Binding and functional studies of the binding parameters, antagonist profile and delta-opioid (subtype)specificity of a new peptidomimetic, Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH

Ph.D. Thesis

Erika Lehoczkyné Birkás

Szeged
2011.

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Ph.D. Thesis

Szeged, 2011.

List of Thesis-Related Publications

I. Birkas, E., Kertesz. I., Toth, G., Bakota, L., Gulya, K., Szucs, M., 2008. Synthesis and pharmacological characterization of a novel, highly potent, peptidomimetic δ-opioid radioantagonist, [^{3}H]Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH. Neuropeptides 42, 57-67. (IF_{2008}: 2.438)

II. Birkas, E., Bakota, L., Gulya, K., Wen, T., Pintar, J., Toth, G., Szucs, M., 2011. A comprehensive study on the putative δ-opioid receptor (sub)types using the highly selective δ-antagonist, Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH. Neurochemistry International; *doi: 10. 1016/j.neuint.2011.04.015* (IF_{2009}: 3.541)

List of Thesis-Unrelated Publications

I. Keresztes, A., **Birkas, E.,** Páhi, A., Tóth, G., Bakota, L., Gulya, K., Szűcs, M., 2011. Pharmacology of a new tritiated endomorphin-2 analog containing the proline mimetic *cis*-2-aminocyclohexanecarboxylic acid. Peptides 32, 722-728. (IF_{2009}: 2.705)

II. Kekesi, O., Tuboly, G., Szucs, M., **Birkas, E.,** Morvay, Z., Benedek, Gy., Horvath, Gy., 2011. Long-lasting, distinct changes in central opioid receptor and urinary bladder functions in models of schizophrenia in rats. European Journal of Pharmacology; 661, 35-41. (IF_{2009}: 2.585)

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List of abbreviations

B-endorphin	Tyr-Gly-Gly-Phe-Met-ThrSer-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Lly-Glu
B_{max}	receptor density
BNTX	7-benzylidenenaltrexone
BSA	bovine serum albumin
CTAP	H-D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2
CTOP	D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2
cyprodime	N-cyclopropil-3,14-dimethoxymorphinan-6-on
DADLE	[D-Ala^2,D-Leu^5]enkephalin
DAMGO	Tyr-D-Ala-Gly-ME-Phe-Gly-ol
deltorphin II	Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2
dermorphin	Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2
DOR	δ-opioid receptor
DOR-1	δ-opioid receptor
DOR-KO	δ-opioid receptor knock-out
DPDPE	Tyr-c[D-Pen-Gly-Phe-D-Pen]-OH
DSLET	Tyr-D-Ser-Gly-Phe-Leu-Thr
Dynorphin-A	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln-OH
ED_{50}	the concentration of the agonist required to achieve 50 % of the maximal stimulation
EGTA	ethylene-bis(oxyethylenenitrilo) tetraacetic acid

EKC	ethylketocyclazocine
E_{max}	maximal stimulation
endomorphin-1	Tyr-Pro-Trp-Phe-NH_2
endomorphin-2	Tyr-Pro-Phe-Phe-NH_2
GDP	Guanosine 5′-diphosphate
GPCRs	G-protein-coupled receptors
GTP-γ-S-Li_4	Guanosine 5′-[γ-thio]triphosphate tetralithium salt
hDOR-CHO	Chinese hamster ovary cells stably transfected with the human δ-opioid receptors
IC_{50}	the concentration of the ligand required to achieve 50 % inhibition
i.t.	*intrathecal*
k_a	association rate constant
kbp	kilobasispairs
k_d	dissociation rate constant
K_D	equilibrium dissociation constant
K_e	apparent antagonist affinity constant
K_i	inhibitory constant
k_{obs}	observed pseudo-first-order rate constant
KOR	κ-opioid receptor
Leu-enkephalin	Tyr-Gly-Gly-Phe-Leu
Met-enkephalin	Tyr-Gly-Gly-Phe-Met
MOR	μ-opioid receptor
%MPE	percent maximal possible effect
naloxone	http://en.wikipedia.org/wiki/Synonym17-allyl- 4,5α-epoxy- 3,14-

	dihydroxymorphinan- 6-one
naltrindole	17-Cyclopropylmethyl-6,7-dehydro-4,5-epoxy -3,14-dihydroxy-6,7,2',3'-indolomorphinan
naltriben	17-(Cyclopropylmethyl)-6,7-didehydro-3,14β-dihydroxy-4,5α-epoxy-6,7-2',3'-benzo[*b*]furanomorphinan mesylate
NOR-BNI	17,17'-(dicyclopropylmethyl)-6,6',7,7'-6,6'-imino- 7,7'-binorphinan-3,4',14,14'-tetrol
Tic	1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid
TIPP	H-Tyr-Tic-Phe-Phe-OH
TIPP[psi]	H-Tyr-Tic[CH_2NH]-Phe-Phe-OH
TM	transmembrane
Tris	Tris(hydroxymethyl)-aminomethane hexahydrate
U-50488	2-(3,4-dichlorophenyl)-N-methyl-N-[(1*R*,2*R*)-2-pyrrolidin-1-yl-cyclohexyl]acetamide
U69593	*N*-methyl-2-phenyl-*N*-[(5*R*,7*S*,8*S*)-7-(pyrrolidin-1-yl)-1-oxaspiro[4.5]dec-8-yl]acetamide
[^{35}S]GTPγS	Guanosine-5'-*O*-(3-[^{35}S]thio)triphosphate

