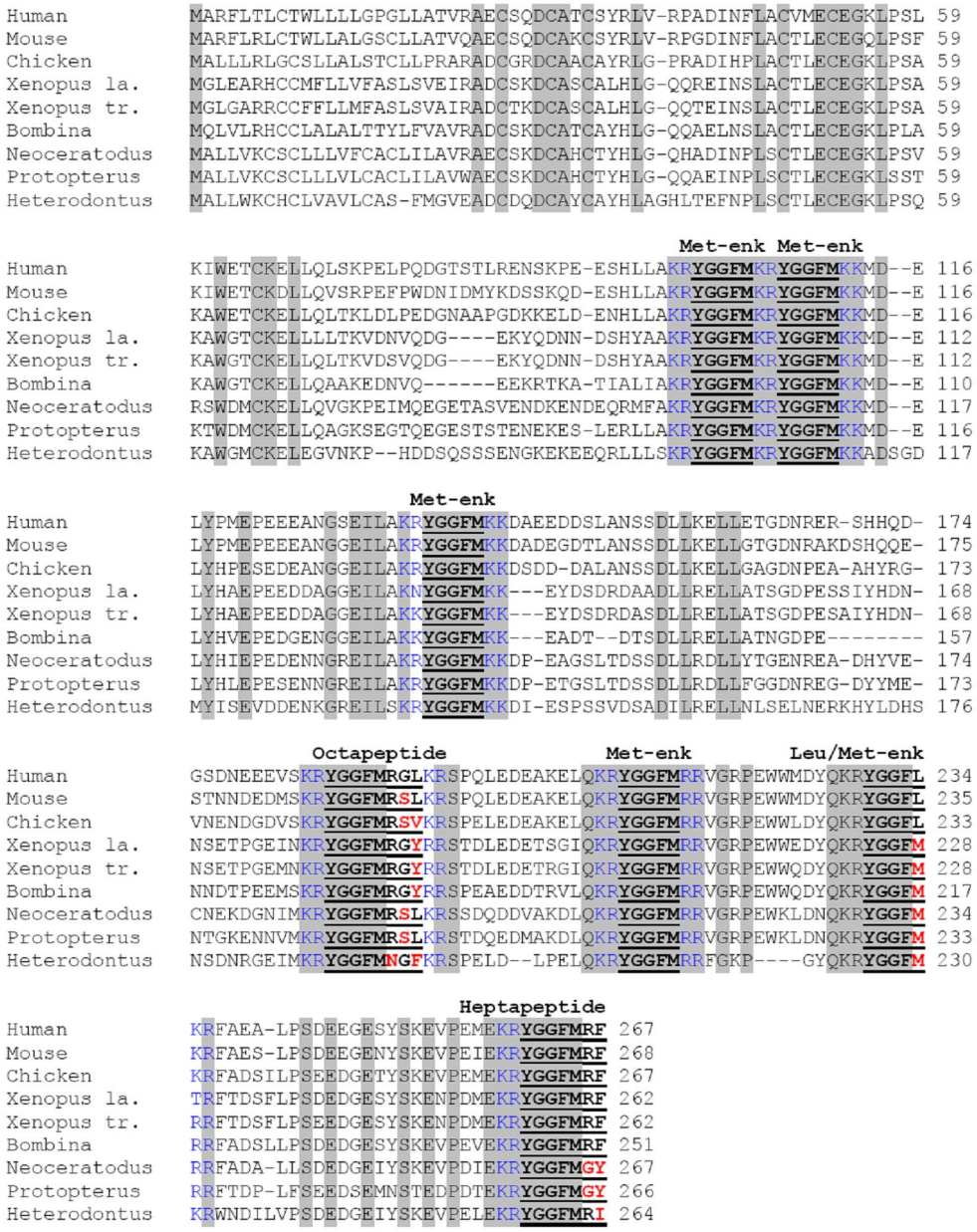

Fig. 4. *Alignment of human, mouse, chicken, xenopus, lungfish and shark proenkephalin sequences. Human (Homo sapiens; NP_001129162), Mouse (Mus musculus; NP_001002927), Chicken (Gallus gallus; XP_419213), Frog (Xenopus laevis; AAB20686 and Xenopus tropicalis; ENSXETP00000012480), Bombina (Bombina orientalis; AAU95755) Lungfish (Neoceratodus forsteri; AAF44658 and Protopterus annectens; AAF44657) and Shark (Heterodontus portusjacksoni; AAU95733). The opioid positions are labelled and sequences are bold and underlined. Modified amino acid positions in comparison with humans are coloured with red. Endoproteolytic cleavage sites are coloured with blue. Only 100% identical amino acid positions are shaded in gray.*

Fourteen distinct orthologues have been identified in the case of PENK derived octapeptides amongst 55 species. The ubiquitous YGGFMRGL sequence is present in 26 mammals including the human, although more other mammalian octapeptides exist, e.g., YGGFMRGY (*Xenopus laevis, Ornithorhyncus anatinus)*, YGGFMRSY (rainforest opossum, *Monodelphis domestica*), YGGFMRAL (cat and dog), YGGFMRSL (mouse), YGGFMRGV (European rabbit, *Oryctolagus cuniculus* and American pika, *Ochotona princeps*). Of the other vertebrate animals tested, including ten fish species, seven amphibians, four reptiles and two birds, even more variability in the structure of the octapeptide is observed (due to space limitation, alignments of all available PENK and PDYN sequences are not shown).

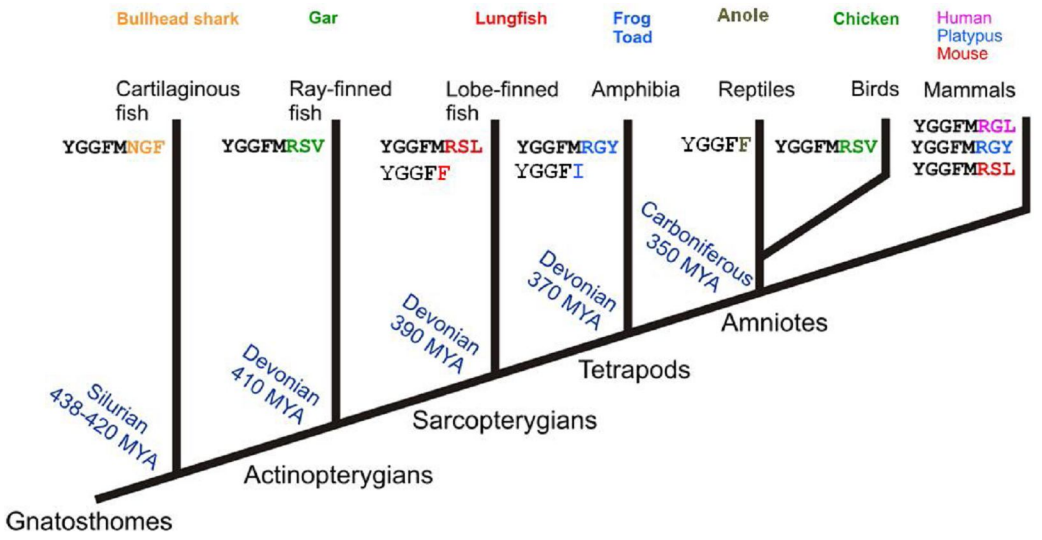

Fig. 5 Evolution scheme of the major groups within superclass Gnathostomata, exhibiting various PENK and PDYN sequences studied here. Nomenclature: vertebrates (subphylum Vertebrata), gnathostomes (jawed animals), actinopterygians (class Actinopterygii), sarcopterygians (class Sarcopterygii), tetrapods (subclass Tetrapoda), amniotes (subclass Amniota). The phylogenetic three is adapted from Dores et al., (2002).

Four different sequences have been selected for the chemical synthesis: The 'mouse sequence' YGGFMRSL (1) is unique among mammals, but is also present in the Australian lungfish (*Neoceratodus forsteri*) and African lungfish (*Protopterus annectens*) proenkephalins *(Sollars et al., 2000; Dores et al., 2001)*. The double substituted YGGFMNGF (2) sequence distinctively found in a cartilaginous fish species *(Komorowski et al., 2004)*, the bullhead shark (*Heterodontus portusjacksoni*), is the only one in that the Arg^{6} residue is replaced (R→N). Moreover, the shark octapeptide ends with an aromatic phenylalanine (Phe^{8}) side chain as a consequence of L→F substitution. The structure of YGGFMRGY (3) represents another

orthologous peptide bearing aromatic (Tyr^8) C-terminus due to L→Y replacement. In addition to the platypus, this sequence also occurs in two pipide frog species, the tetraploid *Xenopus laevis (Martens and Herbert, 1994)* and the diploid *Xenopus tropicalis*, and in the fire-bellied toad, *Bombina orientalis (Dores et al., 2001)*. The YGGFMRSV (4) octapeptide is included in the chicken proenkephalin (prediction from genomic sequence, NCBI), moreover in the incompletely sequenced PENK of the florida gar (*Lepisosteus platyrhynchus*) fish species *(Khalap et al., 2005)*. All full-size propeptides carrying various octapeptide components can well be aligned to the human sequence (Fig. 4).

2. Receptor binding assays

The mammalian and non-mammalian enkephalins and octapeptides were first tested in [^{3}H]naloxone binding assays. Naloxone is an opioid antagonist with high affinity and capable of interacting with all types of opioid receptors. Each novel analogue displayed moderate, and more or less similar affinity in heterologous competition experiments with [^{3}H]naloxone, while the homologous ligand unlabelled naloxone exhibited the highest affinity in these experiments *(Fig. 6, left panel; Fig. 7)*.

