Structure-activity studies of novel, conformationally restricted delta opioid-receptor selective tetrapeptides

Ph.D. thesis

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LIST OF PUBLICATIONS

This thesis is based upon the following publications:

I.   **_Enikő Ioja_**_, Géza Tóth, Sándor Benyhe, Dirk Tourwe, Antal Péter, Csaba Tömböly, Anna Borsodi._ Opioid receptor binding characteristics and structure-activity studies of novel tetrapeptides in the TIPP (Tyr-Tic-Phe-Phe) series. **_Neurosignals_** 2005; 14(6):317-328

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Brain Res. Bull. 2007; 74(1-3):119-29

ABBREVIATION

ACTH - adrenocorticotrop hormone

B_{max} - maximal number of binding sites

BNTX - benzylidenenaltrexone

BSA - bovine serum albumin

BRET - bioluminescence resonance energy transfer

cAMP - adenosine 3',5'-cyclic mono-phosphate

CHO - Chinese hamster ovary

CNS - central nervous system

COS - monkey kidney cells

cpm - counts per minute

CTAP - D-Phe-cyclo-(Cys-Tyr-D-Trp-Arg-Thr-Pen)-Thr-NH_2

DAMGO - [D-Ala^2,(N-*Me*)Phe^4,Gly^5-ol] enkephalin

Dmt - 2',6'-dimethyltyrosine

EC_{50} - 'potency'effective concentration 50%; the concentration of a drug that gives 50% of maximal response

E_{max} - 'efficacy', the maximal effect that an agonist can elicit in a given preparation

FRET - fluorescence resonance energy transfer

g - relative centrifugal force

GDP - guanosine-5'-diphosphate

GI - gastrointestinal tract

G-protein - guanine nucleotide binding protein

GPCRs - G protein-coupled receptors

GTP - guanosine-5'-triphosphate

GTPγS - guanosine-5'-O-(γ-thio) tri-phosphate

HEK - human embryonic kidney cells

IC_{50} - the concentration of a drug that inhibits 50% of the specific binding of a competing ligand

i.c.v - intracerebroventricular

Ile^{5,6}- deltorphin-II - Tyr-D-Ala-Phe-Glu-Ile Ile-Gly-NH_2

i.th. - intrathecal

K_d - equilibrium dissociation constant

K_i - equilibrium inhibition constant

KO - knock out (gene inactivation by homologous recombination)

K_{on} - association rate constant

KOP - κ opioid receptor

PBS - phosphate buffered saline

(pClPhe^4)-DPDPE – *cyclic* [D-Pen^2,4'-ClPhe^4, D-Pen^5]-enkephalin

PCP - phenylcyclidine

Pen - penicillamine

PTX - pertussis toxin

MOP - μ opioid receptor

mRNA - messenger RNA

N/OFQ - Nociceptin/Orphanin FQ

SEM - standard error of the mean

TIPP - Tyr-Tic-Phe-Phe

TIPPψ - Tyr-Ticψ[CH_2NH]-Phe-Phe

TM - transmembrane

Tris - tris-(hydroxymethyl)-aminomethane

I. REVIEW OF THE LITERATURE

1.1. Opioid receptors

1.1.1. Opioid receptors: discovery and heterogeneity

Opioid systems are responsible for a variety of processes in organisms, the most well characterized of which is analgesia. Classical opiate pharmacology derives largely from the isolation and characterization of alkaloids (including morphine) of the opium poppy plant *Papaver somniferum*. Early observation showed that different opioid compounds produced different effects, but the mechanisms for these were unclear. For many years it was thought that opioid drugs act on specific receptor types but only in 1973 three separate research groups reported their identification [1-3]. Radioligand binding studies revealed the suspected existence of multiple opioid receptors [4;5] and provided the evidence for classification of the opioid receptors into three separate types [6;7] these findings having a major impact on the opioid research as well as on the understanding of the mechanisms of analgesia. By systematic investigation of the effects produced by different opioid drugs (*e.g.* morphine, ketocylazocine) three major classes of opioid receptors have been defined: μ, κ and δ. Delta opioid receptors were identified by the effects produced by endogenous enkephalins and endorphins [8].

Previously it had been suggested that PCP and its analogues exert their psychotomimetic effects via a common receptor namely the σ-opioid receptor [9]. Later on several reports demonstrated the significant differences between the binding of PCP and the SKF 10,047 a sigma opioid agonist, deducting distinct PCP and sigma opioid receptors [10]. Other studies showed that the σ-agonist ±SKF 10,047 induced effects is not blocked by the general opioid antagonist naloxone [11]. It is now known that the psychotomimetic effects of PCP reflect blockage of the NMDA subtype of glutamate receptors. Sigma binding proteins, subsequently purified and cloned [12-14] do not appear to represent pharmacologically defined opioid receptors.

The β-endorphin mediated inhibition of the electrically induced contraction of the rat vas deferens suspected and postulated the existence of a fourth opioid receptor, the ε-opioid receptor [15-18]. β-endorphin activity in the rat *vas deferens* was antagonized by naloxone and also cDNA encoding ε-receptors were cloned from human genomic library [19], the expression of these clones in cell lines should further clarify this putative receptor.

Yet, another opioid receptor-like species has been cloned, namely, the opioid receptor-like protein$_1$ (ORL$_1$) later named NOP receptor [20;21].

According to the nomenclature suggested by the International Union of Pharmacology Nomenclature for Opioid Receptors (http://www.iuphar.org), the opioid receptors are referred to as MOP, DOP, KOP and NOP receptors for the mu (μ), delta (δ), kappa (κ) and nociceptin receptors, respectively.

1.1.2. Neurobiology of opioid receptors

Opioid receptors are widely distributed throughout the brain and peripheral tissues. Distribution of the μ-, δ- and κ- receptors and their quantity varies between different species and various major anatomical regions [22-24]. In general these receptors are localized throughout the somatosensory systems and extrapyramidal system as well as in the limbic region [23;24] where they are implicated in correlation with other systems in reward behavior [25], limbic seizure [26], long-term potentiation [27] and in epilepsy [28]. Autoradiography revealed dense concentrations of opioid receptors in different nuclei, such as locus coeruleus, substantia gelatinosa of the spinal cord and brain stem, vagal nuclei *etc.* The μ-opioid receptors are also involved in the thalamic and hypothalamic neurons hyperpolarization under the influence of estrogen [29]. Reduction of these receptor densities accompanies the degenerative symptoms of Alzheimer's disease [30]. There are various evidence that opioids are implicated in numerous homeostatic and physiological functions, such as the regulation of neurohormone secretion through the pituitary-adrenal axis *e.g.* anterior pituitary hormones [31], somatostatin [32], dopamine [33] as well as oxytocin and vasopressin [34]. Agonists acting on μ- and δ- opioid receptors are involved in the attenuation of acute and chronic pain [35-37]. Furthermore opioid antagonists are involved and find clinical applications in alleviating addiction to narcotic alkaloids [38], modulating the behavioral effects of amphetamines [39], relieving alcohol dependence [40-42] also in immunoppression [43] during organ transplantation [44], treating autism [45] and Tourette's syndrome [46]. Moreover different autonomic responses such as respiratory depression [47;48], nausea, bradycardia, thermal regulation [49] *etc.*, are partly mediated through opioid receptors.

1.1.3. Molecular cloning of opioid receptors

The first opioid receptor to be cloned was the δ-opioid receptor. Two groups independently cloned the mouse δ-receptor by preparing an expression library from mouse neuroblastoma × rat glioma hybrid cell line and transferring the library into COS cells [50;51]. A cDNA sequence encoding a 372-amino acid protein was identified. Later Yasuda *et al.* [52] isolated a mouse δ-opioid receptor clone from a brain cDNA library and confirmed the sequence identified by these investigators. The rat δ opioid receptor was also cloned consisting of a number of 372 amino acids with 97% homology to the mouse δ-receptor, the 3% differences lying in the NH_2- and COOH- terminal sequences and one in the second extracellular loop [53]. cDNA for human δ-receptor was cloned from human striatum and SH-SY5Y human neuroblastoma cell lines [54;55]. The cloned receptor shown 93% homology with both mouse and rat δ-opioid receptors most of the differences in amino acid sequences were in the N- and C-terminals but some other differences are also present.

Chen et al. described the first μ-opioid receptor cDNA clone identified from rat brain cDNA library [56]. The rat μ-opioid receptor consists of 398 amino acids and has 64% amino acid identity to the mouse δ-opioid receptor. The mouse [57] and the human [58] μ-opioid receptor genes have also been cloned. The protein sequence has 95% amino acid identity to the rat μ, 59% to the rat κ, and 62% to the rat δ-opioid receptors. Significant levels of μ opioid receptor mRNA were found in several brain regions *e.g.* thalamus, cerebral cortex, striatum *etc* [59]. Recent studies have identified a variety of mRNA splice variants for the μ-opioid receptor gene and some of the resulting proteins differ substantially in their agonist selective membrane trafficking. The haphazard cloning of the mouse κ-opioid receptor occurred during efforts to obtain mouse somatostatin receptor subtypes using somatostatin receptor cDNA probes [60]. This receptor consist of 380 amino acids and has 61% identity to the mouse δ-opioid receptor. Rat [61], guinea pig [62] and human [63] κ-receptors were also cloned. The human κ-opioid receptor consist of 380 amino acids with 91% identity to the cloned rat κ-opioid receptor.

1.1.4. Opioid receptor subtypes

There is little agreement in the exact classification of the opioid receptor subtypes. Regarding the κ-opioid receptor a number of subtypes have been described (κ_1, κ_2, κ_3) binding different ligands but there is a strong suggestion for at least one major molecule with very good affinity to the benzomorphan class of opioid alkaloids [64]. Data about δ-opioid receptor subtypes are very interesting and controversial. Early evidence, regarding the possibility of multiple delta opioid receptors came from binding studies [65] and stronger evidence derived from behavioral studies [66;67] led to the suggestion of two receptor sites the δ_1 and δ_2. However, no δ-subtypes have been cloned, and there remains no definite molecular evidence for distinct subtypes of the δ-opioid receptor. In the case of μ-opioid receptors a subclassification was proposed on the basis of behavioral and pharmacological studies defining μ_1 and μ_2 subtypes [68]. The μ_1 site is thought to be a high affinity receptor with similar affinities for the classical mu and delta ligands. Accordingly to this model both μ_1 and μ_2 receptors are involved in producing analgesia, euphoria is produced mainly through μ_1, and respiratory depression through μ_2 receptors. The different receptor subtypes may result from different posttranslational modifications of the gene product (glycosylation, palmytoylation, phosphorylation, etc), or from receptor oligomerization [69;70].

1.1.5. Molecular biology of opioid receptors

Gene knockdown is carried out by using short sequences of oligodeoxynucleotide that are complementary to a portion of mRNA that codes for a particular gene product. Antisense oligonucleotides (AS oligos) are potentially valuable pharmacological tools especially when no selective antagonists are available. They were first used in the inhibition of the expression of a specific cannabinoid receptor protein *in vivo* [71] thus achieving practically the same final result as receptor blockade [72;73]. Pretreatment of experimental animals with AS oligos to the δ-opioid receptor resulted in reduced response to δ- but not to κ- or μ- selective receptor agonists [74;75]. AS oligos complementary to opioid receptor mRNAs have been successfully used to implicate δ, κ and μ receptors in the development of opioid tolerance and dependence [76], β-endorphin induced antinociception [77] and in a series of other physiological processes. AS oligo knockdown of opioid receptor expression is reversible.

Knockout (KO) strategy involves generation of transgenic mice possessing a discrete gene deletion that result in failure to express a particular gene product. In μ-opioid receptor

deficient mice the analgesic effects of morphine and its metabolites were drastically reduced [78], the gastrointestinal transit was inhibited [79], thermally induced nociception [80], chronic morphine treatment induced physical dependence and withdrawal symptoms [81] are also reduced in these animals. κ-Opioid receptor deficient mice revealed that κ receptors do not contribute to morphine analgesia and withdrawal but participate in the expression of morphine abstinence [82]. Transgenic μ-opiod receptor knockout mice have also been generated to study the interactions between δ- and μ- opioid receptors in the CNS [83]. Findings from these experiments indicated that the δ receptor mediates antinociception in the absence of the μ-opioid receptor, similar findings being reported by Matthes *et al* [84]. The central role of δ-receptors in the dissipation of analgesic tolerance to morphine was also proved in KO mice [85]. From the responses of triple knockout mice simultaneously lacking μ, δ and κ receptors to exogenous opiates it was clarified the role of each receptor type in the main actions of opiate drugs. The μ-opioid receptors have an essential role in morphine analgesia and addiction. The κ-opioid receptors mediate both dysphoric and analgesic activities of classical κ-agonists. δ-receptors produce analgesia and do not mediate opioid reward and dependence. Finally, μ- and δ-receptors may interact functionally while κ-receptors act rather independently.

