Biochemical, functional and pharmacological characterization of novel hexapeptide ligands targeting nociceptin/orphanin FQ receptor, *in vitro* and *in vivo* studies

Ph.D. thesis

by

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LIST OF PUBLICATIONS

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3. Ligeti, M., Bősze,Sz., Csámpai, A., **Gündüz, Ö.**, Al-Khrasani, M., Rónai, A.Z., Medzihradszky-Schweiger H, Benyhe, S., Borsodi, A., Hudecz, F., Magyar, A. **(2006)**. Synthesis of enzymatically resistant Nociceptin-related peptides containing a carbamic acid residue. *Journal of Peptides Sciences*, in press.

4. Ligeti, M.*, **Gündüz, Ö.***, Magyar, A., Kató, E., Rónai, A.Z., Vita, C., Varga, I., Hudecz, F., Tóth, G., Borsodi, A., Benyhe, S. **(2005)**. Synthesis and Biological Studies of Nociceptin Derivatives Containing the DTPA Chelating Group For Further Labeling With Therapeutic Radionuclides, *Peptides*, 26(7):1159-1166.
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5. Kocsis, L., Orosz, Gy., Magyar, A., Al-Khrasani, M., Kató, E., Rónai, A.Z., Bes, B., Meunier, J.C., **Gündüz, Ö.**, Tóth, G., Borsodi, A., Benyhe, S. **(2004)**. Nociceptin antagonism: Probing the receptor by N-Acetyl oligopeptides, *Regulatory Peptides*, 122(3):199-207.

Self citations are indicated as **bold** in the text.

ABBREVIATIONS

Aib	Achiral alpha-amino isobutyric residue
Ac	Acetyl
AcH	Acetylcholine
ANOVA	One-Way Analyses of Variance
APN	Aminopeptidase N
A.U.C.	Area under curve
B_{max}	Maximal binding capacity of an experimental binding system, usually a preparation containing receptors (membranes, cells). The magnitude is most often expressed in number of receptors per cell or molar concentration of receptors per milligram protein.
BSA	Bovine serum albumin
Bz	Benzoyl
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' cell line
$CHO_{IND}NOP_h$	CHO cells stably expressing the ecdysone-inducible mammalian expression system containing the human NOP receptor.
Cit	Citrulline
ClAc	Chloro-acetyl
CNS	Central nervous system
CR	'Concentration rate'; the ratio between the EC_{50} (nM) values of the agonist in the presence and absence of antagonist
2-D and 3-D	2-dimension and 3-dimension
DA	Dopamine
DNA	Deoxyribo nucleic acid
DTPA	Diethylenetriaminepentaacetic acid
EC_{50}	'Potency'; the agonist molar concentration that produces 50% of the maximal possible effect of that agonist.
EDTA	Ethylenediamine-tetraacetic acid
EL	Extracellular
E_{max}	'Efficacy'; the maximal effect that an agonist can elicit in a given tissue/preparation
EP 24.11	Endopeptidase 24.11
EP 24.15	Endopeptidase 24.15
FCS	Fetal calf serum
For	Formyl
g	Gravitation force
GABA	γ-aminobutyric acid
GDP	Guanosin diphosphate
GI	Gastrointestinal tract
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-^{35}S]thiophosphate
5-HT	Serotonin
Hcrts/Oxs	Hypocretins and orexins
HEPES	2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid

HTS	High throughput screening
IC_{50}	'Affinity'; the concentration of the competitor producing 50 % displacement in the competion radioligand binding assay.
i.c.v.	Intracerebroventricular
III-BTD	(3S,6S,9R)-2-oxo-3-amino-7-thia-1-aza-bicyclo[4.3.0]nonane-9-carboxylic acid;
IL	Intracellular
i.t.	Intrathecal
IUPHAR	an acronym for International Union of Pharmacology, a nongovernmental organization of national societies functioning under the International Council of Scientific Unions
J-113397	(±)trans-1-[1-cyclooctylmethyl-3-hydroxymethyl-4-piperidyl]-3-ethyl-1,3-dihydro-2H-benzimidazol-2-one
JTC-801	N-(4-amino-2-methylquinolin-6-yl)-2-(4-ethylphenoxymethyl)benzamide hydrochloride
K_d	Equilibrium dissociation constant of a radioligand-receptor complex
K_i	equilibrium inhibition constant
KOP	κ-opioid peptide receptor
L	Lumbar segment of the spinal cord
[L]	Labelled ligand concentration
MAP	Mitogen-activated protein
MC	Mouse colon
MCH	Melanin-concentrating hormone
MOP	μ-opioid peptide receptor
Ms	Mesyl
mRNA	Messenger ribonucleic acid
mVD	Mouse *vas deferens*
NMU	Neuromedin U
NA	Noradrenaline
NANC	Nonadrenergic-noncholinergic
NNC-63-0532	(8-Naphthalen-1-ylmethyl-4-oxo-1-phenyl-1,3,8-triaza-spiro[4.5]dec-3-yl)-acetic acid methyl ester
N/OFQ	Nociceptin/Orphanin FQ
$NOP^{-/-}$ and $NOP^{+/+}$	NOP receptor knockout and wildtype
NOP receptor	Nociceptin/Orphanin FQ peptide receptor
NPB	Neuropeptide B
NPS	Neuropeptide S
NPW	Neuropeptide W
NRM	Nucleus raphe magnus
oGPCR	orphan G-protein coupled receptor
***Oprl 1* gene**	NOP receptor gene
ORL-1 receptor	Opioid receptor-like 1
UII	Urotensin II
PAG	Periaqueductal gray
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
PCR	Polymerase chain reaction
PEI	Polyethyleneimine

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.
Piv	Pivaloyl
pK_B	'Antagonist potency'; when one single concentration of antagonist is used.
pK_i	antilogarithm of the equilibrium inhibition constant
ppN/OFQ	Prepronociceptin
ppN/OFQ$^{-/-}$	Prepronociceptin gene deficient
Ro-65-6570	(8-acenaphthen-1-yl-1-phenyl-1,3,8-triaza-spiro[4,5]decan-4-one)
Ro-64-6198	[(1S,3aS)-8-(2,3,3a,4,5,6-hexahydro-1H-phenalen-1-yl)-1-phenyl-1,3,8-triaza-spiro[4,5]decan-4-one]
PrRP	Prolactin-releasing peptide
PTX	Pertussis toxin
r^2	Correlation coefficient
rpHPLC	Reverse phase High Performance Liquid Chromatography
RT-PCR	Reverse Transcriptase Polymerase chain reaction
SB-612111	(-)-cis-1-methyl-7-[[4-(2,6-dichlorophenyl)piperidin-1-yl]methyl]-6,7,8,9-tetrahydro-5H-benzocyclohepten-5-ol
SEM	Standard error of the mean
TEM	TrisHCl-EGTA-$MgCl_2$ buffer
TM	Transmembrane
TTX	Tetrodotoxin
Tris	Tris-(hydroxymethyl)-aminomethane
TRK-820	((-)-17-cyclopropylmethyl-3,14b-dihydroxy-4,5a-epoxy-6b-[N-methyl-trans-3-(3-furyl)acrylamide]morphinan hydrochloride)
UFP-101	[Nphe^1,Arg^{14},Lys^{15}]nociceptin/orphanin FQ-NH_2
UFP-102	[(pF)Phe^4,Arg^{14},Lys^{15}]N/OFQ-NH_2
ZP-120	Ac-RYYRWKKKKKKK-NH_2

Standard one- and three-letter amino acid assignations were used

Some definitions

Agonist A molecule that produces physiological response through activation of a receptor.

Full Agonist A name given to an agonist that produces the full system maximal response (E_{max}). A system-dependent phenomenon and should not necessarily be associated with a particular agonist, as an agonist can be full agonist in some systems and a partial agonist in others.

Partial Agonist Whereas a full agonist produces the system maximal response, a partial agonist produces a maximal response that is below that of the system maximum (and that of a full agonist). As well as producing a submaximal response, partial agonists produce antagonism of more efficacious full agonists.

Antagonist A molecule that interferes with the interaction of an agonist and a receptor protein or a molecule that blocks the constitutive elevated basal response of a physiological system.

I. INTRODUCTION

1. Orphan G-protein coupled receptors (oGPCR) and the reverse pharmacology approach

Nociceptin/orphanin FQ (N/OFQ) and its receptor (NOP) constitute a novel peptide-receptor system, widely expressed centrally and in the periphery. N/OFQ is involved in pain transmission, anxiety and response to stress, learning and memory, food intake and locomotor activity. Additionally, N/OFQ controls some functions of the renal, cardiovascular, respiratory and gastrointestinal systems. Therefore, it is an emerging molecular target for the development of novel therapeutics for several disease states.

G-protein coupled receptors (GPCR) are one of the largest family of proteins that are the main modulators of intercellular interactions and regulate activities in the human body and particularly in the central nervous system (CNS). They are targets of most of the primary messengers including the neurotransmitters, all the neuropeptides, the glycoprotein hormones, lipid mediators and other small molecules; thus have considerable pharmaceutical interest. Drugs that are acting on GPCRs are used to treat numerous disorders. More than 30 % of the approximately 500 clinically used drugs, are modulators of GPCR function, representing around 9 % of the global pharmaceutical sales, making GPCRs the most successful of any target class in terms of drug discovery [1].

After the completion of the Human Genome Sequencing Project, many genes (approximately 720) that belong to the GPCR superfamily have been identified. Half of these encode chemosensory receptors (*e.g.*, olfactory, gustatory, chemokine and chemoattractant), while the remaining half regulate responses involving endogenous transmitters. From the latter, for approximately 210 receptors had the individual natural ligands been identified, and the remaining (150) receptors are called orphan GPCRs (oGPCRs) with unidentified endogenous ligand or unknown function. Identification of a novel transmitter that naturally activates an oGPCR provides more information about the function of that receptor. There are several experimental approaches to identify a transmitter: screening of putative small molecule and peptide ligands, reverse pharmacology, and the use of bioinformatics to predict candidate ligands. The first deorphanization activities began in the mid 80s by testing the potential transmitters, and the method was named the reverse pharmacology approach. A variation of the reverse pharmacology approach was developed in 1995: instead of screening potential transmitters, extracts of tissues were used as sources of new transmitters. The orphan receptor strategy was proven to be successful with the discovery of a new neuropeptide, nociceptin/orphanin FQ (N/OFQ) as the transmitter of the oGPCR ORL-1 [2, 3]. In this strategy,

by the help of functional genomics, oGPCRs are expressed in a recombinant expression system, such as mammalian cells, yeast, or *Xenopus* melanophores. Following expression, candidate ligands (including small molecules, peptides, proteins, lipids, or tissue extracts) are screened against the receptor to identify molecules capable of interacting specifically with that receptor. The identification of a candidate ligand is detected according to the activation of an intracellular signalling cascade. An activating ligand (or a hit molecule) will be identified by its ability to cause a concentration-dependent increase in the activity of a signalling cascade. Once identified, the ligand may be further tested against other GPCRs to determine its activity and selectivity profile prior to being used in a cell-based, tissue and in some cases whole-animal experiments, in order to study the physiological role of the receptor.

The first successful example of the novel approach (the discovery of N/OFQ) was followed by the identification of other novel bioactive peptides such as: hypocretins and orexins (Hcrts/Oxs), prolactin-releasing peptide (PrRP), apelin, ghrelin, melanin-concentrating hormone (MCH), urotensin II (UII), neuromedin U (NMU), metastin, neuropeptide B (NPB), neuropeptide W (NPW) and neuropeptide S (NPS). Each of these discoveries was a landmark in its field [4].

2. NOP receptor

2.1. Molecular cloning of NOP receptor

Molecular cloning of the δ-opioid peptide receptor [5, 6] was soon followed by the cloning of the μ- [7] and the κ-opioid peptide (MOP and KOP) receptors [8]. Further attempts to clone additional opioid receptor types and/or subtypes, by hybridization screening at low stringency with opioid receptor cDNA probes, or using probes generated by selective amplification of genomic DNA with degenerative primers led several research groups to isolate a cDNA encoding a homologous protein with a high degree of sequence similarity to the opioid receptors [9]. The novel clones displayed approximately 50 % identity with the traditional opioid receptors overall, with the transmembrane regions showing even higher homologies of up to 80 %. Despite their close homology to the other opioid receptors, opioids displayed very low affinities towards the ORL-1 (Opioid Receptor Like-1) receptor, thus it was considered an orphan receptor until the discovery of the endogenous ligand. ORL-1 was subsequently named NOP (Nociceptin/Orphanin FQ peptide) receptor according to the IUPHAR nomenclature [10]. NOP receptor was cloned from several species like human: ORL1 [11]; mouse: KOR-3 ref. [12, 13], MOR-C [14]; rat: LC132 ref. [15], XOR1 ref. [16, 17], Ratxor1 ref. [18], C3 ref. [19], ROR-C [20, 21]; pig: NOP receptor [22].

The human NOP receptor protein consists of 370 amino acids [11] and contains seven trans-membrane (TM) domains (Fig. 1). Like most GPCRs, human NOP receptor displays many

putative consensus sites for post-translational modifications like i) three asparagine residues within Asn-Xxx-Ser/Thr motifs for N-linked glycosylation, ii) a pair of conserved cysteine residues which may form a disulphide bridge between extracellular loops (EL) 1 and 2 and iii) putative palmitoylation sites downstream of TM segments. Also all cytoplasmic domains contain a number of Ser/Thr residues representing potential sites for phosphorylation, a process known to be involved in desensitization [11].

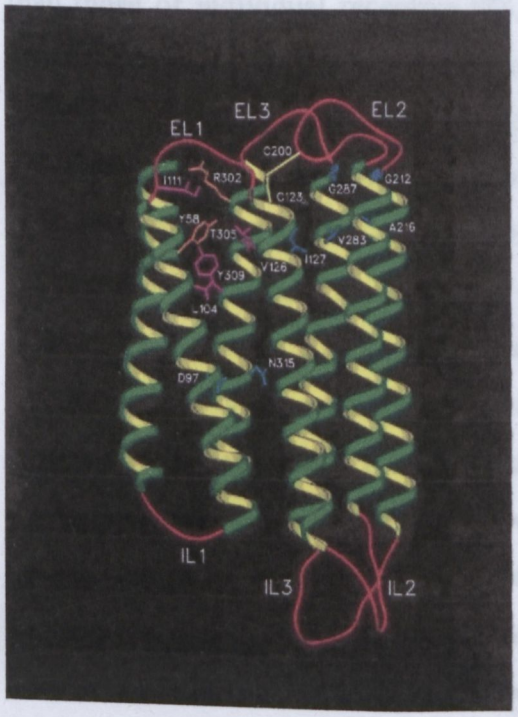


Fig. 1: The NOP receptor model from Topham et al 1998 [29].

2.2. The NOP receptor gene

The NOP receptor gene, *Oprl1*, is located at the q13.2-13.3 region of the human chromosome 20 ref. [23]. In terms of intron-exon organization, the NOP receptor gene is nearly identical to that of the δ-, μ-, and κ-opioid receptors, suggesting that the four genes have evolved from a common ancestor and hence belong to the same family [24]. Indeed, the NOP receptor appears to be evolutionary as old as the opioid receptors, since NOP receptor-like genomic sequences have been reported in teleost [25], in cartilaginous fish [26], in sturgeon [27] and in zebra fish [28].

2.3. NOP receptor heterogeneity

There are some indications for the existance of multiple NOP receptors, which originates from the pharmacological (the biphasic effects of the N/OFQ peptide/NOP receptor system on pain and locomotion) and receptor binding studies (presence of high and low affinity states of the NOP receptor in mouse brain homogenates [30, 31]). Although the pharmacological studies have not firmly established the existence of NOP receptor subtypes [32], the NOP receptor heterogeneity is still an open question. The receptor subtypes may arise from splice variants ([16, 33, 34]) of the NOP receptor cDNA [9]. It has been investigated that the *Oprl1* gene transcript undergoes alternative splicing. So far, five splice variants of NOP receptor mRNA have been isolated. Except one splice variant that encodes a functional receptor which has been isolated from human tissue [23], none of the other variants encode a full-length receptor [35], but rather non-functional truncated receptors.

Alternatively, apparent NOP receptor heterogeneity may arise from posttranslational modifications, expression of an yet unknown receptor gene or functional interaction of the NOP

receptor with other receptors or proteins [36]. The use of NOP receptor deficient (knockout) mice devoid of any N/OFQ binding [37] revealed the extinction of a separate NOP receptor encoded by an independent gene. Thus, the functional modification of the NOP receptor has to be elucidated at the protein level. Receptor oligomerization (formation of homo and hetero oligomers) in particular, heterodimerization of distinct but structurally related GPCRs occurs in plasma membrane and generates pharmacological and signalling properties that are different than those of individual receptors [38, 39]. The synergic or antagonistic interactions between different GPCRs that are co expressed in the same cell may be due to dimerization. Heterodimerization of the NOP receptor with opioid receptors has been investigated and so far just the formation of NOP-MOP receptor heterodimers were reported within the studies in cell lines co-transfected with NOP and MOP receptors. It can be postulated that NOP-MOP receptor heterodimerization and the resulting impairment of the MOP receptor activated signalling pathways contributes to the NOP receptor mediated antiopioid effects in the brain [40, 41]. Further studies are necessary in particular to investigate the possible oligomerization with κ-opioid (KOP) receptors. Due to the structural homologies of the endogenous ligands and the reasonable affinity of dynorphin derivatives (Dyn R8) for inhibition of forskolin-stimulated cAMP accumulation at the NOP receptor [42] and at the [^{125}I](Tyr^{10})N/OFQ(1-11) binding site in mouse brain [43], heterodimer formation with KOP receptor can be expected.