1. Introduction

1.1. The opioid receptors as G-protein-coupled receptors

Opioids are one of the oldest drugs, which are extracted from the plant *Papaver somniferum*. The opioid ligands exert their pharmacological effects, most importantly analgesia, *via* opioid receptors located in the central nervous system (Pert and Snyder, 1973; Simon *et al*., 1973; Terenius, 1973). Opioid receptors belong to the large superfamily of G protein-coupled receptors (GPCR) characterized by seven transmembrane (TM) domains with extracellular N-terminal, and intracellular C-terminal regions (Eguchi, 2004). The TM domains are connected by extra- and intracellular loops, out of those the second and third intracellular loops have been proposed to interact with G_i/G_o proteins (Harrison *et al*., 1998). The G-proteins are heterotrimers of α -, β - and γ-subunits and the α-subunit is bound to GDP in the basal state (Gilman, 1986). When a ligand activate the receptor, the G-protein binds to the receptor and the α-subunit exchanges the GDP to GTP resulting conformational changes in the G-protein, thereby activating the α-subunit. Therefore, the G-protein dissociates from the receptors, as well as the subunits from each other, issuing α-GTP monomer and βγ dimer. The α-GTP-subunit binds to an effector molecule and activates it, than hydrolyse the bound GTP to GDP. The α-GDP-subunit dissociates from the effector, reassociate with the βγ dimer and the cycle is back to the basal state (Koski and Klee, 1981).

1.2. Different types and subtypes of GPCRs: possible explanations of the existence of these subtypes

GPCRs can be devided into 6 classes based on sequence homology and functional similarity. Each class has more subclasses, for example the rhodopsin-like receptors have 19 subclasses (Joost and Methner, 2002). Furthermore, subtypes of many types of GPCRs could be defined based on molecular or functional studies. There are many possible explanations for the existence of these subtypes. One of the most common explanations is that the different subtypes of the receptors are encoded by different genes, as in the case

of the α1 adrenergic receptors, which have 3 different subtypes encoded by 3 different genes (Docherty, 1998; Zhong and Minneman, 1999).

It is possible that although only one gene has been found and the different subtypes represent either alternatively spliced variants of the same gene, or distinct protein products from the same mRNA that have undergone different posttranslational modifications. For example, many of the dopamine receptor types have different transcript variants encoded by the same gene (Fu *et al*., 1995; Giros *et al*., 1991; Zhang *et al*., 2007).

Very distinct receptors may form a homo- or hetero-oligomer complex that displays altered ligand binding and signaling properties from those of the individual receptors (George *et al*., 2002). $GABA_B$ receptors have 2 subunits, which are encoded by 2 different genes. These subunits are $GABAB_1$ and $GABAB_2$, which form a heterodimer, and this new receptor is the functional $GABA_B$ receptor (Marshall and Foord, 2010).

1.3. Different types of the opioid receptors

Three opioid receptor types (μ, δ and κ) have been defined by means of radioligand binding, pharmacological assays and molecular cloning (Evans *et al*., 1992; Kieffer *et al*., 1992; Waldhoer *et al*., 2004). The main differences between the three opioid-receptor types are summarized in Table 1.

Although μ-, δ- and κ-opioid receptors show highly conserved homology in their structures, each opioid receptor have different glycosylation and phosphorylation sites (Table 1).

The three opioid-receptor types could be distinguished by their different affinity for binding the opioid ligands (Eguchi, 2004). The well-known μ-selective ligands are the opioid alkaloid morphine and its derivatives as well as peptides, such as DAMGO (Tyr-D-Ala-Gly-ME-Phe-Gly-ol); dermorphin (Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2), cyprodime (N-cyclopropil-3,14-dimethoxymorphinan-6-on), CTAP (H-D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2) and CTOP (D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2).

Table 1. The main features of the μ- (MOR), δ- (DOR), and κ- opioid receptors (KOR)

	MOR	**DOR**	**KOR**
Structural information	400 aminoacids	372 aminoacids	380 aminoacids
Gene	~ 53 kbp	~ 32 kbp	~ 16 kbp
Chromosomal localization	6q24-25	1p34.3-36.1	8q11.2
Glycosylation sites	5	2	2
Phosphorylation sites	3 ~ 4	4 ~ 7	5 ~ 7
Localization in the CNS	thalamus locus coeruleus dorsal horn of spinal cord neocortex nucleus accumbens amygdala	bulbus olfactorius caudate putamen neocortx nucleus accumbens amygdala	hypothalamus hypophysis epiphysis neocortx nucleus accumbens amygdala
Signal transduction	cAMP ↓ Ca^{2+} channel ↓ K^{+} channel ↑	cAMP ↓ Ca^{2+} channel ↓ K^{+} channel ↑	cAMP ↓ Ca^{2+} channel ↓ K^{+} channel ↑
Physiological effects	antinociception euphoria respiratory depression PRL-release increased nutrition obstipatio	antinociception sedation flexor-reflex inhibiton ADH-release pupil-constriction	stress-antinociception dysphoria GH-release hypotension
Endogen peptides	endomorphin-1 and -2 β-endorphin	Leu-enkephalin Met-enkephalin	dynorphin-A
Selective agonists	morphine DAMGO dermorphin	DPDPE deltorphin II	EKC U-50488
Selective antagonists	cyprodime CTAP and CTOP	naltrindole TIPP BNTX, naltriben	NOR-BNI
Nonselective antagonist	naloxone	naloxone	naloxone