1.1.6. Structure of the opioid receptors

Opioid receptors belong to the class A (Rhodopsin) family of G_i/G_o protein coupled receptors with an extracellular N-terminal domain (Fig. 1), 7 TM helical domains connected by three extracellular and three intracellular domains and an intracellular C-terminal tail, possibly forming a fourth intracellular loop with its putative palmitoylation site(s). On the basis of the 2.8 Å resolution 3-D structure of rhodopsin [86], it is assumed that the seven transmembrane helices of opioid receptors are arranged sequentially in a counterclockwise fashion forming a tight helical bundle with the extracellular domains, which provides a dynamic interface for the binding of various opioid ligands. There is a high sequence homology between the three opioid receptor types (Fig. 1. - indicated by gray and black circles). The transmembrane helices show ~70% identity, and the loops show ~60% identity [87]. However, sequence identity is dependent on the region of the receptor being analyzed *e.g.* while the intracellular loops share ~90% homology, the N-terminus and the C-terminus share little to no homology [88].
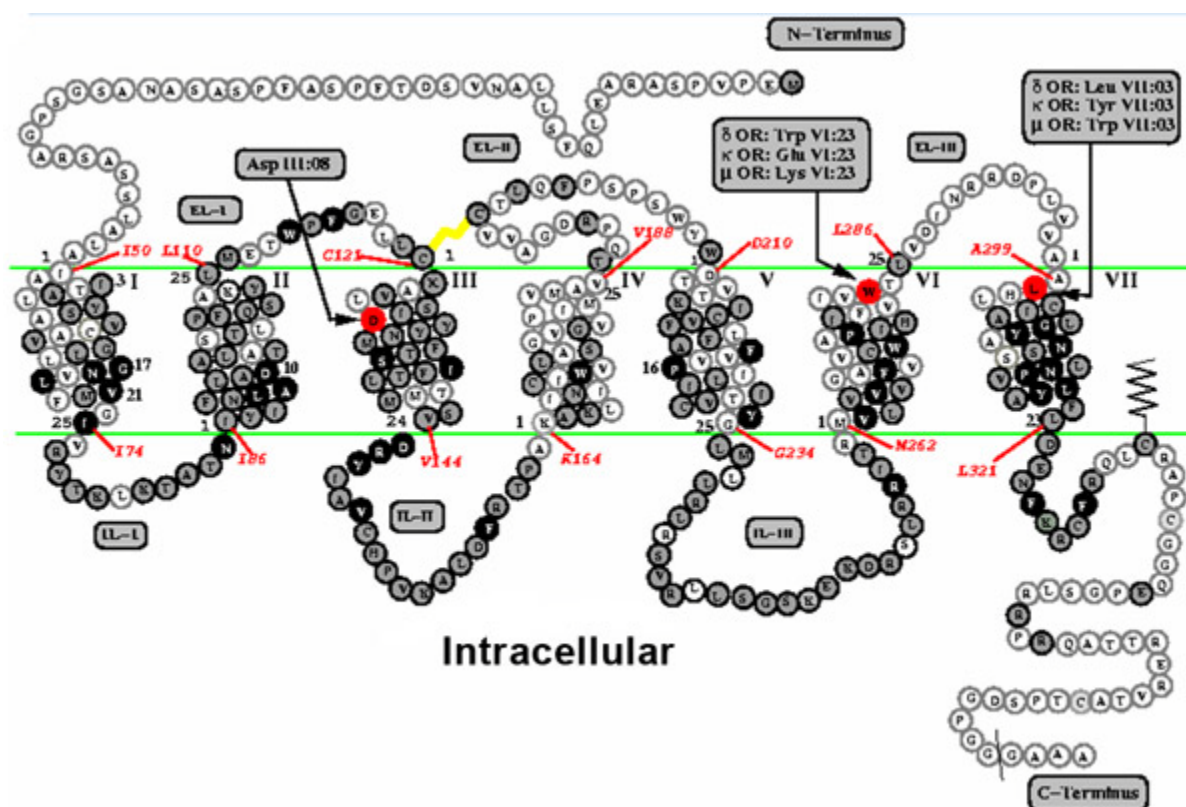
Fig. 1. Serpentine model of the δ-opioid receptor [89]. Circles contain the one-letter code for the given amino acid. The gray circles indicate the residues that are conserved among all 3 receptor types (μ, δ and κ), while the black circles indicate the residues that are highly conserved among the rhodopsin subclass of G-protein coupled receptors. Each TM region is indicated by a roman numeral. At the beginning and end of each helix there are Arabic numbers denoting the position of the residue within the helix.

All four opioid receptors possess two conserved cysteine residues in the frist and second extracellular loops. The TM3 contains an aspartate residue (Asp III:08, see figure 1.) which is conserved among all biogenic amine receptor families, including β-adrenergic and opioid receptors [90;91] and seems to have importance in the formation of a salt bridge beetwen the ligand and receptor [92]. This conserved aspartic residue seems to play role in the binding of amines and other other ligands [93]. The third intracellular loop has been implicated in the binding of G-proteins, and the high homology at this portion of the receptor suggests that various opioid receptors interact with similar G-protein complexes. The third intracellular loop also has consensus sequences for phosphorylation, which might be involved in regulatory processes. At the N-termini, consensus sites are present for asparagine-linked (*N*-linked)

glycosylation, though the different opioid receptors have different number of glycosylation sites [94].

Regions involved in mediating receptor function have been identified primarily by the construction of chimeric receptors, site directed mutagenesis of specific amino acid residues within the receptors and by the construction of truncated or deletion mutants. Results of these techniques pointed to the common features of opioid receptors defining an opioid-binding pocket as well to the divergent aspects of the receptors conferring the high affinity and selectivity for receptor type-specific ligands [95-98]. It has been suggested that all opioid receptors share a common binding cavity that is situated in an inner interhelical conserved region comprising transmembral helices 3, 4, 5, 6, and 7. This cavity is partially covered by extracellular loops. These highly divergent extracellular loops, together with residues from the extracellular ends of the TM segments, play a role in ligand selectivity, especially for peptides, allowing them to discriminate between the different opioid receptor types. It has been further suggested that the extracellular loops might act as a gate that allows for the passage of certain ligands while excluding others, however particular elements of the loops may have unfavourable interactions with selective ligands and thus preventing their binding [99]. Agonist ligand selectivity for μ, δ and κ receptors has been attributed to the first and third extracellular loop for the μ receptor [100], the second for the κ receptor [101;102] and the third extracellular loop for the δ receptor [103;104].

1.1.7. Opioid receptor oligomerization

Recent studies have shown that G-protein coupled receptors may form dimers or higher-order oligomers [105-107]. In some cases receptor oligomerization is essential for receptor function, in other cases, oligomerization has been shown to play a modulatory role. Dimerization of opioid receptors has been shown to alter the binding profile and activation by opioid ligands and receptor trafficking in cell culture model system [108-110] and *in vivo* [111].

Heterodimerization of the fully functional κ- and δ- opioid receptors resulted in a novel receptor complex exhibiting ligand binding and functional properties that are distinct from the those of either receptor [112]. The δ-κ opioid receptor heterodimer displayed novel signaling and regulatory properties. Coexpressing the μ- and δ-opioid receptors, the highly selective synthetic agonists for each had reduced potency and altered rank order, while the endogenous opioid peptides endomorphin-1 and Leu-enkephalin had enhanced affinity suggesting the formation of a novel binding pocket [110]. The coexpressed receptors showed insensitivity to

pertussis toxin in contrast with the individually expressed μ- and δ- receptors. Later on it was demonstrated that μ- and δ- receptors heterodimerize only at the cell surface and that the oligomers of opioid receptors and heterotrimeric G-protein are the bases for the observed μ-δ heterodimer phenotypes [113]. It was also shown that $β_2$-adrenergic receptors can also form heteromeric complexes with both δ and κ receptors when coexpressed [114]. The existence of δ-opioid and thyrotropin-releasing hormone receptor oligomers were also confirmed in intact cells by FRET and BRET techniques [115;116]. Detection of FRET and BRET even in the absence of added agonist, unambiguously shows that many GPCRs can form constitutive homodimers in intact living cells and that GPCR dimerization is not an artefact [117].

1.1.8. G protein - effector mechanisms

The opioid receptor family belongs to the pertussis toxin sensitive G_i/G_o-coupled superfamily of receptors. However, the coupling to pertussis toxin-insensitive G_s and G_z proteins has also been recorded [118-120]. Regardless of the specific α, β, and γ subunits that may comprise a G protein heterotrimer, the activation of G protein-coupled receptors by agonist results in the dissociation of GDP from the α subunit, followed by association of GTP with the open nucleotide binding site [121;122]. The binding of GTP to the α subunit induces a conformational change that results in dissociation of the heterotrimer into α and βγ subunits. Upon receptor activation both G-protein α and βγ subunits interact with multiple cellular effector systems, inhibiting adenylyl cyclase and voltage-gated Ca^{2+} channels and stimulating G protein-activated inwardly rectifying K^+ channels and phospholipase Cβ [123]. These signals are terminated when the endogenous GTPase activity of the α subunit hydrolyzes the bound GTP to GDP and inorganic phosphate. The α subunit/GDP complex then reassociates with the βγ subunits to form the heterotrimeric G protein again. Analysis of μ-receptors and G-protein levels stimulated following μ-opioid receptor occupation in C6 μ cells suggested an average "compartment" consisting of a ratio of one receptor to four G proteins. In SH-SY5Y cells μ-opioid receptors preferentially couple to $Gαi_3$ while δ-opioid receptors preferentially activate $Gαi_1$ [124] suggesting that any compartmentalization is limited to receptors and G protein. Many GPCRs display a basal level of signaling activity thus can stimulate G proteins in the absence of agonists [125]. δ-Opioid receptors display a certain basal level of activity in various cell lines [126]. μ-Opioid receptors have also been shown to exhibit basal signaling activity in SH-SY5Y and HEK cells [127] and display a more enhanced constitutive activity following chronic exposure to

morphine [128]. This phenomenon with others *e.g.* desensitization, endocytosis, downregulation has been suggested to contribute to the development of tolerance and dependence.

1.2. Opioid Ligands

1.2.1. Endogenous opioid ligands

Endogenous opioid peptides are small molecules that are biologically synthesized and excised from individual precursor proteins. These peptides produce the same effects as the chemicals known as classic alkaloid opiates, which include morphine, codeine and heroin. Endogenous opioid peptides function both as hormones and neuromodulators thus by these two mechanisms endogenous opioid peptides produce many effects, ranging from preventing diarrhea to inducing euphoria and pain relief. Opioid peptides are mainly derived from four precursors, pro-opioimelanocortin (POMC), pro-enkephalin, pro-dynorphin [129-131] and pronociceptin/orphanin FQ [132;133]. Active peptides derived from these precursors except for nociceptin/orphanin FQ consist of a pentapeptide sequence Tyr-Gly-Gly-Phe-Met/Leu (YGGFM/L) at their N-termini. POMC gives rise to endorphins, and to the non-opioid ACTH and MSH [134]. Proenkephalin contains one copy of Leu-enkephalin four copies of Met-enkephalin and two copies of the extended Met-enkephalin. Prodynorphin gives rise to dynorphin A and B and α-, β-neoendorphin. The two enkephalins were the first endogenous opioid peptides identified from pig brain by Hughes and Kosterlitz [135]. Although naturally occurring peptides usually have some binding preferences they are able to bind to all three types of opioid receptors. For the highly selective μ-opioid receptor peptides endomorphin-1 and-2 [136], no putative precursor proteins for these peptides have yet been identified, and these tetrapeptides are structurally unrelated to all other endogenous opioids containing the unusual Tyr-Pro dipeptide motif (Table 1.).

The skin of some amphibians contains two families of D-amino acid-containing peptides (dermorphins, deltorphins) that are μ- or δ-receptor selective. The distinctive feature of these peptides is the presence of naturally occurring D-amino acid. Whereas dermorphins [137] are μ-opioid agonists the deltorphins: deltorphin (dermenkephalin), [D-Ala^2]-deltorphin I and [D-Ala^2]-deltorphin II are highly selective for δ-receptors [138]. Even more interesting is the fact that the μ-selective dermorphin and the δ-opioid preferring deltorphins contains a similar N-terminal sequence (Tyr-D-Xaa-Phe) while their C-terminal "address" domains determine receptor selectivity [139].

Opioid peptides act through opioid receptors and transmit messages that primarily inhibit the secondary systems, such as pain perception. In addition they can induce different other effects, such as: decrease respiration, stimulate or depress cardiovascular functioning, decrease the motility of GI tract, decrease susceptibility to seizures, induce euphoria, affect certain behaviors (*e.g.* food and alcohol consumption). Endogenous opioid peptides are produced and released often with other neurotransmitters. Although the function of the co-release of peptide-neurotransmitters pairs is not always clear, evidence suggests that opioid peptides can alter the release of other classic neurotransmitters thus they might contribute to complex behaviours (*e.g.* alcohol consumption).

Table 1. Main features of the endogenous opioid peptides

Precursor	Opioid peptide	Structure	Specificity
POMC	β-endorphin	YGGFMTSEKQTPLVTLFKNAIIK NAYKKGE	μ, ε>δ>>κ
Proenkephalin	[Leu^5]-enkephalin	YGGFL	δ>μ>>κ
	[Met^5]-enkephalin	YGGFM	μ=δ>>κ
	[Met^5]-enkephalin-Arg^6-Phe^7	YGGFMRF	κ$_2$
Prodynorphin	Dynorphin A(1-17)	YGGFLRRIRPKLKWDNQ	κ>μ=δ
	α-neoendorphin	YGGFLRKYPK	
	β-neoendorphin	YGGFLRKYP	
(Frog skin peptides) Prodermorphin Prodeltorphin	dermorphin	YaFGYPS-NH$_2$	μ
	deltorphin	YmFHLMD-NH$_2$	δ
	[D-Ala^2]deltorphin-I	YaFDVVG-NH$_2$	δ
	[D-Ala^2]deltorphin-II	YaFEVVG-NH$_2$	δ
Unknown	endomorphin-1	YPWF-NH$_2$	μ>>δ>κ
	endomorphin-2	YPFF-NH$_2$	μ>>δ>κ

1.2.2. Delta opioid ligands

With few exceptions all delta opioid receptor agonists are peptides, derived chemically from enkephalins or belonging to the class of amphibian skin opioids (Table 1.). Most of the currently used opioid drugs produce their analgesic effects via μ-opioid receptors (*e.g.*, morphine, fentanyl *etc.*). These μ-type opiates are highly effective in alleviating severe pain but they produce side effects, such as tolerance, dependence, respiratory depression, constipation *etc.* κ-Opioid agonist have been shown to be potent analgesics, however they also cause psychotomimetic and dysphoric effects [140;141]. δ-Opioid agonists are known to produce analgesic effects and there are evidences that they induce less tolerance and dependence, no respiratory depression and few adverse gastrointestinal effects [142;143]. [D-Pen^2,D-Pen^5]-enkephalin (DPDPE) produced only a weak, centrally mediated analgesic effect after systemic administration, indicating that it does not cross the blood-brain barrier [144]. [D-Ala^2,D-Leu^5]-enkephalin (DADLE) was initially found to be a selective agonist at δ-receptors, however it only had two-fold greater affinity for δ-receptors than for μ-receptors. A hexapeptide, DSLET (Tyr-D-Ser-Gly-Phe-Leu-Thr) was found to have at least 20-600-fold selectivity for δ- over the κ-, and μ- receptors. A related compound, DTLET (Tyr-D-Thr-Gly-Phe-Leu-Thr) displayed even higher affinities compared with DSLET. The most selective δ-agonists known to date are deltorphins among them [D-Ala^2]-deltorphin-II shows highest δ-selectivity. Other highly δ-selective opioid peptide agonists have also been synthesized such as, DSTBULET (Tyr-D-Ser(OtBu)-Gly-Phe-Leu-Thr) [145] and its analogues, BUBU (Tyr-D-Ser-(OtBu)-Gly-Phe-Leu-Thr(OtBu)), BUBUC (Tyr-D-Cys(StBu)-Gly-Phe-Leu-Thr-(OtBu)) [146] *etc.*

Several nonpeptide compounds showing potent and selective δ agonist activity *in vitro* were developed. These include TAN-67 [147], the racemic compound BW373U86 [148], and its chemically modified enantiomer SNC 80 [149]. These compounds produced *in vivo* analgesic effects when administered *i.th.* or *i.c.v.* but showed no analgesic activity when given systemically.