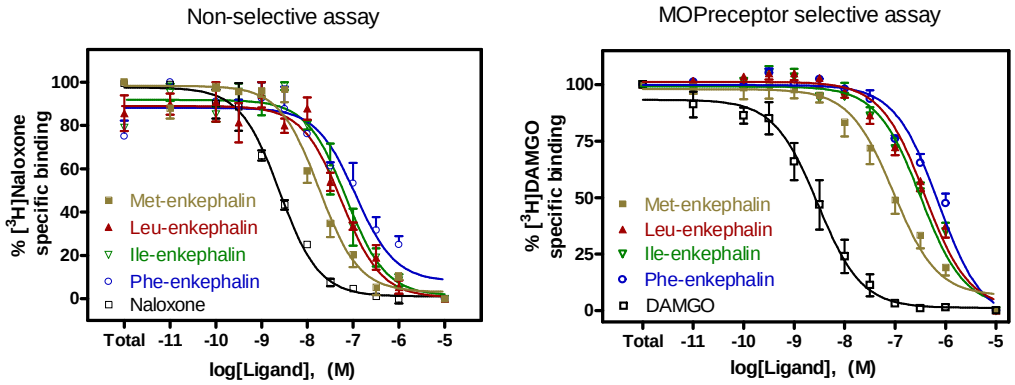

Fig. 6. *Equilibrium competition binding of enkephalins at the MOP receptor sites. Left panel: [^{3}H]naloxone binding (0.8-1.2 nM, 60 mins, 0-4°C); Right panel: [^{3}H]DAMGO binding (0.9-1.2 nM, 45 mins, 24°C); Points represent the means ±SEM of at least three different experiments each performed in duplicate.*

Using a MOP receptor specific radioprobe, the peptide [^{3}H]DAMGO, all of the novel sequences could displace the radiprobe in concentration deppendent manner, with homologous unlabelled ligand DAMGO displaying the best affinity. Lungfish Phe-

enkephalin, and frog Ile-enkephalin displaced effectively the bound radioligand from the receptor *(Fig. 6 right panel).*

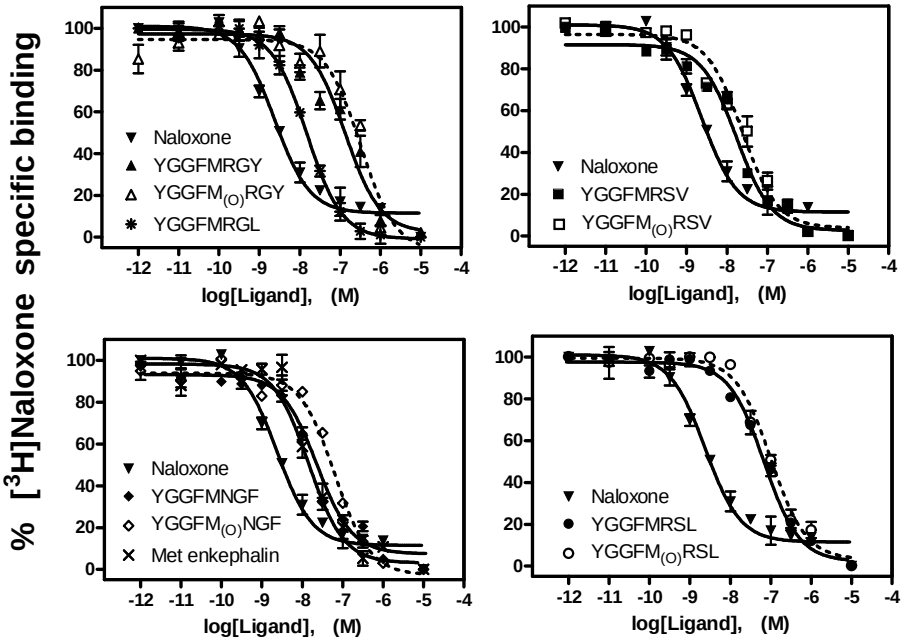

Fig. 7. *Equilibrium competition binding of octapeptides at the non selective opioid receptor sites, using [^{3}H]naloxone. (0.6-1.1 nM, 60 mins, 0-4°C); Points represent the means ±SEM of at least three different experiments each performed in duplicate.*

In the case of octapeptides, YGGFMRSL (mouse) and YGGFMRSV (chicken) were most effective while YGGFMRGY (frog) and YGGFMNGF (shark) were weaker for activation of the MOP receptor under the conditions used, meaning that the novel peptides had relatively good or moderate potencies in competing reversibly with the MOP binding sites under the conditions used *(Fig. 8).*

Binding properties of the compounds at the DOP receptors were measured with a radiolabelled frog skin peptide ligand analogue, Ile^{5,6}deltorphin-2 ([^{3}H]Tyr-D-Ala-Phe-Glu-Ile-Ile-Gly-NH_2). Although mammalian enkephalins are preferentially DOP receptor selective compounds, yet in our assays both the lungfish and frog enkephalins failed to effectively compete for the labelled deltorphin sites, therefore Ile- and Phe- enkephalins turned out to be weaker ligands at the opioid δ-sites *(Fig. 10, left panel).* The rank order of potency was Ile^{5,6}deltorphin-2 > Met-enkephalin > Leu-enkephalin > Ile-enkephalin > Phe-enkephalin.

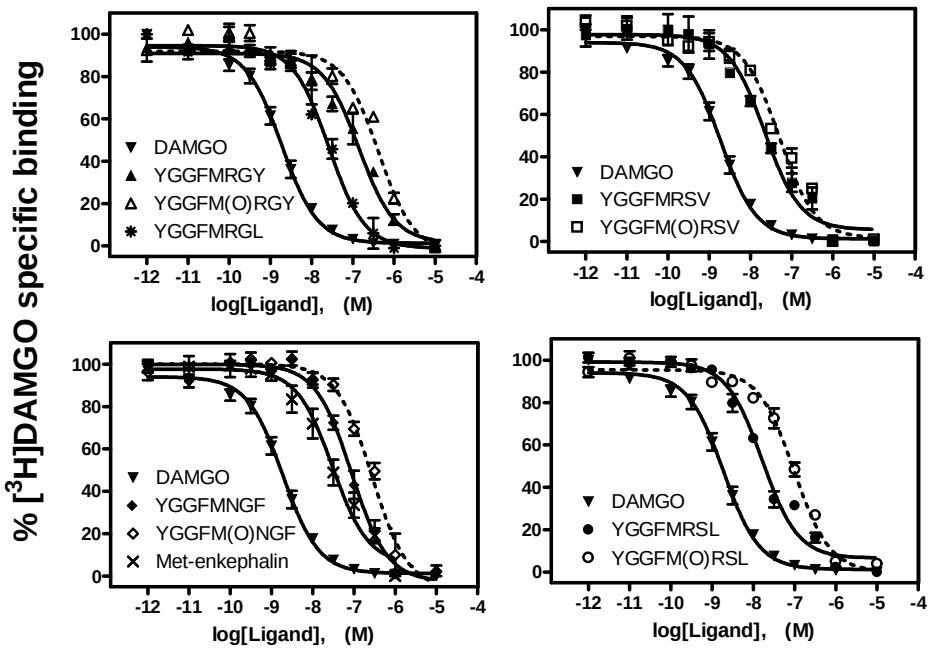

Fig. 8. *Equilibrium competition binding of octapeptides at the MOP receptor sites, using [^{3}H]DAMGO. (0.7-1.1 nM, 45 mins, 24°C); Points represent the means ±SEM of at least three different experiments each performed in duplicate.*

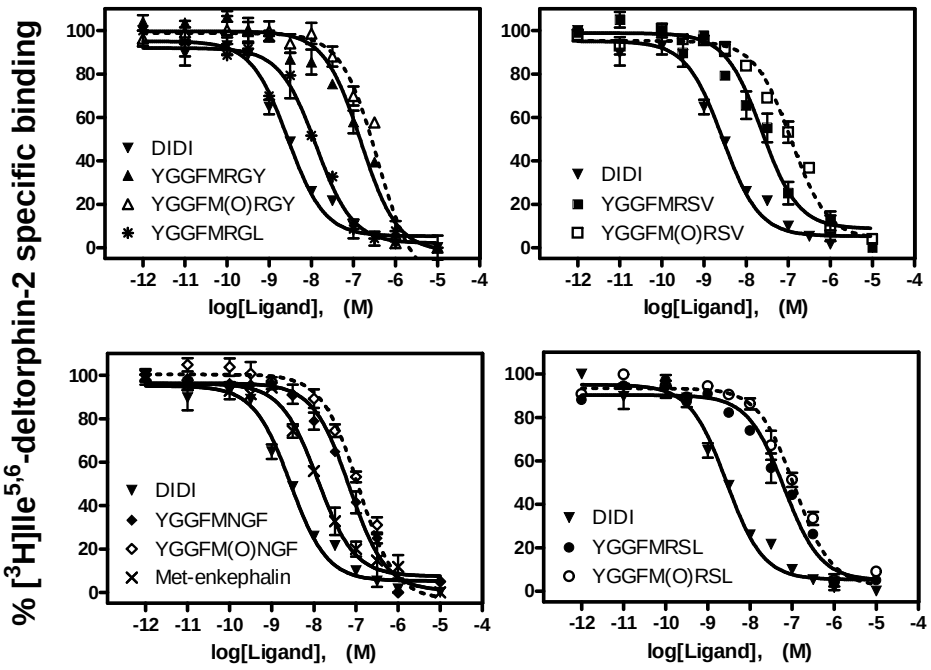

Fig. 9. *Equilibrium competition binding of octapeptides at the DOP receptor sites, using [^{3}H]Ile^{5,6}deltorphin-2. (0.9-1.4 nM, 45 mins, 24°C); Points represent the means ±SEM of at least three different experiments each performed in duplicate.*

Octapeptides sequences similar to the MOP assay could displace the [^{3}H][^{3}H]Tyr^1,Ile^{5,6}-deltorphin-2 radiprobe in a concentration dependent manner from the opioid δ-sites. Again, YGGFMRGY (Frog) and YGGFMNGF (Shark) were less potent, while YGGFMRSV (chicken) and YGGFMRSL (mouse) were more effective in the sense of DOP receptor activation *(Fig. 9)*.