Based on Eguchi (2004), Mansour *et al*. (1998), Satoh and Minami (1995), Waldhoer *et al*. (2004).

The δ-selective ligands are DPDPE (Tyr-c[D-Pen-Gly-Phe-D-Pen]-OH), deltorphin II (Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2), naltrindole (17-Cyclopropylmethyl-6,7-dehydro-4,5-epoxy-3,14-dihydroxy-6,7-2',3' indolomorphinan), BNTX (7-benzylidenenaltrexone), naltriben (17-(Cyclopropylmethyl)-6,7-didehydro-3,14β-dihydroxy-4,5α-epoxy-6,7-2',3'-benzo[*b*]furanomorphinan mesylate) and TIPP (H-Tyr-Tic-Phe-Phe-OH). The κ-selective ligands are dynorphin-A (Tyr-Gly-Gly-Phe-Leu-Arg-

Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln-OH), U-50488 (2-(3,4-dichlorophenyl)-N-methyl-N-[(1*R*,2*R*)-2-pyrrolidin-1-yl-cyclohexyl]acetamide), EKC (etilketociklazocin) and NOR-BNI (17,17'-(dicyclopropylmethyl)-6,6',7,7'-6,6'-imino- 7,7'-binorphinan-3,4',14,14'-tetrol) (Table 1). The effects of all the opioid ligands could be blocked by the nonselective antagonist naloxone (17-allyl- 4,5α-epoxy- 3,14-dihydroxymorphinan- 6-one).

The opioid receptors are located in the central nervous system, mainly in those areas, which play a role in the passing on and processing of pain and in the limbic system (Mansour *et al.*, 1998). Each opioid receptor type has a different localization in the central nervous system, consequently different brain areas display different μ:δ:κ ratios showing the different function of the brain areas (Mansour *et al*., 1995; Petrillo *et al*., 1992; Pradhan and Clarke, 2005). All receptor types could be found in the areas of neocortex, nucleus accumbens and amygdala. High densities of μ-opioid receptors could be shown in the thalamus, locus coeruleus and in the dorsal horn of the spinal cord. The δ-opioid-specific areas are the bulbus olfactorius and the caudate putamen, while the κ-opioid receptors have high numbers in the hypothalamus, hypophysis and epiphysis (Table 1).

The main physiological effect of the opioid receptors is the antinociception, which is mediated mainly *via* μ-opioid receptors. Unfortunately, μ-opioid receptors have several serious side effects (*e.g.* tolerance, physical and psychic dependence), which are limiting their clinical applications in pain management (Higashida *et al*., 1998; Horvath *et al*., 1999; Shen *et al*., 2000; Spreekmeester and Rochford, 2000; Stone *et al*. 1997). Tolerance means that after repeated administration of the drug, the effects of the drugs are decreasing, namely larger doses of the drug are required to achieve the same effect. Dependence means that after chronic administration of the drug, the organism of the patient adapted the presence of the drug and in the absence of the drug it is not able to do its normal function (Harrison *et al*., 1998). Dependence has physical (respiratory depression, diarrhea, vomiting, cramps, insomnia) and psychic symptoms (craving for drug, depression, anxiety), which could summarize as withdrawal symptoms.

Nowadays, δ-opioid receptors got into the focus of the research, because they also have analgesic effect, but show less side-effect than μ-opioid receptors. Selective

agonists of the δ-opioid receptors have been shown to produce both spinal and supraspinal antinociception *via* the δ-opioid receptor with less physical dependence, less respiratory depression and less constipation than morphine (Cowan *et al*., 1988; Heyman *et al*., 1987; Maldonado *et al*., 1992). However, the analgesic response mediated by δ-opioid receptors is weaker than μ-opioid antinociception (Scherrer *et al*., 2004), thus δ-opioid agonists efficacious enough are still to be developed. It was found in an elegant study using knock-out animals that μ-agonists preferentially reduce heat pain, while δ-agonists reduce mechanical pain (Scherrer *et al*., 2009). In contrast to these results, Wang *et al.* (2010) showed the coexistence of δ- and μ-opioid receptors in dorsal root ganglia neurons suggesting direct interaction of opioid receptors in opioid antinociception.

A new and promising direction is to use compounds with mixed μ-agonist/δ-antagonist profile (for a review see Schiller *et al*., 1999). This is based on the observation that when morphine was co-administered with a δ-receptor antagonist, then increased antinociception with an improved side-effect profile (tolerance and dependence) was observed (Abdelhamid *et al.*, 1991). Morphine was shown to retain its μ receptor-mediated analgesic activity without producing tolerance in δ-opioid receptor knockout mice suggesting that δ-receptors had a major role in the development of tolerance (Zhu *et al*., 1999).