1.2.3. Development of peptide ligands

Numerous efforts are made to obtain more selective opioid receptor ligands through synthesis of opioid peptide analogues that are developed on the basis of the following design principles:

> ➢ substitution, deletion or addition of natural or artificial amino acids

- concept of conformational restriction of opioid peptides through various appropriate intramolecular cyclizations

- design of bivalent compounds containing two opioid receptor ligands separated by a spacer of appropriate length and able to interact simultaneously with two receptor binding sites

Regarding these applications many efforts have been made to develop more selective opioid receptor ligands through synthesis of linear opioid peptides *e.g.* hundrends of enkephalin analogues have been prepared by substituting one or several amino acids. Because small linear peptides such as the enkephalins are flexible molecules this structural flexibility may be one of the reasons for the lack of receptor selectivity. Another approach of analogue design is based on conformational restriction through peptide cyclization via side chains of substituted amino acids *e.g.* DPDPE [150]. The replacement of special parts of the peptide backbone with other desirable structural elements might eliminate susceptibility to enzymatic degradation and to increase their absorption *e.g.* crossing through blood-brain barrier. Such compounds generally are referred to as peptide mimetics. An example is the peptide mimetic derived from the cyclic enkephalin analog H-Tyr-*cyclo*[D-Orn-Gly-Phe-Leu-] [151]. To bridge two opioid receptor binding sites is another application in the ligand design. By this technique various bivalent ligands containing two enkephalin-related peptides linked via their C-terminal carboxyl group through flexible spacers of varying length have been synthesized and pharmacologically tested. It has been observed that, at a certain spacer length the affinity and selectivity for δ-receptors increased drastically [152]. These bivalent opioid peptide ligands might represent valuable tools for peptide research but are less attractive candidates for drug development because of their increased molecular size.

1.2.4. Delta opioid antagonists

δ-Opioid antagonists are of interest not only as pharmacological tools but they also may have therapeutic potential as well as immunosuppressants [44] and for the treatment of cocaine and alcohol addiction [153;154]. The first antagonists shown to exhibit significant selectivity for δ-opioid receptors were enkephalin analogues. The enkephalin analogue containing diallyltyrosine ICI 174864 was the first useful peptide antagonist for the opioid receptors [155]. This compound was also proved to be an inverse δ-agonist [156]. A widely used nonpeptide δ-opioid antagonist is the naltrexone derivative naltrindole (NTI) [157] which has a subnanomolar affinity but only limited δ-selectivity. In higher doses this compound acts as an agonist at μ-receptors in mice.

1.2.5. The TIPP opioid peptides

Recently, a new series of highly potent, δ-receptor selective opioid antagonists have been developed based on the N-terminal message domain of the endogenous enkephalins (Tyr-Gly-Gly-Phe) and amphibian derived dermorphins and deltorphins (Tyr-D-Met/Ala-Phe). The substitution of the 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic) in the second position of these peptides led to the development of the so called TIP(P) peptides [158]. Two prototypes in this class are TIPP (Tyr-Tic-Phe-Phe) and TIPP-ψ (Tyr-Ticψ[CH$_2$NH]-Phe-Phe) [159;160].

The importance of Tic is threefold: to constrain the peptide backbone, to restrain the side-chain and to provide an additional aromatic and hydrophobic residue in the peptide. Substitution of the L-Tic^2 in [Leu^5]-enkephalin, dermorphin and the κ-ligand dynorphin A$_{(1-11)}$ [161] converted these peptide agonists into antagonists, and remarkably the μ agonist dermorphin became a δ antagonist [162]. The aromaticity afforded by Phe was considered essential for opioid tetra- and heptapeptides [163;164], its elmination from TIP(P) led to the development of di- and tripeptide [165] antagonists. Importance of Tic was further investigated through systematic replacement but all changes were ineffective suggesting that the aromatic ring of Tic is necessary for δ-opioid receptor recognition. Augmentation of hydrophobicity and restriction of the aromatic ring of Tyr inspired the addition of methyl groups at the 2' and 6' position of tyrosine forming Dmt-Tic di- and tripeptides [166]. These peptides have elevated δ-affinity and selectivity. Relevance of the Dmt-Tic pharmacophore is evident in the heightened activity of Dmt-containing peptides. Further enhancement of the hydrophobic properties produced ligands with variable δ-opioid antagonist activities. Amidation of the di- and tripeptides enhanced μ binding with modest changes in δ affinity. This observation provided evidence on their potential application as bifunctional probes for δ and μ receptors [167]. The first known compound with mixed properties (μ-agonist/δ-antagonist) was the tetrapeptide amide Tyr-Tic-Phe-Phe-NH$_2$ (TIPP-NH$_2$) [168]. The formation of diketopiperazine can occur spontaneously under the acidic conditions during peptide synthesis and storage in peptides containing imino acids such as Pro or Tic. Several natural diketopiperazines are biologically active, including the morphiceptin-derived *cyclo*(Tyr-Pro), which had a modest μ affinity and weak agonist bioactivity [169] comparable to *cyclo*(Dmt-Tic). These data further substantiate the importance of the methyl groups on the phenol ring of Dmt to align the other ligand substituents with the receptor-binding domain, suggesting that the methyl groups influence the solution conformation of a peptide [170]. Diketopiperazines offer rigid scaffold for the generation of new

classes of opioid antagonists that lack charged groups and posses higher hydrophobic properties. The aromatic ring-to-ring distance within the peptide could also be important in the receptor-bound conformation of δ-opioid antagonists and provides the basis for the development of other lead compounds. Regarding the aromatic rings an analogous model was postulated for the parallel placement of aromatic rings of Dmt and Tic in a low energy model of H-Dmt-Tic-OH (Fig. 2.).

The successful strategy that enhanced affinity and biological potency was the substitution of Dmt for Tyr [166]. Furthermore the small size, overall shape and hydrophobic nature of Dmt-Tic pharmacophore compounds are advantageous features for therapeutic applicability. Their potential clinical application involves the ability to elicit δ-antagonism after systemic administration [171] *in vivo* because small opioid antagonists are known to act centrally [172].

Fig. 2.
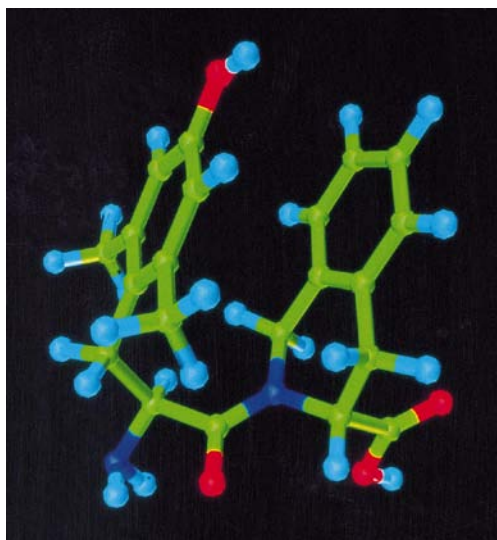
Low-energy model of Dmt-Tic-OH representing the pharmacophore features of a prototypic δ-opioid receptor antagonist. Those features are close proximity and approximate parallel orientation of the aromatic rings, cis orientation of the peptide bond (N-C') and gauche$^-$ (−60°) and gauche$^+$ (60°) orientation about C_α-C_β bonds of Dmt and Tic, respectively. Carbon, hydrogen, oxygen and nitrogen atoms are represented by green, light blue, red, and dark blue, respectively. Dmt, 2',6'-dimethyltyrosine-3-carboxilic acid. [173]

II. AIMS OF THE STUDIES

δ-Opioid antagonists are of interest not only as pharmacological tools but they might also have therapeutic potential as immunosuppressants or in the treatment of cocaine or alcohol addiction. The concept of conformational restriction has been successfully applied to the development of opioid peptide analogues with certain desired activity profiles. The incorporation of conformational restraints in opioid peptides has been shown to enhance their selectivity for a distinct receptor type as well to increase their stability against enzymatic degradation.

Systematic conformational restrictions of the amino acid residues lead to the discovery of a new series of δ-antagonist peptides, the so called TIPP peptides containing 1,2,3,4 tetrahydroisoquinoline-3-carboxilic acid (Tic) residue at the second position. Further modifications of these ligands resulted in more complex analogues which beside the high δ-antagonist profiles displayed µ-agonist/δ-antagonist characters as well. The aim of the present study was to test biochemically and functionally several newly developed TIPP derived peptide analogues acting mainly through δ-opioid receptors as well as to study their structure-activity relationship.

In the first part a structurally novel [^{3}H]Dmt-Tic-(2S,3R)βMePhe-Phe diastereomeric TIPP derived radiolabeled analogue was biochemically analysed. The main goals were:

➢ To have a novel opioid radioligand with improved activity and specificity.
➢ To measure its opioid activity in kinetic, equilibrium and competition binding studies.
➢ To compare its opioid binding properties with those of other well-known compounds labeling opioid receptors.

Furthermore the novel TIPP derived nonlabeled diastereomeric ligands were biochemically and functionally analysed:

➢ To investigate their binding properties to the native receptors.
➢ To examine the post-binding effects (G-protein activation) in the functional biochemical [^{35}S]GTPγS binding assay.
➢ To elucidate their intrinsic characteristics in native as well as in different cellular systems.

III. MATERIALS AND METHODS

3.1. Chemicals

3.1.1. Radiochemicals

[^{3}H]Dmt-Tic-($2S,3R$)βMePhe-Phe (58.06 Ci/mmol) [174] and [^{3}H]Ile^{5,6}-deltorphin-II (27 Ci/mmol) were prepared in the Isotope and Peptide Laboratory of the Biological Research Center, Szeged [175]. [^{3}H][D-Ala^2,N-MePhe^4,Gly^5-ol]enkephalin (DAMGO; 51 Ci/mmol) was purchased from DuPont de Nemours (Wilmington, Del., USA). Guanosine-5'-O-(3-γ[^{35}S]thio)triphosphate ([^{35}S]GTPγS) (37-41 Tbq/mmol) was purchased from the Isotope Institute Ltd. (Budapest, Hungary).

3.1.2. Peptides

The stereoisomeric TIPP derived (βMeCha^3 or βMePhe^3) peptides and Ile^{5,6}-deltorphin-II were synthesized in the Isotope Laboratory of the Biological Research Center (Szeged, Hungary) or in the Department of Organic Chemistry of the Free University of Brussels (Belgium). Unlabelled DAMGO was purchased from Bachem Feinbiochemica, (Budendorf, Switzerland).

3.1.3. Other chemicals

Bovine serum albumin (BSA), ethylenediamine-tetraacetic acid (EDTA), Guanosine 5'-diphospate (GDP), Guanosine-5'-O-(3-thiotriphosphate) GTPγS, Dulbecco's modified Eagle's medium were products of Sigma-Aldrich (St. Louis, MO, USA). All other reagents used in this study were of the highest purity available.

3.2. Animals

Inbred Wistar rats (250-300 g body weight, Animal House of the Biological Research Center 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/EEC) and the Hungarian Act for the Protection of Animals in Research (1998/XXVIII.tv. 32.§).

3.3. Cell lines

CHO cell line stably transfected with the human μ-opioid receptor (MOR) gene, 3.5 pmol/mg membrane protein [176] and CHO cells stably expressing mouse δ-opioid receptor (DOR), 4.3 pmol/mg membrane protein, have been described earlier [177].

3.4. Membrane preparations

3.4.1. Preparation of rat brain membranes

A crude membrane fraction from Wistar rat forebrains were prepared according to a method of Pasternak with small modifications [178]. Briefly, the brains without cerebella were rapidly 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 40000 g for 20 min at 4°C and the resulting pellet was resuspended in fresh buffer and incubated for 30 min at 37°C to remove any endogenous opioids. 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. Before use the membranes were thawed and resuspended in 50 mM Tris-HCl buffer (pH 7.4) and centrifuged (40000 g at at 4ºC, for 20 min) to remove sucrose and used immediately in binding assays.

3.4.2. Membrane preparation from cell cultures

Chinese hamster ovary (CHO) cells stably expressing the cloned human μ- and mouse δ- opioid receptors were grown in Dulbecco's modified Eagle's medium (catalog no. D 7777, Sigma) with F12 (Ham's) nutrient mixture HEPES modification (cat. no. N 4138 Sigma) 1:1 mix (DMEM:F12; 1:1) supplemented with 10% fetal calf serum (FCS), 2 mmol/l L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin and 0.4 mg/ml geneticin G418 (cat. no. A 1720 Sigma). Cells were maintained in culture at 37°C in a CO_2 incubator with 5% CO_2. Membranes were prepared from confluent cultures. Cells were harvested and homogenized in PBS for 15s with a polytron homogenizer in an icebath, followed by centrifugation at 1,000 g for 10 min The pellet was resuspended in 20 ml TEM (0.1 mM TrisHCl, 5 mM EDTA, 1 mM mercaptoethanol

pH 7.6) and centrifuged at 38,000 *g* for 20 min The final pellet was reconstituted in a small volume of a 50 mM Tris-HCl pH 7.6 buffer and stored in aliquots at −70°C until use.

3.5. *In vitro* biochemical and functional characterization of the stereomeric TIPP peptides

3.5.1. Radioligand binding assay

Radioligand binding assays are a relatively simple but extremely powerful tool for studying receptors. They allow an analysis of the interaction of hormones, neurotransmitters, growth factors, and related drugs with the receptors, studies of receptor interactions with second messenger systems, and characterization of regulatory changes in receptor number, subcellular distribution, and physiological function [179].

Two basic types of assays utilize radioligands. The first, direct binding assays, measure the direct interaction of a radioligand with a receptor. The second, indirect binding assays, measure the inhibition of the binding of a radioligand by an unlabelled ligand to deduce indirectly the affinity of receptors for the unlabelled ligand.