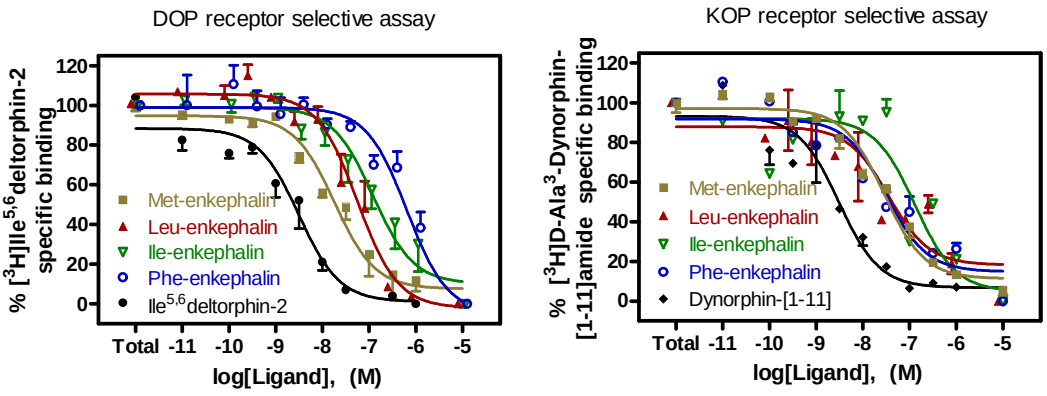

Fig. 10 . *Equilibrium competition binding of enkephalins at the DOP and KOP receptor sites. Left panel: [^{3}H]Ile^{5,6}deltorphin-2 binding (0.8-1.3 nM, 45 mins, 24°C); Right panel: [^{3}H]D-Ala^3-dynorphin-[1-11]amide binding (0.4-0.7 nM, 30 mins, 24°C) Points represent the means ±SEM of at least three different experiments each performed in duplicate.*

Enkephalin analogues were further studied at the KOP receptor sites labelled by a tritiated synthetic dynorphin A analogue, D-Ala^3-dynorphin[1-11] *(Lung et al., 1995)*. Predictably all enkephalins were less potent in this assays comparing with the affinities observed in the DOP receptor sensitive assays *(Fig. 10, right panel)*. Nevertheless, the affinity of Phe-enkephalin was the best in this KOP receptor selective assay system. The other enkephalins investigated here were roughly equipotent, but the experimental errors were higher in this assay. This is likely due to the enzymatic sensitivity and adsorptive properties of dynorphin peptides, although several endopeptidase inhibitors, such as EDTA, trasylol, bacitracin, and phenylmethylsulfonyl fluoride (PMSF) were also included in the assays.

On the other hand, KOP receptor selectivity of octapeptides were studied with a nonpeptide benzeneacetamide compound, U-69,593 which is among the most potent and selective ligand for the specific labelling of KOP receptors *(Rahmani et al., 1991)*. In guinea pig membranes none of the octapeptide sequences displayed any affinity in this system. Opioid activity of all these peptides was inactive in the KOP system *(Fig. 11)*.

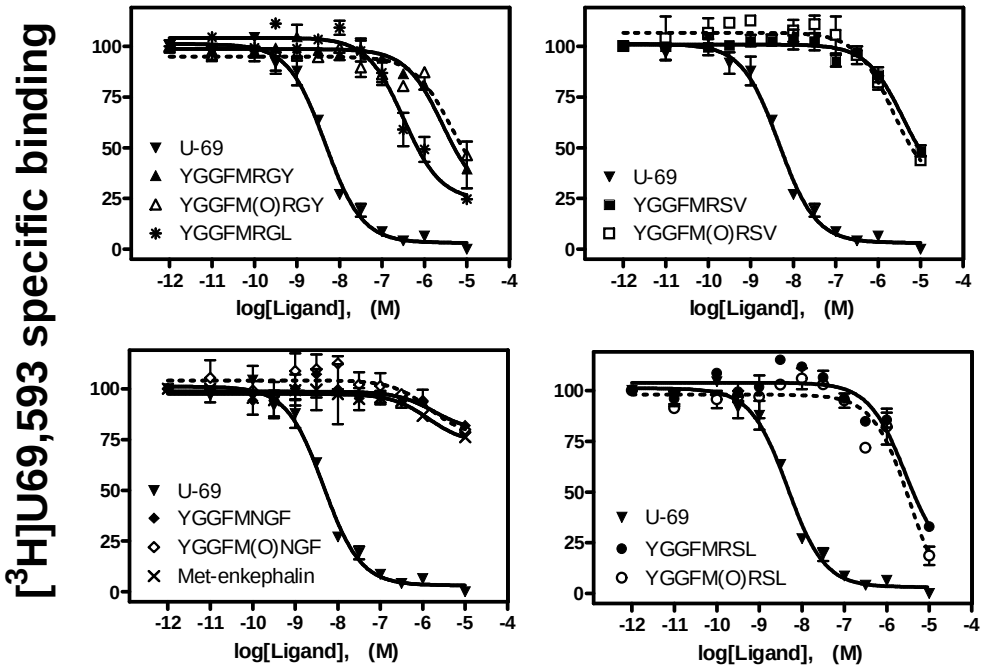

Fig. 11 . Equilibrium competition binding of octapeptides at the KOP receptor sites, using [^{3}H]U-69,593. *(0.6-1.1 nM, 60 mins, 24°C)*; Points represent the means ±SEM of at least three different experiments each performed in duplicate.

The oxidation of the Met^5 residue reduced the affinity of the enkephalins by shifting the competition curves to the right. In each receptor binding assay, all the oxidized form of the octapeptides was less potent (Figs 7-8-9-11).

Table 4 is summarizing the binding data of the novel opioid sequences studied, together with the relative affinities of the peptides towards the opioid receptors.

Ligand	Equilibrium affinity logIC_{50}±S.E.M. (K_i, nM)[a]				
	Non-selective	**DOP assay, δ**	**MOP assay, μ**	**KOP assay, κ**	**% Relative affinity[b] δ: μ: κ**
Naloxone	8.62±0.07 (1.54)	-	-	-	-
DIDI	-	8.55±0.07 (1.92)	-	-	-
DAMGO	-	-	8.72±0.04 (1.28)	-	-
U-69,593	-	-	-	8.33±0.05 (4.61)	-
Dynorphin-[1-11]	-	-	-	8.51±0.12 (2.73)	-
Met-enkephalin	7.74±0.08 (11)	7.91±0.08 (8.6)	7.52±0.08 (20)	5.86±0.5 (1117)	69:30:1
Ile-enkephalin *(Frog)*	7.20±0.1 (29)	6.92±0.1 (20.8)	6.45±0.07 (211)	6.880.2± (125)	79:8:13
Phe-enkepahlin *(Fish)*	6.98±0.1 (52)	6.19±0.1 (111)	6.14±0.06 (432)	7.49±0.2 (30.4)	20:6:74
YGGFM**RGL** *(Human)*	7.82±0.04 (9.9)	7.88±0.09 (9.1)	7.58±0.06 (17)	6.48±0.09 (269)	64:34:2
YGGFM**RGY** *(Frog)*	6.87±0.1 (87)	6.82±0.08 (104)	6.87±0.07 (95)	5.57±0.1 (2211)	47:51:2
$YGGFM_{(O)}$**RGY**	6.47±0.1 (213)	6.42±0.08 (258)	6.37±0.1 (258)	5.38±0.3 (3363)	51:45:4
YGGFM**RSV** *(Chicken)*	7.76±0.06 (11)	7.62±0.08 (16)	7.65±0.08 (15)	5.41±0.1 (3204)	48:51:1
$YGGFM_{(O)}$**RSV**	7.62±0.01 (15)	6.92±0.07 (81)	7.29±0.08 (34)	5.67±0.1 (1763)	30:69:1
YGGFM**NGF** *(Shark)*	7.74±0.07 (12)	7.15±0.05 (48)	7.05±0.05 (59)	5.74±0.3 (1494)	54:44:2
$YGGFM_{(O)}$**NGF**	7.19±0.06 (41)	6.93±0.06 (79)	6.55±0.07 (189)	5.87±0.4 (1137)	67:28:5
YGGFM**RSL** *(Fish)*	7.11±0.04 (49)	7.11±0.07 (45)	7.78±0.06 (14)	5.55±0.2 (2285)	24:75:1
$YGGFM_{(O)}$**RSL**	7.04±0.06 (58)	6.93±0.06 (81)	7.01±0.06 (67)	5.44±0.2 (2952)	45:54:1

[a] Calculated by the Chenc-Prusoff equation

[b] % Relative affinity calculated according to Kosterlitz and Paterson, 1980 [% Relative affinity=$\{(1/K_i\kappa)/(1/K_i\delta+1/K_i\mu+1/K_i\kappa)\}$x100]

Table 4. *Completion of the novel opioid sequences in a set of opioid receptor type-selective radioligand binding assays in brain membrane fractions*

3. [^{35}S]GTPγS binding experiments

[^{35}S]GTPγS binding assays represent functional tests determining agonist-induced, receptor-mediated G-protein activation. The assay is based on the increase in guanine nucleotide exchange at G-proteins upon agonist stimulation. All enkephalins effectively stimulated the G-proteins on rat brain membranes indicating the agonist properties of the ligands tested *(Fig. 12)*. In terms of maximal stimulation, Met-enkephalin caused the highest effect, but all other peptides were also decidedly effective.

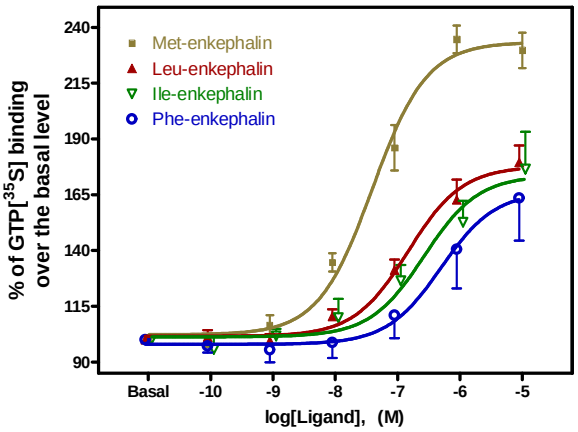

Fig. 12. *Agonist-induced activation of G-proteins in rat brain membranes. Stimulation is given as a percentage of the nonstimulated (basal) level. Basal activity (basal = 'total binding' – 'nonspecific binding') is taken as 100%. Nonspecific binding is determined in the presence of 10 μM unlabeled GTPγS. Incubations were carried out with 0.05 nM [^{35}S]GTPγS for 60 mins at 30°C with gentle shaking. Points represent the means ±SEM of at least three independent determinations each performed in triplicate.*

Novel octapeptides as well effectively stimulated the G-proteins on rat brain membranes *(Fig. 13)*. In terms of maximal stimulation, again Met-enkephalin caused the highest effect, but all other octapeptide analogues were also very effective in G-protein activation. While potency values (pEC$_{50}$) were higest in YGGFMRSV and YGGFMRSL, maximum stimulation (efficacy, %E$_{max}$) was relatively similar in all the peptides used for the [^{35}S]GTPγS binding assays. Oxidized analogues of the octapeptides were again less efficient for G-protein activation. The maximal stimulation levels (efficacy, %E$_{max}$) and the potency (pEC$_{50}$) values of the ligands are summarized in Table 5.