1.4. The putative subtypes of the δ-opioid receptors

Classical *in vivo* pharmacological studies have suggested the existence of different subtypes of each of the three opioid types (Jiang *et al*., 1991; Mattia *et al*., 1991; Paul *et al*., 1989; Sofuoglu *et al*., 1991; Vanderah *et al*., 1994; Zukin *et al*., 1988). The first opioid receptor cloned was the mouse δ-opioid receptor (Evans *et al*, 1992; Kieffer *et al*., 1992). The classification of the δ-opioid receptor subtypes is based on data from analgesic assays in mice, showing that the effect of DPDPE was blocked by BNTX and this subtype of the δ-opioid receptor was defined as the δ_1-opioid receptor (Portoghese *et al*., 1992; Sofuoglu *et al*., 1993). The effects of deltorphin II were antagonized by naltriben and it was as signed as the δ_2-opioid receptor (Sofuoglu *et al*., 1991; Takemori *et al*., 1992). The lack of antinociceptive tolerance between DPDPE and [D-

Ala^2]deltorphin II provided further support for the existence of δ-receptor subtypes (Mattia *et al*., 1991). Since naltriben displayed a higher affinity than BNTX for the cloned δ-opioid receptor (DOR-1) both in binding and functional experiments (Law *et al*., 1994), the cloned δ-opioid receptor was thought to correspond to the pharmacological δ_2-subtype (Raynor *et al*., 1994). The existence of receptor subtypes was further supported by adenylyl cyclase regulation (Buzas *et al*., 1994; Olianas and Onali, 1995) and antisense mapping (Rossi *et al*., 1997; Standifer *et al*., 1994).

However, these proposed δ-opioid receptor subtypes could not be distinguished at the molecular level (Allouche *et al*., 2000; Mansour *et al*., 1995; Zaki *et al*., 1996). Only one δ-opioid receptor gene (DOR-1) has been cloned from the amphibian *Rana pipiens* (Stevens *et al*., 2007), mouse (Zhu *et al*. 1999), rat (Fukuda *et al*., 1993) and human (Knapp *et al*., 1994) brain so far. Zhu *et al*. proposed that the DOR-1 gene encodes both the δ_1 and δ_2 subtypes (Zhu *et al*., 1999). Contrary, two δ-opioid receptor genes have been cloned from *zebrafish* (Barrallo *et al*., 1998; Pinal-Seoane *et al*., 2006), but they did not seem to correspond to the proposed δ-opioid receptor subtypes (Gonzalez-Nunez *et al*., 2007). One splice variant has been revealed by mRNA analysis in mouse brain (Gavériaux-Ruff *et al*., 1997), but the existence of this splice variant at the protein level remains to be demonstrated.

Receptor binding studies performed with various ligands and various tissues resulted in conflicting results showing only one (Connor *et al*., 1997; Toll *et al*., 1997) or heterogeneous (Fang *et al*., 1994; Kim *et al*., 2001) δ-opioid sites. Receptor autoradiography using proposed δ_1- and δ_2-selective agonists either has not revealed a discrete distribution for the two receptor subtype (Gouarderes *et al*., 1993), or has shown that the binding sites of [^{3}H]DPDPE and [^{3}H]DSLET displayed differences in some single anatomical structures (Hiller *et al*., 1996). It was demonstrated that the selectivity of some agonists for δ-opioid receptor differs in different species. For example, β-endorphin, [Leu^5]enkephalin, DSLET (Tyr-D-Ser-Gly-Phe-Leu-Thr) and DADLE ([D-Ala^2,D-Leu^5]enkephalin) are selective agonists for the δ-opioid receptor in mouse, but not in human, cells (Raynor *et al*., 1994; Toll *et al*., 1997)**.** The issue is further complicated by the observation that selectivity of a ligand *in vitro* (seen in binding studies) does not always correspond to its specificity *in vivo* and *vice versa*. The use of various antagonists

or antisense oligonucleotides, as well as co-administration and cross-tolerance studies suggested that μ-receptors may be involved, with possible functional μ-δ interactions, at least in some δ-opioid functions (Rozenfeld *et al*., 2007; Traynor and Elliot, 1993; Zaki *et al*., 1996).

Some of the results suggest that δ-opioid receptors can form heterooligomers with μ-opioid receptors (George et al., 2000; Hasbi et al., 2007; Traynor and Elliot, 1993). Riba *et al*. propose that during tolerance, a change occurs in the conformation of either μ- and/or δ-opioid receptors, changing their physiological interaction (Riba et al., 2002). It was also shown that δ-opioid receptor antagonists could enhance the morphine-mediated *intrathecal* analgesia, which provides the possibility of the interaction between DOR and MOR as a heterodimer (Gomes et al., 2004). Therefore, the heterodimeric associations between μ-δ opioid receptors can also be used as a model for the development of novel multi-target analgesics with favorable side-effect profile.