3. The Endogenous Ligand Nociceptin/Orphanin FQ

3.1. Isolation

Soon after the cloning of the orphan opioid receptor-like 1 ('NOP' according to the current IUPHAR nomenclature) receptor, Chinese Hamster Ovary (CHO) cells transfected with the orphan receptor were used to fish the endogenous ligand. Because the ORL1 transcripts were particularly abundant in the hypothalamus, two independent research groups used crude extracts from rat brain [3] and porcine hypothalamus [2] to screen the crude tissue fractions for the adenylyl cyclase inhibitory activity as a functional assay in the cell lines stably transfected with ORL1. The fractions that were able to inhibit the adenylyl cyclase activity, were further fractionated through reverse-phase high-performance liquid chromatography (rpHPLC). The purification and mass spectrometry analyses yielded to identify a heptadecapeptide (Fig.2), the sequence of the peptide was determined and synthesized. The synthetic peptide was shown to inhibit the forskolin-stimulated accumulation of cAMP in the CHO cells transfected with the 'orphan' receptor, while showing no activity in non-transfected control cells. *In vivo* studies revealed that this peptide following intracerebroventricular (i.c.v.) administration in mice produced hyperalgesia in the hot plate [3] and tail flick [2] tests. Decrease in the locomotor activity but no analgesia was also observed in the hot plate test [2]. The french group named this

peptide 'nociceptin' to denote its pronociceptive activity, while the swiss authors called it 'orphanin FQ' due to its high affinity to the orphan receptor with the indications of the peptide's first and last amino acids phenylalanine (Phe, F) and glutamine (Gln, Q), respectively. The latest terminology of this peptide given by IUPHAR is nociceptin/orphanin FQ (N/OFQ) [10]. Both N/OFQ and the NOP receptor share sequence homologies with the opioid peptide ligand Dynorphin A and the κ- opioid receptor, respectively. In Fig. 2, the structural similarities of the peptides are shown. Despite the similarities, these peptides are functionally quite distinct. N/OFQ has no significant affinity for any of the opioid receptors [42].

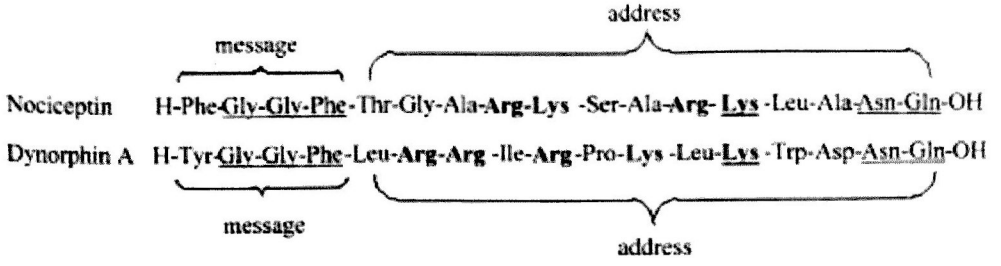


Fig. 2: Structural similarities between dynorphin A and N/OFQ amino acid sequences.

3.2. Precursor Protein

Like all neuropeptides, N/OFQ is also derived from a larger polypeptide precursor (the length is 176 amino acids in human) prepronociceptin (ppN/OFQ) [44, 45]. The ppN/OFQ genes were shown to be highly conserved in three species from which they had been isolated. The ppN/OFQ gene is located in the human chromosome 8 (8p21) ref [44]. Moreover the organizational features that are strikingly similar in the precursors of endogenous opioid peptides, suggests that the four genes belong to the same family hence they have a common evolutionary origin [44, 45].

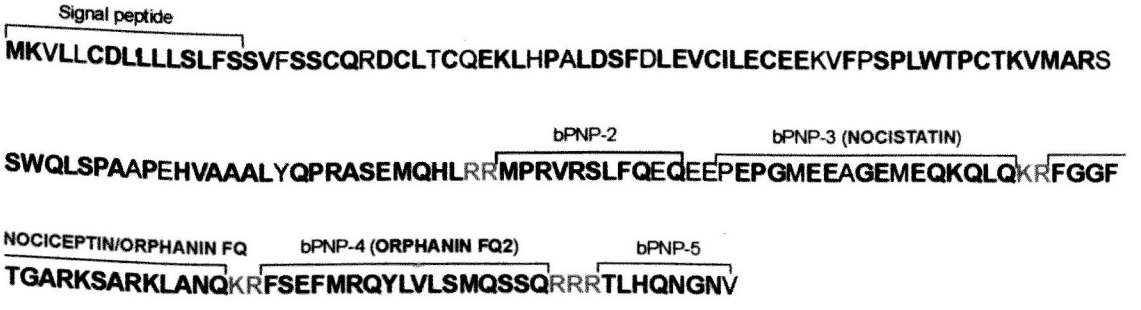


Fig. 3: The amino acid sequence of the human precursor protein of N/OFQ (ppN/OFQ). The signal peptide and putative peptides derive from the same precursor (eg. N/OFQ, N/OFQ II and nocistatin) are indicated by upper lines. The conserved amino acids are shown as **bold** and the putative proteolytic cleavage motifs are shown in blue.

The primary structure of the precursor protein contains the typical structural elements of a neuropeptide precursor: an amino terminus signal peptide necessary for its secretion, several

pairs of basic amino acids that represent possible sites of cleavage for precursor maturation or for transcriptional regulation [46]. Several biologically relevant peptides may derive from the N/OFQ precursor (Fig. 3). Apparently two additional peptides are excised from the same precursor: orphanin FQ2 or N/OFQ II and nocistatin [47]. None of them bind to NOP receptor [44, 45], and until now specific receptors for them have not been identified, although an unidentified receptor was proposed for N/OFQ II [48]. Orphanin FQ2 is a heptadecapeptide terminating with a FQ couple similar to N/OFQ. It has been found to be biologically active, stimulating locomotor activity in mice [49], inducing antinociception both supraspinally and spinally [50] and inhibiting gastrointestinal transit [50]. Nocistatin which antagonizes several functions of N/OFQ, is the other peptide derived from the same precursor [47]. It has been found in many studies to be inactive *per se* but was able to reverse several effects of N/OFQ such as induction of allodynia after spinal administration in mice [47, 51], inhibition of glutamate release from rat brain slices [52], impairment of learning and memory in mice [53]. However it has been reported that nocistatin applied to rats i.c.v. or i.t. can cause antinociception in the carrageenan test [54] or in the formalin test [55]. Interestingly nocistatin or its C-terminal hexapeptide exerts anxiogenic-like effect in mice [56].

3.3. Metabolism of N/OFQ

Once N/OFQ is released from the neurons, it is metabolised to inactive fragments by different types of proteolytic enzymes such as aminopeptidase N (APN) generating N/OFQ (2-17) that does not bind to NOP receptor. Endopeptidase 24.15 (EP 24.15) [57] acts on the peptide bonds Ala^{7}-Arg^{8}, Ala^{11}-Arg^{12}, Arg^{12}-Lys^{13} and releases inactive compounds, Endopeptidase 24.11 (EP 24.11) acts on the cleavage site (Lys^{13}-Leu^{14} bond) and plays a major role in the initial stage of N/OFQ metabolism in mouse spinal cord [58]. N/OFQ(1-13) is the active metabolite that has less affinity than N/OFQ to the NOP receptor [59]. Also the carboxypeptidase B acting on the arginine, lysine and ornithine residues in the C-terminal may play a minor role in the degredation of N/OFQ [57, 58]. The schematic illustration of the degredation of N/OFQ can be seen in Fig. 4.

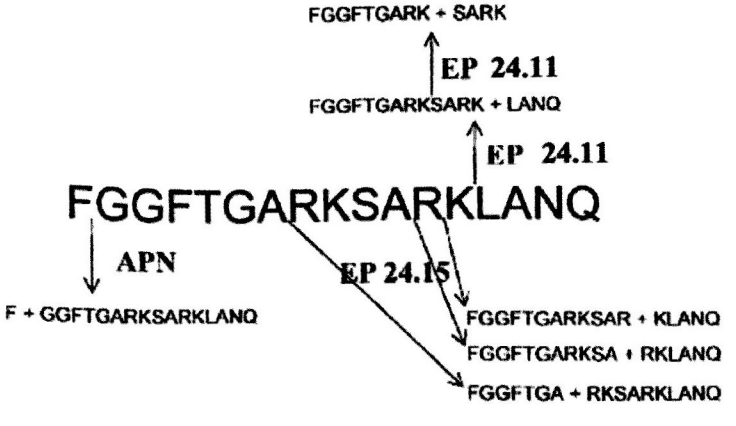


Fig. 4: N/OFQ metabolism and the metabolites. APN, aminopeptidase N; EP, endopeptidase.

3.4. Transgenic Mice

As an alternative to the pharmacological tools such as stable NOP receptor antagonists, the developments in molecular biology provided the molecular tools for specific gene deleting. Up to date transgenic 'knockout' mice devoid of N/OFQ precursor [60, 61] as well as mice lacking the NOP receptor [37, 62] have been generated and studied. NOP receptor knockout ($NOP^{-/-}$) mice do not display differences in nociceptive threshold or anxiety-related behaviours when compared with wild-type animals [62, 63], however NOP knockout mice display an antidepressant-like phenotype [64]. While the knockout mice for the ppN/OFQ gene ($ppN/OFQ^{-/-}$) presents an elevated basal nociceptive threshold and an anxiogenic-like phenotype [60]. This discrepancy between phenotypes could possibly arise from the existence of NOP receptor subtypes, or the different genetic backgrounds of mice. However it should not be underestimated that, the biological activities of nocistatin or orphanin FQ2 that are mediated by a receptor other than NOP receptor, could contribute to the phenotype differences [65].

4. Tissue distribution of NOP receptor and N/OFQ

Before identification of the endogenous ligand, the distribution of the NOP receptor transcripts in murine tissues was investigated by *in situ* hybridization [11, 15, 17, 19, 20], Northern blot [18] and RT-PCR [16] analyses. Immunolocalization of the receptor protein, using specific antibodies provided a detailed mapping of the rat brain [66] and spinal cord [67]. Following the identification of N/OFQ, the distribution of the NOP receptor was confirmed with autoradiographic studies based on agonist stimulated [^{35}S]GTPγS binding [68] and binding with labelled N/OFQ analogs [69, 70]. Additionally, NOP receptor binding is compared and shown to be in good correlation with mRNA hybridization. Regions with NOP receptor binding typically express NOP mRNA as well, although the levels of mRNA and NOP binding do not always match very closely. [71]

The NOP receptor is widely expressed in the CNS, in particular in the forebrain (cortical areas, olfactory regions, limbic structures: hippocampus and amygdala, thalamus), throughout the brainstem (central periaqueductal gray, substantia nigra, several sensory and motor nuclei), and in both dorsal and ventral horns of the spinal cord [71, 72]. Regions only light immunohistochemical labelling for NOP receptors are observed in the caudate-putamen and the cerebellum [66] (in contrast to rodents, in humans a considerable amount of NOP receptors have been found in the cerebellum [36]). The NOP receptor has also been identified in the peripheral nervous system and several other organs. Thus, the NOP receptor is expressed in smooth muscles, in peripheral ganglia and in the immune system. It has been detected in rat intestine and *vas deferens*, porcine gastrointestinal tract and kidney, also in rat retina and heart [72].

It is worth to mention that NOP receptors co-express with MOP receptors in the dorsal horn of the spinal cord, the hippocampal formation and the caudate putamen [66, 73] in the midbrain periaqueductal gray (PAG) and the nucleus raphe magnus (NRM) [74].

The localization of N/OFQ-immunoreactive fibers and terminals and/or the localization of the N/OFQ peptide precursor mRNA is more limited, compared with that of the NOP receptor. Limbic areas, however, highly express N/OFQ, in particular the bed nucleus of the stria terminals, and the amygdala nuclei [75, 76]. A matching pattern of N/OFQ and NOP receptor expression in the human and rodent central nervous system has been observed [23, 36, 77]. In the periphery mRNA of N/OFQ was detected in rat ovary, human spleen, lymphocytes, and fetal, but not adult kidney [44, 45]. Moreover, under physiological conditions N/OFQ is present in the plasma of humans (~ 10 pg/ml) [78], in several pathological conditions such as female fibromyalgia syndrome [79], postpartum depression [80], Wilson's disease [81], hepatocellular carcinoma [82], pain and chronic pain [83], cluster headache [84], migraine without aura [85]. N/OFQ levels were detected to be either increased or decreased (in the case of the latter two) in the plasma.

Analysis of N/OFQ and opioid stimulated [^{35}S]GTPγS binding autoradiography in guinea pig brain showed a unique distribution of the NOP receptor which might explain the different, or sometimes opposite pharmacological effects of N/OFQ and opioids [68]. Due to the widespread distribution of N/OFQ peptide and NOP receptor, this novel system is associated with a large number of physiological responses and probably contributes to the homeostasis by modulating neuronal circuitry [72]. This might explain why the NOP receptor knockout mice has not an obvious impact (else than the unrestrained nociceptive response and disregulation of hearing ability) [62] and also why pharmacological effects of N/OFQ are sometimes contradictory (e.g. in pain and locomotor tests) depending on the locus of injection and dose [72].

5. Cellular Actions of N/OFQ

Since N/OFQ and opioid receptors are members of GPCR superfamily, they are capable of interacting with specific GTP binding proteins (*i.e.*, G-proteins). G-proteins are membrane bound/associated heterotrimeric proteins composed of α, β, γ subunits. Because of the high sequence homology in the putative intracellular loops (IL) of NOP and opioid receptors, it was anticipated that the NOP receptor couples to the same G-protein regulated cellular effectors as do the opioid receptors. Early postreceptor events induced by N/OFQ are:

i) inhibition of forskolin-stimulated cellular cAMP accumulation. Forskolin is used to directly activate adenylyl cyclase and consequently increase cAMP production.

ii) inhibition of voltage-gated N-type calcium channels

iii) activation of inwardly-rectifying potassium conductance

There is evidence for N/OFQ mediated activation of additional signalling pathways. It has been shown that NOP receptor activation stimulates phospholipase C via a pertussis toxin (PTX)-sensitive G-protein ($G_{i/o}$ family), to increase intracellular Ca^{2+} concentration which then activates phosholipase C [86]. Both NOP and MOP receptors were reported to mediate PTX-sensitive mitogen-activated protein (MAP) kinase activation via signaling pathway using the $Gi_{\beta\gamma}$ [87]. However, these effects were observed only in recombinant cells, thus, further studies are required especially in non-recombinant (*i.e.*, native cellular) systems.

6. N/OFQ and neurotransmitter release

N/OFQ-NOP receptor system has an important role in neurotransmitter release. N/OFQ reduces neuronal excitability and presynaptic transmitter secretion primarily by acting on ion conductances [24]. In the **central nervous system**: studies using synaptosomes and brain slices revealed that N/OFQ inhibits the release of noradrenaline (NA), serotonine (5-HT), dopamine (DA), acetylcholine (AcH), γ-aminobutyric acid (GABA), and glutamate (nicely reviewed [88]). In the **peripheral nervous system**: studies showed the general modulatory effects (mostly inhibitory) of N/OFQ on neurotransmitter release from sympathetic, parasympathetic, nonadrenergic-noncholinergic (NANC) and sensory nerve endings (presynaptical inhibitory function). On the respiratory, cardiovascular, genitourinary and gastrointestinal systems N/OFQ exerts inhibitory effects [89].

7. Other Biological Actions of N/OFQ

Due to the widespread distribution of N/OFQ and NOP receptor, this peptidergic system is involved in a broad spectrum of biological actions. The role of this peptidergic system has been explored intensely with the pharmacological and biological tools available, such as i) antisense oligonucleotides targeting NOP receptor or ppN/OFQ gene, ii) antibodies directed against N/OFQ, iii) transgenic mice in which the receptor or the peptide precursor genes have been genetically eliminated, iv) available stable and highly potent antagonists (preferably non-peptide) [32]. Some of the central effects of N/OFQ can be seen in Table 1.

On the central level N/OFQ modulates: nociception [2, 3, 90], spontaneous locomotor activity [2], responses to stress and anxiety [91], food intake [92], rewarding actions of opioids [93] and ethanol [94], addiction [95, 96], learning and memory processes [97]. On the peripheral level N/OFQ has effects like: inhibition of gastrointestinal motility [98], inhibition of contractions of bronchus and trachea in the airway [99, 100, 101], broncho constriction induced by capsaicin [102], inhibition of coughing reflex [103], bradycardia and hypotension [104, 105], increase blood pressure and heart rate [106] in the cardiovascular system, diuretic and antinatriuretic responses on the renal function [107, 108], suppression of micturition reflex [109].

Table 1: Central effects of N/OFQ.