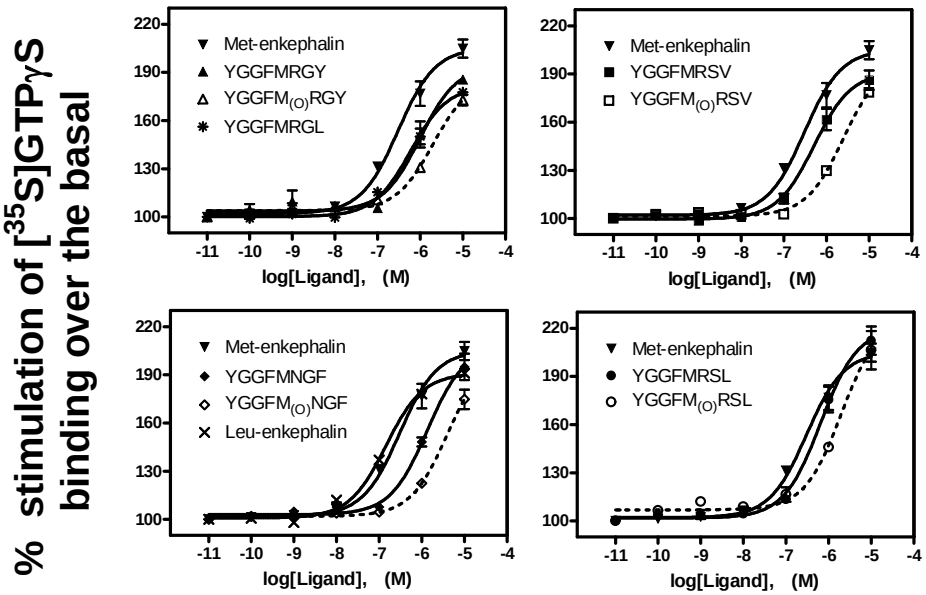

Fig. 13. *Agonist-induced activation of G-proteins in rat brain membranes. Nonspecific binding is determined in the presence of 10 μM unlabeled GTPγS. Incubations were carried out with 0.05 nM [^{35}S]GTPγS for 60 mins at 30°C with gentle shaking. Points represent the means ±SEM of at least three independent determinations each performed in triplicate.*

Peptides	**Potency logEC_{50}±S.E.M (EC_{50}, nM)**	**Efficacy (E_{max}), % stimulation ±S.E.M over the basal**
Met-enkephalin	7.35±0.08 (60)	234±3.8
Leu-enkephalin	6.84±0.08 (145)	178±6
Ile-enkephalin	6.63±0.23 (237)	176±18
Phe-enkephalin	6.49±0.11 (323)	168±19
YGGFM**RGL** *(Human)*	6.22±0.07 (594)	182±2.8
YGGFM**RGY** *(Frog)*	6.03±0.12 (993)	194±6.3
YGGFM$_{(O)}$**RGY**	5.7±0.08 (1990)	186±4.6
YGGFM**RSV** *(Chicken)*	6.28±0.09 (515)	191±4.1
YGGFM$_{(O)}$**RSV**	5.6±0.6 (2465)	197±4.0
YGGFM**NGF** *(Shark)*	5.87±0.08 (1335)	207±5.4
YGGFM$_{(O)}$**NGF**	5.4±0.1 (3958)	203±8.2
YGGFM**RSL** *(Fish)*	6.18±0.08 (653)	220±5.5
YGGFM$_{(O)}$**RSL**	5.7±0.12 (1979)	226±9.7

Table 5. *G-protein activation by opioid peptides in the rat brain membranes*

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

1. Receptor binding assays

The radioligand binding studies were performed with [^{3}H]Ac-RYYRIK-ol using rat brain membranes. Binding studies were done using N/OFQ and the hexapeptide alcohols Ac-RYYRIK-ol, Ac-RYYRIR-ol. All three peptides displaced [^{3}H]Ac-RYYRIK-ol in a concentration dependent manner. Ac-RYYRIR-ol was relatively the weakest and Ac-RYYRIK-ol showed the highest affinity to the NOP receptor in the competition binding experiments *(Fig. 14).* The pK_i values were as follows; Ac-RYYRIK-ol 8.85, N/OFQ 8.69 and Ac-RYYRIR-ol 8.26.

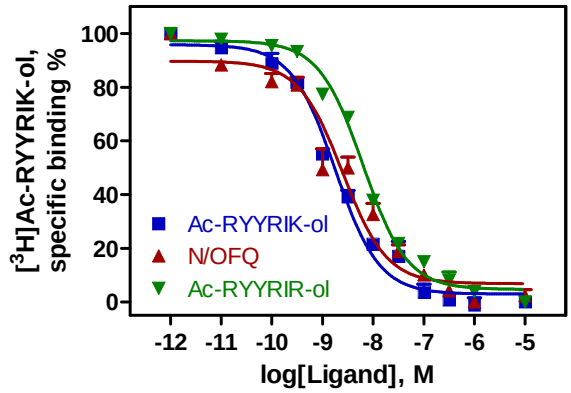

Fig. 14. *Equilibrium competition binding of the Ac-RYYRIK-ol, N/OFQ and Ac-RYYRIR-ol at NOP receptor using [^{3}H]Ac-RYYRIK-ol in rat brain membranes. The nonspecific binding was measured with 10 μM Ac-RYYRIK-ol. Data are means ±SEM of 3 experiments performed in duplicate.*

Receptor binding experiments done with [^{3}H]DAMGO and [^{3}H]Ile^{5,6}deltorphin-II revealed that Ac-RYYRIR-ol has no any affinity toward these opioid receptors. The novel hexapeptide could not displace both radioprobes, even up to very high concentrations (*i.e.* 10 μM, data not shown).

2. [^{35}S]GTPγS binding assays

In the [^{35}S]GTPγS binding assays N/OFQ effectively stimulated G-proteins in rat brain membranes (E_{max} 64 ± 2%, pEC_{50} 7.04) defining the full agonist effect at the NOP receptors *(Fig. 15, left panel).* Ac-RYYRIR-ol could also stimulate the G-protein activation with similar potency (pEC_{50} 6.73) but far less efficacy (E_{max} 18 ±2

%, α = 0.28) compared to N/OFQ *(Fig. 15, left panel).* Stimulatory effect of both N/OFQ and Ac-RYYRIR-ol could be inhibited using specific NOP receptor antagonist UFP-101 *(Figure 15, left panel)* and pA_2 value of 6.9 vas calculated. In the inhibition experiments, the effects of increasing concentration of Ac-RYYRIR-ol were tested against the stimulatory effect produced by a fixed concentration of N/OFQ (1 μM). Ac-RYYRIR-ol effectively inhibited in a concentration dependent manner the stimulatory effect of 1 μM N/OFQ with a maximal inhibitory effect similar to that estimated in agonist type experiments. A pK_B value of 7.29 was derived from these experiments *(Fig 15 , right panel).*

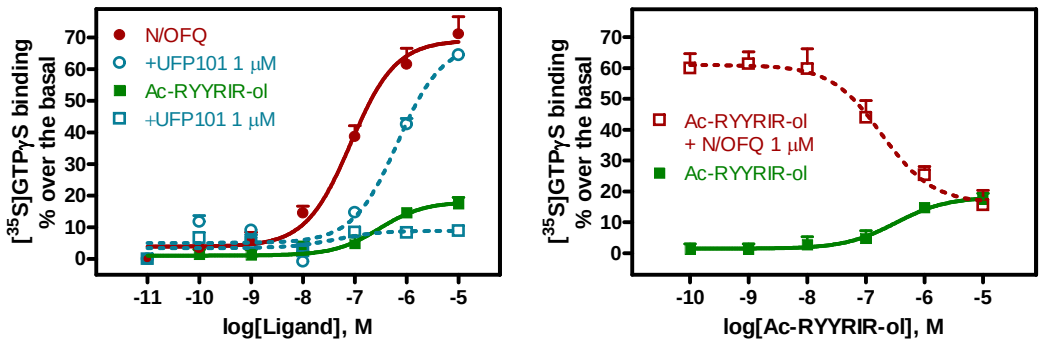

Figure 15. *Stimulation of [^{35}S]GTPγS binding in rat brain membranes. Left: UFP-101 antagonizing response versus N/OFQ and Ac-RYYRIR-ol, in the [^{35}S]GTPγS binding assay. Data are means ±SEM of 3 experiments performed in triplicate. Right: Inhibition concentration response curves of Ac-RYYRIR-ol against the effects evoked by 1 μM N/OFQ.*

In order to study the possible antagonist action of Ac-RYYRIR-ol in a separate series of experiments using [^{35}S]GTPγS binding assay, the hexapeptide was challenged at increasing concentration against the concentration response curve to N/OFQ. From this experiment Ac-RYYRIR-ol potency as an antagonist towards the NOP receptors has been determined and pA_2 was found to be 8.33 with the correlation coefficient of 0.99 *(Fig 16).*