Recently, however, it was reported that the putative δ_1- and δ_2-agonists have opposing and synergist effects on ethanol consumption. Authors postulated that while the δ_1-opioid receptor is a δ-μ heterodimer, the δ_2-opioid receptor is a δ-δ homodimer (van Rijn and Whistler, 2009). Thereby, the authors have reinforced the idea that δ_1- and δ_2-opioid receptors are distinct molecular targets (van Rijn and Whistler, 2009).

Opioid effects within the *canine* sinoatrial node, which regulates the normal cardiatic rhytm, were also shown to be bimodal in character, namely low doses are vagotonic, acting on δ_1-receptors, and higher doses are vagolytic, acting on δ_2-receptors (Farias *et al*., 2003a, b). δ_1-opioid receptors have been implicated in reducing myocardial structure injury, while the δ_2-opioid receptors in raising the postischemic myocardial mechanical functions, both δ-opioid receptor subtypes attenuating myocardial injury by targeting the mitochondrial permeability transition pore (Zeng *et al*., 2010). These results suggest that the yet hypothetical δ-opioid receptor subtypes may participate in distinct physiological effects.

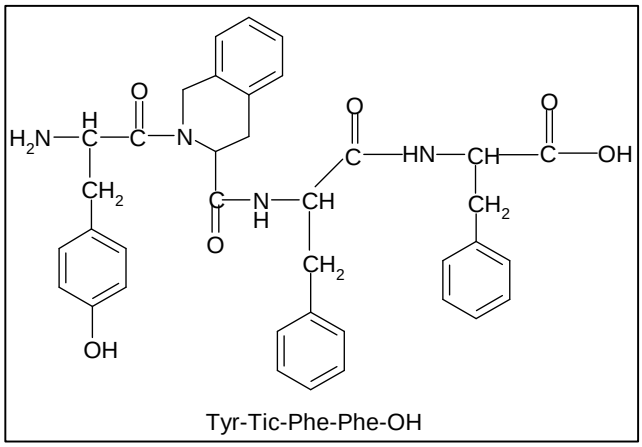
1.5. Structurally modified peptides, the TIPP family

The unique physiological role of the individual receptor types, however, is not fully understood, mainly due to the paucity of selective antagonists. Highly potent and selective δ-opioid receptor antagonists are not only essential pharmacological tools for ascertaining the biological processes mediated by δ-opioid receptors but may also have therapeutic applications to regulate δ-receptor function in various clinical disorders, including drug addiction (for a review, see Bryant *et al.*, 1998). The availability of δ-opioid receptor antagonists with high potency and receptor selectivity may facilitate delineation of receptor types and subtypes. Using antagonists is advantageous, since the receptors might have different affinity states due to different G-protein coupling when agonist binding is studied. These distinct conformational states might be misinterpreted as receptor subtypes.

There is high claim for having new analgesics with less side effects. It was found that δ-opioid antagonists, such as naltrindole or TIPP[psi] (H-Tyr-Tic[CH_2NH]-Phe-Phe-OH) significantly decreased the morphine-induced tolerance and dependence (Abdelhamid *et al.*, 1991; Fundytus *et al.*, 1995). It suggests that using μ-agonist and δ-antagonist ligands together, could be a useful treatment for chronic pain. Therefore the main direction of the opioid research is to develop ligands with μ-agonists/δ-antagonists profile.

H-Tyr-Tic-Phe-Phe-OH (TIPP; Tic=1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid), represents the prototype of a new class of highly potent and selective, conformationally constrained δ-opioid antagonists (Schiller *et al.*, 1992). Modifications of TIPP resulted in a series of δ-antagonists with moderate to high bioactivity and δ-selectivity (Bryant *et al.*, 1998; Ioja *et al.*, 2005, 2007; Marsden *et al.*, 1993; Nevin *et al.*, 1995; Schiller *et al.*, 1993, 1999; Tóth *et al.*, 2007). A systematic study was earlier performed in which each of the four amino acids of TIPP was substituted by all stereoisomers of the corresponding β-methyl amino acid (Tourwe *et al.*, 1998). Alicyclic β-amino acids have two chiral centers, which are defined as *R* or *S* resulting four possible enantiomers (*R,R; R,S; S,S; S,R*) (Fülöp, 2001). The effects of methylation of the β-carbon of a side chain on the biological properties of a peptide depends on the chiralities

of the stereoisomers. These structural modifications might have profound effects on the potency, selectivity and pharmacological features of the parent peptide, especially agonist/antagonist character of the ligand. Among the new analogs, Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH displayed the highest affinity and selectivity to δ-opioid receptors in receptor binding assays, and very high δ-antagonist potency in bioassays (Tourwe *et al.*, 1998). The favorable properties of the new ligand warrant its wild application in further pharmacological studies. This will be facilitated by the availability of the ligand in a radioactive form.