Nociception	
Pronociceptive and antiopioid (supraspinal) [3, 90]Pronociceptive (spinal) [110, 111]	Antinociceptive (spinal) [111, 112]
Spontoneus locomotor activity	
Hypolocomotion (supraspinal) [2, 62]	Stimulation of locomotion (low doses) (supraspinal) [113] related to anxiolytic effect [91]
Anxiety	
Anxiolytic-like action (supraspinal) [91] [60]	Anxiogenic-like action (supraspinal) [114]
Food intake	
Hyperphagia (supraspinal) [92]	
Reward and Addiction	
Abolishes rewarding property of ethanol [94] and morphine [115] Suppression of methamethamine induced conditioned place preference [96]	
Learning and memory	
Impairs spatial learning and memory retention (supraspinal) [97, 116]	

8. Pharmacology of NOP receptor

The novel N/OFQ peptide/NOP receptor system is considered to be a 'non-opioid branch of the opioid family' due to its close structural and transductional similarities and on the other hand its pharmacological and functional differences with the classical opioid systems [10]. From the numerous modulatory actions of N/OFQ in several neurological pathways, one thing is obvious that this novel peptidergic system represents an important molecular target for the development of novel therapeutics for several neurological and other pathological conditions [117]. Thus, this system is highly attractive for drug design purposes. In fact, there is a tremendous interest from pharmaceutical companies in developing both agonists and antagonist non-peptide ligands for the NOP receptor as potential drugs for various human disorders [118]. Some of the therapeutic applications of NOP receptor agonists, partial agonists and antagonists are summarized in Table 2.

Table 2: Potential therapeutic applications of NOP receptor ligands

AGONISTS	PARTIAL AGONISTS	ANTAGONISTS
Anxiolytics Stimulants of food intake Antitussives Anti-epileptics Spinal analgesics Suppressants of drug abuse For management of hyponatremia and water retention	Aquaretics (peripherally acting) for managements of hyponatremia and water retention	Analgesics (alone/in combination with opiates) Antidepressants Anorectics Nootropic agents Antiparkinson

Until now there has been a number of compounds identified that represent useful pharmacological tools for clarification of physiological and pathophysiological roles of N/OFQ-NOP receptor system. Currently available NOP receptor ligands can be classified as the following:

8.1. N/OFQ related peptides

Peptides that belong to this group are developed by **classical structure-activity relationship (SAR) studies**. Amidation of the C-terminus (N/OFQ-NH_2) maintained full potency and activity [59]. Deletion of up to four amino acids from the C-terminus gives N/OFQ(1-13) analogues with different activities and receptor affinities depending on the C-terminal end chemical function. N/OFQ(1-13)-NH_2 becoming the smallest active fragment that restores potency and agonist activity comparable with N/OFQ-NH_2, whereas the free acid, N/OFQ(1-13)-OH, has considerably less affinity and potency [59, 119]. Replacement of Phe^1 by Tyr^1 resulted in Tyr^1-N/OFQ and Tyr^1-N/OFQ(1-13)-NH_2 that binds also to opioid receptors [120, 121]. The N-terminal FGGF is essential for the activity and Phe^4 is important for receptor activation. The replacement of the Phe^1-Gly^2 amide bond with a pseudopeptide (CH_2-NH) bond resulted in the discovery of [$Phe^1\psi(CH_2$-NH)Gly^2]N/OFQ(1-13)-NH_2 ([F/G]N/OFQ(1-13)-NH2 [121]. This ligand was previously purported to be the first NOP receptor antagonist, but later on it became evident that this peptide acts as an antagonist, partial agonist or even as a full agonist, depending on the tissue preparation [122]. Further modifications of the N-terminus by transposition of the Phe^1 side chain from the α-carbon of Phe^1 to the N-terminal amino-nitrogen atom led to the design of the first NOP receptor peptide antagonist [$Nphe^1$]N/OFQ(1-13)-NH_2 [123]. Retro-N/OFQ methylester which has an oppositely directed structure to that of N/OFQ, is a low potency NOP antagonist [124]. [Arg^{14}, Lys^{15}]N/OFQ was designed for interaction with more strength and became the first peptide agonist more potent than N/OFQ and named as a superagonist [125]. [Arg^{14},$Lys(DTPA)^{15}$]nociceptin(1-17)-NH_2 and its cross-linked dimer are synthesized for further radiolabelling to get a radiopharmaceutical that can be used diagnostically **[126]**. Modifications on Phe^4 residue of N/OFQ(1-13)NH_2, revealed that *para*-substituted electron-withdrawing groups such as *p*F and *p*NO_2 increase the binding affinity and potency and *in vivo* duration of action [127-129]. A cyclic peptide having an intramolecular disulphide bridge: c[$Cys^{10,14}$]N/OFQ(1-14)NH_2, was reported to be the first conformationally restricted derivative among the N/OFQ analogues. This peptide has an agonist activity and may serve as a good template for studying the bioactive conformation of N/OFQ [130]. Combination of the cyclic analog with $Nphe^1$ which previously led to pure antagonism, provided a partial agonist c[$Nphe^1$,$Cys^{10,14}$]N/OFQ(1-14)NH_2 [131]. High affinity peptides containing α-helix-promoting

conformational constrains, such as [Aib^{7},Aib^{11}]N/OFQ-NH_2 are also reported [132]. A series of structure-activity studies [133] led to the identification of a very potent antagonist, [Nphe^{1}, Arg^{14}, Lys^{15}]N/OFQ-NH_2 (UFP-101) ref.[134], the most potent agonist [(pF)Phe^{4},Arg^{14},Lys^{15}]N/OFQ-NH_2 (UFP-102) ref.[135] and the most potent partial agonist [Phe$^{1}\psi$(CH_2-NH)-Gly^{2},(pF)Phe^{4},Arg^{14},Arg^{15}]N/OFQ-NH_2 [133]. Recently a detailed SAR study with combination of different modifications has been performed [136] and a very potent agonist [(pF)Phe^{4},Aib^{7},Aib^{11},Arg^{14},Lys^{15}]N/OFQ-NH_2 and an antagonist (the most potent until now) [Nphe^{1},(pF)Phe^{4},Aib^{7},Aib^{11},Arg^{14},Lys^{15}]N/OFQ-NH_2 was reported [137].

8.2. Small peptides

The small peptides in this group are identified by screening of **synthetic peptide combinatorial libraries**. Peptide III-BTD was identified from a combinatorial library of β-turn constrained peptides [138]. This conformationally restricted peptide is a mixed NOP antagonist / opioid agonist [139]. Five hexapeptides (Ac-RYYRIK-NH_2, Ac-RYYRWK-NH_2, Ac-RYYRWR-NH_2, Ac-RYYLWR-NH_2, Ac-RYYKWK-NH_2) with high affinity and selectivity to the NOP receptor were identified from a peptide library containing about 52 millions of compounds made considering all the natural amino acids except cysteine [140]. Similar to [F/G]N/OFQ(1-13)-NH_2, these peptides were partial agonists whose final effects vary from full agonist to antagonist depending on the tissue and system used in the study [140-145]. These hexapeptides, the shortest peptide sequences interacting with the NOP receptor, have been used as chemical templates for SAR studies. The head to tail cyclization of Ac-RYYRWK-NH_2 produced a drastic decrease in binding affinity [146] while the N-terminal acylation with a pentanoyl group [147] or the replacement of the Tyr^{2,3} residues with (pF)Phe [148] led to the discovery of high affinity low efficacy NOP receptor ligands. The N-terminal alkylation of the central core YYRW with groups bearing a guanidine function generated a NOP receptor agonist [149]. Modifications on the Trp (W) were also preformed [150]. The substitution of the C-terminal amide with an alcoholic function produced an antagonist derivative **[151]** which was further shown to behave as a partial agonist **[152]**. Moreover the C-terminal addition of a polylysine sequence generated the peptide Ac-RYYRWKKKKKKK-NH_2 (ZP-120) which behaves similarly to the reference compound as a NOP receptor selective partial agonist but displays a higher affinity, higher metabolic stability and higher *in vivo* duration of action [153]. ZP-120 was patented [154] as a drug candidate (for indications such as aquaresis) especially for its selectivity to produce renal but not cardiovascular effects [155, 156].

8.3. Non-peptide ligands

The non-peptide ligands are generally discovered via **high-throughput screening (HTS)** in pharmaceutical industry laboratories. Since the NOP receptor represents the non-opioid branch

of the opioid family, the search for non-peptide ligands was initiated by examining small-molecule opiate ligands. Among these compounds: i) σ-receptor ligands carbetapentane and rimcazole [157]; ii) MOP receptor ligands lofentanil (an anilidopiperidine) and etorphine (an oripavine derivative) [158]; iii) anilidopiperidines, morphinans and benzomorphan classes of opiate ligands [159]; iv) MOP receptor ligand buprenorphine [160]; v) naloxonebenzoylhydrazone (NalBzoH) [161]; vi) the 5-HT partial agonist spiroxatrine. These compounds have thus far provided useful leads for the design of selective NOP receptor ligands (agonist and antagonist). The non-peptide NOP receptor ligands can be broadly divided into five structural classes. It is noteworthy that many of these ligands were first reported in the patent literature (patents from Pfizer, Banyu Pharm., Hoffmann La Roche, EuroCeltique S.A., NovoNordisk, Schering, Smith Kline Beecham, Japan Tobacco Inc, Toray Industries, etc). However, the biological data of some of them is still not available, thus making it difficult to define clearly the structural requirements for NOP receptor affinity and selectivity.

i) *Morphinan-based ligands*: Among this group TRK-820 was reported in collaboration with Toray Industries, Japan [162].

ii) *Benzimidazopiperidines*: The first non-peptide NOP receptor antagonist was a benzimidazolinone, J-113397 [163] was patented by Banyu Pharmaceutical Co. [164] and followed by several other patents.

iii) *Spiropiperidines*: Hoffmann La Roche patented a series of compounds. Among them Ro 65-6570 and Ro 64-6198 were two of those ligands widely used as pharmacological tools [165]. This was followed by other patents such as NovoNordisk (NNC-63-0532) [166].

iv) *Aryl piperidines*: Designing compounds in this group led several pharmaceutical companies (Schering, Roche etc) to get patents. Recently SB-612111 was patented by GlaxoSmithKline [167].

v) *4-Aminoquinolines*: Japan Tobacco Inc. patented JTC-801 [168]. This compound, reported to be a competitive antagonist [169], was chosen as the clinical candidate for clinical trials for analgesia due to its oral bioavailability profile. Interestingly, the benzimidazolinone J-113397, also a NOP receptor antagonist, has no analgesic effects *per se*, in similar models of nociception.

Recently a preliminary 2-D pharmacophore useful for understanding the structural parameters that play a role in determining the binding, selectivity and the intrinsic activity of small-molecule NOP receptor ligands was reported [170]. This pharmacophore consists of 3 elements i) a heterocyclic moiety that has a hydrogen bond acceptor group. This moiety is an important determinant of binding affinity and selectivity vs. the opioid receptors. ii) a basic-nitrogen-containing moiety. iii) a lipophylic moiety on the basic nitrogen that plays a role in

optimal binding and in intrinsic activity, thus changing the character from agonist to antagonist. For effective NOP receptor agonists and antagonists that can be developed as therapeutics ligand-based analysis has to be complemented with the site-directed mutagenesis of the NOP receptor as well as the 3-D model of the NOP receptor. Some of the available NOP receptor ligands are summarized in Table 3.

Table 3: Some of the NOP receptor ligands available.

	AGONISTS	PARTIAL AGONISTS	ANTAGONISTS
N/OFQ-RELATED PEPTIDE ANALOGUES	1. N/OFQ [2, 3] 2. N/OFQ-NH_2 [59] 3. [Tyr^1]N/OFQ [120] 4. N/OFQ(1-13)-NH_2 [59, 119] 5. [Arg^{14},Lys^{15}]N/OFQ [125] 6. [(pF)Phe^4]N/OFQ(1-13)-NH_2 [127] 7. [(pF)Phe^4,Arg^{14},Lys^{15}]N/OFQ-NH_2 *(UFP-102)* [135] 8. [(pF)Phe^4,Aib^7,Aib^{11},Arg^{14},Lys^{15}] N/OFQ-NH_2 [137]	1. [$Phe^1\psi(CH_2\text{-}NH)$-$Gly^2$]N/OFQ(1-13)-$NH_2$ [121] 2. [$Phe^1\psi(CH_2\text{-}NH)$-$Gly^2$,(pF)$Phe^4$,$Arg^{14}$, Lys^{15}]N/OFQ-NH_2 [133]	1. [$Nphe^1$]N/OFQ(1-13)-NH_2 [123] 2. Retro N/OFQ methylester [124] 3. [$Nphe^1$,Arg^{14},Lys^{15}]N/OFQ-NH_2 *(UFP-101)* [134] 4. [$Nphe^1$,(pF)Phe^4,Aib^7,Aib^{11}, Arg^{14},Lys^{15}]N/OFQ-NH_2 [137]
SMALL ARTIFICIAL PEPTIDES		1. Ac-RYYRWK-NH_2 [140] 2. Ac-RYYRIK-NH_2 [140] 3. Ac-RYYRIK-ol [151] 4. Ac-RYYRWKKK KKKK-NH_2 *(ZP 120)* [153]	1. Peptide-IIIBTD [138] 2. Pentanoyl-RYYRWK-NH_2 [147]
NON-PEPTIDES	1. Ro 65-6570 [165] 2. NNC-63-0532 [166] 3. Ro 64-6198 [165]		1. NalBzoH [161] 2. J-113397 [163] 3. JTC-801 [168] 4. SB-612111 [167]

II. AIM OF THE STUDIES

Nociceptin/orphanin FQ (N/OFQ) and its receptor (NOP) constitute a novel peptide-receptor system, widely expressed centrally and in the periphery. N/OFQ is involved in pain transmission, anxiety and response to stress, learning and memory, food intake, locomotor activity. Additionally, N/OFQ controls some functions of the renal, cardiovascular, respiratory and gastrointestinal systems. Therefore, it is an emerging molecular target for the development of novel therapeutics for several disease states.

Until now, relatively few ligands (agonists, antagonists and partial agonists) have been identified that have high NOP receptor selectivity and potency. Thus, novel ligands are still needed both as research tools for further investigations on the N/OFQ – NOP receptor systems and as drug prototypes to establish the therapeutic potential of drugs, that selectively interact with this receptor. The present study was dedicated to biochemical, functional and pharmacological characterization of NOP receptor ligands in particular novel hexapeptide derivatives. This study includes the basic characterization of a novel synthetic hexapeptide by *in vitro* and *in vivo* analyses, and the structure-activity relationship studies performed on this compound.

The main goals of the study were:

- To identify and characterize putative, selective, potent and stable NOP receptor ligands ***in vitro*** and ***in vivo***.
- To investigate the binding properties to the native receptors of **rat brain membranes** and to **recombinant human NOP receptors**.
- To examine the post-binding effects (G-protein activation) in the functional biochemical **[^{35}S]GTPγS binding assay**.
- To examine the biological effects in the native receptors localized in N/OFQ sensitive tissues that are isolated from **mouse *vas deferens*** and **mouse colon**.
- To characterize the novel hexapeptide *in vivo* by i) **analgesiometric assay** such as tail withdrawal, to see its action on **supraspinal** and **spinal** level; ii) **spontaneous locomotor activity test;** iii) **food intake studies;** iv) **forced swimming test**.

III. MATERIAL AND METHODS

1. Chemicals

1.1. Radiochemicals

[*leucyl*-^{3}H]N/OFQ (160 Ci/mmol) was purchased from Amersham, UK. In some of the preliminary studies [^{3}H]N/OFQ-NH_2 (25 Ci/mmol), [^{3}H]N/OFQ(1-13)-NH_2 (30 Ci/mmol), [^{3}H]Tyr^1-N/OFQ (40 Ci/mmol) were used. These radioligands were radiolabelled by Dr Judit Farkas and Dr Géza Tóth. [^{3}H]Naloxone (28 Ci/mmol) was prepared as described [171]. Guanosine-5'-[γ-^{35}S]-triphosphate (1204 Ci/mmol) was purchased from the Isotope Institute Ltd. (Budapest, Hungary) and from Amersham, UK with a specific activity of 1033 Ci/mmol.

1.2. Peptides

N/OFQ, N/OFQ(1-13)NH_2 were obtained from Bachem, Bubendorf, Switzerland. UFP-101 ([Nphe^1,Arg^{14},Lys^{15}]N/OFQ-NH_2) was kindly provided by Dr Remo Guerrini, Department of Pharmaceutical Sciences and Biotechnology Centre, University of Ferrara, Italy. Dr György Orosz, Reanal Finechemical Co. Budapest, kindly provided Tyr^1-N/OFQ and N/OFQ-NH_2. Endomorphin-1 was purchased from Tocris, U.K. Ac-RYYRIK-NH_2, Ac-RYYRIK-ol and all the other modified hexapeptides were synthesized accordingly to [151] in the laboratory of Dr Anna Magyar (Research Group of Peptide Chemistry, Eötvös Loránd University, Budapest, Hungary) using solid-phase peptide synthesis techniques.

1.3. Other chemicals

$MgCl_2$, KCl, ethylenediamine-tetraacetic acid (EDTA), polyethyleneimine (PEI), Tris-hydroxymethyl-aminomethane, protease-free bovine serum albumin (Protease-free BSA, Fraction V), Guanosine 5'-diphospate (GDP), Guanosine-5'-o-(3-thiotriphosphate) GTPγS, carbachol were purchased from Sigma-Aldrich (St. Louis, MO, USA). All the tissue culture media and supplements were from GIBCO-Invitrogen. Other reagents used in this study were of the highest purity available.