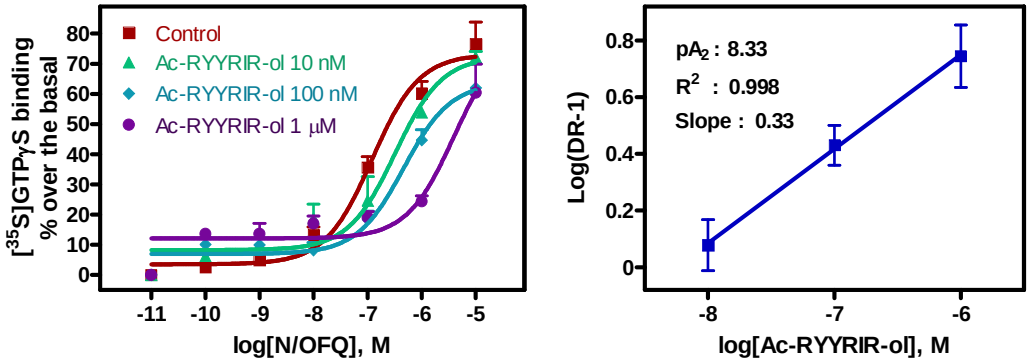

Fig. 16. *Stimulation of [^{35}S]GTPγS binding to rat brain membranes. Concentration response curve of N/OFQ, obtained in the absence (control) and presence of increasing concentrations of Ac-RYYRIR-ol (10–1000 nM). On the right panel the corresponding Schild plot is shown. Data are means ±SEM of 3 experiments performed in triplicate.*

3. Electrically stimulated isolated tissues and mouse colon bioassay

In the mouse vas deferens, N/OFQ inhibited the twitch response to electrical field stimulation in a concentration dependent manner showing an E_{max} of 90±5% and a pEC_{50} of 7.60 *(Fig. 17, left panel).* In this preparation Ac-RYYRIR-ol was completely inactive at 10 and 100 nM while at 1 µM it produced a slight inhibition of the electrically induced twitch in some but not all tissues. However, Ac-RYYRIR-ol tested over the concentration range of 10–1000 nM shifted the concentration response curve to N/OFQ to the right in a concentration dependent manner. Curves obtained in the presence of Ac-RYYRIR-ol were parallel to the control and reached similar maximal effects even in the presence of the highest concentration of compound. The corresponding Schild plot was linear and slope of the regression line was found to be 0.91 with a pA_2 value of 7.99 *(Fig. 17, right panel).* N/OFQ could also inhibit the stimulation caused by the electrical impulses in rat vas deferens showing an E_{max} of 87±2% and a pEC_{50} of 7.29, while again Ac-RYYRIR-ol was inactive until 1 µM concentration (Data not shown). In rat vas deferens assay 100 nM Ac-RYYRIR-ol could inhibit the effect of N/OFQ in these preparations. pA_2 value of 8.20 was calculated for rat vas deferens.

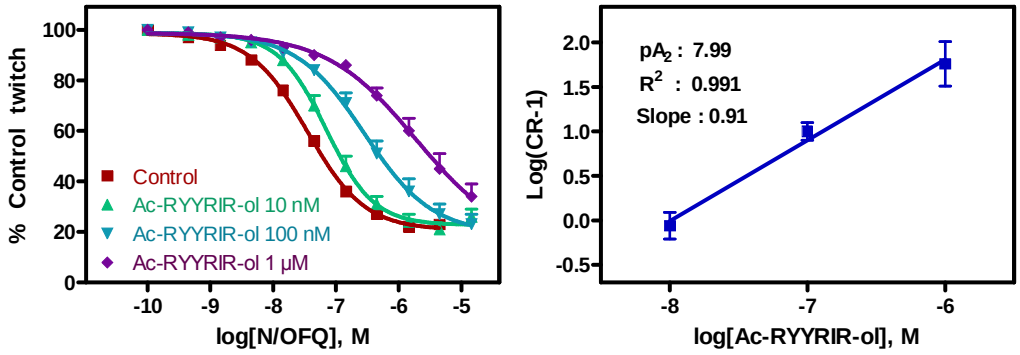

Fig. 17. *Inhibitory effect of the hexapeptide in the electrically stimulated mouse vas deferens. Concentration response curve of N/OFQ, obtained in the absence (control) and presence of increasing concentrations of Ac-RYYRIR-ol (10–1000 nM). On the right panel the corresponding Schild plot can be seen. Data are means ±SEM of 3 experiments performed in duplicate.*

In the isolated mouse colon, N/OFQ evoked concentration dependent contractile effects showing a pEC_{50} of 8.82 and an E_{max} of 32 ± 4 %. This effect was mimicked by Ac-RYYRIR-ol which displayed a similar maximal effects (28 ± 3 %) and a slightly higher value of potency (pEC_{50} 9.09) *(Fig. 18).*

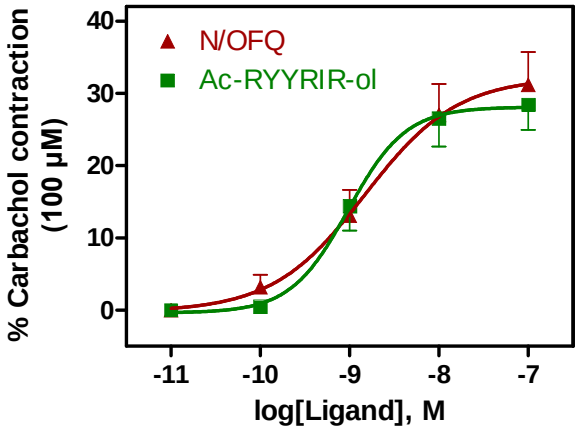

Fig. 18. *Concentration response curves of N/OFQ and Ac-RYYRIR-ol in the isolated mouse colon. Data are mean ± SEM of 3 experiments performed in duplicate.*

In order to examine delta and mu opioid receptor potency of Ac-RYYRIR-ol in the electrically stimulated tissues, we have studied the concentration response curves to DPDPE in the mouse vas deferens and to dermorphin in the guinea pig ileum. Curves were obtained in the absence and presence of a single concentration of Ac-RYYRIR-ol (1 µM). The hexapeptide was found to be completely inactive in both preparations.

4. 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 $G\alpha_{qi5}$. Results demonstrated that both N/OFQ and Ac-RYYRIR-ol could stimulate in a concentration-dependent manner calcium mobilization with pEC_{50} values of 9.19 and 7.94, and E_{max} of 240±16% and 161±21% over the basal levels, respectively *(Fig. 19)*. Thus, Ac-RYYRIR-ol behaved as a partial agonist, showing a reduction in maximal effects compare to N/OFQ.

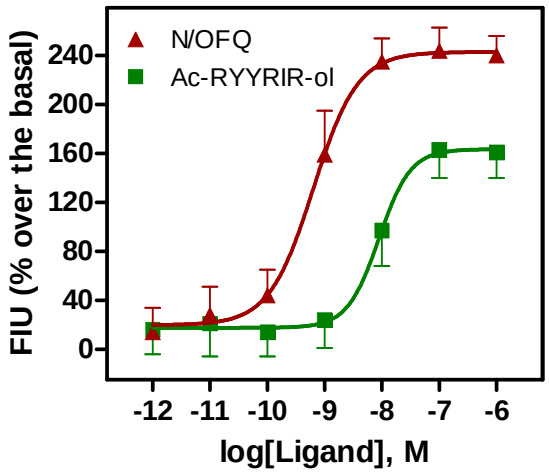

Fig. 19. *Concentration response curves of N/OFQ and Ac-RYYRIR-ol in CHO cells expressing the human NOP receptor and the chimeric protein $G\alpha_{qi5}$. Changes in intracellular Ca^{2+} ion levels were expressed as % increase of fluorescence intensity units (FIU) over basal. Data are mean ± SEM of 3 experiments performed in duplicate.*

5. Internalization of the NOP receptors

Prototype hexapeptide Ac-RYYRIK-ol and novel analogue Ac-RYYRIR-ol was not efficient to promote NOP receptor internalization in cultured human embryonic kidney (HEK_{293}) cells expressing the fusion construct $_{h}$NOP-GFP (green fluorescent protein), while the full agonist N/OFQ internalized the majority of the receptor within 1 hour *(Fig. 20 white arrows)*.

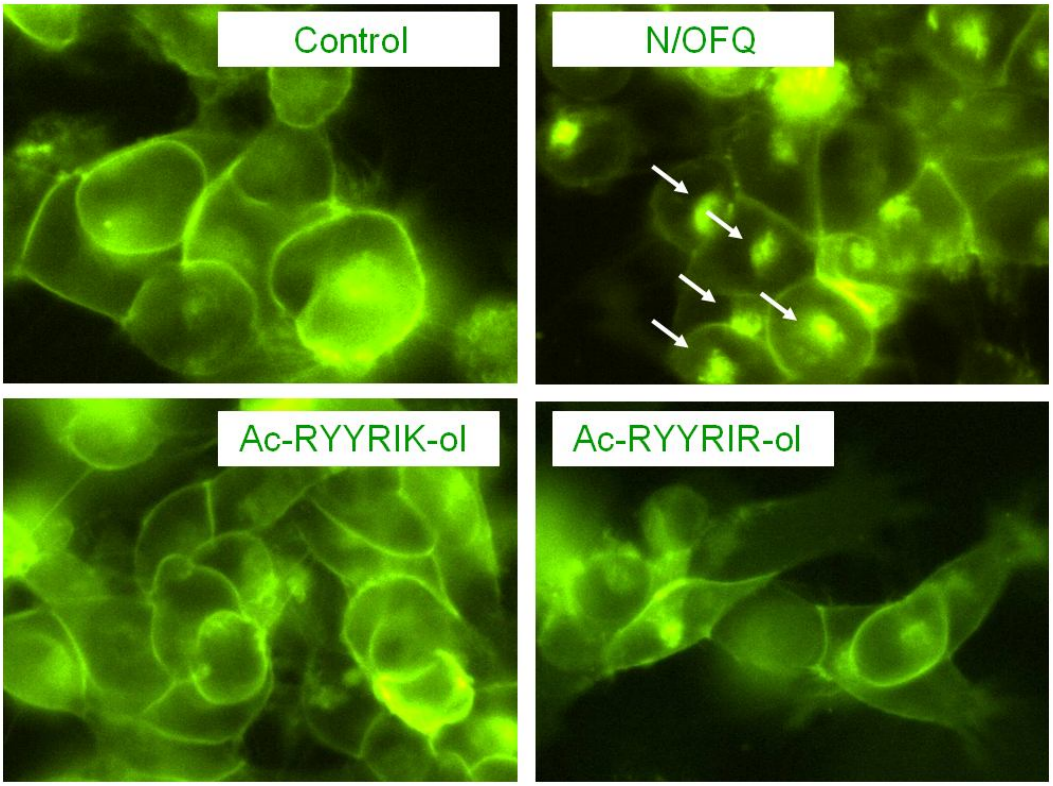

Fig. 20 *Olympus Cell-R fluorescence microscope was used with GFP filter set combination and 20 x objectives.*