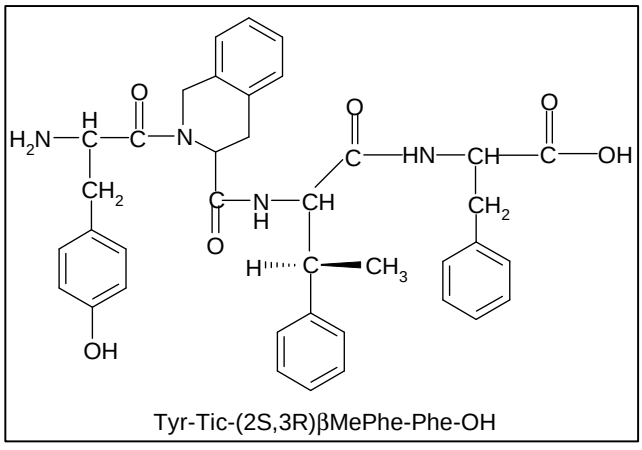


Figure 1. Structures of TIPP (Tyr-Tic-Phe-Phe-OH) and Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH.

2. Aims and scope

Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH was synthesized in unlabeled and tritiated form (53.7 Ci/mmol) in the Isotope Laboratory of the BRC as published (Birkas *et al*., 2008). The aims of our work were to:

- fully characterize the new radioligand in *in vitro* receptor binding experiments;
- visualize its binding sites by receptor autoradiography;
- investigate the *in vivo* specificity of *intrathecal* Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH in tail-flick analgesic assay;
- study the signaling and agonist/antagonist feature using the [^{35}S]GTPγS functional assay;
- set up conditions to measure putative δ_1- and δ_2-opioid receptor functions *in vitro;*
- check if Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH is able to distinguish among δ-receptor subtypes *in vitro*.

The experiments were performed in membranes of rat brain, as well as wild type (wt) and δ-opioid receptor knock out (DOR-KO) mouse brain, and Chinese Hamster Ovary, CHO cells stably transfected with recombinant human δ-opioid receptors (hDOR-CHO), respectively.

3. Materials and methods

3.1. Chemicals

[^{3}H]Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH (53.7 Ci/mmol) and [^{3}H]Ile^{5,6}-deltorphin II (49.5 Ci/mmol) were synthesized and tritiated in the Isotope Laboratory of the Biological Research Centre (Szeged, Hungary) as published (Nevin *et al.*, 1994). The tritiated compound was stored as a 37 MBq/cm^3 solution in ethanol at -80 ºC. No diketopiperazine formation was detected by HPLC under these conditions (data not shown). Guanosine-5'-*O*-(3-[^{35}S]thio)triphosphate ([^{35}S]GTPγS) (37–42 TBq/mmol) was purchased from the Isotope Institute Ltd. (Budapest, Hungary). Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH, TIPP, Ile^{5,6}-deltorphin II, D-Ala^2-dynorphin-NH_2 and deltorphin II were synthesized in the Isotope Laboratory of the Biological Research Centre (Szeged, Hungary) as published (Buzas *et al.*, 1992; Lung *et al*., 1995; Nevin *et al*., 1994). Naloxone and naltrindole were kindly provided by Dr. S. Hosztafi (Semmelweis University, Budapest, Hungary). DPDPE was from Bachem AG (Bubendorf, Germany). BNTX and naltriben were purchased from Tocris Bioscience (Ellisville, MO, USA). Guanosine 5′-diphosphate sodium salt (GDP), Guanosine 5′-[γ-thio]triphosphate tetralithium salt (GTP-γ-S-Li_4), magnesium chloride hexahydrate, Tris(hydroxymethyl)-aminomethane (Tris, free base), bacitracin, NaCl, ethylene-bis(oxyethylenenitrilo) tetraacetic acid (EGTA), Kodak Sigma Fixer, Kodak D-19 Developer and Kodak X-OMAT AR films were from Sigma-Aldrich Kft. (Budapest, Hungary). Bradford reagent and bovine serum albumin (BSA) were from Bio-Rad Laboratories (Hercules, CA, USA). The purities of the peptides, alkaloids and solvents were of at least 95% or analytical grade.

3.2. Rat and mouse brain membrane preparation

Adult male rats (Wistar, 2-3 months old) and wild type mice (C57Bl/6J, 2-3 months old) were handled in accordance with the European Communities Council Directives (86/609/ECC) and the Hungarian Act for the Protection of Animals in Research (XXVIII.tv. Section 32). They were housed in a temperature- and light-controlled room.

Lighting was ensured in a 12-h cycle, and food and water were available *ad libitum*. δ-opioid receptor knock-out, DOR-KO mice was generated by replacing exon 2 with a neomycin resistance cassette as published (Zhu *et al*., 1999). Whole brains (without cerebellum) were dissected and homogenized in 30 volumes (v/w) of ice-cold 50 mM Tris-HCl buffer (pH 7.4) with a teflon-glass Braun homogenizer as published (Bozo *et al*., 1997). The homogenate was centrifuged at 20,000 x g for 25 min at 4 °C, the resulting supernatant was carefully discarded and the pellet was taken up in the original volume of Tris-HCl buffer. After homogenization with an all-glass Dounce, the homogenate was incubated at 37 °C for 30 min in a shaking water-bath. Centrifugation was then repeated as described above. The final pellet was suspended in 5 volumes of 50 mM Tris-HCl pH 7.4 buffer containing 0.32 M sucrose, frozen in liquid N_2 and stored at -80 °C. Prior to the experiments, an appropriate aliquot was melted, diluted with 5-fold Tris-HCl buffer and centrifuged at 20,000 x g for 25 min to remove sucrose. The protein content of the membrane preparation was determined by the method of Bradford, BSA being used as a standard (Bradford, 1976).