2. Animals

Inbred Wistar rats (250-300g, Animal House of the Biological Research Centre, Szeged, Hungary) were used throughout the *in vitro* receptor binding part of this study. Rats were kept in groups of four, allowed free access to food and water and maintained on a 12/12-h light/dark cycle until the time of sacrifice. Animals were treated according to the European Communities Council Directives (86/609/ECC) and the Hungarian Act for the Protection of Animals in Research (XXVIII.tv. 32.§).

Male Swiss mice weighing 20-25 g were used throughout the *in vitro* isolated tissue assays and *in vivo* part of this study (Morini, Reggio Emilia, Italy). Mice were housed in 425 x 266 x

155-mm cages (Techniplast, Milan, Italy), eight animals/cage, under standard conditions (22°C, 55 % humidity, 12-h light/dark cycle, light on at 7:00 am) with food (MIL, standard diet; Morini, Reggio Emilia, Italy) and water ad libitium for at least 3 days before experiments began. Each mouse was used only once. In addition, each mouse received only one injection (i.c.v. or i.t.). Mice were handled according to guidelines published in the European Communities Council directives (86/609/EEC) and Italian national regulations (D.L. 116/92).

Intracerebroventricular injections (i.c.v., injection volume 2 μl) to the left ventricule was done according to the procedure described by Laursen and Belknap [172] Briefly, a 27-gauge needle attached via a polyethylene tube to a 10-μl Hamilton syringe was used for the injection at an angle approximate angle of 45°, at 2 mm lateral to the bregma midline.

Intrathecal injections (i.t., injection volume 5 μl) were adapted according to the method of Hylden and Wilcox [173]. A 28-gauge stainless steel needle attached to a 50-μl Hamilton microsyringe was inserted in the spinal subarachnoid space between the L5 and L6 segments in mice.

3. Isolated tissues and preparation

In vitro experiments were performed on mouse *vas deferens* (mVD) and mouse colon (mC). On the day of the experiment, the animals were killed by decapitation and the tissues were isolated and prepared immediately. The prostatic portion of mouse *vas deferens* was isolated from male Swiss Mice (20-25 g, Morini, Reggio Emilia, Italy) and prepared according to [174]. The segments, approximately 1 cm length, of mouse colon (Swiss Mice 20-25 g, Morini, Reggio Emilia, Italy) were prepared as described in [144] to record the isometric smooth muscle contraction. Briefly, the mouse segments of transverse and descending colon (1 cm length) were obtained.

A separate series of experiments were performed in colon tissues taken from CD1/C57BL6/J-129 wild type ($NOP^{+/+}$) and NOP receptor knockout ($NOP^{-/-}$) mice weighing 20-25 g. These mice were genotyped by PCR. Details of the generation and breeding of mutant mice have been published previously [62, 175].

4. Membrane Preparations

4.1. Preparation of rat brain membranes

Crude brain membrane from Wistar rats were prepared as previously described **[126, 151]**. Briefly, rats were decapitated and the brains without cerebellum were quickly removed and washed several times with chilled 50 mM Tris-HCl buffer (pH 7.4). Weighed tissues were transferred to ice-cold buffer, homogenized using a Braun teflon-glass homogenizer (10-15 strokes) and filtered through four layers of gauze to remove large aggregates. The homogenate was centrifuged (Sorvall RC5C centrifuge, SS34 rotor) at 40,000 × g for 20 min at

4°C and the resulting pellet was resuspended in fresh buffer and incubated for 30 min at 37°C. The centrifugation step was repeated, and the final pellet were resuspended in 50 mM Tris-HCl buffer (pH 7.4) containing 0.32 M sucrose and stored at -70°C until use.

4.2. Preparation of CHO-NOP_h cells

Chinese hamster ovary (CHO) cells stably expressing the wild type human nociceptin (NOP_h) receptor protein was kindly provided by Dr Jean-Claude Meunier, Toulouse, France. Cells were cultured in a medium containing Nut Mix F-12 (HAM) with l-glutamine (GIBCO-Invitrogen) and 25 mM Hepes, 10 % FCS, 100 UI/ml penicillin, 100 µg/ml streptomycin and 0.4 mg/ml G418 at 37°C in a humidified atmosphere consisting of 5 % CO_2 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 crystallisation 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 4°C 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 4°C. 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 -70°C 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 $MgCl_2$) pH 7.4 buffer. And membrane fractions were aliquoted (~ 240 µg protein per series = ~ 10 µg protein/sample) and stored at -70°C until use.

5. *In vitro* biochemical and functional characterization of hexapeptides

5.1. Radioligand Binding Experiments

5.1.1. Saturation Binding Experiments

Aliquots of frozen rat brain membranes were thawed, washed by centrifugation (40 000 × g, 20 min, 4°C) to remove the sucrose and pellets were suspended in 50 mM Tris-HCl buffer (pH 7.4) up to 0.3-0.4 mg/ml protein. Aliquots of CHO-NOP_h membranes were thawed and homogenised with a syringe and used directly in the binding assay. Membranes were incubated with gentle shaking for 1 h, 24°C in a final volume of 1 ml with increasing concentrations of [*leucyl*-^{3}H]N/OFQ (0.01 - 1 nM) that was prepared in 1 mg/ml protease-free bovine serum albumine solution. Non-specific binding was determined in the presence of 1 µM of unlabelled N/OFQ. The reaction was terminated by rapid filtration under vacuum (Brandel M24R Cell Harvester), and washed three times with 5 ml ice-cold 50 mM Tris-HCl (pH 7.4) buffer through Whatman GF/C glass fibers that are presoaked for 30 min in 0.3% Polyethyleneimine (PEI)

solution (pH 10). After filtration, filter fibers were dried and bound radioactivity was measured in UltimaGold™ scintillation cocktail using Packard Tricarb 2300TR Liquid Scintillation Analyzer. Total binding was measured in the presence of the radioligand, non-specific binding was determined in the presence of 1 μM unlabeled N/OFQ and subtracted from total binding to calculate the specific binding. Protein concentration was measured photometrically by the Bradford method with bovine serum albumine as standard [176].

5.1.2. Competition Binding Experiments

Aliquots of frozen rat brain membranes were thawed washed by centrifugation (40 000 x g, 20 min, 4°C) to remove sucrose and pellets were suspended in 50 mM Tris-HCl buffer (pH 7.4) up to 0.3-0.4 mg/ml protein. Aliquots of CHO-NOP_h membranes were thawed and homogenised with a syringe and used directly in the binding assay. Membranes were incubated with gentle shaking for 1 h, 24°C in a final volume of 1 ml with unlabelled compounds (10^{-5} to 10^{-11} M), and ~ 0.05 nM [*leucyl*-^{3}H]N/OFQ which was prepared in 1 mg/ml protease-free bovine serum albumin solution. Non-specific binding was determined in the presence of 1 μM of unlabelled N/OFQ. The reaction was terminated and bound radioactivity was measured in the same way as described above.

5.1.3. [^{35}S]GTPγS Binding Experiments

Membranes (~ 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}–10^{-5} M) of peptides (for concentration response curves) and various concentrations (10^{-8}, $3x10^{-8}$, 10^{-7}) of Ac-RYYRIK-ol (for Schild plot analyses) tested in the presence of 30 μM GDP in a final volume of 1 ml. Total binding was measured in the absence of the tested 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. Bound radioactivity was detected in UltimaGold™ scintillation cocktail (Packard). [^{35}S]GTPγS binding experiments were performed in triplicates and repeated at least three times.

5.2. Isolated tissue assays

5.2.1. Mouse *vas deferens* bioassay

The mouse *vas deferens* tissues were suspended in 5 ml organ bath containing Mg^{2+} free, and 1.8 mM $CaCl_2$ containing Krebs buffer (in mM: 118.1 NaCl, 4.7 KCl, 2.5 $CaCl_2.2H_2O$, 1.2 KH_2PO_4, 25 $NaHCO_3$, and 5 Glucose) at 33°C and incessantly gassed with 5% CO_2 and

95% O_2. A resting tension of 0.3 g was applied. The mVD tissues were continuously stimulated through two platinum ring electrodes with supramaximal voltage rectangular pulses of 1 ms duration and 0.05 Hz frequency. Electrically evoked contractions (twitches) were measured isotonically with a strain gauge transducer (Basile 7006) and recorded with the PC based acquisition system Autotrace 2.2 (RCS, Florence, Italy). Following an equilibration period of about 60 min, the contractions induced by electrical field stimulation were stable subsequently the cumulative concentration-response curves to N/OFQ were performed (0.5 log unit steps) in the absence or in the presence (30 min preincubation time) of hexapeptides. For Schild plot analyses increasing concentrations of Ac-RYYRIK-ol (10 - 1000 nM) was used.

5.2.2. Mouse colon Assay

Segments of mouse colon 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. The concentration-response relationships were determined non-cumulatively by adding different concentrations of peptides to the bath every 20 min followed by washing.

6. *In vivo* pharmacological characterization of hexapeptides

6.1. Tail Withdrawal Assay

All experiments were started at 10.00 a.m. and performed according to the procedures previously described [177]. Briefly, the mice were placed in a holder and the distal half of the tail was immersed in water at 48°C. Withdrawal latency time was measured by an experienced observer blind to the drug treatment. A cut off time of 20 s was chosen to avoid tissue damage. Four mice were randomly assigned to each experimental group and the experiment was repeated at least 3 times. Tail withdrawal latency was determined immediately before and at 5, 15, 30, and 60 min after i.c.v. or i.t. injection of saline (control), and Ac-RYYRIK-ol (0.01 - 1 nmol).

6.2. Locomotor Activity Assay

Experiments were performed starting at 10.00 am, following the procedures previously described [178]. Briefly, the mice were routinely tested 5 min after i.c.v. injection of Ac-RYYRIK-ol (0.01 - 1 nmol). Locomotor activity was assessed using Basle activity cages, which consist of a four-channel resistance detector circuit which converts the bridges "broken" by the animal paws into pulses that are summed up by an electronic counter every 5 min. Total number of impulses were recorded every 5 min for 60 min. Mice were not accustomed to the cages before drug treatment and the experiment was performed in a quiet and dimly illuminated room. Four mice were randomly assigned to each experimental group and the experiment was repeated at least 3 times.

6.3. Food Intake Assay

The experiments were carried in freely feeding and drinking mice. The experiments took place at 10:00 a.m. Mice were individually housed in a cage and saline or Ac-RYYRIK-ol (0.001 - 0.1 nmol) were injected i.c.v. Food intake was measured at 30 and 60 min following drug injection. Food intake was expressed as g/kg of body weight.

6.4. Forced Swimming Test

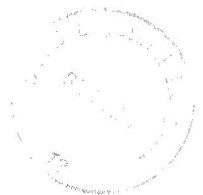
Experiments were performed following the procedures previously described [175]. The test consists in placing mice, individually, in a Plexiglas cylinders (18.5 cm high, 12.5 cm diameter, water 13.5 cm deep) partially filled with water (24-26 °C), for two swimming sessions: an initial 15-min training session, which was followed by a 5-min test session 24 h later. The time each animal remained immobile (immobility time) during the 5-min test session was recorded. Animals were judged immobile when they ceased struggling/swimming and remained floating motionless in the water, making only those movements necessary to keep their heads above the water line. At the end of each swimming session, the animal was removed from the cylinder, dried with paper towels, placed in an individual cage for rest and recovered over 15 min, and then returned to its collective home cage. Ac-RYYRIK-ol (0.001 - 0.1 nmol) was injected i.c.v 5 min before the test. In some experiments, Ac-RYYRIK-ol 0.1 nmol and UFP-101 10 nmol was co-injected i.c.v. 5 min before the test.

7. Data Analysis and Terminology

All data are expressed as means ± standard error of the mean (S.E.M.) of at least 3 experiments. All the binding experiments were performed in duplicates and the [^{35}S]GTPγS binding assays were performed in triplicates. Data was analyzed by GraphPad Prism (version 3.0 and 4.0 San Diego, CA, USA). Hyperbolic saturation binding curves were fitted by non-linear regression using one-site binding model and equilibrium dissociation constant (K_d) and maximal number of binding sites (B_{max}) were calculated. Sigmoid displacement curves were also analysed to yield affinity values expressed as IC_{50} by non-linear regression using the one-site competition fitting option. The equilibrium inhibition constant (K_i) was calculated according to the Cheng-Prusoff equation: $K_i = IC_{50}/(1+[L]/K_d)$ [179] where [L] designates the labelled ligand concentration and IC_{50} is the concentration of the competitor producing 50 % displacement. pK_i is the antilogarithm of the K_i values obtained after the calculations. [^{35}S]GTPγS binding data were analysed by sigmoid dose-response curve fit option of Prism 3.0. Stimulation is given as percent of the specific [^{35}S]GTPγS binding observed in the absence of receptor ligands (basal activity). 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. E_{max} of agonists is expressed as the maximal stimulation of [^{35}S]GTPγS over the basal (for [^{35}S]GTPγS binding), % of inhibition of the control twitch (for the electrically stimulated *vas deferens*), % of the contraction elicited by 100 μM carbachol (for mouse colon data). 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 [180]. pA_2 values were calculated by Schild's linear regression, that correlates the log of concentrations of antagonist (x-axis) to the log of (CR-1) (y-axis), where CR is the ratio between the EC_{50} (nM) values of the agonist in the presence and absence of antagonist. If the slope of the regression line is not significantly different from the unity, the value of x for y = 0 represents the pA_2 value, and the slope not significantly different from the unity refers to competitive antagonism. When one single concentration of antagonist is used, the antagonist potency was indicated as pK_B value and calculated with Gaddum Schild Equation (pA_2 = -log{(CR-1)/[Antagonist]}).

In vivo data have been statistically analyzed with the Student's *t*-test or one way ANOVA followed by the Dunnett test, as specified in figure legends; p values less than 0.05 were considered statistically significant. *In vivo* data from studies in mice were analyzed as follows: for tail withdrawal experiments raw data were converted to the area under the time versus tail withdrawal latency curve (AUC min s^{-1}) as described by [181]. The AUC data for the interval of time (0-60 min) was calculated and these values were used for statistical analysis. Locomotor activity data were statistically analyzed using the data expressed as cumulative impulses over the 60 min observation period.



IV. RESULTS

1. *In vitro* biochemical and functional characterization of hexapeptides

In this part of the study *in vitro* biochemical and functional characterization of newly synthesized series of NOP receptor ligands (summarized in Table 4) are described. These studies include receptor binding, functional [^{35}S]GTPγS binding and isolated tissue assays.

Table 4: Compounds used in this study.

No	Amino Acid Sequence	Structure / Abbreviated Name
1	Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-Leu-Ala-Asn-Gln	**FGGFTGARKSARKLANQ** (N/OFQ)
2	Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-Leu-Ala-Asn-Gln-NH_2	**FGGFTGARKSARKLANQ-NH_2** (N/OFQ-NH_2)
3	Tyr^1-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-Leu-Ala-Asn-Gln	**YGGFTGARKSARKLANQ** (Tyr^1-N/OFQ)
4	Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-NH_2	**FGGFTGARKSARK-NH_2** (N/OFQ(1-13)NH_2)
5	[$Nphe^1$,Arg^{14},Lys^{15}]N/OFQ-NH_2	**FGGFTGARKSARKRKNQ-NH_2** (UFP-101)
6	Acetyl-Arg-Tyr-Tyr-Arg-Ileu-Lys-NH_2	**Ac-RYYRIK-NH_2** "parent compound"
7	Acetyl -Arg-Tyr-Tyr-Arg-Ileu-Lys-ol	**Ac-RYYRIK-ol**
8	Acetyl -Cit-Tyr-Tyr-Cit-Ileu-Lys-NH_2	**Ac-Cit-YY-Cit-IK-NH_2**
9	Acetyl -Cit-Tyr-Tyr-Arg-Ileu-Lys-NH_2	**Ac-Cit-YYRIK-NH_2**
10	Acetyl -Arg-Tyr-Tyr-Cit-Ileu-Lys-NH_2	**Ac-RYY-Cit-IK-NH_2**
11	Acetyl -Cit-Tyr-Tyr-Cit-Ileu-Lys-ol	**Ac-Cit-YY-Cit-IK-ol**
12	Acetyl -Cit-Tyr-Tyr-Arg-Ileu-Lys-ol	**Ac-Cit-YYRIK-ol**
13	Acetyl -Arg-Tyr-Tyr-Cit-Ileu-Lys-ol	**Ac-RYY-Cit-IK-ol**
14	Chloro-acetyl -Arg-Tyr-Tyr-Arg-Ileu-Lys-ol	**ClAc-RYYRIK-ol**
15	Benzoyl-Arg-Tyr-Tyr-Arg-Ileu-Lys-ol	**Bz-RYYRIK-ol**
16	Formyl-Arg-Tyr-Tyr-Arg-Ileu-Lys-ol	**For-RYYRIK-ol**
17	Pivaloyl-Arg-Tyr-Tyr-Arg-Ileu-Lys-ol	**Piv-RYYRIK-ol**
18	H-Arg-Tyr-Tyr-Arg-Ileu-Lys-ol	**H-RYYRIK-ol**
19	Mesyl-Arg-Tyr-Tyr-Arg-Ileu-Lys-ol	**Ms-RYYRIK-ol**

1.1. Radioligand Binding Experiments

1.1.1. Saturation Binding Experiments

Saturation binding experiments measure specific binding at equilibrium at various concentrations (often 6-12) of the radioligand to determine receptor number and affinity. Specific binding is plotted against the concentration of the radioligand. The linear transformation of the saturation curve "Scatchard plot" [182] was used to calculate the B_{max} (maximal number of binding sites) and K_d (equilibrium dissociation constant) values. The identification of the dissociation constant (K_d) of [*leucyl*-^{3}H]N/OFQ is crucial for further characterization of the hexapeptides (*i.e.*, to determine the inhibitory constant, K_i values of the hexapeptides).