According to the experimental design, the following setups of radioligand assays were used:

3.5.2. Saturation binding experiments

Saturation binding experiments measure specific binding at equilibrium at various concentrations of the radioligand to determine the receptor number and affinity. Specific binding is plotted against the concentration of radioligand 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 the radioligand is also crucial fur further characterization of the unlabeled peptide analogues. Mostly the analysis of radioligand binding experiments is based on a simple model, called the law of mass action, which describes ligand-receptor intractions [180]. Because these kind of experiments used to be and are analyzed with linear Scatchard plots (more accurately attributed to Rosenthal), they are also called "Scatchard experiments" [181;182].

In this assay 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 δ_mCHO membranes were thawed and homogenized with a syringe and used directly in the binding assay. Membranes

were incubated for 35 min, 25°C in a final volume of 1 ml with increasing concentrations of [^{3}H]($2S,3R$)βMePhe^3-DYTPP (0.05 - 8 nM). Non-specific binding was determined in the presence of 10 μM of unlabelled naloxone. 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. After filtration filter fibers were dried and bound radioactivity was measured in UltimaGold™ scintillation cocktail using Packard Tricarb 2300TR Liquid Scintillation Analyzer with 58% counting efficiency. Total binding was measured in the presence of the radioligand, non-specific binding was determined in the presence of 10 μM unlabeled naloxone and subtracted from total binding to calculate the specific binding. Protein concentration was measured photometrically by the Bradford method with bovine serum albumin as standard [183].

3.5.3. Competition binding experiments

Competitive binding experiments measure the binding of a single concentration of labeled ligand in the presence of various concentrations of unlabelled ligand. In these experiments the equilibrium inhibition constants (K_i) are determined for the unlabelled ligand. This value can be obtained from the IC_{50} value using Cheng-Prusoff equation $K_i = IC_{50}/(1+[L]/K_d)$ where [L] is the concentration of radioactive ligand used and K_d is the affinity of the radioactive ligand for the receptor [184].

The frozen rat brain membrane aliquots were thawed in these assays and washed by centrifugation (40 000 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. Membranes were incubated with gentle shaking for 35 min, 25°C in a final volume of 1 ml with unlabelled compounds (10^{-5} to 10^{-11} M), and ≈ 1 nM radioligand. Non-specific binding was determined in the presence of 10 μM of unlabeled naloxone. The reaction was terminated by washing three time with ice could 5-ml portions of ice-cold 50 mM Tris-HCl buffer and bound radioactivity was measured in the same way as described above. All assays were performed in duplicate and repeated several times and data are expressed as means ± standard error of the mean (S.E.M). Experimental data were analyzed by GraphPad Prism (version 3.0 and 4.0) program [185].

3.6. [^{35}S]GTPγS Binding Experiments

Agonist stimulated [^{35}S]GTPγS binding assay is a widely used functional and biochemical *in vitro* assay used for determination of basic pharmacological characteristics and relative efficacy of ligands. The key step in this process is induced guanine nucleotide exchange on the G-protein α-sunbunit. This results in replacement of GDP by GTP followed by conformational rearrangements and dissociation of the G-protein α-subunit from the βγ complex. This exchange process can be monitored by using a non-hydrolysible analogue of GTP that contains γ-thiophosphate bond ([^{35}S]GTPγS). Guanine nucleotide exchange is a very early event in the signal transduction cascade thus represents a good tool for monitoring because is less influenced by other cellular processes. [^{35}S]GTPγS binding assay is mostly feasible using for GPCRs interacting with pertussis-toxin-sensitive G_i family G proteins. The accumulation of [^{35}S]GTPγS on the membrane allows the measurement by counting of the amount of [^{35}S]-label incorporated [186].

Cellular membrane fractions ($\approx$ 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 μM NaCl; pH 7.4) containing [^{35}S]GTPγS (0.05 nM) and increasing concentrations (10^{-9}-10^{-5} M) of the compounds tested in the presence of 30 μM GDP in a final volume of 1 ml. Binding was determined using rat brain- or CHO cell membranes expressing human μ- (μ$_h$CHO cells) or mouse δ-receptors (δ$_m$CHO cells). Total [^{35}S]GTPγS 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 the addition of [^{35}S]GTPγS and terminated by vacuum filtration through Whatman GF/B filters with a Brandel M24R cell harvester where bound and free [^{35}S]GTPγS were separated. Filters were washed with 3x5 ml ice-cold 50 mM Tris-HCl buffer (pH 7.4) and the bound radioactivity was detected in Packard UltimaGOLD™ scintillation cocktail with a Packard TriCarb 2300TR counter. Stimulation is given as percent of the specific [^{35}S]GTPγS binding observed in the absence of receptor ligands (basal activity). Data were calculated from three independent experiments performed in triplicates, and analyzed by sigmoid dose-response curve fit option of GraphPadPrism [185]. Specific binding in cpm values were taken as 100%, defining basal G-protein activity. Agonist potencies were expressed as EC_{50} or 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 cellular system. E_{max} of agonists is expressed as the maximal stimulation of [^{35}S]GTPγS over the basal in percentage.

IV. RESULTS

4.1. *In vitro* biochemical characterization of TIPP (Tyr-Tic-Phe-Phe) derived tetrapeptides

Ten novel analogues from the potent δ-opioid receptor selective tetrapeptide family (TIPP series), were studied *in vitro* and their biochemical and functional characterizations are described. Detailed structural information of the peptides, including Cahn-Ingold-Prelog (CIP) configurations of the βmethyl amino acids are summarized in Table 2. These studies include radioligand and competition binding as well as functional $[^{35}S]GTP\gamma S$ binding assays in different cellular systems.

Table 2. The novel tetrapeptide analogues used in this study.

No.	Amino acid sequence	Chirality	Abbreviated name
	Reference compound *H-Tyr-Tic-Phe-Phe-OH*		**TIPP**
1	H-Dmt-Tic-(2R,3S)βMePhe-Phe-OH	D-*threo*	(2R,SR)βMePhe[3]-**DYTPP**
2	H-Dmt-Tic-(2S,3R)βMePhe-Phe-OH *In tritiated form correspondingly*	L-*threo*	(2S,3R)βMePhe[3]-**DYTPP**
3	H-Dmt-Tic-(2R,3S)βMeCha-Phe-OH	D-*threo*	(2R,3S)βMeCha[3]-**DYTCP**
4	H-Dmt-Tic-(2S,3R)βMeCha-Phe-OH	L-*threo*	(2S,3R)βMeCha[3]-**DYTCP**
5	H-Dmt-Tic-(2R,3R)βMeCha-Phe-OH	D-*erythro*	(2R,3R)βMeCha[3]-**DYTCP**
6	H-Dmt-Tic-(2S,3S)βMeCha-Phe-OH	L-*erythro*	(2S,3S)βMeCha[3]-**DYTCP**
7	H-Dmt-Tic-(2R,3S)βMeCha-Phe-NH$_2$	D-*threo*	(2R,3S)βMeCha[3]-**DYTCP**-amide
8	H-Dmt-Tic-(2S,3R)βMeCha-Phe-NH$_2$	L-*threo*	(2S,3R)βMeCha[3]-**DYTCP**-amide
9	H-Dmt-Tic-(2R,3R)βMeCha-Phe-NH$_2$	D-*erythro*	(2R,3R)βMeCha[3]-**DYTCP**-amide
10	H-Dmt-Tic-(2S,3S)βMeCha-Phe-NH$_2$	L-*erythro*	(2S,3S)βMeCha[3]-**DYTCP**-amide

4.1.1. Radioligand Binding Experiments

4.1.1. Determination of appropriate conditions for equilibrium binding studies with $[^3H](2S,3R)\beta$MePhe-DYTPP

To determine appropriate conditions for equilibrium binding studies of $[^3H](2S,3R)\beta$MePhe[3]-DYTPP, the temperature dependence was first studied using rat brain membrane homogenates. Specific binding and specific/non-specific binding ratio of $[^3H](2S,3R)\beta$MePhe[3]-DYTPP at 0°C and 35°C was much less than at 25°C (data not shown), therefore all subsequent binding experiments were conducted at 25°C.

4.1.2 Kinetic experiments

Time-dependence of binding interaction was examined by incubating the rat brain membrane preparations with [^{3}H](2S,3R)βMePhe^3-DYTPP until equilibrium has been achieved. Kinetic studies revealed relatively rapid, monophasic association of the labeled tetrapeptide to the opioid receptors in the membrane preparations used (Fig. 3.). The association rate constant (K_{on}) for [^{3}H](2S,3R)βMePhe^3-DYTPP was determined by performing the association binding experiments at several different concentration of radioligand with same protein concentrations in each experiments and was calculated using non-linear regression plots, affording an association rate constant (K_{on}) of   0.122 ± 0.01 min^{-1} nM^{-1} (data not shown). The specific binding of [^{3}H](2S,3R)βMePhe^3-DYTPP to rat brain membranes reached the steady state condition after 35 min of incubation at 25°C.

Non-specific binding remained unchanged within the time intervals tested. Only specific binding data are shown.
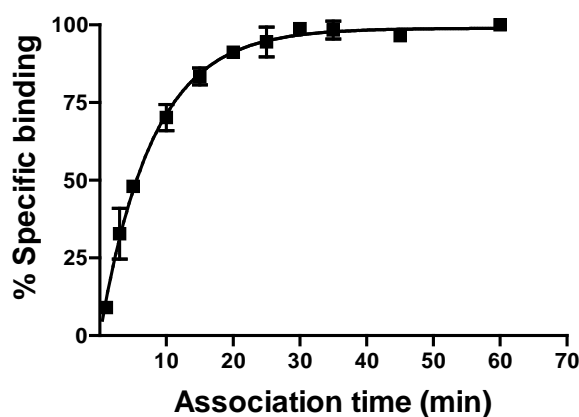
Fig. 3. Association kinetics of [^{3}H](2S,3R)βMePhe^3-DYTPP binding determined at 25C. Membranes from rat brain were incubated with 0.5 nM radioligand for various periods of time. Specific binding values (cpm) were converted into % specific binding, binding value at 60 min was taken as 100%. Points represent the means ± S.E.M. of three separate experiments each performed in duplicates.

4.1.3. Saturation binding experiments

In the equilibrium saturation experiments, various concentrations (0.05-8 nM) of the radioligand were incubated with the membrane. Binding of [^{3}H](2S,3R)βMePhe^3-DYTPP to rat brain

membranes (Fig. 4. left panel) and δ_mCHO (Fig. 4. right panel) at 25°C was saturable and of high affinity. The equilibrium dissociation constant (K_d), which measures the affinity of a ligand for a receptor and the maximal number of binding sites (B_{max}) were calculated by non-linear regression analysis of the saturation binding data (Fig. 4.).
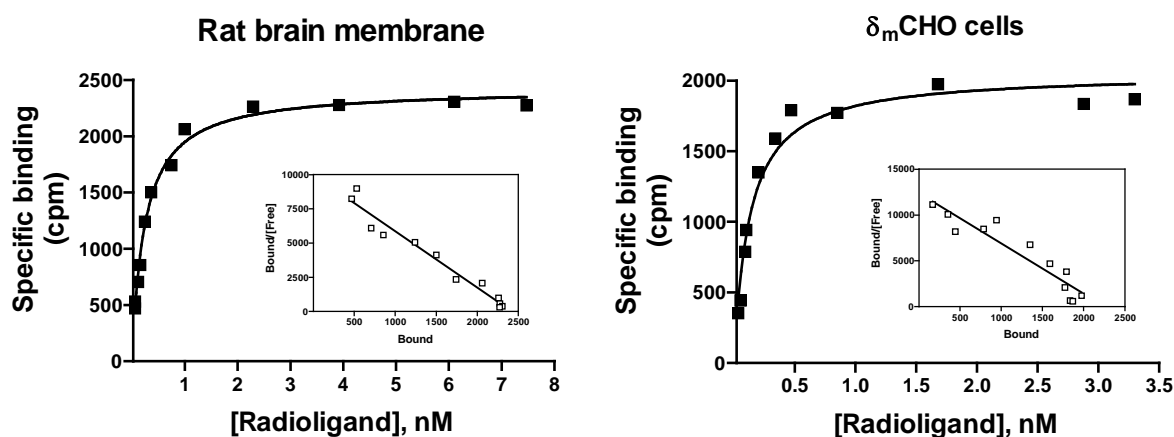
Fig. 4. Saturation binding isotherms and Scatchard plots (inset) of [^{3}H](2*S*,3*R*)βMePhe^3-DYTPP binding in rat brain membranes (left panel) and membranes of δ_mCHO cells expressing δ-opioid receptors (right panel). Membranes were incubated with increasing concentrations of the radioligand in the absence or presence (for non-specific binding) of 10 μM naloxone for 35 min at 25°C. Points illus trate duplicate values of specific binding in a single experiment that was repeated three times in both preparations.

The results showed the existence of a single binding component with a K_d value of 0.3 ± 0.1 nM in rat brain membrane and $K_d = 0.14 \pm 0.02$ nM in δ_mCHO membrane preparations. The binding capacity (B_{max}) was 127.3 ± 23.6 fmol/mg protein and 2730 ± 90 fmol/mg protein for rat brain membranes and δ_mCHO cell membranes respectively. These results are in good agreement with the literature data [187;188]. The B_{max} value of δ_mCHO is significantly higher (p<0.05, unpaired t-test) than of rat brain membranes. Non-specific binding of [^{3}H](2*S*,3*R*)βMePhe^3-DYTPP to rat brain preparations was less than 30% of total binding at radioligand concentrations close to the K_d value.