3.3. hDOR-CHO cell membranes

Membranes of Chinese Hamster Ovary, CHO cells stably transfected with the human δ_2-opioid receptors (hDOR-CHO, Malatynska *et al*., 1995) were purchased from PerkinElmer (Boston, USA). They were suspended in 50 mM TRIS-HCl (pH 7.4), 5 mM $MgCl_2$ and 10% sucrose and stored at -80 ºC until use. Prior the [^{35}S[GTPγS functional assay, they were melted and diluted with 50 mM Tris-HCl buffer (pH 7.4) to yield in 10 µg protein/tube. Prior the saturation experiments, an appropriate aliquot was melted, diluted with 5-fold Tris-HCl buffer and centrifuged at 20,000 x g for 25 min to remove sucrose. The protein content of the membrane preparation was determined by the method of Bradford, BSA being used as a standard (Bradford, 1976).

3.4. Analgesia measurement

All procedures have been approved by the Institutional Animal Care and Use Committee of UMDNJ (Piscataway, NJ, USA). Mice (C57Bl/6J, both genders, 2-3 months old) were lightly anesthetized by isoflurane. *Intrathecal* (*i.t.*) lumbar puncture was performed using a modified version of the method of Hylden and Wilcox (1980) as reported (Porreca and Burks, 1983). All drugs were dissolved in physiological saline and administered to 7-16 mice/group. Animals were injected *intrathecally* with 5 µl saline or Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH (10 µg,13 nmol) immediately followed by 2 µl of either DPDPE (8 µg, 12 nmol), Ile^{5,6}-deltorphin II (15 µg,19 nmol) or DAMGO (6 ng, 12 pmol). The site of injection was chosen to be between L5-L6 areas, which minimize the possibility of the spinal damage (Hylden and Wilcox, 1980). Analgesic latency was assessed by the tail-flick method 15 minutes later. The radiant heat tail-flick assay was performed as published using a light intensity that produced baseline latencies ranging from 2-3 seconds and a 10 seconds cut-off time (Zhu *et al*., 1999). The percent maximal possible effect (% MPE) was calculated using the formula: (measured value – baseline value) / (cut-off time – baseline value) x 100%. Group comparisons were performed by two-tailed *t*-test. After the experiments, cursory examination of the injected mice for 1-2 h (Hylden and Wilcox, 1980) showed no evidence of over motor impairment.

3.5. Receptor binding assay

Preliminary experiments revealed that silanization of the pipette tips and the use of polystyrene reaction tubes was necessary to minimize the radioactivity loss due to adsorption. The time course of association was measured by incubating (0.7 nM) [^{3}H]Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH with the protein for the indicated times. To assess the dissociation rate constant, the radioligand was incubated with the protein for 90 min, which was followed by the addition of 10 µM naloxone, and the dissociation of the radioligand was subsequently assessed for 120 min. All binding experiments were performed at 25 °C for 90 minutes in a final volume of 1 ml of 50 mM Tris-HCl buffer (pH 7.4). Saturation binding experiments were performed with increasing concentrations (0.05-5 nM) of [^{3}H]Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH in the absence or in the presence of 100 mM NaCl and rat or mouse brain (≈100 µg protein/tube) or hDOR-CHO (≈ 25 µg

protein) membranes. Competition binding experiments were performed by incubating rat or mouse brain membranes in the absence (total binding) or in the presence of various concentrations of unlabeled opioid ligands and [^{3}H]Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH (0.7 or 1.5 nM) by increasing concentrations of appropriate μ- (DAMGO, endomorphin-2, naloxone), δ- (TIPP, naltrindole, DPDPE, Ile^{5,6}-deltorphin II, BNTX, naltriben) and κ-opioid ligands (U50,488, D-Ala^2-dynorphin-NH_2) and rat or mouse brain membranes at ≈250 or ≈150 μg protein/tube, respectively. The nonspecific binding was determined with 10 μM naloxone in rat brain or 1 μM naltrindole in mouse brain membranes and subtracted from the total values to give the specific binding. The reaction was stopped by diluting the samples with 5 ml of ice-cold Tris buffer, followed by rapid filtration through Whatman GF/C glass fiber filters (Whatman LTD, Maidstone, England) with a Brandel M24-R Cell Harvester (Gaithersburg, MD, USA). Filters were washed twice with 5 ml of ice-cold TRIS buffer, air-dried and counted in a toluene-based scintillation cocktail in a Wallac 1409 Counter (Wallac, Turku, Finland). All assays were performed in duplicate and repeated at least three times.