In the equilibrium saturation experiments, various concentrations (0.01-1 nM) of the radioligand were incubated with the membrane. Binding of [*leucyl*-^{3}H]N/OFQ to rat brain membranes (Fig. 5 left panel) and CHO-NOP$_h$ (Fig. 5 right panel) at 25°C was saturable and of high affinity. The equilibrium dissociation constant (K_d) and the maximal number of binding sites (B_{max}) were calculated by nonlinear regression analysis of the saturation binding data.

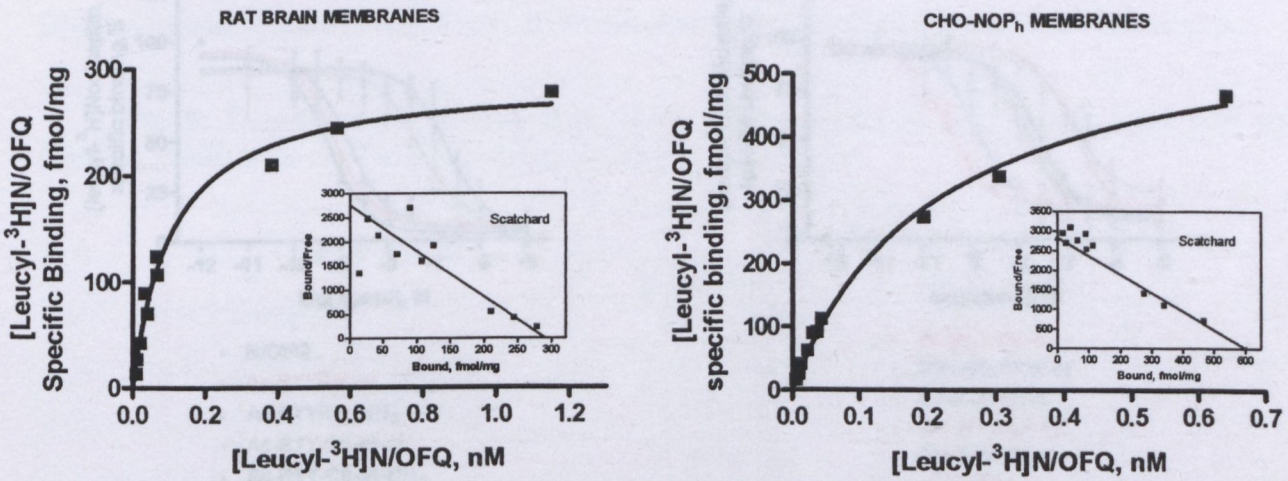


Fig. 5: Saturation binding curves with [*leucyl*-^{3}H]N/OFQ and inserted scathard plots. Left panel: [*leucyl*-^{3}H]N/OFQ binding on native NOP receptors of rat brain membrain. Right panel: [*leucyl*-^{3}H]N/OFQ binding on CHO-NOP$_h$.. Data is representative of at least 3 experiments.

The results showed the existence of a single binding component with K_d values of 0.22 ± 0.04 nM and 0.105 ± 0.01 nM and the maximal binding capacity (B_{max}) values were 604.75 ± 17.05 fmol/mg and 290.4 ± 12.03 fmol/mg protein for CHO-NOP$_h$ and rat brain membranes, respectively. The B_{max} value of CHO-NOP$_h$ is twice as much as that of rat brain, which is a significant difference ($p<0.05$, unpaired t-test). However the K_d values were not significantly different from each other ($p>0.05$, unpaired t-test). Non-specific binding of [*leucyl*-^{3}H]N/OFQ to the membrane preparations was less than 10 % of total binding at a

radioligand concentration close to the K_d values. These results are in good agreement with the literature data [183]. Until now, there is a disparity among the reported K_d and the B_{max} values calculated not only from saturation binding experiments but also from the homologous displacement binding experiments. Furthermore no complete correlation can be established between affinities observed and radioligand, or receptor source used [183].

1.1.2. Competition Binding Experiments

Competitive or displacement binding experiments measure the binding of a single concentration of labelled ligand in the presence of various concentrations of unlabelled ligand to determine the equilibrium inhibitory constant K_i, affinity of the drug to the receptor. Competition binding experiments were used to determine the ability of the new peptides competing for the NOP receptor binding sites.

Receptor binding experiments were performed with [*leucyl*-^{3}H]N/OFQ on membranes from rat brains (Fig. 6 left panel) and cells expressing human NOP receptor (Fig. 6 right panel). In this figure the hexapeptides are shown in two panels to avoid the crowd on the graphic.

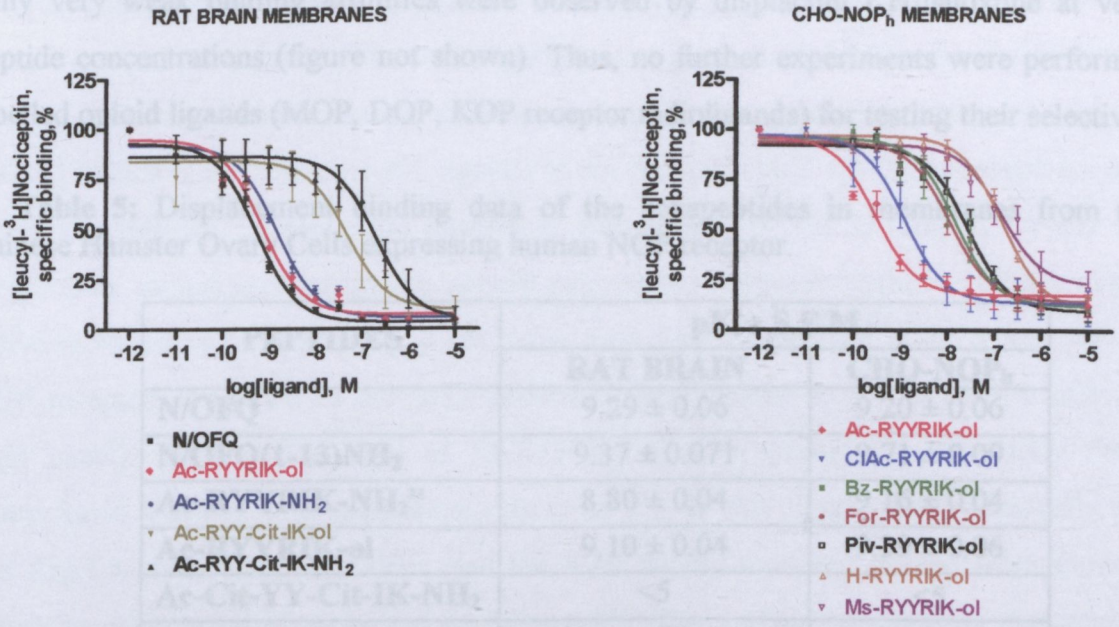


Fig. 6: Competition binding curves with [*leucyl*-^{3}H]N/OFQ. Left panel: displacement curves for C-terminally modified and Arg/Cit replaced ligands on native NOP receptors of rat brain membrain. Right panel: displacement curves for N-terminally modified ligands on CHO-NOP_h. Hexapeptides are shown in rank order of affinity. Points indicate means and vertical lines the S.E.M. of at least 3 experiments.

All the compounds were effectively competed [*leucyl*-^{3}H]N/OFQ in a concentration dependent manner. Heterologous competition curves for each compound were satisfactorily fitted according to the single-site binding model. The pK_i values are shown in Table 5. Among the hexapeptides Ac-RYYRIK-ol, the hexapeptide alcohol, showed the highest affinity towards the NOP receptor (pK_i = 9.10 and 9.39 M, rat brain and CHO-NOP_h, respectively).

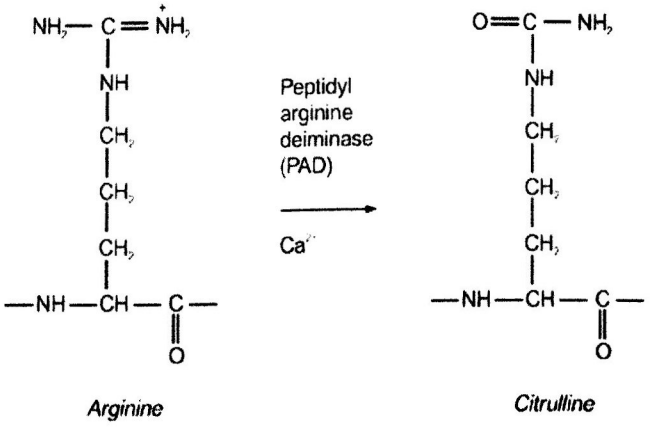


Fig. 7: Structure of arginine and citrulline.

Replacement of the arginine residue with citrulline (an uncharged α-amino acid structurally similar to arginine shown in Fig. 7) at 1st and/or 4th position indicated that the positively charged arginine residue at 1st position is crucial for the hexapeptide structure. When the positively charged arginine residue was replaced with citrulline the binding affinity was lost regardless of the C-terminal modification. However, the lower but still acceptable binding affinities show that the citrulline replacement at the 4th position was more tolerable. The N-terminal modifications led to compounds having lower affinities to the NOP receptor. All hexapeptides were also tested for competing against [^{3}H]naloxone, to estimate their affinity towards opioid binding sites, if any. Only very weak binding affinities were observed by displacing [^{3}H]naloxone at very high peptide concentrations (figure not shown). Thus, no further experiments were performed with labelled opioid ligands (MOP, DOP, KOP receptor radioligands) for testing their selectivity.

Table 5: Displacement binding data of the hexapeptides in membranes from rats and Chinese Hamster Ovary Cells expressing human NOP receptor.

PEPTIDES	$pK_i \pm$ S.E.M	
	RAT BRAIN	CHO-NOP_h
N/OFQ	9.29 ± 0.06	9.20 ± 0.06
N/OFQ(1-13)NH_2	9.37 ± 0.071	9.71 ± 0.09
Ac-RYYRIK-NH_2"	8.80 ± 0.04	9.16 ± 0.04
Ac-RYYRIK-ol	9.10 ± 0.04	9.39 ± 0.06
Ac-Cit-YY-Cit-IK-NH_2	<5	<5
Ac-Cit-YYRIK-NH_2	<5	<5
Ac-RYY-Cit-IK-NH_2	6.63 ± 0.2	6.32 ± 0.12
Ac-Cit-YY-Cit-IK-ol	<5	<5
Ac-Cit-YYRIK-ol	<5	<5
Ac-RYY-Cit-IK-ol	7.30 ± 0.13	7.39 ± 0.15
ClAc-RYYRIK-ol	8.35 ± 0.29	8.86 ± 0.08
Bz-RYYRIK-ol	7.43 ± 0.26	7.78 ± 0.23
For-RYYRIK-ol	8.00 ± 0.11	7.93 ± 0.17
Piv-RYYRIK-ol	7.63 ± 0.31	7.52 ± 0.12
H-RYYRIK-ol	6.39 ± 0.06	6.81 ± 0.05
Ms-RYYRIK-ol	6.78 ± 0.13	6.80 ± 0.18

1.1.3. [^{35}S]GTPγS Binding Experiments

This test is a widely used functional biochemical assay for determining the pharmacological property of ligands *in vitro*. GPCRs couple to $G\alpha_{i/o}$, $G\alpha_s$, $G\alpha_q$, however, the G_i coupling is the most abundant. In the inactive state of the receptor the membrane bound G-protein exists as $G_\alpha(GDP)_{\beta\gamma}$. Activation of receptor by an agonist leads to the dissociation of GDP from G_α, allowing endogenous GTP to bind to G_α. Consequently, G_α-GTP and $G_{\beta\gamma}$ subunits are dissociated and are able to interact with the effector systems. The G-protein heterotrimer is reformed by the GTPase activity of the G_α subunit, forming G_α-GDP and allowing G_α and $G_{\beta\gamma}$ to re-combine. Using the [^{35}S]GTPγS binding assay is mostly suitable for receptors coupled to the abundant $G\alpha_{i/o}$ proteins and measures the functional consequence of receptor occupancy by agonists at one of the earliest receptor-mediated events. This process can be easily monitored by using a non-hydrolysable analogue of GTP that contains a γ-thiophosphate bond ([^{35}S]GTPγS). Since heterotrimer formation will be delayed, the [^{35}S]GTPγS will accumulate in the membrane and thus can be measured by counting the amount of [^{35}S]-label incorporated [184].

The pharmacological parameters like potency (EC_{50}) and efficacy (E_{max}) as well as the relative intrinsic activities of the hexapeptides were determined on membranes from rat brains and CHO cells expressing human NOP receptor. These results were compared with those of N/OFQ, which is the reference full agonist at the NOP receptor. All the hexapeptides displayed weak to moderate concentration dependent stimulations of the G-proteins on the rat brain membranes, indicating their partial agonist properties. The weak intrinsic efficacy observed in native receptors of rat brain (Fig. 8 left panel) was clearly amplified at the recombinant human NOP receptors therefore all the hexapeptides exerted agonist to partial agonist effects (Fig.8 right panel). Experimental data of N/OFQ and the hexapeptides were clearly fitted with monophasic sigmoid dose-response curves (Fig. 8). In this figure, the C-terminally modified and Arg/Cit replaced hexapeptides, and the N-terminally modified hexapeptides shown in two panels in order to avoid the crowd on the graphic. The maximal stimulation percentage values (E_{max}) and the potency (pEC_{50}) of the ligands are summarized in Table 6.

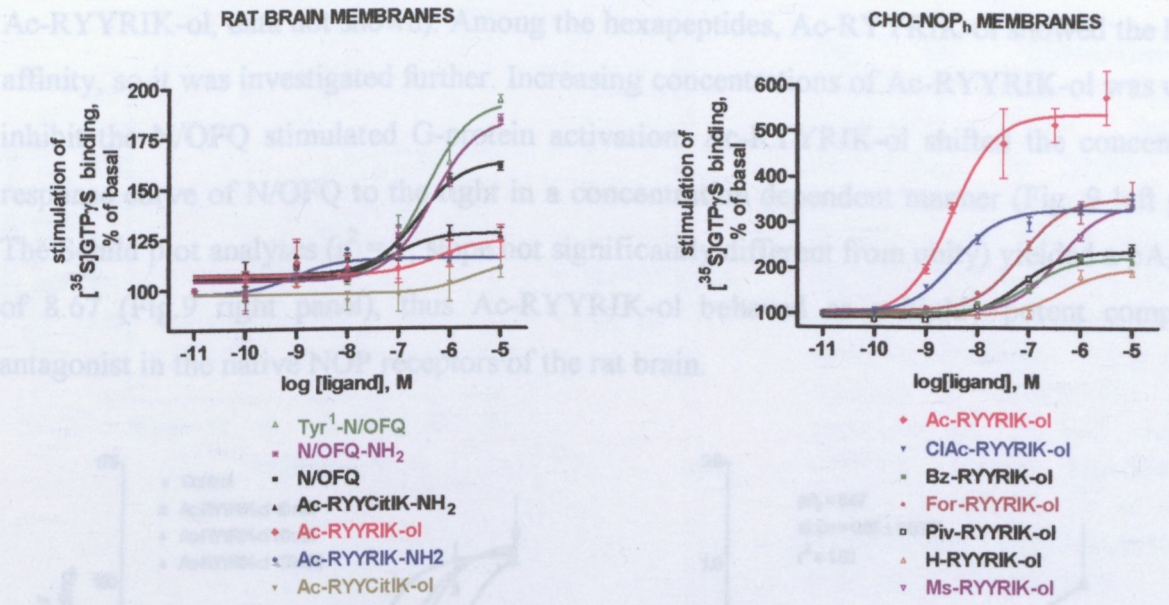


Fig. 8: [^{35}S]GTPγS binding experiments. Left panel: stimulation of G-protein activation by N/OFQ analogs, C-terminally modified and Arg/Cit replaced ligands on native NOP receptors of rat brain membrain. Right panel: stimulation of G-protein activation by N-terminally modified ligands on CHO-NOP$_h$. Points indicate means and vertical lines the S.E.M. of at least 3 experiments performed in triplicates.

Table 6: [^{35}S]GTPγS binding data, the stimulation of G-proteins by the hexapeptides in native NOP receptors of rat brain and recombinant human NOP receptor. The pEC_{50} values are not determined due to the shallow concentration-response curves (nd referes to not determined).