4.1.4. Stereoselectivity of [³H](2*S*,3*R*)βMePhe³-DYTPP

Stereoselectivity of [³H](2*S*,3*R*)βMePhe³-DYTPP binding sites was observed using optically active enantiomeric compounds (−)levorphanol and (+)dextrorphan (Table 3) as well as (−)N-allyl-normetazocine (SKF10,047) and (+)N-allyl-normetazocine in heterologous competition studies (Fig. 5.). The opiate agonist (−)levorphanol exhibited high affinity (K_i value of 31 nM) toward the [³H](2*S*,3*R*)βMePhe³-DYTPP binding sites, whereas its pharmacologically inactive dextrorotatory stereoisomer displayed only very low affinity (Ki > 10000 nM).
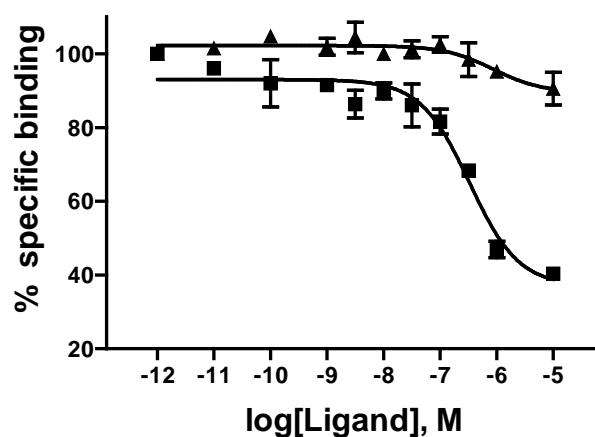
Fig. 5. Stereospecifity of [³H](2*S*,3*R*)βMePhe³-DYTPP binding. Rat brain membranes were incubated with 0.5 nM [³H](2*S*,3*R*)βMePhe³-DYTPP in the presence of increasing concentrations of (−)N-allyl-normetazocin (■) and (+)N -allyl-normetazocin (▲). Each value represents the means ± S.E.M. of at least three experiments each performed in duplicates.

4.1.5. Competition experiments by the use of various unlabeled ligands to displace bound [³H](2S,3R)βMePhe³-DYTPP

The equilibrium competition experiments were performed by the use of various unlabeled ligands selective for μ-, δ-, or κ-receptors to displace bound [³H](2S,3R)βMePhe³-DYTPP (Fig. 6.).
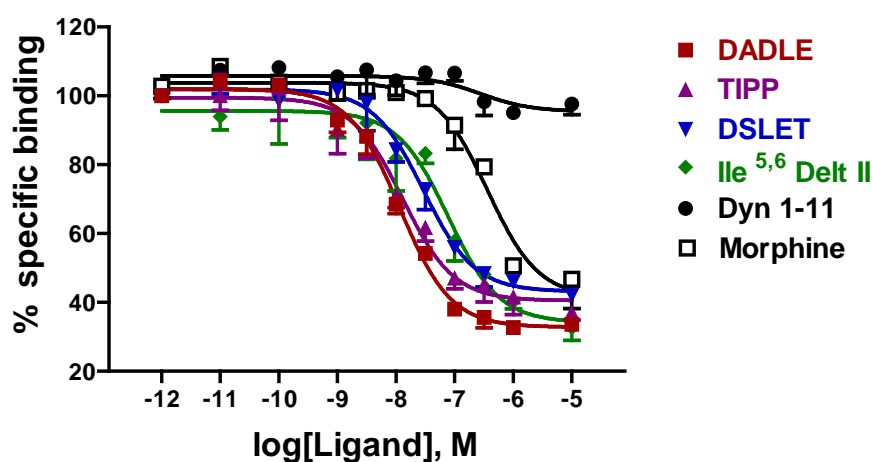
Fig. 6. Inhibition of [³H](2S,3R)βMePhe³-DYTPP binding to rat brain membranes by various ligands. Rat brain membranes were incubated with 0.5 nM [³H](2S,3R)βMePhe-DYTPP in the presence of increasing (10^{-12} to 10^{-5} M) concentrations of unlabeled (■) [D-Ala², D-Leu⁵]enkephalin (DADLE); (▲) Tyr-Tic-Phe-Phe-OH (TIPP); (▼) [D-Ser², Leu⁵]enkephalin-Thr⁶ (DSLET), (♦) Ile⁵,⁶-Deltorphin-II, (●) Dynorphin 1-11 and (□) Morphine for 35 min at 25 ˚C. Each value represents the means ± S.E.M. of at least three experiments each performed in duplicates.

Several δ-selective opioid ligands such as the prototype agonist peptide Leu-enkephalin [189], the frog skin opioid deltorphin II [138;190], the selective TIPP peptides and Naltrindole (NTI) [157] competed for [³H]Dmt-Tic-(2S,3R)βMePhe³-DYTPP binding sites in rat brain membrane preparations with much higher affinities than the μ- selective ligands such as DAMGO [191], endomorphin-1 and -2 [136], and the κ-specific ligands norbinaltorphimine (nor-BNI) [136;192] and D-Ala³-Dynorphin-(1-11)-NH₂ [193]. The data represented as equilibrium inhibition constant (K_i) values are shown in Table 3.

Table 3. Potencies of various opioid ligands in inhibiting [^{3}H]($2S,3R$)βMePhe^3-DYTPP binding to rat brain membranes.

Ligands		Selectivity	Ki (nM)
Peptides			
Dmt-Tic-(2S,3R)βMePhe-Phe;	(L-*threo*)	(δ-antagonist)	0.75 ± 0.05
Dmt-Tic-(2R,3S)βMePhe-Phe;	(D-*threo*)	(δ-antagonist)	1.92 ± 0.1
Dmt-Tic-(2R,3S)βMeCha-Phe;		δ-antagonist	19.4 ± 0.15
Dmt-Tic-(2S,3R)βMeCha-Phe;		δ-antagonist	27 ± 5.5
Tyr-Tic-Phe-Phe;	TIPP	δ-antagonist	5 ± 0.05
Tyr-Tic-Ψ[CH$_2$-NH]Phe-Phe;	TIPPψ	δ-antagonist	8.3 ± 1.2
Tyr-Gly-Gly-Phe-Leu;	Leu-enkephalin	δ-agonist	89 ± 36
[D-Ser^2,Leu^5,Thr^6]enkephalin;	DSLET	δ-agonist	9.5 ± 0.4
[D-ala^2, D-Leu^5]-enkephalin;	DADLE	δ-agonist	4.1 ± 0.1
Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH$_2$;	Deltorphin II	δ-agonist	6.5 ± 0.2
Ile^{5,6}-deltorphin-II		δ-agonist	37.9 ± 5
cyclo[D-Pen^2,D-Pen^5]enkephalin;	DPDPE	δ-agonist	18.6 ± 0.05
[D-Ala^2,$_{(N-Me)}$Phe^4,Gly^5-ol]enkephalin;	DAMGO	μ-agonist	541 ± 8
Tyr-Pro-Trp-Phe-NH$_2$;	Endomorphin-1	μ-agonist	>10000
Tyr-Pro-Phe-Phe-NH$_2$;	Endomorphin-2	μ-agonist	>10000
Dmt-DALDA		μ-agonist	166 ± 28
D-Ala^3-Dynorphin-(1-11)-NH$_2$		κ-agonist	>10000
Non-peptide ligands			
Naltrindole		δ-antagonist	1.1 ± 0.1
Cyprodime		μ-antagonist	505 ± 377
Morphine		μ-agonist	539 ± 41
(−)Levorphanol		μ-agonist	31 ± 4.4
(+)Dextrorphan		Inactive stereomer	>10000
Norbinaltorphimine		κ-antagonist	>10000
(−)N-allyl-normetazocine		κ/σ agonist/antagonist	196 ± 6.5
(+)N-allyl-normetazocine		Inactive stereomer	>10000

Rat brain membranes were incubated for 35 min at 25˚C with 0.5-1 nM [^{3}H]($2S,3R$)βMe-Phe^3-DYTPP in the presence of increasing concentrations of various opioid ligands. IC_{50} values obtained by nonlinear regression were converted into equilibrium inhibitory constant (K_i) values on the basis of the Cheng-Prusoff equation. Values represent the means ± S.E.M. of at least three experiments each performed in duplicates.

4.1.6. Equilibrium competition experiments

Opioid affinities of the nonlabeled DYTCP analogues (βMeCha[3]) for δ- and μ-opioid receptors were performed with the highly δ-selective [3H]Ile[5,6]-deltorphin-II [175], while for μ-opioid receptors the affinities were determined by displacing the equilibrium binding of the prototype μ-specific [3H]DAMGO [194] using rat brain membrane fractions. The βMeCha[3]-DYTCP analogues competed reversibly with [3H]DAMGO (Fig. 7. left panel) but they were more potent in competing [3H]Ile[5,6]-deltorphin-II binding (Fig. 7. right panel) exhibiting K_i values in the nanomolar range.

Fig. 7. Competitive inhibition of [3H]DAMGO (left panel) and [3H]Ile[5,6]-deltorphin-II (right panel) binding to rat brain membranes by unlabelled βMeCha[3]-DYTCP peptides. Membranes were incubated with 0.9-1.8 nM [3H]DAMGO and 0.5-1 nM [3H]Ile[5,6]-deltorphin-II for 45 min at 35 °C in the presence of various (10^{-12} - 10^{-5} M) concentrations of the competitors. Data shown on the figure were normalized by the protein content and expressed as percent specific binding. Each value represents the means ± S.E.M. of at least three independent experiments performed in duplicates.
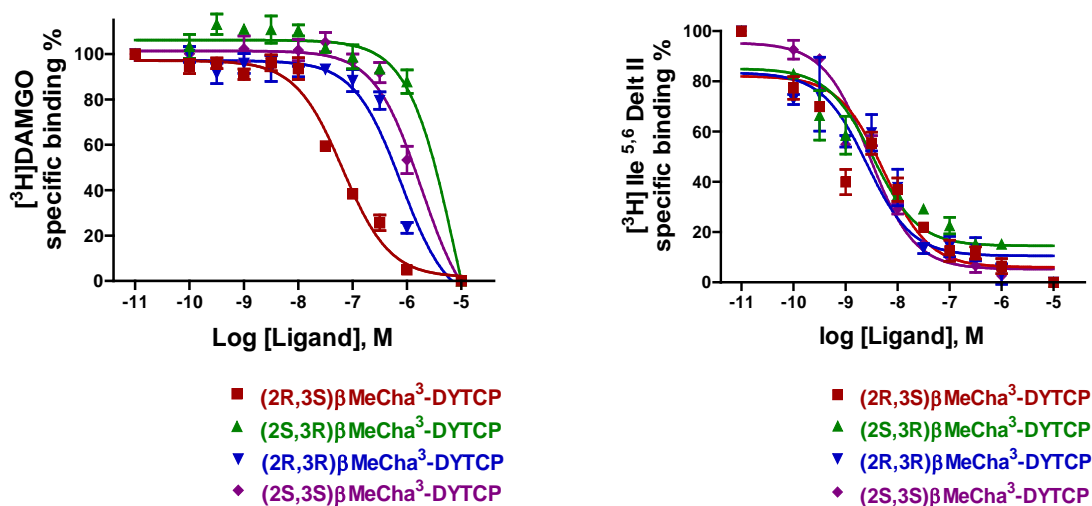
As for the βMeCha[3]-DYTCP-amide analogues they were competing more efficiently with [3H]DAMGO, being at least two times more potent compared to those retaining an unmodified C-terminus end. All of the above mentioned competition curves were satisfactorily fitted to the single-site binding model. The K_i values are shown in Table 4.

Table 4. Displacement binding data of the DYTCP tetrapeptides in rat brain membranes.

LIGANDS	Receptor binding (K_i, nM)		
	K_i^μ $N=3$	K_i^δ $N=3$	K_i^μ/K_i^δ
H-Tyr-Tic-Phe-Phe-OH (TIPP)	244 ± 13	32 ± 1	13.7
(2R,3S)βMeCha[3]-DYTCP	55 ± 0.63	1.49 ± 0.14	36.9
(2S,3R)βMeCha[3]-DYTCP	5776 ± 1636	60 ± 0.95	92.2
(2R,3R)βMeCha[3]-DYTCP	575 ± 79.6	2 ± 0.58	287.5
(2S,3S)βMeCha[3]-DYTCP	1342 ± 149	0.48 ± 0.05	2796
(2R,3S)βMeCha[3]-DYTCP-amide	202 ± 36.6	7 ± 1.3	28.8
(2S,3R)βMeCha[3]-DYTCP-amide	168 ± 10.9	4.1 ± 0.7	40.9
(2R,3R)βMeCha[3]-DYTCP-amide	289 ± 99.2	12.8 ± 2.6	22.5
(2S,3S)βMeCha[3]-DYTCP-amide	327 ± 106	4 ± 1.4	81.7

Receptor binding data are presented as the mean ± S.E.M. of two or three numbers of repetition from independent assays (**N**) listed in parentheses. K_i^μ**:** [³H]DAMGO. K_i^δ**:** [³H]Ile.[5,6]-deltorphin-II. The ratio of the affinities for δ and μ receptors (K_i^μ/K_i^δ) shows δ selectivity.

Interestingly enough in the case of two (2*S*,3*R*) and (2*R*,3*S*)βMeCha[3]-DYTCP analogues the competition curves with [³H]Ile[5,6]-deltorphin-II could be significantly better described (F-test comparison) by fitting to th two-site binding model (Fig. 8. left panel). The same phenomenon could be observed in the case of homologous competition of (2*S*,3*R*)βMePhe[3]-DYTPP in rat brain membranes (Fig. 8. right panel). The apparent presence of a second binding component suggests either interaction with physically different receptor types *e.g.* μ or δ, the presence of receptor homo- or heterodimerization or different affinity states *e.g.* G-protein coupled and uncoupled forms of the same receptor population. The existence of different affinity states is more likely, since the addition of specific G-protein reagent, non-hydrolysable GTP analogue 5'-guanylyl-imidodiphosphate (GppNHp; 1 μM) was able to inhibit the ultrahigh affinity sites by transforming the receptors into a lower, although still high affinity states. Thus, dose response curves are also shifted to the right and successfully analyzed by the single-site binding model.