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

Heterologous competition experiments with [^{3}H]Ac-RYYRIK-ol

Competition assays were performed using various NOP receptor related ligands such as the full-size nociceptin (N/OFQ$_{(1-17)}$), Tyr^1-N/OFQ$_{(1-17)}$, the NOP receptor antagonist peptide UFP-101, the non-peptide antagonist J-113397, several acetyl-hexapeptides, *e.g.*, Ac-RYYRIK-ol, Ac-RYYRIK-NH$_2$ (parent compound, or Dooley's-peptide), the Arg^6-derivative Ac-RYYRI**R**-ol, the citrulline substituted (Cit^4) analogues Ac-RYY*Cit*IK-NH$_2$ and Ac-RYY*Cit*IK-ol (*Gündüz et al., 2006-07*). Some opioid compounds including the general opioid receptor antagonist naloxone, KOP receptor agonist/MOP receptor antagonist buprenorphine, naloxone-benzoylhydrazone, an antagonist compound known to interact with both KOP and NOP receptors, the MOP receptor selective peptide agonist [D-Ala^2,*N*MePhe^4,Gly^5-ol]enkephalin (DAMGO) and the KOP receptor agonist peptide D-Ala^3-dynorphin$_{(1-11)}$ were also studied to determine their displacing capability in rat brain membranes. All NOP receptor ligands efficiently displaced [^{3}H]Ac-RYYRIK-ol binding with varying affinities, in concentration dependent manner (Fig. 21). Opioid compounds exhibited less potency in competing reversibly for the [^{3}H]Ac-RYYRIK-ol recognition sites, although moderate affinities were obtained with buprenorphine, naloxone-benzoylhydrazone and D-Ala^3-dynorphin$_{(1-11)}$ (Fig. 21). Rank order of the affinity was: Ac-RYYRIK-ol > N/OFQ > Tyr^1-N/OFQ$_{(1-17)}$ > Ac-RYYRIK-NH$_2$ > Ac-RYYRIR-ol > UFP-101 > Ac-RYY*Cit*IK-ol > Ac--RYY*Cit*IK-NH$_2$ > J-113397 > naloxone-benzoylhydrazone > buprenorphine > D-Ala^3-dynorphin$_{(1-11)}$ > I$_2$Tyr^1-N/OFQ$_{(1-17)}$-NH$_2$ > naloxone and DAMGO. The latter two opioids were unable to produce even 20% inhibition of the specific [^{3}H]Ac-RYYRIK-ol binding. The equilibrium inhibition constant (K_i) values are summarized in Table 6.

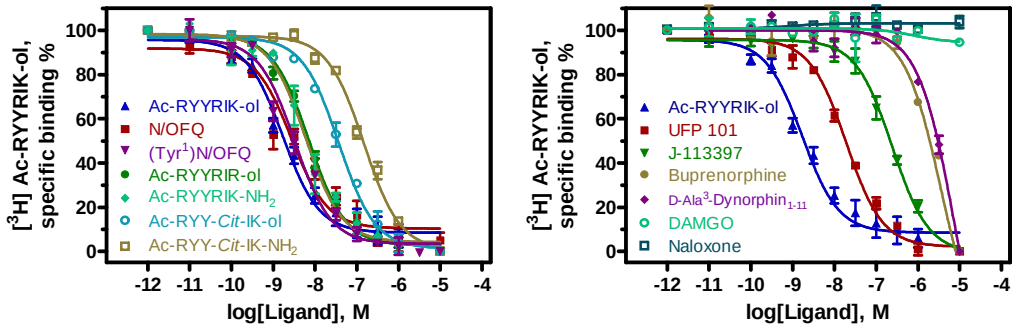

Fig. 21 *Equilibrium competition binding of various nociceptin (Left Panel) and opioid (Right Panel) ligands at the [^{3}H]Ac-RYYRIK-ol sites in rat brain membranes. [^{3}H]Ac-RYYRIK-ol (0.2–0.4 nM) was incubated in the presence of increasing concentrations of homologous or heterologous competitors, compound names are indicated in the figure. Points represent the means ± S.E.M. of at least three different experiments each performed in duplicate. Homologous competition curves are shown in both panels for comparison.*

[^{3}H]Ac-RYYRIK-ol binding was also studied in cultured Chinese Hamster Ovary (CHO) cells stably transfected with recombinant human NOP receptor. Competition studies were performed with [^{3}H]Ac-RYYRIK-ol and [^{3}H]N/OFQ$_{(1–17)}$ (Amersham) permitting comparisons for the NOP receptor sites labeled by the hexapeptide and heptadecapeptide radioligands. In homologous competition studies performed on $_h$NOP-CHO cell membranes almost the same binding affinities were measured for Ac-RYYRIK-ol either with [^{3}H]Ac-RYYRIK-ol or with [^{3}H](Leu^{14})nociceptin. Results of the cross- and self-displacement studies ('competition tetrad analyses') are shown in Fig. 22.

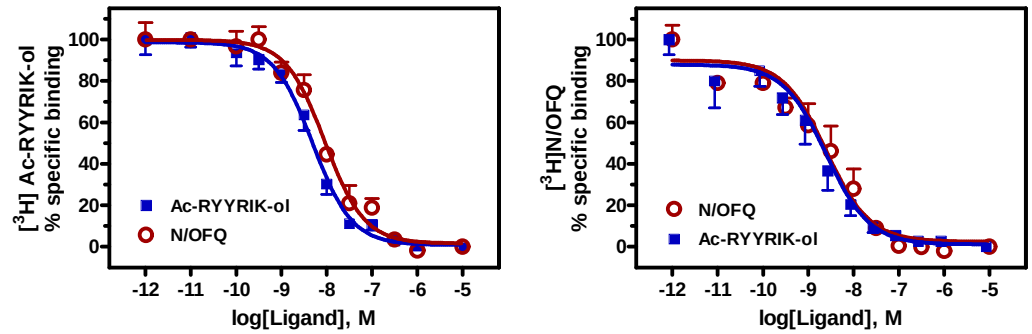

Fig. 22. *Cross- and self-displacement studies with two radioligands in Chinese Hamster Ovary cells expressing recombinant human nociceptin receptor ($_h$NOP-CHO). (Left Panel) [^{3}H]Ac-RYYRIK-ol binding (0.2–0.3 nM, 30 min, 24ºC). (Right Panel) [^{3}H](Leu^{14})N/OFQ$_{(1–17)}$ (nociceptin) binding (0.05–0.15 nM, 30 min, 24ºC).*

Compound	Property	[^{3}H]Ac-RYYRIK-ol	
		pIC_{50}±S.E.M	**K_i (nM) ***
N/OFQ$_{(1-17)}$	NOP agonist (endogenous sequence)	8.56±0.09	2.3
Tyr^1-N/OFQ$_{(1-17)}$	Tyr^1-substituted analogue	8.50±0.07	2.6
I_2Tyr^1-N/OFQ$_{(1-17)}$-NH_2	Iodinated analogue	5.04±0.40	7726
Ac-RYYRIK-ol	NOP partial agonist hexapeptide	8.77±0.07	1.3
Ac-RYYRIK-NH_2		8.22±0.07	5.1
Ac-RYYRIR-ol	NOP hexapeptide analogues	8.14±0.03	6.1
Ac-RYY*Cit*IK-ol		7.43±0.04	31
Ac-RYY*Cit*IK-NH_2		6.83±0.04	126
UFP-101	NOP antagonist peptide	7.71±0.04	16
J-113397	Non-peptide NOPr antagonist	6.61±0.05	206
Naloxone-benzoylhydrazone	Partial opioid agonist / NOPr antagonist	5.85±0.07	1196
Buprenorphine	Opioid agonist-antagonist	5.34±0.11	3827
D-Ala^3-Dynorphin$_{(1-11)}$	KOP selective opioid agonist peptide	5.12±0.11	6399

Table 6 *Competition of [^{3}H]Ac-RYYRIK-ol binding in rat brain membranes. *Calculated by Cheng-Prusoff equation*

DISCUSSION

In this thesis detailed biochemical characterization of several PENK and PDYN derived penta- and octapeptide enkephalin sequences have been described and their selectivity for the opioid receptors has been evaluated. Other than opiod sequences, a novel NOP receptor hexapeptide ligand Ac-RYYRIR-ol has been tested in both biochemical and in-vitro pharmacological assays. Furthermore a full characterization of our novel radiolabeled hexapeptide analogue [^{3}H]Ac-RYYRIK-ol, selective for the NOP receptor has been described by the use of neuronal and cultured epithelial cell membrane preparations containing native or recombinant proteins.

Identification of the Novel Naturally Occurring Opioid Peptides

Interspecies variability of opioid peptides and their precursors point to the significance of evolutionary changes and the need of comparative research. Altogether 39 PDYN sequences representing 36 animal species have so far been collected by database searching. Non-allelic PDYN gene duplicates have been identified in the tetraploid *Xenopus laevis (Pattee et al., 2003)*. Organization and peptide sequence variability appears more complex among PDYNs, since both the number and the length of the opioid units are rather different within this precursor.