PEPTIDES	RAT BRAIN		CHO-NOP$_h$ CELL	
	E_{max} (Stim. %)	pEC_{50} ± S.E.M	E_{max} (Stim. %)	pEC_{50} ± S.E.M
N/OFQ	162.9 ± 1.81	6.60 ± 0.14	669.7 ± 66.03	8.76 ± 0.09
Ac-RYYRIK-NH$_2$	115.5 ± 1.7	nd	517.3 ± 97.21	8.36 ± 0.18
Ac-RYYRIK-ol	122.7 ± 4.66	nd	540.9 ± 82.70	8.52 ± 0.05
Ac-RYY-Cit-IK-NH$_2$	127.7 ± 3.30	nd	427.1 ± 40.01	6.81 ± 0.04
Ac-RYY-Cit-IK-ol	107.0 ± 9.04	nd	331.5 ± 3.41	6.86 ± 0.23
ClAc-RYYRIK-ol	112.4 ± 4.56	nd	330.9 ± 25.43	8.43 ± 0.04
Bz-RYYRIK-ol	100.8 ± 4.79	nd	223.5 ± 5.252	7.06 ± 0.22
For-RYYRIK-ol	129.2 ± 5.63	nd	356.1 ± 48.48	7.20 ± 0.14
Piv-RYYRIK-ol	108.9 ± 2.56	nd	237.9 ± 20.71	7.19 ± 0.07
H-RYYRIK-ol	101.7 ± 0.38	nd	201.2 ± 16.06	6.40 ± 0.14
Ms-RYYRIK-ol	112.2 ± 1.44	nd	325.3 ± 25.32	6.55 ± 0.18

Due to their weak intrinsic activities seen in the native NOP receptors of the rat brain, these hexapeptides were tested to antagonise the N/OFQ stimulated G-protein activation. At 1 μM effective concentration the hexapeptides could inhibit the N/OFQ stimulated G-protein activation in rat brain membranes with low antagonist potencies (pA_2< 7.0, except for

Ac-RYYRIK-ol, data not shown). Among the hexapeptides, Ac-RYYRIK-ol showed the highest affinity, so it was investigated further. Increasing concentrations of Ac-RYYRIK-ol was used to inhibit the N/OFQ stimulated G-protein activation. Ac-RYYRIK-ol shifted the concentration response curve of N/OFQ to the right in a concentration dependent manner (Fig. 9 left panel). The Schild plot analyses ($r^2 = 1$, slope not significantly different from unity) yielded a pA_2 value of 8.67 (Fig.9 right panel), thus Ac-RYYRIK-ol behaved as a highly potent competitive antagonist in the native NOP receptors of the rat brain.

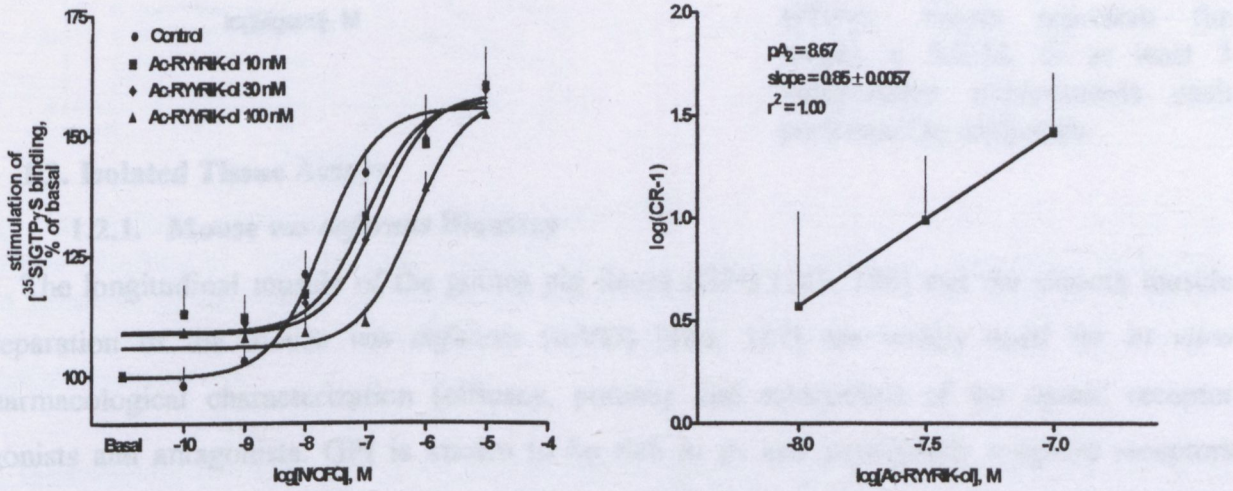


Fig. 9: Stimulation of [^{35}S]GTPγS binding on native NOP receptors of rat brain membranes. Left panel: Concentration response curve of N/OFQ obtained in the absence (control) and presence of increasing concentrations of Ac-RYYRIK-ol (10-100 nM). Right panel: corresponding Schild plot. Points indicate the means and vertical lines the S.E.M of at least 3 separate experiments that were performed in triplicates. CR: the ratio between the EC_{50} (nM) values of the agonist in the presence and absence of antagonist.

However, when these ligands were tested to stimulate the G-protein activation on CHO-NOP_h cells, they all turned out to be agonists or more evidently partial agonists. The indistinguishable intrinsic activities of some of the hexapeptides were amplified by using CHO cells expressing human NOP receptors with a binding capacity of 604.75 fmol/mg (twice as much as that of native receptors of rat brain), and these ligands showed pharmacological properties changing from full agonist to partial agonists (figure not shown, see Table 6 for pEC_{50} and E_{max} values). Not surprisingly, the purported antagonist Ac-RYYRIK-ol behaved almost as the full agonist N/OFQ with a similar potency and efficacy. The well known NOP receptor antagonist, the peptide UFP-101 ref. [134] showed almost no intrinsic agonist activity when used in these cells to stimulate G-protein activation. (Fig. 10).

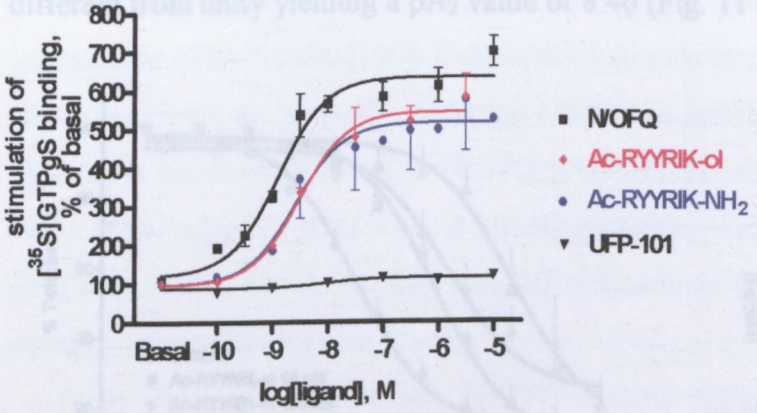


Fig. 10: Stimulation of [^{35}S]GTPγS binding on CHO cells expressing NOP_h receptors. Stimulation is given as a percentage of the non-stimulated (basal) level. Basal activity is taken as 100 % and calculated as: Basal = 'Total binding' – 'Non-specific binding'. Non-specific binding is determined in the presence of 10 μM unlabeled GTPγS. Points represent the means ± S.E.M. of at least 3 independent expreriments each performed in triplicates.

1.2. Isolated Tissue Assays

1.2.1. Mouse *vas deferens* Bioassay

The longitudinal muscle of the guinea pig ileum (GPI) [185, 186] and the smooth muscle preparation of the mouse *vas deferens* (mVD) [186, 187] are widely used for *in vitro* pharmacological characterization (efficacy, potency and selectivity) of the opioid receptor agonists and antagonists. GPI is known to be rich in μ- and particularly κ-opioid receptors (MOP and KOP), while mVD contains all three opioid receptors, however it is mostly sensitive to δ-opioid receptor (DOP) [188]. N/OFQ was shown to inhibit the electrically stimulated contractions both in mVD [174, 188, 189] and in GPI [174] [188]. In these preparations, the twitch response to the electric field stimulation due to nerve activation and subsequent release of neurotransmitter occurs (that is blocked by tetrodotoxin, TTX). The contractions of mouse and rat *vas deferens* derive largely from the release of noradrenaline from the sympathetic nerves, since they are blocked by α_1-adrenergic receptor antagonist prazosin. In the guinea pig ileum, atropine and tetrodotoxin sensitive contractions derive from the release of acetylcholine from cholinergic terminals of the myenteric plexus.

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 11 left panel). In this preparation, Ac-RYYRIK-ol was completely inactive at 10 and 100 nM while at 1 μM it produced a slight inhibition of the electrically induced twitch in 5 out of 8 tissues. Ac-RYYRIK-ol tested over the concentration range of 10 – 1000 nM shifted the concentration response curve of N/OFQ to the right in a concentration dependent manner. Curves obtained in the presence of Ac-RYYRIK-ol were parallel to the control and reached similar maximal effects even in the presence of the highest concentration of compound (Fig. 11

left panel). The corresponding Schild plot was linear (r^2=0.99) with a slope not significantly different from unity yielding a pA_2 value of 8.46 (Fig. 11 right panel).

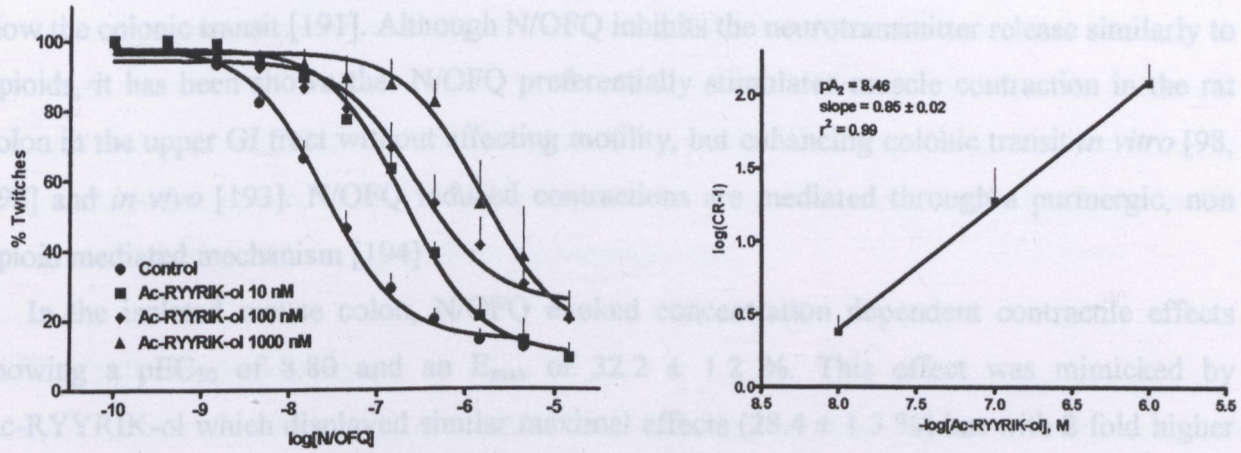


Fig. 11: Effects of N/OFQ and Ac-RYYRIK-ol in the electrically stimulated mouse *vas deferens*. Left panel: concentration response curves to N/OFQ obtained in the absence (control) and presence of increasing concentrations of Ac-RYYRIK-ol (10 – 1000 nM). Right panel: Corresponding Schild plot. Points indicate the means and vertical lines the S.E.M. of at least 4 separate experiments.

N-terminally modified and Arg-Cit replaced hexapeptides were also tested in mVD. In an attempt to determine the agonistic activity, the structurally modified hexapeptides were tested alone for their ability to inhibit the twitch response to the electric stimulation. A slight inhibition (<50%) was recorded only at high concentrations (*i.e.*, 1 μM). Thus, the hexapeptides tested were indicated as 'variable agonist effect' in the Table 7. On the other hand, these compounds could antagonize the effect of N/OFQ on the mVD at 1 μM concentration or even at 100 nM in the case of ClAc-RYYRIK-ol. Table 7 shows the antagonist potencies (pK_B) of the modified hexapeptides.

Table 7: Effects of the hexapeptides on the electrically stimulated mouse *vas deferens*.

PEPTIDES	Agonist		Antagonist
	pEC_{50} ± S.E.M	E_{max} (Inh. %)	pK_B ± S.E.M
N/OFQ	7.6 ± 0.05	90 ± 5 %	/
Ac-RYY-Cit-IK-NH_2	variable agonist effect		6.34 ± 0.21
Ac-RYY-Cit-IK-ol	variable agonist effect		6.56 ± 0.14
ClAc-RYYRIK-ol	variable agonist effect		8.22 ± 0.11
Bz-RYYRIK-ol	variable agonist effect		6.05 ± 0.3
Piv-RYYRIK-ol	variable agonist effect		7.02 ± 0.26
H-RYYRIK-ol	variable agonist effect		6.05 ± 0.25

1.2.2. Mouse colon Bioassay

Principally MOP and KOP receptors are highly expressed in the myenteric plexus and nerve terminals in colon tissues [190]. Despite their stimulatory action on colonic contractions, opioids slow the colonic transit [191]. Although N/OFQ inhibits the neurotransmitter release similarly to opioids, it has been shown that N/OFQ preferentially stimulates muscle contraction in the rat colon in the upper GI tract without affecting motility, but enhancing colonic transit *in vitro* [98, 192] and *in vivo* [193]. N/OFQ induced contractions are mediated through a purinergic, non opioid mediated mechanism [194].

In the isolated mouse colon, N/OFQ evoked concentration dependent contractile effects showing a pEC_{50} of 8.80 and an E_{max} of 32.2 ± 1.2 %. This effect was mimicked by Ac-RYYRIK-ol which displayed similar maximal effects (28.4 ± 1.3 %) but with 2 fold higher potency (pEC_{50} 9.09) (Fig. 12 left panel). The effects of Ac-RYYRIK-ol were also investigated in colon tissues taken from $NOP^{+/+}$ and $NOP^{-/-}$ mice. 100 nM of N/OFQ and Ac-RYYRIK-ol produced a contraction of colon tissues of $NOP^{+/+}$ mice amounting to 37± 7% and 32 ± 5% of contraction induced by 100 µM of carbachol, respectively. These two peptides were found completely inactive when tested in tissues taken from $NOP^{-/-}$ mice (Fig. 12 right panel). On the contrary, the selective MOP receptor agonist endomorphin-1 (tested at 1 µM) produced similar contractile effects in tissues from $NOP^{+/+}$ and $NOP^{-/-}$ animals (Fig. 12, right panel). Thus, these results show the NOP receptor selectivity of the action of N/OFQ and Ac-RYYRIK-ol in the mouse colon.

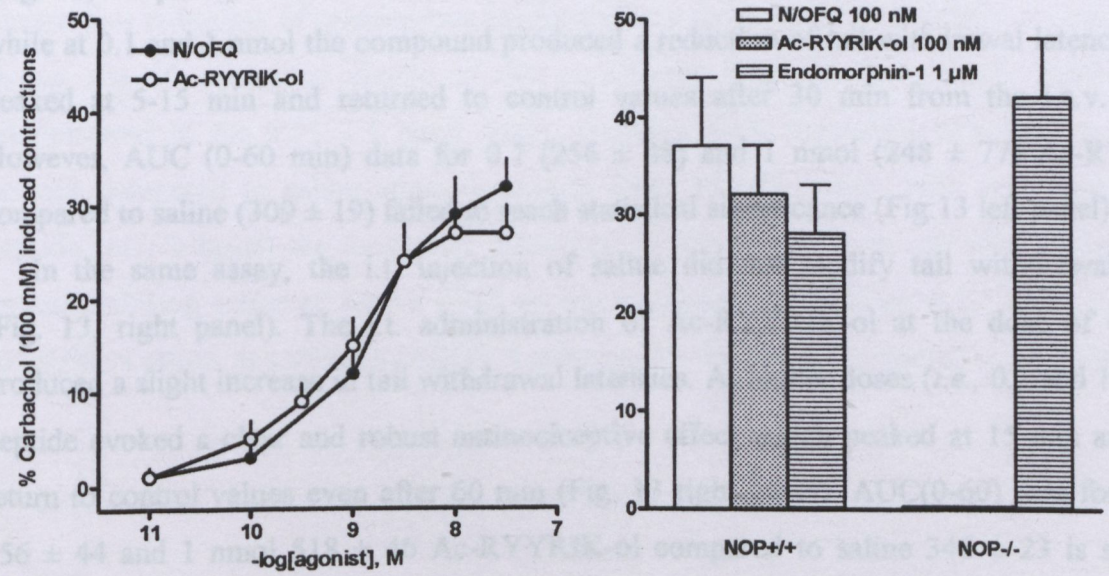


Fig. 12: Effects of N/OFQ and Ac-RYYRIK-ol in the isolated mouse colon. Left panel: concentration response curve to N/OFQ and Ac-RYYRIK-ol in tissues taken from male Swiss mice. Right panel: effect of single concentrations of N/OFQ, Ac-RYYRIK-ol, and EM-1 on mouse colon tissues taken from $NOP^{+/+}$ and $NOP^{-/-}$ mice. Points indicate the means and vertical lines the S.E.M. of at least 3 separate experiments.

2. *In vivo* pharmacological characterization of hexapeptide alcohol

The hexapeptide alcohol, Ac-RYYRIK-ol, appeared to be a potent and high affinity NOP receptor ligand. This interesting compound was aimed to be characterized further, particularly in the *in vivo* assays: i) to estimate if the novel compound is efficient *in vivo*, and if so, ii) to determine what kind of pharmacological behaviour the novel compound has, iii) if the behaviour changes due to the different ways of administration, or if it differs in peripheral/central level. In this part briefly the pharmacological assay results are summarized and compared with the results obtained by N/OFQ and analogues from the literature data.

2.1. Tail withdrawal assay

I.c.v. injection of 0.01 nmol of Ac-RYYRIK-ol in mice did not induce any effect on gross behaviour. In contrast, mice treated with 0.1 and particularly with 1 nmol Ac-RYYRIK-ol displayed a decrease in locomotor activity, ataxia and loss of the righting reflex, similarly to what was observed and reported by various groups after the i.c.v. injection of high doses (> 1 nmol) of N/OFQ [2, 177, 178]. However, whilst the effects of N/OFQ on gross behaviour appeared immediately after i.c.v. injection, those produced by Ac-RYYRIK-ol were slower to develop becoming evident only after 15-20 min. No effects were observed in the gross behaviour of the mice after i.t. injection of Ac-RYYRIK-ol.