Fig. 8. Heterologous competition curves of (▲) (2*S*,3*R*) and (■) (2*R*,3*S*)βMePhe[3]-DYTCP (left panel) and homologous competition curves for (■) (2*S*,3*R*)βMePhe[3]-DYTPP binding (right panel) in rat brain membranes. Samples were incubated with 0.9 nM [3H]Ile[5,6]-deltorphin-II for 45 min at 35 °C or 0.4 nM [3H](2*S*,3*R*)βMePhe[3]-DYTPP for 35 min at 25 °C in the presence of increasing (10^{-12} to 10^{-5} M) concentrations of unlabeled ligands. Data were normalized by the protein concentration and transformed into percent specific binding. Competition curves represent the results of the two-site fits (red filled symbols) and the competitions described by the one-site model (empty symbols) carried out in the presence of G-protein reagent (10^{-6} M, GppNHp). Mean values ± SEM of at least three experiments are given in each point, each performed in duplicate.
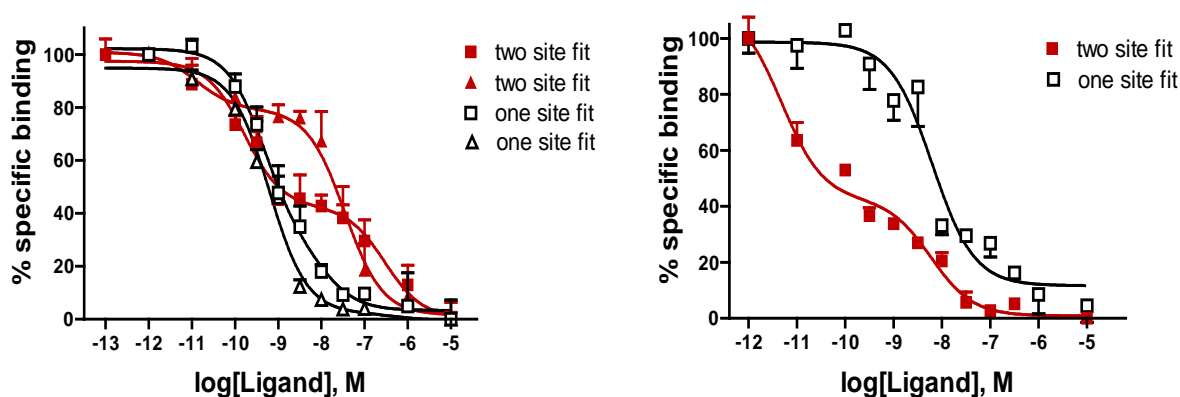
Affinities of the two βMePhe[3]-DYTPP ligands were also assessed in competition binding assays using [3H]DAMGO [195] as a μ-specific ligand and [3H]Ile[5,6]-deltorphin-II [175] as a δ-selective ligand. Both ligands displaced labeled [3H]Ile[5,6]-deltorphin-II from the δ-opioid sites, affording *Ki* values in nanomolar range. Competing with [3H]DAMGO the two stereomeric derivatives displayed rather different affinities toward μ-receptor binding sites as marked out from the K_i^{μ}/K_i^{δ} ratios. The (2*R*,3*S*)βMePhe[3]-DYTPP diastereomer displayed the [3H]DAMGO with higher potency comparing with the (2*S*,3*R*)βMePhe[3]-DYTPP stereomer, thus being 18 fold more δ-selective than the latter one. The K_i values for the compounds are presented in Table 5.

Table 5. Different affinities of diastereomeric βMePhe³-DYTPP tetrapeptides in displacement radioligand binding assays using rat brain membranes.

Compound	Receptor binding (K_i, nM)		
	K_i^{δ} $N=3$	K_i^{μ} $N=3$	K_i^{μ}/K_i^{δ}
(2S,3R)βMePhe³-DYTPP	0.55 ± 0.1	90 ± 0.5	164
(2R,3S)βMePhe³-DYTPP	0.47 ± 0.1	4.2 ± 0.1	9

Rat brain membranes were incubated with [³H]Ile⁵,⁶-deltorphin-II (0.8-1.2 nM, 45 min, 35°C) for δ receptor affinities, while μ receptors were tested with [³H]DAMGO (0.6-0.8 nM, 45 min, 35°C). Points represent means ± SEM of three independent repetitions of duplicate assays.

4.2. [³⁵S]GTPγS Binding Stimulation Assays

Activation of G-proteins by the agonist occupation of the G-protein coupled 7 TM receptors can be determined by *in vitro* GTPγS binding assays [196]. The pharmacological parameters like potency (EC$_{50}$) and efficacy (E$_{max}$) as well as the relative intrinsic activities of the tetrapeptides were determined on membranes from rat brains and CHO cell membranes stably expressing either μ- or δ-opioid receptors. These results were measured and compared to the effects exerted by the prototype μ-opioid receptor selective DAMGO [197;198] and the delta agonist Ile⁵,⁶-deltorphin-II [199;200] or (pClPhe⁴)-DPDPE [201;202]. All the tetrapeptides displayed very weak, insignificant stimulations of the G-proteins on the rat brain membranes, indicating their mostly antagonist properties and also the need of a more sensitive assay to distinguish their different partial agonist, inverse agonist or neutral antagonist properties. Therefore Chinese hamster ovary (CHO) cell lines transfected with either human μ- or mouse δ-opioid receptors were used. This cellular system proved to be more sensitive compared with those from rat brain membranes as the ligands exhibited very distinct profiles depending on their structural and stereomeric forms. In δ$_m$CHO cell homogenates expressing mouse δ-opioid receptors the βMeCha³-DYTCP tetrapeptide ligands displayed inverse agonist properties, the acidic C-termini derivatives showed more pronounced inverse agonist profiles (Fig. 9.). Interestingly both βMePhe³-DYTPP compounds exerted partial agonist effects in the δ-opioid receptor system

compared with the full-agonist (pClPhe[4])-DPDPE (not shown) which caused a maximal stimulation of [^{35}S]GTPγS binding of 334 ± 4 % E_{max} value. (Fig. 10.).
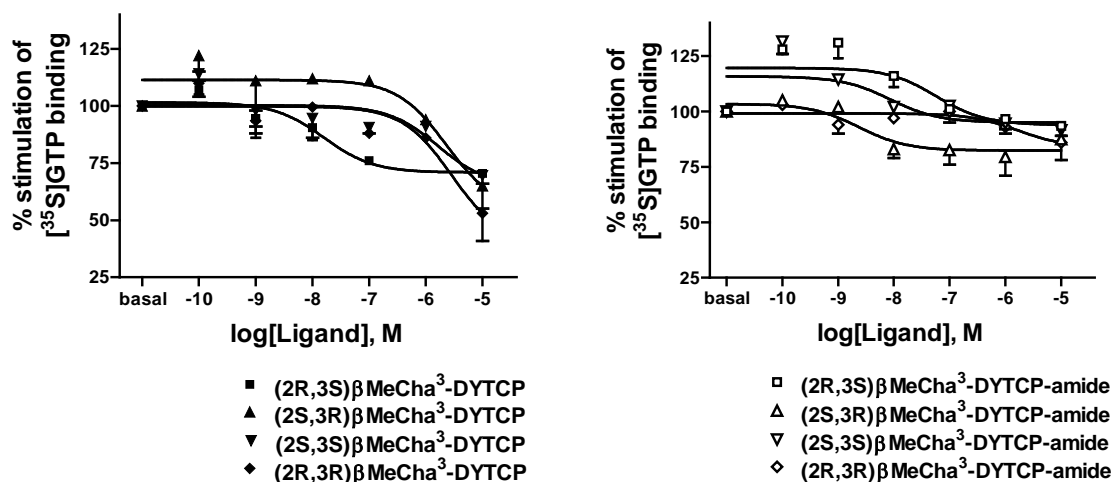
Fig. 9. Inverse agonist profiles of βMeCha[3]-DYTCP (■, ▲, ▼, ●) and βMeCha[3]-DYTCP-amide tetrapeptide ligands (□ △, ▽, ○) in δ$_m$CHO cell membranes stably expressing mouse δ-opioid receptors. Basal activity measured in the absence of receptor ligands is taken as 100% (Basal= 'Total binding' – 'Non-specific binding'). Non-specific binding is determined in the presence of 10 μM unlabelled GTPγS. Incubations were carried out for 60 min at 30°C. Points represent the means ± S.E.M. of at least three independent experiments, carried out in triplicates. Results of sigmoid dose-response curve fitting are plotted.

Concerning the two βMePhe[3]-DYTPP tetrapeptide derivatives on δ-receptor system the (2*R*,3*S*)βMePhe[3]-DYTPP induced a higher stimulation of the G-proteins affording an E_{max} value of 134% but comparing with the reference compound (pClPhe[4])-DPDPE still displayed a weak partial agonism.

Fig. 10. Stimulation of [^{35}S]GTPγS binding by various concentrations of (2*R*,3*S*)βMePhe^3-DYTPP (■) and (2 *S*,3*R*)βMePhe^3-DYTPP (▲) in δ$_m$CHO cell homogenates (left panel) and the same data shown with the reference compound (pCl-Phe^4)-DPDPE (▼) (right panel). Incubations were carried out for 60 min at 30°C. Note the scale differences in the Y-axis (indicated by bars). Points represent means ± SEM of at least three independent experiments, each performed in triplicates.
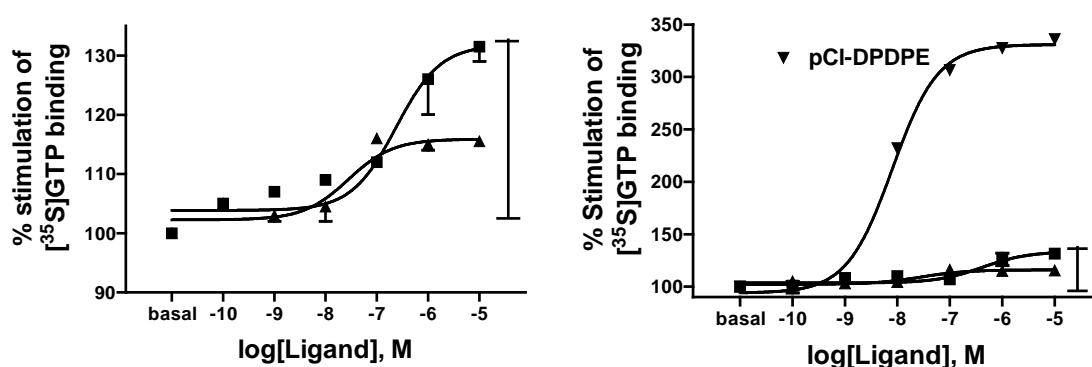
The Chinese hamster ovary (CHO) lines transfected with the human μ-opioid receptors were also used to determine whether the novel analogues do carry μ-agonist effects besides their δ-antagonist and weak δ-agonist characters. In the [^{35}S]GTPγS binding tests all βMeCha^3-DYTCP analogues displayed weak-to-moderate agonist properties (Fig. 11. left and right panel). The amidated βMeCha^3-DYTCP analogues are expected to exhibit higher μ-opioid receptor affinities which is best described in the case of (2*S*,3*R*)βMeCha^3-DYTCP affording an E_{max} value of 200%. The reference μ-receptor selective DAMGO ligand in this system exhibited a maximal stimulation of 280% above the basal (non-stimulated) value thus all the βMeCha^3-DYTCP compounds can be considered as partial agonists. The rank order of the efficacy in the case of both amidated and non-amidated tetrapeptides was (2*S*,3*R*)βMeCha^3-DYTCP-amide > (2*R*,3*S*)βMeCha^3-DYTCP-amide > (2*R*,3*R*)βMeCha^3-DYTCP-amide > (2*S*,3*S*)βMeCha^3-DYTCP-amide. The βMeCha^3-DYTCP derivatives with unchanged C-termini stimulated the [^{35}S]GTPγS binding with less potency and weaker maximal stimulating effects (highest stimulation - 170%).
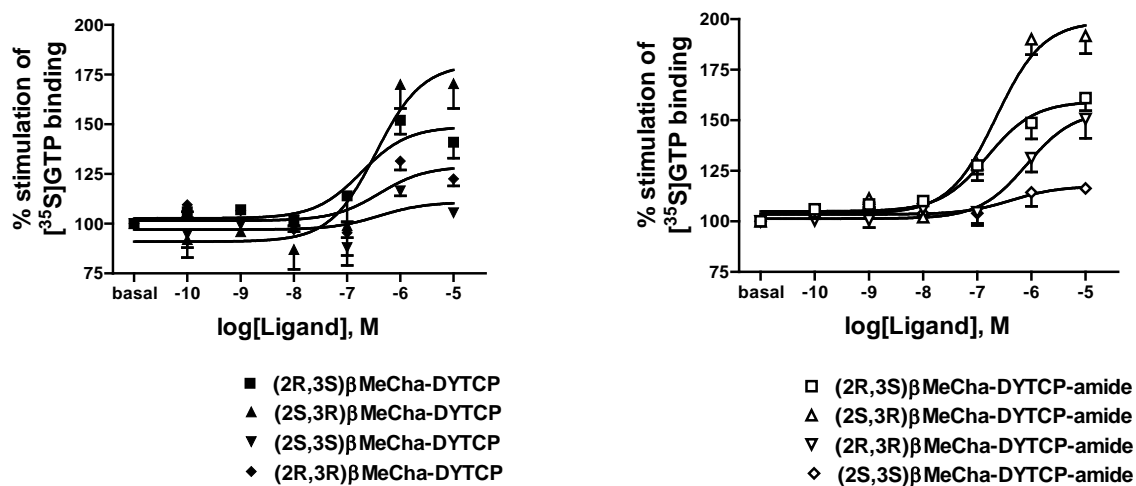
Fig. 11. Ligand-induced activation of [^{35}S]GTPγS specific binding in μ_hCHO cell membranes stably expressing human μ-opioid receptors by the stereomeric βMeCha^3-DYTCP tetrapeptides (filled symbols = carboxyl acid ends; empty symbols = amidated ends). Stimulation is given as a percentage of the non-stimulated (basal) level. Basal activity (Basal= 'Total binding' – 'Non-specific binding') is taken as 100%. Non-specific binding is determined in the presence of 10 μM unlabeled GTPγS. Incubations were carried out for 60 min at 30 °C. Points represent the means ± S.E.M. of at least three independent experiments, carried out in triplicates.

The maximal stimulations (E_{max}) and the potencies (EC_{50}) produced by the βMeCha^3-DYTCP stereomeric tetrapeptides in μ_hCHO cell lines are summarized in Table 6.

Table 6. [^{35}S]GTPγS binding data, stimulation of the G-proteins by the βMeCha^3-DYTCP tetrapeptides.