Isoleucine enkephalin is N-terminally located within the structure of xendorphin-1A. This is the first opioid segment from the 5' end of *Xenopus* PDYN-A cDNA sequence encoding one of the two prodynorphin precursor polypeptides in this frog species *(Pattee et al., 2003)*. It is assumed, that Ile-enkephalin can be a naturally occurring peptide in these animals, because an analogous 'biosynthetic' route producing Leu-enkephalin by sequential enzymatic hydrolysis of larger PDYN fragments have been reported in mammals *(Traynor, 1987; Dixon and Traynor, 1993)*. Since the Ile-enkephalin motifs are flanked by basic dipeptide repeats in each species listed in, the formation of Ile-enkephalin may proceed through regular enzymatic cleavage. The *Xenopus laevis* hexadecapeptide xendorphin-B1 produced naloxone reversible anti-nociception measured by the acetic acid pain test in frogs *(Stevens et al., 2009)*. In addition, xendorphin-B1 displayed notable κ-opioid receptor affinity in radioligand binding assays *(Stevens et al., 2007)*. The heptapeptide YGGFMGY derived from the zebrafish PENK has been shown to bind differentially to duplicate (ZFOR1 and ZFOR4) DOP receptors from *Danio rerio (Gonzalez-Nunez et al., 2007)*. Little is

known about the biological properties of the recently discovered atypical opioids. Since there were no more data available on the bioactivity of the lately identified endogenous opioids, we determined the binding and signaling properties of the two novel enkephalins, i.e., the 'frog' Ile-enkephalin and the 'lungfish' Phe-enkephalin. Such studies provide relatively quick and precise estimates on the basic characteristics of the ligands without performing detailed pharmacological assays.

The unique structure of Phe-enkephalin has been detected in the brain cDNA sequence encoding the African lungfish (*Protopterus annectens*) PDYN *(Dores et al., 2004)*. The *Protopterus* PDYN encodes for two copies of Leu-enkephalin and one-one single copy of Met- and Phe-enkephalin. A full-length proenkephalin cDNA *(Protopterus* PENK) was also cloned from the same species *(Dores et al., 2000)*.

In our hand, these non-mammalian enkephalins showed slightly varying affinities in different radioligand binding assays. All four enkephalins displayed high to moderate affinities in the DOP receptor selective binding assays. As far as the general opioid nature is concerned, Met-enkephalin seems the most potent, while Phe-enkephalin the least efficacious peptide. Consequently, the KOP receptor preference of this peptide, if any, needs further evaluations. The potency and the efficacy of Met-enkephalin was by far the best in the [^{35}S]GTPγS assays, but the overall differences among the potencies of the peptides tested were smaller than in the competition binding experiments.

In spite of the numerous reports on the correct chemical and immunohistological identification of enkephalin-like peptides in invertebrates, there is no genomic or cDNA sequence data describing the complete structure of any invertebrate enkephalin precursor(s). However, enkephalin biosynthesis is well established in mammals (Noda et al., 1982). Mammalian and other vertebrate enkephalins are regularly produced by posttranslational enzymatic processing of their primary precursor proenkephalin (PENK). Prodynorphin (PDYN) propeptides might be secondary sources of enkephalins *(Traynor, 1987; Bojnik et al., 2009a)*. Opioid peptides, including enkephalins, also form families of closely related peptides, where several members may occur in one animal species *(Dores et al., 2002)*. This is due to gene or exon duplications followed by mutations. Gene splicing and posttranslational processing ultimately decides the gene product in a single cell of given species *(Holmgren and Jensen, 2001)*. The ancestral opioid receptor gene duplications remarkably coincide with the origin of the four opioid peptide precursor genes

(Dreborg et al., 2008; Dores et al., 2002). Thus, the complete vertebrate opioid system was already established in the first jawed vertebrates (Gnathostoma).

The organization of PENK is well conserved *(Roberts et al., 2007),* seven individual enkephalin motifs exist in almost all PENK sequences of the 55 vertebrate species studied so far. Our bioinformatical search with PENK has focused on the phylogenetic diversity of the octapeptide enkephalin segment placed in the fourth position (IVth) of the repetitive opioid units. The typical mammalian PENK octapeptide is YGGFMRGL, but five other point-mutated orthologues have been collected by analyzing mammalian PENK sequences. The mutations occurred within the C-terminal tripeptide region of the octapeptides. No mutations on the enkephalin pentapeptide unit was observed, so the opioid message region *(Schwyzer, 1986)* carrying the structural basis of the biological activity is indeed the most conserved part as suggested by Holmgren and Jensen (2001).

The amphibian *Xenopus* and *Bombina* octapeptides have the same structure as in the platypus. However, the most abundant mammalian sequence YGGFMRGL is apparently absent in the non-mammalian vertebrate taxa. Instead, data collected from 2 birds, 4 reptiles, 7 amphibians and 10 fish species reveal the occurrence of at least 7 such motifs those are not found in the mammalian PENKs. The octapeptide structure, for instance, is YGGFMRSV in the chicken and in the Florida gar fish species. An identical sequence is evidently present in one of the zebrafish (*Danio rerio*) PENKs, although here the C-terminal cleavage site is missed *(Gonzalez Nuñez et al., 2003).* Since the other, paralogous copy of the zebrafish PENK raised by gene or genom duplication (*Taylor et al., 2001*) contains YGGFM sequence directly followed by the RR endopeptidase recognition motif in the aligned position, no matured octapeptide enkephalin is expected in this species. The PENK octapeptide motifs are flanked by basic dipeptide repeats (KR or RR) in all other species, permitting the formation of the mature oligopeptide by sequential enzymatic cleavage.

Four of the 14 different octapeptide enkephalins found so far in protein sequence databases have been chemically synthesized and studied along with the human sequence. Three novel structures are rather ubiquitous, as they are ranged in different animals, while the fourth sequence of YGGFMNGF is solely present in a cartilaginous fish species. From chemical aspects the $Leu^8 \rightarrow Tyr^8$ (frog) and $Leu^8 \rightarrow Phe^8$ (shark) mutations are consistent with an aliphatic to aromatic change. The $Arg^6 \rightarrow Asn^6$ replacement in the shark PENK is acconpanied by the loss of the

positively charged residue. The lungfish octapeptide YGGFMRSL contains a single $Gly^7 \rightarrow Ser^7$ rather neutral mutation, while the YGGFMRSV peptide holds the Gly^7-$Leu^8 \rightarrow Ser^7$-Val^8 duplicate point mutations.

The serine (Ser^7) containing peptides displayed higher affinities in radioligand binding assays, while the frog sequence (YGGFMRGY), also present in the platypus PENK, seems to have lesser affinity. The opioid receptor-type selectivity pattern supports that endogenous agonists are not by necessity the most specific ligands for a particular receptor. The oxidation of the Met^5 residue considerably reduced the affinity of the enkephalins by shifting the competition curves to the right. Methionine is a key amino acid that has numerous roles in essential vital processes. Moreover, methionine oxidation might be biologically important during conditions of oxidative stress (*Bobrowski et al., 2008*). Methionine oxidation in enkephalins (*Turkall et al., 1982*) generally results in the decrease in biological response, although enhanced antinociception of the oxidized enkephalin analogue [$_D$-Ala^2,$Met^5{}_{sulfoxide}$]enkephalin-amide was also reported (*Kiritsroy et al., 1983*). The potency of Leu-enkephalin used as control was the highest in the [^{35}S]GTPγS assays, whereas Met-enkephalin and the C-terminally extended octapeptides effectively stimulated the binding of [^{35}S]GTPγS to $G_{i/o}$ proteins in rat brain membranes. Consistent with their full agonist effect, the maximal stimulation values (% E_{max}, efficacy) of the octapeptides were around two times higher over the basal activity.

As a conclusion for the newly recognized opioid sequences, the receptor binding and G-protein activating properties of several novel, PDYN and PENK derived non-mammalian opioid peptides were described, and compared to those of the well known Met-enkephalin-Arg^6-Gly^7-Leu^8 and the pentapeptide Met-enkephalin. Among the pentapeptide enkephalin structures tested, Ile- 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. Research on the non-conventional neuropeptide structures is essential from both phylogenetic and chemical-biochemical

viewpoints. One significance of these mutationally variable sequences is that they represent a natural 'combinatorial' peptide library emerged by the evolution. The various peptides evolved by gene mutations and natural selection offer template sequences for structure-activity relationship studies. Inasmuch as the availability of the genome sequencing data grows rapidly, an increasing impact of the phylogenetic and bioinformatic studies on experimental biology is expected.

Pharmacological Characterization of the Ac-RYYRIR-ol

Intensive research has focused on the development of potent and selective NOP receptor ligands, and resulted in various peptide and non-peptide agonist and antagonist compounds (*Zaveri et al., 2003*; *Lambert 2008*). These discoveries have been of great help for understanding the role of the N/OFQ system in a variety of physiological processes, such as pain modulation, anxiety and depression, locomotor activity, food intake, control of the cardiovascular and renal system, and so on *(Lambert, 2008, Jenk et al., 2000)* .

In this thesis a novel NOP receptor targeting peptide analogue, Ac-RYYRIR-ol (Arg^6 replacement in Ac-RYYRIK-ol; *Kocsis et al., 2004*) has been synthesized and tested in both biochemical and pharmacological assays. The idea for replacing the C-terminal lysine with an arginine unit was that the guanidinium group of Arg, in addition to the electrostatic interaction, is capable of forming two supplementary hydrogen bonds with an adjacent carboxylate group *(Bajusz et al., 1980)*; therefore higher binding affinities and receptor activation were expected. The significance of the hexapeptides in the NOP receptor field is that they can elucidate the role of the N-terminal phenylalanine in the N/OFQ heptadecapeptide and to investigate how nociceptin and hexapeptide derivatives, which have completely different sequences, can activate NOP receptor.

In receptor binding experiments done with [^{3}H]Ac-RYYRIK-ol, the novel hexapeptide Ac-RYYRIR-ol could effectively displace the radiolabelled analogue with pK_i value of 8.26, not very different from NOP receptor endogenous ligand N/OFQ which was 8.69. Ac-RYYRIR-ol was not able to bind to MOP and DOP receptors even up to high concentrations as demonstrated by the lack of displacement of [^{3}H]DAMGO and [^{3}H]DIDI from rat brain membranes. Similar results were obtained in the electrically stimulated tissues, where concentration response curves to DPDPE in the mouse vas deferens (for DOP receptor selectivity) and to dermorphin in

the guinea pig ileum (for MOP receptor selectivity) were not affected in the presence of Ac-RYYRIR-ol. In addition the high NOP selectivity of Ac-RYYRIR-ol is suggested by the results obtained with UFP-101 that fully prevented the stimulatory effects of Ac-RYYRIR-ol in GTP binding experiments performed in rat brain membranes. Altogether these findings converge indicating that Ac-RYYRIR-ol behaves as a high affinity and highly selective NOP receptor ligand. Ac-RYYRIR-ol could stimulate the [^{35}S]GTPγS binding using rat brain membranes. Efficacy of Ac-RYYRIR-ol was much less compared to N/OFQ as it was previously described for parent compound Ac-RYYRIK-ol under the same experimental conditions *(Kocsis et al., 2004)*. Stimulatory effect of both N/OFQ and Ac-RYYRIR-ol could be antagonized with UFP-101, which is highly specific NOP receptor antagonist. This result indicates that Ac-RYYRIR-ol stimulates selectively the G-proteins bound to the NOP receptor. Ac-RYYRIR-ol similar to previously described NOP specific hexapeptides produced both agonist and antagonist like effects in various assays used in this study.