In the tail withdrawal assay, the i.c.v. injection of saline did not modify tail withdrawal latencies, which remained stable around 5 s for the time course of the experiments (Fig. 13, left panel). Ac-RYYRIK-ol at the dose of 0.01 nmol failed to alter the animal response while at 0.1 and 1 nmol the compound produced a reduction of tail withdrawal latencies, which peaked at 5-15 min and returned to control values after 30 min from the i.c.v. injection. However, AUC (0-60 min) data for 0.1 (256 ± 48) and 1 nmol (248 ± 77) Ac-RYYRIK-ol compared to saline (309 ± 19) failed to reach statistical significance (Fig.13 left panel).

In the same assay, the i.t. injection of saline did not modify tail withdrawal latencies (Fig. 13, right panel). The i.t. administration of Ac-RYYRIK-ol at the dose of 0.01 nmol produced a slight increase in tail withdrawal latencies. At higher doses (*i.e.*, 0.1 and 1 nmol) the peptide evoked a clear and robust antinociceptive effect which peaked at 15 min and did not return to control values even after 60 min (Fig. 13 right panel). AUC(0-60) data for 0.1 nmol 556 ± 44 and 1 nmol 518 ± 46 Ac-RYYRIK-ol compared to saline 346 ± 23 is statistically significantly $p<0.05$ according to ANOVA followed by the Dunnett's test.

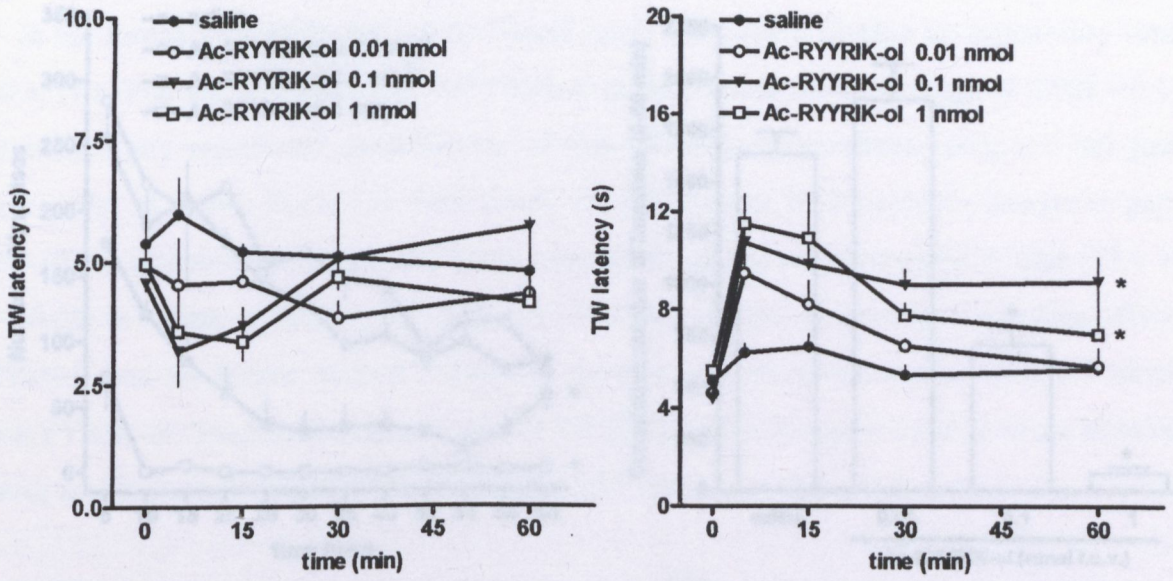


Fig. 13: Effects of Ac-RYYRIK-ol in the mouse tail withdrawal assay. Left panel: dose response curve to Ac-RYYRIK-ol (0.01 - 1 nmol) injected i.c.v. (AUC(0-60): saline 309 ± 19; 0.01 nmol 289 ± 25; 0.1 nmol 256 ± 48; 1 nmol 248 ± 77). Right panel: dose response curve to Ac-RYYRIK-ol (0.01 - 1 nmol) injected i.t. (AUC(0-60): saline 346 ± 23; 0.01 nmol 419 ± 13; 0.1 nmol 556 ± 44*; 1 nmol 518 ± 46*, *$p<0.05$ according to ANOVA followed by the Dunnett's test). Points indicate the means and vertical lines the S.E.M. of 4 experiments.

2.2. Locomotor activity assay

In the locomotor activity assay, mice injected with saline (2 µl/mouse, i.c.v.) displayed a progressive reduction of spontaneous locomotor activity from 255 ± 6 to 84 ± 7 impulses/5 min during the 60 min time course of the experiment (Fig. 14, left panel). Ac-RYYRIK-ol administered in the 0.01 - 1 nmol range, caused a dose dependent reduction of locomotor activity compared to saline injected animals (Fig. 14 left panel). With the highest dose of peptide (*i.e.*, 1 nmol) the locomotor behaviour of the animals was virtually suppressed. The motor inhibiting action of Ac-RYYRIK-ol was statistically significant at 0.1 and 1 nmol doses. ($p< 0.05$ vs saline, according to ANOVA followed by the Dunnett's test) (Fig. 14 right panel).

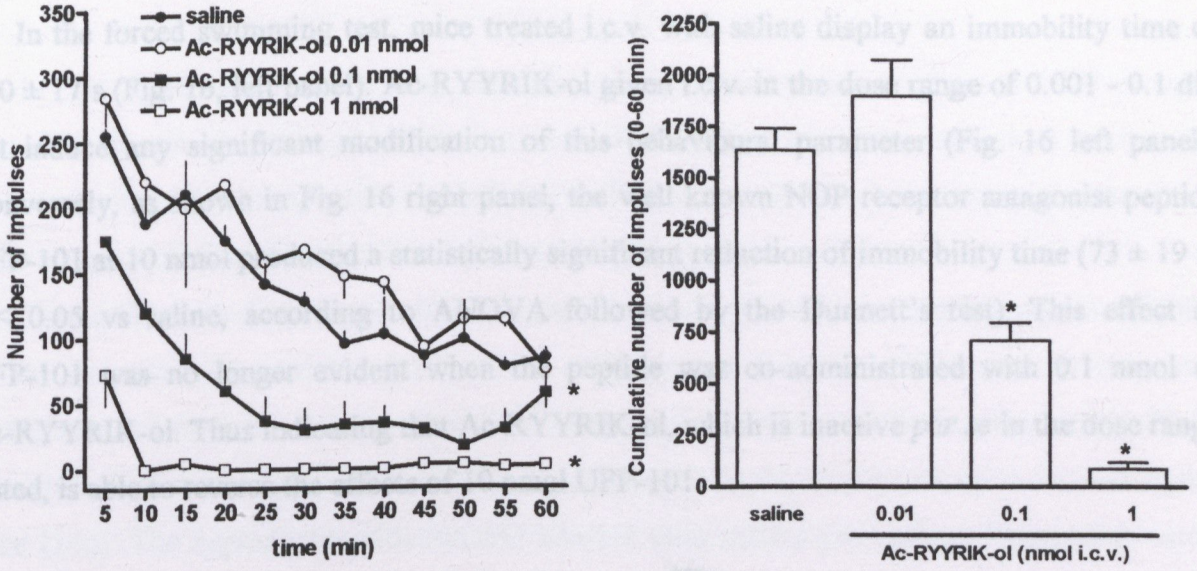


Fig. 14: Effects of Ac-RYYRIK-ol in the locomotor activity in mice. Left panel: dose response curve to Ac-RYYRIK-ol (0.01 - 1 nmol) injected i.c.v. Points indicate the means and vertical lines the S.E.M. of 4 separate experiments. Right panel: Data expressed as cumulative impulses over 60 min were used for statistical analysis. *p< 0.05 vs saline, according to ANOVA followed by the Dunnett's test.

2.3. Food intake assay

In food intake studies, the i.c.v. injection of 0.001 nmol of Ac-RYYRIK-ol did not modify food intake of sated mice (Fig. 15). On the contrary, when the peptide was administrated at 0.01 and 0.1 nmol it produced a statistically significant stimulation of food intake (p< 0.05 vs saline, according to ANOVA followed by the Dunnett's test.) Interestingly, in this assay Ac-RYYRIK-ol behaved as a very potent stimulant of food intake at 0.01 nmol (Fig. 15).

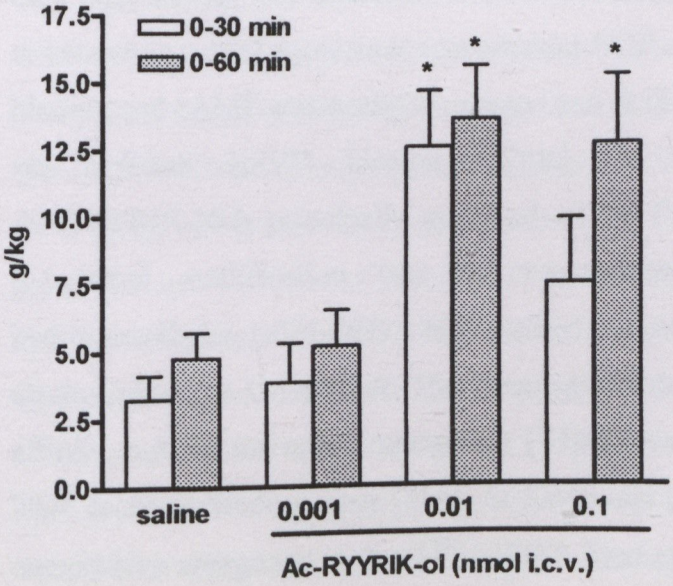


Fig. 15: Cumulative 30 and 60 min food intake following i.c.v. injection of saline or Ac-RYYRIK-ol (0.001, 0.01 and 0.1 nmol). Values are means ± S.E.M. of 12 - 15 animals. Ordinate: grams / kg body weight. *p < 0.05 vs saline according to ANOVA followed by the Dunnett' s test.

2.4. Forced swimming test

In the forced swimming test, mice treated i.c.v. with saline display an immobility time of 190 ± 17 s (Fig. 16, left panel). Ac-RYYRIK-ol given i.c.v. in the dose range of 0.001 - 0.1 did not induce any significant modification of this behavioural parameter (Fig. 16 left panel). Conversely, as shown in Fig. 16 right panel, the well known NOP receptor antagonist peptide UFP-101 at 10 nmol produced a statistically significant reduction of immobility time (73 ± 19 s) ($p < 0.05$ vs saline, according to ANOVA followed by the Dunnett's test). This effect of UFP-101 was no longer evident when the peptide was co-administrated with 0.1 nmol of Ac-RYYRIK-ol. Thus indicating that Ac-RYYRIK-ol, which is inactive *per se* in the dose range tested, is able to reverse the effects of 10 nmol UFP-101.

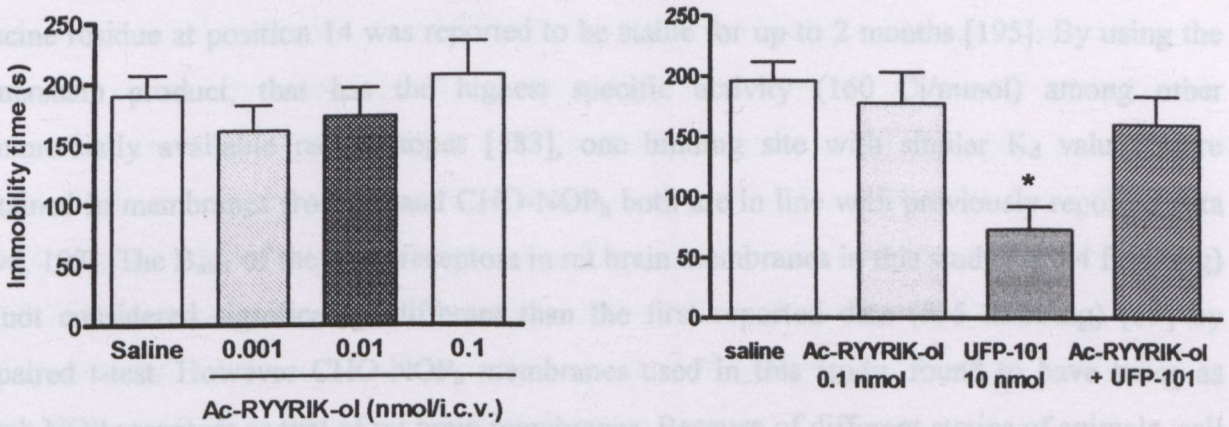


Fig. 16: Effects of Ac-RYYRIK-ol in the mouse forced swimming test. Left panel: dose response curve to Ac-RYYRIK-ol (0.001 – 0.1 nmol) injected i.c.v. Right panel: effects of 0.1 nmol Ac-RYYRIK-ol and 10 nmol UFP-101 injected alone and co-injected on immobility time of Swiss mice submitted to the test. Values are means ± S.E.M. of 9 - 13 animals. *p <0.05 vs saline according to ANOVA followed by the Dunnett's test.

V. DISCUSSION

In this thesis, biochemical and pharmacological characterization of novel NOP receptor ligands is described, particularly focusing on Ac-RYYRIK-ol by the use of various *in vitro* and *in vivo* studies. This hexapeptide alcohol was studied further for structure-activity relations by *in vitro* receptor binding, functional and pharmacological assays.

The equilibrium dissociation constants and the maximal number of binding sites for N/OFQ in the literature are variable [183] which might come from the differences in the assay conditions and from the stability alterations of the radioligands used. In fact, the actual affinity of a given ligand in displacement binding experiments is highly dependent upon the radioligand used [146]. The heptadecapeptide N/OFQ labelled with tritium (Amersham Pharmacia) on the leucine residue at position 14 was reported to be stable for up to 2 months [195]. By using the Amersham product, that has the highest specific activity (160 Ci/mmol) among other commercially available radioisotopes [183], one binding site with similar K_d values were obtained in membranes from rat and CHO-NOP_h both are in line with previously reported data [196, 197]. The B_{max} of the NOP receptors in rat brain membranes in this study (290.4 fmol/mg) is not considered significantly different than the first reported data (535 fmol/mg) [59] by unpaired t-test. However CHO-NOP_h membranes used in this study, found to have twice as much NOP receptors as that of rat brain membranes. Because of different strains of animals, cell lines, experimental conditions, radioligand source used in various studies no clear correlation can be established neither of the K_d nor the B_{max} values reported.

A series of hexapeptides of the general formula Ac-RYY-R/K-W/I-R/K-NH_2 were identified by Dr Toll's group in 1997 from a synthetic combinatorial hexapeptide library [140]. Among these peptides Ac-RYYRIK-NH_2 and Ac-RYYRWK-NH_2 are the most used compounds due to their high affinity and selectivity for the NOP receptor [122]. These hexapeptides were reported to behave as partial agonists at recombinant NOP receptors expressed in CHO cells ([^{35}S]GTPγS binding and cAMP accumulation assays) and at the native NOP receptor expressed in the mouse *vas deferens* (mVD bioassay) [140]. In this work, structure-activity studies on Ac-RYYRIK-NH_2 principally modified on the C- and the N-terminal were summarized. The C-terminal modification was the replacement of the carboxyamide residue with a hydroxymethylen (-CH_2OH) which yielded a novel hexapeptide, Ac-RYYRIK-ol with a primer alcohol group at C-terminal. The hexapeptide alcohol displaced [*leucyl*-^{3}H]N/OFQ with high affinity, but not the opioid antagonist [^{3}H]naloxone, indicating the selectivity of the ligand for NOP receptor binding sites [151]. In functional assays this peptide was first reported to be a competitive antagonist in the [^{35}S]GTPγS binding performed in the rat brain membranes and

CHO cells expressing human NOP receptor as well as in the mouse *vas deferens* bioassay [**151**]. However, detailed *in vitro* and *in vivo* pharmacological characterization of the compound indicated that the pharmacological profile of Ac-RYYRIK-ol is more complex most likely due to its low efficacy agonist nature [**152**]. The hexapeptide alcohol interacted specifically with the NOP receptors in rat brain membranes and recombinant cells expressing human NOP receptors conferring high affinity in subnanomolar range (pK_i = 9.10 and 9.39 M, respectively). In rat brain membrane the hexapeptide alcohol was able to stimulate G-proteins with a low efficacy, furthermore the efficacy of the peptide was low enough to competitively antagonize (pA_2 8.67) the N/OFQ stimulated G-protein activation. However, in membranes from CHO cells expressing human NOP receptor, the hexapeptide alcohol showed a clear partial agonism with a high potency (pEC_{50} 8.52) and maximal stimulation of 540.9 ± 82.70 % over the basal level. Most likely due to the high density of the NOP receptors expressed in the cells used in the study.

In the electrically stimulated mouse *vas deferens* the hexapeptide alcohol competitively antagonized (pA_2 8.46) the inhibitory effect of N/OFQ. However, on the mouse colon tissues Ac-RYYRIK-ol behaved as an agonist with high potency (pEC_{50} 9.09). The contractile effect was no longer evident in colon tissues taken from mice knockout for the NOP receptor gene ($NOP^{-/-}$) demonstrating that the effect is derived from the exclusive activation of the NOP type. This result also confirms the previous findings from the binding experiments [**151**] that the hexapeptide is highly selective for the NOP receptor.