Compound	EC_{50} (nM)	% E_{max} *
DAMGO (full μ-agonist)	85.4 ± 11.5	280 ± 19
Ile^{5,6}-deltorphin-II (full δ-agonist)	no stim. [&]	No stim.
(2R,3S)βMeCha^3-DYTCP	299 ± 99 [&]	148.7 ± 7
(2S,3R)βMeCha^3-DYTCP	362.5 ± 83.5 [&]	169 ± 2.3
(2R,3R)βMeCha^3-DYTCP	447.5 ± 178.5 [&]	129.3 ± 1.2
(2S,3S)βMeCha^3-DYTCP	418.5 ± 205.5 [&]	111 ± 0.5
(2R,3S)βMeCha^3-DYTCP-amide	102 ± 10 [&]	161 ± 9.5
(2S,3R)βMeCha^3-DYTCP-amide	222 ± 4 [&]	199.7 ± 14
(2R,3R)βMeCha^3-DYTCP-amide	478.5 ± 158.5 [&]	159.7 ± 14
(2S,3S)βMeCha^3-DYTCP-amide	301.5 ± 42.5 [&]	121 ± 1.2

Data are presented as the mean ±SEM of two or three numbers of repetition from independent assays. Data were converted to the percentage of [^{35}S]GTPγS binding in the presence of increasing ligand concentrations, compared with basal binding in the absence of any drug.

* Per cent stimulation over the basal (100 %) level

& Measured in μ$_h$CHO cell membranes

In another set of experiments the βMePhe^3-DYTPP derivatives were tested in μ$_h$CHO cell lines stably expressing human μ-opioid receptors where the reference compound DAMGO behaved as a full agonist producing an E$_{max}$ value of 395%. Referring to this compound the two βMePhe^3-DYTPP tetrapeptides behaved as partial agonists displaying moderate maximal stimulations (Fig. 12.). The E_{max} and EC_{50} values from the [^{35}S]GTPγS assays are presented in Table 7.

Table 7. [^{35}S]GTPγS binding data, stimulation of the G-proteins by the βMePhe^3-DYTPP tetrapeptides.

Compound / preparation	EC_{50} (nM)	% E_{max} *
DAMGO (full μ-agonist)	87.5 ± 10.5 [&]	395 ± 6.3
(2S,3R)βMePhe^3-DYTPP	74.7 ± 3.2 [&]	195.4 ± 4
(2R,3S)βMePhe^3-DYTPP	512 ± 15 [&]	172.9 ± 0.05
pClPhe^4-DPDPE (full δ-agonist)	8 ± 0.8 [§]	331 ± 3.8
(2S,3R)βMePhe^3-DYTPP	27.7 ± 1.7 [§]	117.5 ± 0.7
(2R,3S)βMePhe^3-DYTPP	6.61 ± 4.7 [§]	133.6 ± 11.7

Data are presented as means ±SEM of two or three numbers of repetitions from independent assays. Data were converted to the percentage of [^{35}S]GTPγS binding in the presence of increasing ligand concentrations, compared with basal binding in the absence of any drug.

*   Per cent stimulation over the basal (100 %) level

&   measured in μ$_h$CHO cell membranes

§   measured in δ$_m$CHO cell membranes
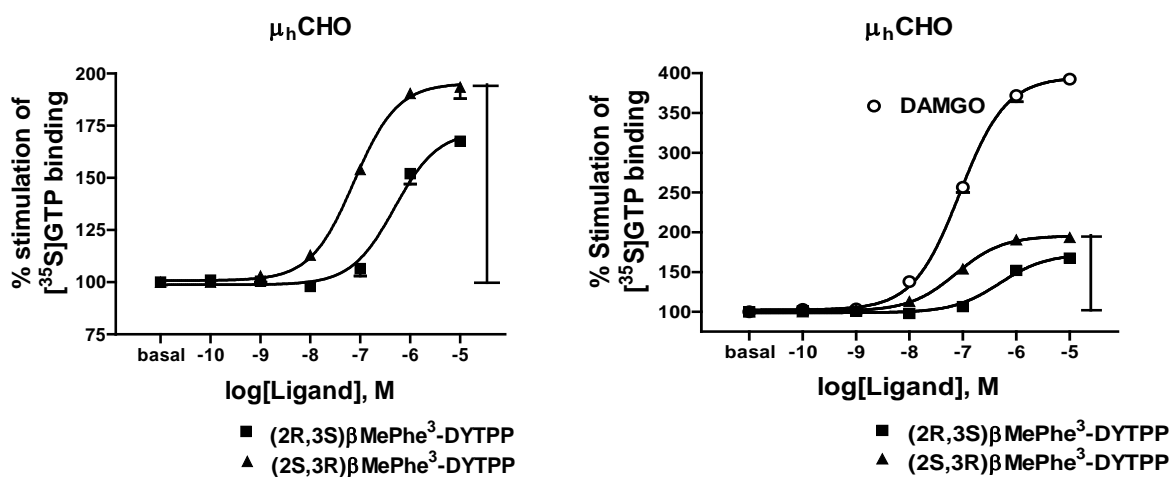
Fig. 12. Ligand-induced activation of [^{35}S]GTPγS specific binding in μ$_h$CHO cell membranes (left panel) by the two stereomeric βMePhe^3-DYTPP tetrapeptides and the same data with the reference compound DAMGO included (right panel). Stimulation is given as a percentage of the non-stimulated (basal) level. Basal activity (Basal= 'Total binding' – 'Non-specific binding') is taken as 100%. Non-specific binding is determined in the presence of 10 μM unlabeled GTPγS. Note the scale differences in the Y-axis (indicated by bars). Incubations were carried out for 60 min at 30°C. Points represent the means ± S.E.M. of at least three independent experiments, carried out in triplicates.

4.2.1. [^{35}S]GTPγS Binding Competition Assays

To elucidate the possible competitive antagonist properties of the novel tetrapeptide derivatives in the functional G-protein activation test, competition like experiments were performed. In these experiments the inhibition of agonist-stimulated [^{35}S]GTPγS binding was measured by co-administering with the stereomeric tetrapeptide ligands. Experiments were carried out in CHO cell membranes transfected with either human μ- or mouse δ-opioid receptors by co-incubating the samples with increasing concentrations of the tested tetrapeptides in the presence of a full agonist Ile^{5,6}-deltorphin-II; (pCl-Phe^4)-DPDPE or DAMGO at 10 μM concentration. In δ$_m$CHO cell homogenates expressing mouse δ-opioid receptors the βMeCha^3-DYTCP tetrapeptide derivatives effectively inhibited the δ-agonist stimulated (Ile^{5,6}-deltorphin-II) [^{35}S]GTPγS binding (Fig. 13.) in a concentration-dependent manner. The highest potency was obtained in the case of (2S,3R)βMeCha^3-DYTCP-amide analogue affording an EC$_{50}$ value of 2 ± 0.5 nM.

Concerning the two βMePhe[3]-DYTPP analogues effectively inhibited the δ-agonist (pClPhe[4])-DPDPE stimulated [^{35}S]GTPγS binding in concentration dependent manner in the δ-receptor system. They displayed slightly different 'inhibitory' EC_{50} values but there are no significant differences between the two values (p = 0.93, unpaired t-test) however the (2S,3R)βMePhe[3]-DYTPP seems to be to some extent more potent.
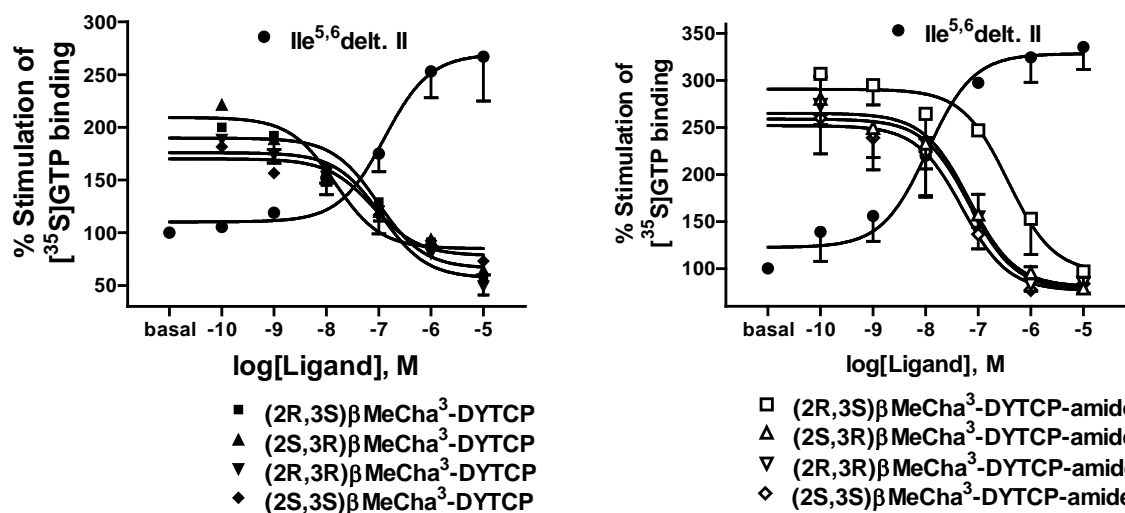
Fig. 13. Competitive inhibition of the agonist-stimulated [^{35}S]GTPγS binding to δ$_{m}$CHO cell membranes. Receptor-mediated G-protein activation was achieved by the addition of the δ-agonist Ile[5,6]-deltorphin-II (10 μM) and the subsequent "displacement" was measured in the additional presence of increasing concentrations of competing βMeCha[3]-DYTCP analogues (left panel) or βMeCha3-DYTCP-amide derivatives (right panel). Basal activity (Basal= 'Total binding' – 'Non-specific binding') is taken as 100%. Non-specific binding is determined in the presence of 10 μM unlabeled GTPγS. Points represent the means ± S.E.M. of at least three independent experiments, carried out in triplicates.

In CHO cell membranes stably expressing human μ-opioid receptors the two βMePhe[3]-DYTPP analogues were able to antagonize to some extent the agonist DAMGO induced G-protein stimulation compared with the βMeCha[3]-DYTCP analogues which only exhibited agonist properties in this system. In Fig. 14. the inhibitory profiles of the two βMePhe[3]-DYTPP derivatives in δ_mCHO (left panel) and μ_hCHO (right panel) cellular systems are presented.
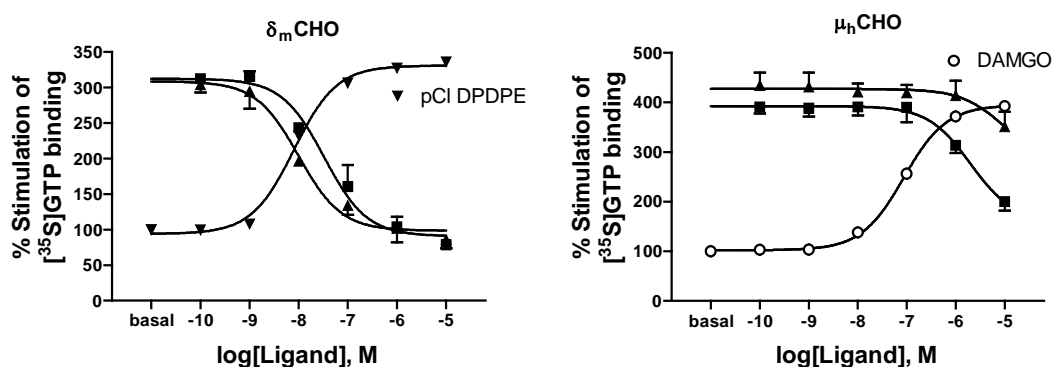
Fig. 14. Competitive inhibition of the agonist-stimulated [^{35}S]GTPγS binding to δ_mCHO (left panel) and μ_hCHO (right panel) cell membranes stably expressing mouse δ- and human μ-opioid receptors. Receptor-mediated G-protein activation was achieved by the addition of the δ-agonist (pClPhe[4])-DPDPE (10 μM) in δ_mCHO and the μ-agonist DAMGO in μ_hCHO cell membranes and the subsequent 'displacement' was measured in the additional presence of increasing concentrations of competing (2R,3S)βMePhe[3]-DYTPP (■) and (2S,3R)βMePhe[3]-DYTPP (▲) analogues. Stimulation is given as a percentage of the non-stimulated (basal) level. Basal activity measured in the absence of receptor ligands is taken as 100% (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 three independent experiments, carried out in triplicates.

V. DISCUSSION

Since endogenous opioid peptides are involved in moderation of a variety of physiological processes *e.g.* analgesia, tolerance and dependence, appetite, renal functions, gastrointestinal motility, learning and memory, respiratory depression *etc.* these processes can be mimicked by other opiates or non-endogenous ligands. Also the fact, that the endogenous ligands exert their effects via at least three different types of opioid receptors (μ, δ and κ) with limited selectivity, raised the consideration that highly selective opioid agonist or antagonist ligands might have therapeutic applications. Thus, if a ligand acts only at one receptor type, the side-effects mediated by other receptors would be minimized. Extensive investigations of endogenous peptide analogues (*e.g.* enkephalins) led to the concept that appropriate modifications of natural opioid peptides can produce active analgesic agents. It was also proven that ligands which exhibit concomitantly different intrinsic activities (*e.g.* agonist/antagonist profiles) and selectivities (μ/δ) might also reduce the development of opioid tolerance and dependence. By prototypic ligand design applying different structural changes (*e.g.* substitution with unnatural, modified or secunder amino acids, peptide bond reduction, side-chain or head-to-tail cyclizations) in the peptide analogues not only their affinities and selectivities are or can be increased but also their intrinsic profile can be changed. In opioid research the focus is on designing of delta-receptor antagonist ligands and also on ligands with more complex intrinsic properties like ones displaying simultaneously agonist/antagonist or inverse agonist characters on different receptor types.

In the present thesis biochemical characterization of a series of novel TIPP (Tyr-Tic-Phe-Phe) related, structurally modified stereomeric tetrapeptide ligands is described, reporting the results of various *in vitro* studies. These novel peptides were designed and synthesized in order to enhance their selectivity for one particular receptor type especially for delta receptors but also to obtain new "chimeric" compounds with bivalent characteristics presenting interaction with more than one receptor type. The first known peptide with mixed μ-agonist/δ-antagonist characters was the C-terminally amidated tetrapeptide Tyr-Tic-Phe-Phe-NH$_2$ [158;168] from the TIPP peptide series. From the novel set of stereoisomeric tetrapeptides included in this study one was chosen for radioligand labeling arising from the consideration that it should display high receptor affinity as well as good receptor selectivity toward delta-receptors. The non-labeled tetrapeptides containing β-methyl amino acid at the third position displaying different side-chain configurations so it is expected that their characters and properties vary in function of their configuration [203]. The importance of side chain residues at the third and fourth position were

studied in the case of H-Tyr-Tic-OH and H-Tyr-Tic-Ala-OH dipeptides whereas they presented weak interactions with delta receptors [204;205]. Moreover the N-terminal substitution of tyrosine by 2',6'-dimethyltyrosine (Dmt) is expected to enhance the potency and selectivity of the ligands acknowledged in dipeptide Dmt-Tic ligands as well [171]. The study of these assumptions was done by SAR (structure-activity relationship) studies using receptor binding and functional assays.