Mouse and rat vas deferens, and [^{35}S]GTPγS binding studies also demonstrated the NOP antagonist properties of Ac-RYYRIR-ol. In mouse vas deferens Ac-RYYRIR-ol competitively antagonized the inhibitory effect and in the [^{35}S]GTPγS binding the stimulatory effect of N/OFQ. Similar results were reported using various hexapeptides such as Ac-RYYRIK-NH_2 and Ac-RYYRIK-ol *(Berger et al., 1999, Gündüz et al., 2006/2007).* It has been also proven that this antagonistic effect is NOP receptor specific as Ac-RYYRIK-NH_2 was not able to inhibit the stimulatory effect evoked by opioid receptor agonists *(Berger et al., 1999).* N/OFQ causes contractions of the mouse proximal colon *(Taniguchi et al. 1998)* by inhibiting the tonic neuronal release of nitric oxide (*Menzies et al. 2000*) and this preparation was used by many authors for characterizing novel NOP related compounds *(Rizzi et al., 1999, Menzies et al., 1999, Gündüz et al 2006*). In the mouse colon bioassay Ac-RYYRIR-ol mimicked effect evoked by N/OFQ thus behaving like a very potent agonist in this assay as it was previously described for the closely related hexapeptide Ac-RYYRIK-ol (*Gündüz et al., 2006/2007).* Agonistic effect of Ac-RYYRIR-ol was also present in the calcium mobilization experiments. Previously a characterization of the calcium signaling via NOP receptor using several ligands including partial agonists was done and shown to have overlapping potency as the Ac-RYYRIR-ol *(Camarda et al., 2009).* Similar results have been shown for the Ac-RYYKWR-NH_2 *(Corbani et al.*

2004). Ac-RYYRIR-ol and Ac-RYYRIK-ol was not able to induce NOP receptor internalization. Partial agonists that can produce response without significant receptor internalization might be good experimental tools in some cellular models of drug tolerance.

Ac-RYYRIR-ol exhibited full (mouse colon) and partial (calcium mobilization, [^{35}S]GTPγS binding) agonist as well as antagonist (mouse and rat *vas deferens*) properties at NOP receptors. These features of Ac-RYYRIR-ol are not surprising. Similar behaviors have been reported for many other NOP receptor related peptides such as; [Phe^{1}ψ(CH_2–NH)Gly_2]-N/OFQ(1–13)–NH_2, Ac-RYYRIK-NH_2, and Ac-RYYRIK-ol *(Guerrini et al., 1998, Mason et al., 2001, Kocsis et al., 2004)*. One explanation is the difference of NOP receptor densities in various preparations *(McDonald et al., 2003)*. Another possibility would be that low efficacy partial agonist ligands might exert different degrees of activation depending on various factors, such as the composition of the G-protein pool in the target tissue. It has been demonstrated that partial agonists are able to produce low to full biological responses *(Berger et al., 2000)*.

Difference in the sensitivity of Ac-RYYRIR-ol in various assays, but not the N/OFQ, might be explained by their likely diverse way of binding and activating the NOP receptors as shown by photo-affinity labelling studies of the NOP receptor with radio-iodinated probes *(Bes and Meunier, 2003, Judd et al., 2004)*. Those findings have indicated that the hexapeptides interacted with a region of Gln^{107}-Gly-Thr-Asp-Ile-Leu-Leu^{113} within the C-terminus of the second transmembrane domain (TM-II) in the NOP receptor *(Bes and Meunier, 2003, Guerrini et al., 1997)*, whereas N/OFQ interacted with the region of Thr^{296}-Ala-Val-Ala-Ile-Leu-Arg^{302}, spanning the C-terminus of EL-III and the N-terminus of transmembrane domain VII *(Mouledous et al., 2000)*. Recently, receptor-bound conformations of hexapeptide – NOP receptor and N/OFQ – NOP receptor complexes have been calculated by computer aided structural modeling *(Akuzawa et al., 2007)*. This model describes how both sorts of the peptides activate NOP receptor and supports that their docking conformations have similar 3D structures. The receptor bound conformation of the N-terminal FGGF message sequence in N/OFQ and the Ac-RYY fragment displayed fairly well overlapping structural arrangements.

In conclusion, Ac-RYYRIR-ol displayed a complex biochemical and pharmacological profile which is likely due to the low efficacy agonist nature of this unique

hexapeptide. It has been suggested that low partial agonism upon receptor – G-protein coupling in native systems may be sufficient to evoke full biologic responses *(Berger et al., 2000).* Exhibiting very good potencies and definite NOP receptor selectivity in various assays makes Ac-RYYRIR-ol an attractive tool for pharmacological and biochemical studies in the N/OFQ peptide receptor system.

Binding Characterization of the [^{3}H]Ac-RYYRIK-ol

Moreover in this thesis in vitro characterization of a novel radiolabeled hexapeptide analogue [^{3}H]Ac-RYYRIK-ol, selective for the NOP receptor has been described. Radioligands are essential tools in the G-protein coupled receptor (GPCR) research, especially the ^{3}H and ^{125}I labelled ligands *(Baker et al., 2000, Thomsen et al., 2000).* Binding characteristics and anatomical localization of the NOP receptors have been studied by using a set of radioligands *(reviewed by Dooley and Houghten, 2000).*

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 highest affinities were obtained with the homologous ligand Ac-RYYRIK-ol and N/OFQ, although the affinity of the Tyr^1-replaced derivative (Tyr^1)N/OFQ was also very good. The iodinated analogue (I_2Tyr^1)N/OFQ, however, displayed only modest affinity (Table 6) confirming the steric effect of the iodine atoms discussed above. Among the opioids tested, naloxone-benzoylhydrazone was the most efficient ($K_i \approx 1$ μM), while the general opioid antagonist naloxone and the MOP receptor agonist DAMGO were completely ineffective. The relative potency of naloxone-benzoylhydrazone in competing reversibly the binding of [^{3}H]Ac-RYYRIK-ol is in line with the peculiar feature of this rather promiscous opiate *(Cox et al., 2005; Olianas et al., 2006; Connor and Kitchen, 2006).* It should also be noted that some non-peptide compounds such as buprenorphine and J-113397 displayed considerably lower affinities than expected. This might be due to a potentially different, or perhaps overlapping binding site(s) for N/OFQ and the hexapeptides within the receptor as discussed below.

The results obtained in the present study revealed that the new tritiated peptide analogue [^{3}H]Ac-RYYRIK-ol recognized and labelled NOP receptors specifically in rat brain membrane preparations and in cellular system expressing the recombinant

human NOP receptors. Importantly, this radioligand satisfied the criteria of reversibility, saturability and relatively low non-specific binding necessary for valuable radioprobes. These characteristics of [^{3}H]Ac-RYYRIK, together with its improved chemical and biological stability, and high specific radioactivity, make this radioligand an inspiring tool for analyzing the properties and function of the NOP receptor.

SUMMARY

Leu- and Met-enkephalin were the first endogenous opioid peptides identified in different mammalian species including the human. Within the PENK structure two Met-enkephalin sections are C-terminally elongated. One octapeptide sequence Tyr-Gly-Gly-Phe-Met-Arg-Gly-Leu (YGGFMRGL) exists at the fourth position, while the seventh, C-terminally located Met-enkephalin extension is present in heptapeptide form. Comparative biochemical and bioinformatic evidence indicate that enkephalins are not limited to mammals. Various PDYN and PENK sequences in lower vertebrates revealed the presence of other endogenous opioid peptides in these precursor polypeptides. 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 has been identified by 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 in receptor binding and G-protein activation assays performed on rat brain membranes.

In various receptors binding assays performed on rat brain membrane preparations both of the enkephalin peptides turned out to be moderate affinity opioids with a weak preference for the DOP receptor sites. Phe-enkephalin of the lungfish displayed rather unexpectedly low affinities toward the MOP and DOP, while exhibited moderate affinity toward the KOP receptor. In receptor-mediated G-protein activation assays measured by the stimulation of [^{35}S]GTPγS binding, Met-enkephalin produced the highest stimulation followed by Leu-enkephalin, Ile-enkephalin and Phe-enkephalin. The overall binding and signalling profile of the novel octapeptides as well revealed moderate opioid agonist activities and a rank order of potencies for the *mu* ~ *delta* >> *kappa* receptor binding sites. Peptides with the oxidized $M_{(O)}$ residue were found to be less potent in both receptor binding and G-protein stimulation studies.

Nociceptin/orphanin FQ (N/OFQ) is an endogenous neuropeptide, which is widely distributed in central and peripheral nervous system. Some N/OFQ sequence unrelated hexapeptides can effectively bind to the NOP receptor and they were used as template for structure activity studies that lead to discovery of the new NOP selective ligands. In this thesis, 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+} 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+} mobilization assay Ac-RYYRIR-ol displayed lower potency. We have concluded that the novel NOP receptor selective hexapeptide Ac-RYYRIR-ol has fine selectivity, high potency, furthermore agonist and antagonist effects toward the NOP receptors which is likely due to its partial agonist pharmacological activity.

Radioreceptor binding studies were of [^{3}H]Ac-RYYRIK-ol was conducted using native neuronal NOP receptor preparation of rat brain membrane fractions and recombinant human nociceptin receptor ($_h$NOP) preparations from cultured CHO cells stably expressing $_h$NOP receptors. Results indicated 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. This new radiprobe [^{3}H]Ac-RYYRIK, with high specific radioactivity, makes this radioligand an inspiring tool for analyzing the properties and function of the NOP receptors.

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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. (IF: 3.556)

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. (IF: 3.228)