The different pharmacological activity of Ac-RYYRIK-ol at NOP receptors expressed in the mouse *vas deferens* (antagonist) and colon (agonist) is not completely surprising. In fact, very similar results were reported not only for the parent compound, Ac-RYYRIK-NH_2, but also for the NOP ligand [$Phe^1\psi(CH_2\text{-}NH)Gly^2$]N/OFQ(1-13)-$NH_2$ ([F/G]N/OFQ(1-13)NH_2). These molecules behaved as NOP antagonists in the *vas deferens* assay [145, **151**, 198] and in the GTPγ[^{35}S] binding assay performed in rat brain membranes and mouse brain sections [143, 145], while they displayed as NOP agonists in the mouse colon [144]. Recently McDonald et al [199] reported the use of an ecdysone-inducible expression system containing $CHO_{IND}NOP_h$ (human NOP receptor expressed in Chinese hamster ovary cells) in order to examine the activity of a range of partial agonists. Interestingly enough, it has been demonstrated that the pharmacological profile of [F/G]N/OFQ(1-13)NH_2 as well as that of Ac-RYYRIK-NH_2 can be manipulated to encompass full and partial agonism along with antagonism in the same cellular environment by changing, as the only variable, NOP receptor density [199]. It is worth of mentioning that the receptor reserve or spare receptor, which defines the ability of a given agonist to produce a maximal system response and also refers to the percentage of receptors not required for the production of a maximal response. The receptor reserve is a property of the

tissue (*i.e.*, the strength of amplification of receptor stimulus inherent to the cells) and it is a property of the agonist (*i.e.*, how much stimulus is imparted to the system by a given agonist receptor occupancy). This latter factor is quantified as the efficacy of the agonist. The actual value of the receptor reserve will be unique to each agonist in the given system. Different cellular backgrounds have different capabilities for the amplification of receptor stimuli. Depending on the efficiency of the stimulus-response coupling apparatus of the cell, a given agonist could produce no response, a proportion of the maximal response, or a maximal response. If the stimulus-response capability is reduced, weak partial agonists can be studied as antagonists to gain measurements of affinity [200]. Hence, it can be suggested that Ac-RYYRIK-ol is, similarly to [F/G]N/OFQ(1-13)NH_2 and Ac-RYYRIK-NH_2, a NOP receptor partial agonist [**152**] whose estimated pharmacological activity (full, partial agonism or antagonism) strongly depends upon the receptor density and coupling efficiency, whereas these parameters are tissue/model dependent, and intrinsic activity in different tissues can vary [201]. In summary, *in vitro* results demonstrated that Ac-RYYRIK-ol is a potent and selective NOP receptor low efficacy agonist [**151, 152, 202**].

As the most interesting compound, Ac-RYYRIK-ol was characterized further in a rather large battery of *in vivo* assays in mice where it mimicked N/OFQ effects that were previously shown to be sensitive to NOP selective antagonists and/or no longer evident in $NOP^{-/-}$ mice. This applies to the effects related to:

1. Algesia: Hyperalgesia induced by N/OFQ in mice was first demonstrated by Meunier et al. [3] using the hot plate test and Reinscheid et al. [2] using the tail flick test. On the contrary it was suggested that N/OFQ reversed the stress induced antinociception which is actually mediated by the endogenous opioid system, and associated with the i.c.v. injection procedure [90]. N/OFQ was shown to reverse the morphine induced analgesia in the tail withdrawal assay in mouse [90] [203] and in the tail flick assay in rat [204]. Dose-dependent but naloxone-insensitive reduction of tail withdrawal latency and reversal of opioid induced analgesia was reported by Calo et al. [177]. Correspondingly to the supraspinal pronociceptive action of N/OFQ, Ac-RYYRIK-ol produced a dose dependent (0.01-1 nmol) hyperalgesia with a similar potency and duration of effect (about 15 to 30 min) that are similar to those of N/OFQ as previously reported [177] [205] [134]. The spinal antinociceptive effect of Ac-RYYRIK-ol was similar to that of N/OFQ [206, 207]. Ac-RYYRIK-ol behaves as an agonist in the *in vivo* analgesiometric tests similarly to the low efficacy agonist [F/G]N/OFQ(1-13)NH_2 [177, 208].

2. Food intake: It has been shown that intracerebroventricular administration of N/OFQ induces hyperphagia in sated rats [92, 209]. The orexigenic effect of N/OFQ is significantly reduced by NOP receptor antagonist [$Nphe^1$]N/OFQ(1-13)NH_2 [210]. The NOP receptor partial

agonist [F/G]N/OFQ(1-13)NH_2 stimulated feeding alone and the co administration with N/OFQ did not reduce the hyperphagic effect of N/OFQ [210] [211]. Ac-RYYRIK-ol induced a dose-dependent stimulation of food intake similarly to N/OFQ and [F/G]N/OFQ(1-13)NH_2 [210]. However, the hexapeptide alcohol was more potent, effects becoming significant at 0.01 nmol and having longer duration of effects when compared to N/OFQ. Thus, Ac-RYYRIK-ol mimicked the hyperphagic effects of N/OFQ and the partial agonist [F/G]N/OFQ(1-13)NH_2 and behaved as an agonist in the central NOP receptors controlling the food intake [**152**]. Recently the hyperphagic effects of N/OFQ was proposed to be related to inhibition of neurons that release alpha-melanocyte stimulating hormone (α-MSH) and oxytocin (OT) by N/OFQ [212], or therefore inhibition of the activity of the neuronal systems that promote termination of feeding are supposedly involved in the mechanism of the orexigenic effect of N/OFQ [213].

3. Locomotor activity: N/OFQ was shown to have a biphasic effect on locomotor activity similar to its effects on pain mechanisms. Hypolocomotion following the i.c.v. administration of N/OFQ (0.1-10 nmol) was first described by Reinscheid et al [2] and pursued by other researchers [214] [62, 178]. On the contrary, stimulation of locomotion and exploratory behaviour in mice has also been reported by Florin et al. However the dose range was much more wider than previously reported and this effect was blocked by dopamine receptor (D1 and D2) antagonists but not by naloxone [113]. The studies on NOP receptor knockout and wild type mice clearly showed the involvement of NOP receptor system especially with 10 nmol N/OFQ. On the other hand, the relative ineffectiveness of the low dose (1 nmol) indicated that the NOP receptor-peptide system is not a major regulator in the control of locomotion [62, 161]. Nevertheless, the biphasic effects were blocked by NOP receptor antagonists [215]. Similarly to the partial agonist Ac-RYYRIK-NH_2 [143] Ac-RYYRIK-ol was also shown to cause a dose dependent (0.01-1 nmol) inhibition of spontaneous locomotor activity in this study. Interestingly [F/G]N/OFQ(1-13)NH_2 behaved as a partial agonist in this assay [178].

4. Mood disorders: Mouse forced swimming test is a model widely used for screening the activity of potential antidepressant drugs [216]. The blockade of N/OFQ signalling obtained with NOP-selective antagonists promotes antidepressant-like effects in the forced swimming and in the tail suspension tests. This was first shown by using two NOP receptor antagonists; the peptide [Nphe^1]N/OFQ(1-13)NH_2, and the non-peptide J-113397 [217]. Later on these findings were confirmed and extended with the NOP receptor peptide antagonist UFP-101 [175, 218]. Similarly to N/OFQ [175, 217, 218] Ac-RYYRIK-ol was inactive *per se* but able to revert the antidepressant-like effects of the well known NOP receptor antagonist peptide UFP-101 in the mouse swimming test [**152**]. It was suggested that NOP receptor antagonists might elicit an antidepressant-like effect by counteracting the inhibitory effects of the endogenous N/OFQ on

monoaminergic systems at both pre- and postsynaptic levels, thus producing a synergistic potentiation of the cortical monoaminergic signal [64].

Thus, in all the *in vivo* assays we performed, Ac-RYYRIK-ol behaved as a NOP agonist. Again, very similar results were obtained in our and other laboratories by testing the partial agonist [F/G]N/OFQ(1-13)NH_2 and Ac-RYYRIK-NH_2 in analgesiometric tests after supraspinal [177, 219, 220] or spinal administration [219, 220], in locomotor activity [143, 178] and food intake [210] studies. These data together with those obtained with Ac-RYYRIK-ol in the present study clearly demonstrated that low efficacy agonists behave as full agonists in these *in vivo* assays.

NOP receptor partial agonists like Ac-RYYRIK-NH_2, Ac-RYYRWK-NH_2 and [F/G]N/OFQ(1-13)NH_2 were shown to function selectively, in particular on cardiovascular and renal functions. Depending on the route of administration, these compounds behave as full agonists (central, *i.e.*, i.c.v.) as well as partial/antagonist (peripheral, *i.e.*, i.v. bolus). Thus, NOP receptor partial agonists, especially the hexapeptides had variable pharmacological profile not only in the *in vitro* isolated tissue or cellular systems, but also in the *in vivo* studies related to cardiovascular and renal function [221]. There is no simple explanation why these partial agonists differ functionally. Splice variants of preproOFQ/N in mouse, rat and human [23, 222-225] were reported. Besides the presumed splice variants, and the possible formation of receptor oliomerization [40, 41], some of the competition and saturation binding results [31, 30, 145] also suggest the heterogeneity of the NOP receptor. However the functional differences of the partial agonists may not be due to diversity of the NOP receptor system such as central and peripheral since until now there is no clear evidence of the claimed NOP receptor subtypes [32]. Therefore the alternative possibility; the different receptor density/coupling efficiencies is more reliable to explain these discrepacencies. It would be interesting, in future studies, to investigate the actions of Ac-RYYRIK-ol on cardiovascular parameters after i.c.v and i.v. administration in order to see if the effects change depending on the route of administration as it was seen with other partial agonists [221].

Further structure-activity studies that we performed on the hexapeptide alcohol provided valuable information on the structural requirements of the hexapeptide alcohol. The newly synthesized acetylated hexapeptide alcohol possesses two positively charged Arg (R) and two adjacent aromatic Tyr (Y) residues. In an attempt to understand the importance of the charged Arg residues, Arg / Cit replacement at first and/or fourth position was done. Consequently, in the binding studies intact Arg residue at the first position turned out to be essential for NOP receptor activation. Our finding is in line with the result observed by Ala-scanning [226]. Interestingly enough, N/OFQ also contains two arginine side chains, at 8^{th} and 12^{th} position. The

Arg^8 / Ala replacement produced a decreased functional property and receptor affinity [119]. Although it is feasible to assume that the hexapeptides interact in a similar manner (*i.e.*, with the negatively charged side chains of EL of the NOP receptor) as N/OFQ, it has been shown by photo-affinity labeling that actually the hexapeptides interact with a region within the C-terminus of the TM-II in NOP receptor: aa. 107-113 (Gln-Gly-Thr-Asp-Ile-Leu-Leu), whereas N/OFQ interacts with the region of the NOP receptor: aa. 296-302 (Thr-Ala-Val-Ile-Leu-Arg), spanning the the C-terminus of EL-III and the N-terminus of transmembrane helix VII ref. [227, 228].

None of the citrulline-replaced hexapeptides showed better affinities than the parent peptide, so it can be concluded that Arg at 1^{st} and 4^{th} position play an important role in the biological activity. Nevertheless complete affinity loss claimed the critical role of Arg at 1^{st} position. Our results support the findings by Kawano et al [226] indicating the importance of Arg at 1^{st} position by residual N-terminal truncation.

The N-terminal acetyl group of the hexapeptide alcohol was further modified to determine its role in the binding process, and to determine to which extent its elimination or subtitution with other moieties is tolerated in terms of biological activity. The N-acetyl group was replaced by i) *chloro-acetyl-* or *pivaloyl* group that increases the steric hinderance; ii) *mesyl* group that increases steric hinderance and changes the character of the attached amide group; iii) *benzoyl* group that increases steric hinderance and confers an aromatic feature, furthermore it permits protonation; iv) *formyl* group that decreases the steric hinderance, and provides more hydrogen bond forming ability v) *Free* amino-terminal (H_2N-) that eliminates steric hinderance and restores the basic character to the N-terminal.

The deacetylated hexapeptide showed the lowest affinity towards the receptor however, this peptide had the minimum intrinsic agonist activity as well. Interestingly, substitution by the bulky pivaloyl (trimethylacetyl) group was less tolerated; on the other hand, the intrinsic activity was lowered with these replacements. Among the polar group-bearing peptides, the chloro-acetylated compound had the best affinity. The complete change of the N-terminal character with the mesyl substitution resulted in a decreased potency. As a consequence, the N-terminal protection with a polar group seems to be important, the N-Acetyl-Arginine group is essential for eliciting the molecular contact or interaction with the receptor. All the modified analogs of Ac-RYYRIK-ol were found to displace the opioid binding sites (by using [^{3}H]naloxone) only at very high concentrations indicating their low affinities with the interaction with opiod receptors.

The novel analogues of the hexapeptide alcohol exhibited low antagonist potencies on the mouse *vas deferens,* when tested with a single concentration to antagonize the twitch response inhibition elicited by N/OFQ.

NOP receptor partial agonists have recently been shown to produce functionally selective effects on cardiovascular and renal function in conscious rat [221]. Moreover NOP partial agonists and antagonists were shown to have less effect than the NOP agonists on the internalization of hNOP receptor [229], thus it is expected for the partial agonists to induce less tolerance [229].

The SAR studies confirmed the structural requirements for the hexapeptide alcohol: i) the positively charged Arg residues at first and fourth positions are important for the biological activity; in particular, the first position is crucial for ligand-receptor interaction. ii) The substitution of the N-terminal with various acyl groups clearly emphasized the necessity of a polar group to protect the N-terminal with an acetyl group.

VI. SUMMARY

Following the discovery of the nociceptin/orphanin FQ (N/OFQ) peptide receptor (NOP) and its endogenous ligand, an extensive search has started to find selective agonists and antagonists, targeting this novel receptor-ligand system, due to their therapeutic potentials. By the help of combinatorial chemistry a series of hexapeptides with a general formula of Ac-RYY-R/K-W/I-R/K-NH_2 were identified and found to have high NOP receptor affinity and selectivity [140]. The present work focused on the biochemical, functional and pharmacological characterization of novel NOP receptor ligands, Ac-RYYRIK-ol and analogues, which appear from the C-terminal modification of one of the previously reported hexapeptide. This work consists of basic characterization of Ac-RYYRIK-ol by *in vitro* and *in vivo* experiments and the structure-activity relationship studies performed on the novel hexapeptide. The main findings are as follows:

1. C-terminal carboxyamide modification to a hydroxymethylene yielded high affinity (pK_i = 9.096 and 9.389 in rat brain membrane and CHO-NOP_h receptors, respectively), selective NOP receptor ligand, Ac-RYYRIK-ol.
2. N-terminal modifications with several acyl groups (ClAc-, pivaloyl-, formyl-, benzoyl-, mesyl-) decreased the affinity of the ligand towards the NOP receptor.
3. The replacement of the positively charged arginine (Arg) residues with an uncharged citrulline (Cit) clearly indicated that Arg at the first posititon is essential for NOP receptor binding and activity.
4. Ac-RYYRIK-ol (10 - 100 nM) competitively antagonized (pA_2 = 8.67) the N/OFQ induced G-protein activation in rat brain membranes. However, in CHO cells expressing NOP receptors with binding capacities (B_{max}) about two-times higher as in rat brain, the hexapeptide alcohol behaved as an agonist with high potency (pEC_{50} 8.52) and efficacy (E_{max} = 540.9 %).
5. Ac-RYYRIK-ol (10 - 1000 nM) competitively antagonized (pA_2 = 8.46), the inhibitory effect of N/OFQ in the electrically stimulated mouse *vas deferens*.
6. Ac-RYYRIK-ol mimicked the contractile effects of N/OFQ in the isolated mouse colon thus behaving as a NOP receptor agonist (pEC_{50} = 9.09). This effect was no longer evident in colon tissues taken from transgenic mice lacking the NOP receptor gene ($NOP^{-/-}$) indicating the NOP receptor selectivity.
7. In a series of *in vivo* experiments performed on Swiss mice, similarly to N/OFQ, Ac-RYYRIK-ol (dose range 0.001 - 1 nmol) was found to produce: i) pronociceptive effects

after intracerebroventricular (i.c.v.) administration and antinociceptive actions when given intrathecally (i.t.) in the tail withdrawal assay; ii) inhibition of locomotor activity and iii) stimulation of food intake after supraspinal administration. In the forced swimming test, Ac-RYYRIK-ol was inactive *per se*, but reversed the antidepressant-like effects elicited by the NOP receptor selective antagonist UFP-101 ([Nphe^{1},Arg^{14},Lys^{15}]N/OFQ-NH_2). Thus in all *in vivo* assays Ac-RYYRIK-ol behaved as a NOP receptor agonist.

In conclusion, Ac-RYYRIK-ol displayed a complex pharmacological profile, which is likely due to the low efficacy agonist nature of this novel ligand of the NOP receptor. It is worthy of mentioning that NOP receptor partial agonists are drug candidates for management of hyponatremia and water retention (for indications such as aquaresis) in particularly because of their selectivity to produce renal, but not cardiovascular effects. The high potency, selectivity of action, and *in vivo* effectiveness make Ac-RYYRIK-ol a useful pharmacological tool for future studies in the field of N/OFQ and its NOP receptor.

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