The binding properties of the new radiolabeled tetrapeptide $[^3H](2S,3R)\beta MePhe^3$-DYTPP were determined in rat brain membranes as well as in δ_mCHO cell lines stably transfected with δ-opioid receptor. The kinetic experiments showed that the formation of ligand-receptor complexes (association) occurred rapidly according to pseudo-first order kinetics. Equilibrium saturation experiments and Scatchard plots revealed that a single class of opioid-binding sites was labeled by this radioligand. The affinity for this site is in good agreement with the kinetically determined values for $[^3H](2S,3R)\beta MePhe^3$-DYTPP specific binding. The affinity and capacity values are in good agreement with those reported by using the parent compound radioprobes $[^3H]$TIPP [187]; $[^3H]$TIPP[ψ] [188] or $[^3H]$TICP[ψ] [206] in rat brain membranes. Similar values were described in rat brain membranes using various δ-opioid receptor selective radioligands such as $[^3H]$deltorphin-II [207]; $[^3H]$Ile^{5,6}-deltorphin-II [175] and $[^3H](pClPhe^4)$-DPDPE [208]. In the δ_mCHO cell membranes used in this study the number of $[^3H](2S,3R)\beta MePhe^3$-DYTPP receptor sites were significantly higher than in the rat brain membranes.

Heterologous competition assays with different opioid compounds revealed that $[^3H](2S,3R)\beta MePhe^3$-DYTPP specific binding is effectively inhibited by δ-selective ligands, whereas κ- and μ-specific ligands displayed lower affinity (the rank order of potency is: $\delta > \mu > \kappa$). This selectivity pattern and the affinities of the side-chain diastereomeric peptides verifies the previous observations obtained in CHO or C6 glioma cell membranes [209]. In the binding assays with $[^3H]$DAMGO and $[^3H]$Ile^{5,6}-deltorphin-II the two $\beta MePhe^3$ substituted non-labeled DYTPP analogues proves high δ affinities and also the high impact of the $\beta MePhe^3$ residue on the μ-affinity. In consideration of the side chain stereochemistry the tetrapeptides practically have similar δ receptor affinities and differing μ-affinities resulted in about 18 fold difference in the δ receptor selectivity.

The beta-methyl substitution of cyclohexylalanine in the third position led to a series of new TIPP (Tyr-Tic-Phe-Phe) derived stereomeric peptides containing 2',6'-dimethyltyrosine at the N-terminus as in the case of above mentioned peptides. The detailed structure-activity

studies of the DYTCP peptides with βMeCha[3] side chain, demonstrated the effects of steric and topographic constraints on the receptor binding affinity and selectivity [210;211]. As some of the earlier TIPP derived analogues containing a C-terminal carboxamide group displayed μ-receptor affinities [212] C-terminal amidation was performed in the case of βMeCha[3]-DYTCP ligands as well. Substitution of the carboxyl group to carboxamide on the C-terminus led to increased μ-receptor affinity and enhanced analgesic effects as shown previously with an enkephalin analogue [213]. The same increase in the μ-receptor affinity was expected in the case of the novel stereomeric compounds.

In the binding assays the tetrapeptide ligands displayed good δ-receptor affinities and moderate to week μ-receptor potencies. Overall, the amidated DYTCP peptide analogues exhibited slightly increased μ-receptor affinities compared with the analogues bearing free carboxyl acid at their C-termini. The (2R,3S)βMeCha[3]-DYTCP analogue presented the highest μ-receptor affinity whereas it displayed very good δ-receptor affinity, thus being an outstanding exception of the series. The mostly δ-receptor selective compounds were the *erythro* isomer (2S,3S)βMeCha[3]-DYTCP with a K_i value of 0.48 nM and the *threo* analogue (2R,3S)βMeCha[3]-DYTCP (K_i 1.49 nM) against [^{3}H]Ile[5,6]-deltorphin-II binding, but with substantially less affinity for μ-opioid sites labeled by [^{3}H]DAMGO in rat brain preparations.

A significant body of evidence indicates that the TIPP opioid peptides act as selective δ-opioid receptor antagonist, demonstrating high affinity, potency and selectivity [214]. The antagonist profile of the TIPP compounds was primarily determined by using the mouse vas deferens assay where these compounds failed to elicit any activity but they were able to block the activity of known delta opioid agonists. Another approach to elucidate their intrinsic activities is the measuring the binding of the nonhydrolizable GTP analogue [^{35}S]GTPγS as a function of ligand concentration [215]. Understanding of the action of δ receptor selective peptides at the cellular level is essential, particularly because compounds active at this receptor may show agonist, antagonist or inverse agonist properties. In the [^{35}S]GTPγS assays the novel tetrapeptide ligands exhibited different intrinsic profiles in diverse *in vitro* systems. As the native membrane preparations made of rat brain usually consist of mixed populations of neuronal cells, each expressing different types and amount of receptors we used other cellular systems as well. Recent cDNA cloning studies of the different opioid receptor types facilitates the study of individual receptors regarding their pharmacological profile, structure-activity analysis and signal transduction mechanisms [216]. Transfection of different cell lines with individual recombinant receptor types represents a powerful tool for the study of opioid signal

transduction at the receptor level. Several epithelial cell lines were transfected with cloned δ-receptor such as monkey kidney cells (COS cells) [177], Chinese hamster ovary cells (CHO) [217], as well as pituitary tumor cells GH_3, human embryonic kidney cells HEK [218] and others. The only disadvantage of using transfected cell line is the possible altered membrane environment, like other sets of G-proteins as well as the receptor number and G-protein density can also influence the extent of agonist-stimulated [^{35}S]GTPγS binding [219].

In our experiments with the novel stereomeric tetrapeptide analogues we used rat brain membrane homogenates and Chinese hamster ovary cell lines stably expressing either μ- or δ-opioid receptors ($δ_m$CHO and $μ_h$CHO cell cultures). The DYTCP ligands did not elevated basal G-protein activity in rat brain membranes none of them activated [^{35}S]GTPγS binding. However, they slightly decreased the basal activity in rat brain membranes at higher peptide concentrations thus displaying more likely as inverse agonist activity. For further evidence of this inverse agonisms $δ_m$CHO cell lines were used, were DYTCP analogues behaved clearly as inverse agonists. Similar findings were demonstrates in the GH3 cells expressing delta-opioid receptors (GH_3DOR) with the parent TIPP (Tyr-Tiy-Phe-Phe) peptide [220] as well as with dipeptide Dmt-Tic analogues [221]. In $μ_h$CHO cells expressing μ-opioid receptors the DYTCP analogues dose-dependently stimulated G-proteins. Maximal stimulation was achieved in the case of (2S,3R)βMeCha^3-DYTCP-amide analogue with an E_{max} value of 200% displaying partial agonist characters compared with the full agonist DAMGO in this system. Differences induced by the stereochemistry of the βMeCha^3 residue was noticeable in this assay also, while the C-terminal carboxamide group had some impact but to a lesser extent on the μ-agonist activity of the analogues. Displaying the ability of inverse agonism competitive antagonist character of the DYTCP analogues in the presence of full agonist was also tested. This was demonstrated in $δ_m$CHO cell membranes expressing delta-opioid receptors whereas the stereomeric DYTCP ligands effectively blocked and inhibited the stimulation of [^{35}S]GTPγS binding induced by the delta agonist Ile^{5,6}-deltorphin-II. These findings suggest that both neutral antagonists (without intrinsic agonist activity) and inverse agonists can inhibit G-protein stimulation induced by a full-agonist.

Functional efficacy of the two βMePhe^3-DYTPP analogues was also tested in different cellular systems. In $μ_h$CHO cell lines transfected with human mu-opioid receptors the two diastereomers dose-dependently stimulated the [^{35}S]GTPγS binding. The extent of stimulation being of 200% in the case of (2S,3R)βMePhe^3-DYTPP similarly to the cyclohexylalanine substituted (2S,3R)βMeCha^3-DYTCP-amide analogue with the same third side chain

configuration, although the carboxamide-end can also have some influence on the ligand profile. Interestingly, in the δ_mCHO cell lines, both compounds activated [^{35}S]GTPγS binding to some extent in contrast with the βMeCha^3-DYTCP analogues wherein they exhibited inverse agonist profiles. Regarding their competitive antagonist profile both tetrapeptides effectively inhibited the (pClPhe^4)-DPDPE induced stimulation of [^{35}S]GTPγS binding in δ_mCHO cell membranes. Similarly, activation of μ-receptors in the μ_hCHO cell system was also decreased in the presence of the two DYTPP analogues but with far less potencies. This antagonism of the μ-agonist DAMGO in the μ-opioid receptor system by the TIPP derived peptides was not yet demonstrated, although some weak μ-antagonist characters of some Dmt-Tic dipeptide analogues were observed in functional bioactivity studies [222;223]. Moreover in guinea pig ileum (GPI) and mouse vas deferens (MVD) functional assays all the βMeCha^3-DYTCP compounds displayed high δ-antagonist activity as well as in the case of (2S,3R)βMeCha^3-DYTCP and (2S,3R)βMeCha^3-DYTCP-amide analogues exposed bivalent partial μ-agonist/δ-antagonist characters [211]. Taken together these findings, we can conclude that the novel TIPP derived peptides can have diverse intrinsic properties under proper conditions and in different cellular systems thus they can be used in exploring the delta receptor activation [224].

The results obtained with the radiolabeled [^{3}H](2S,3R)βMePhe^3-DYTPP compound regarding its reversibility, saturability, stereoselectivity and low non-specific binding features fulfill the main criteria necessary for valuable radioligands. Thus this radioligand with its properties should make it an important and useful reagent in probing δ-opioid receptor mechanisms as well to promote a further understanding of the opioid system at the cellular and molecular level.

Different structural modifications and their consequences on ligand profiles are also highlighted in this study. The incorporation of the topographically constrained unnatural amino acid (Tic) [225], increasing the peptide hydrophobicity by methylation (Dmt) [226], substitutions of the third Phe^3 [227;228] and combination of Dmt and beta-methyl-amino acid substitution [209] alltogether modify the ligands' biochemical and biological properties. The nonlabeled stereomeric compounds may also serve as valuable pharmacological and therapeutic agents as well as probes in order to deduce the requirements for coupling an opioid peptide with the δ or μ-receptors suggested by other detailed structure-activity studies [163;229;230].

VI. SUMMARY

Since it was demonstrated that the delta receptor sites are involved in the process or phenomenon of morphine tolerance and dependence the focus is on the development of potent antagonist ligands with high selectivity for the δ-receptors [231;232]. Recently a new series of highly potent, δ-receptor selective opioid antagonist tetrapeptide ligands have been reported. The first prototype antagonist ligand from these new peptides was TIPP (*Tyr-Tic-Phe-Phe*) which was followed by extensive structure-activity relationship studies. The present study focused on the biochemical and functional analysis of a series of novel analogues from the TIPP series. The novel analogues carry different structural and conformational changes as substitution of the first Tyr by 2',6'-dymethyltyrosine (Dmt), the unnatural 1,2,3,4-tetrahydroisiquinoline-3-carboxylic acid (Tic[2]), β-methyl-amino acid substitution at the third place as well C-terminal modification. In this work we report the combination of Dmt[1] and β-Me-amino acid[3] substitution on the TIPP template structure and analyse the receptor binding and G-protein activating properties of the novel peptide analogues. The main findings are the following:

1. Side chain methylation of Phe by βMePhe[3] yielded diastereomeric pairs of analogues wherein (2*S*,3*R*)βMePhe[3]-DYTPP analogue was a potent δ-selective antagonist compound and the (2*R*,3*S*)βMePhe[3]-DYTPP had higher μ-receptor affinities and less δ-selectivity.

2. Specific binding of [3H](2*S*,3*R*)βMePhe[3]-DYTPP to rat brain membranes and to membrane fractions of CHO cell lines stably expressing δ-opioid receptors was of high affinity, saturable and stereoselective.

3. Kinetic experiments showed that the ligand-receptor association occurred rapidly according to pseudo-first order kinetics. Equilibrium saturation experiments revealed that a single class of nanomolar affinity opioid-binding sites was labeled by this radioligand.

4. Heterologous competition assays with different opioid compounds revealed that the rank order of potency is: δ > μ> κ in displaceing [3H] (2*S*,3*R*)βMePhe[3]-DYTPP specific binding.

5. Side chain methyl substitution by βMeCha[3] resulted in four diastereomeric DYTCP compounds displaying good δ-receptor affinities and different selectivities in function of their diastereomerism.

6. C-terminal amidation of the DYTCP stereoisomers slightly increased their μ-receptor affinity and μ- agonist properties.

7. Combination of Dmt^1 and $\beta MePhe^3$ replacements in the DYTPP ligands resulted in potent and more complex analogues displaying simultaneously δ-antagonist/agonist as well as μ-agonist/antagonist profiles in cell membrane preparations.

8. The $\beta MeCha^3$ substitution together with the Dmt^1 at the first position and C-terminal carboxamide group resulted in DYTCP stereoisomeric ligands with weak inverse agonist activities in rat brain and cell membranes as well as mixed μ-agonist/δ-antagonist properties in cell membrane preparations *in vitro*.

In conclusion the novel tetrapeptide analogues carrying different structural modification by their complex intrinsic properties may serve as valuable compounds in the biochemical and pharmacological research of the opioid system. Furthermore by their increased hydrophobicity imposed by dimethylation of Tyr as well as lipid solubility and low molecular weight might fulfill the suggested criteria required for passage through blood-brain barrier thus they might further offer new compounds for clinical studies. Moreover, analogues from the TIPP family by their structural resemblance with endomorphins (Endomorpin 1: Tyr-Pro-Trp-Phe-NH_2 and endomorphin 2: Tyr-Pro-Phe-Phe-NH_2) might also represent an interesting series for molecular modeling and searching for pharmacophore elements since a single small structural modification can drastically change the ligands intrinsic properties and characteristics.

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