3.6. Ligand-stimulated [^{35}S]GTPγS functional binding

Membranes of hDOR-CHO cells (10 μg protein/tube) were thawed and incubated with [^{35}S]GTPγS (0.1 nM), appropriate concentrations of the ligands tested, 100 mM NaCl and 3 μM GDP in TEM (50 mM TRIS-HCl, 1 mM EGTA and 5 mM $MgCl_2$, pH 7.4) buffer for 60 minutes at 30 ºC in a total volume of 1 ml as published (Cinar and Szucs, 2009). The same conditions were used in wild type and DOR-KO mouse brain membranes, except that the concentration of GDP was 100 μM (Bozo *et al*., 1994; Fabian *et al*., 2002). Nonspecific binding was determined with 10 μM GTPγS and subtracted. Bound and free [^{35}S]GTPγS were separated by vacuum filtration through Whatman GF/F filters with a Brandel M24-R Cell Harvester as above. Basal activities assessed in the absence of opioids were defined as 0%. Data are expressed as % of the basal activities and are the means ± S.E.M. of at least 3 independent experiments performed in triplicate.

3.7. Receptor autoradiography

The rats (Wistar, both genders, 2-3 months old) and mice (C57Bl/6J, both genders, 2-3 months old) were anesthetized with diethyl ether and decapitated. The brains were quickly removed, embedded in Cryomatrix embedding medium (Shandon Scientific, Pittsburgh, PA, USA) and frozen immediately at -80 °C. Serial *coronal* cryostat sections (15 μm) were cut at four or six different levels from the olfactory bulb to the cerebellum, ascertained according to the brain atlas of Paxinos and Watson (1997) or Franklin and Paxinos (2004). Tissue sections were thaw-mounted onto 3-aminopropyltriethoxysilane-coated glass slides, air-dried and stored at -80 °C until further processing. Receptor autoradiography was carried out according to Gulya *et al*. (1986) as it is published by Keresztes *et al.* (2011). In brief, sections were incubated with amounts of the radioligand corresponding to approximately three or four times their K_D value, as determined in the kinetic binding experiments, i.e. 2 nM [^{3}H]Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH and 1.5 nM [^{3}H]Ile^{5,6}-deltorphin II (Nevin *et al.,* 1994). The nonspecific binding was measured by the addition of 1 μM naloxone. Tissue sections were washed three times (10 min each) with Tris-HCl buffer (50 mM, pH 7.4, 4 °C). After the final rinse, the sections were quickly dried and exposed to Kodak X-OMAT films for 5 or 9 months at -80 °C. The films were developed with the use of Kodak D-19 developer solution.

3.8. Data analysis

All curve fittings were performed with the GraphPad Prism 4.0 software (GraphPad Prism Software Inc., San Diego, CA, USA). The kinetic binding parameters k_{obs} (observed pseudo-first-order rate constant) and k_d (dissociation rate constant) were obtained by nonlinear regression analysis. The association rate constant, k_a was calculated *via* the equation $k_a = (k_{obs}–k_d)/[\text{radioligand}]$. The equilibrium dissociation constant, K_D was calculated from the kinetic rate constants as follows: $K_D = k_d/k_a$. Nonlinear regression analysis of the saturation curves was performed to obtain the equilibrium K_D and B_{max} (receptor density) values. IC_{50} (the concentration of the ligand required to achieve 50 % inhibition) values were obtained from the displacement curves.

The inhibitory constant, K_i values were calculated with the equation: $K_i = IC_{50}/(1+[ligand]/K_D)$, where K_D values for Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH were 0.25 nM in rat or 0.18 nM in mouse brain membranes taken from the isotope saturation curves. E_{max} (maximal stimulation) and ED_{50} (the concentration of the agonist required to achieve 50 % of the maximal stimulation) values were determined by nonlinear regression of the dose-response curves in the ligand-stimulated [^{35}S]GTPγS functional assays. Apparent antagonist affinity constant, K_e values were calculated with the equation K_e = [antagonist]/(ED_{50} in the presence of antagonist/ED_{50} in the absence of antagonist)-1 (Kosterlitz and Watt, 1968). Statistical analysis was performed using ANOVA or Student's *t*-test analysis.

Autoradiographic images of the sections were scanned at 600 x 600 dpi resolution and analyzed with the computer program Image J (version 1.32; developed by W. Rasband (National Institutes of Health, Washington DC) and downloaded from the Internet at http://rsb.info.nih.gov/ij). Regions of interest were outlined on the computer screen and their signal intensities were measured. Grey values between 0 (lightest) and 255 (darkest) were assigned to the grayness of the images. The specific gray values were determined by subtracting the nonspecific values from the total ones. The lowest value was considered 0 %, while the highest value, corresponding to the highest receptor density in the external plexiform layer of the olfactory bulb, was accepted as 100 %. Grey scale values between 0-33, 33- 66, 66- 90 and 90-100 % were considered as brain areas with low, medium, high and extremely high receptor densities, respectively.

4. Results

4.1. Kinetic parameters of [^{3}H]Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH in rat brain membranes

Association (Fig. 2A) and dissociation (Fig. 2B) binding experiments were performed with [^{3}H]Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH at 25 °C in rat brain membranes. The specific binding reached the steady state by about 60 min and remained stable for 150 min, the longest time examined. At this radioligand concentration (0.7 nM), under equilibrium conditions, the nonspecific binding was about 30 % of the total binding (data not shown).

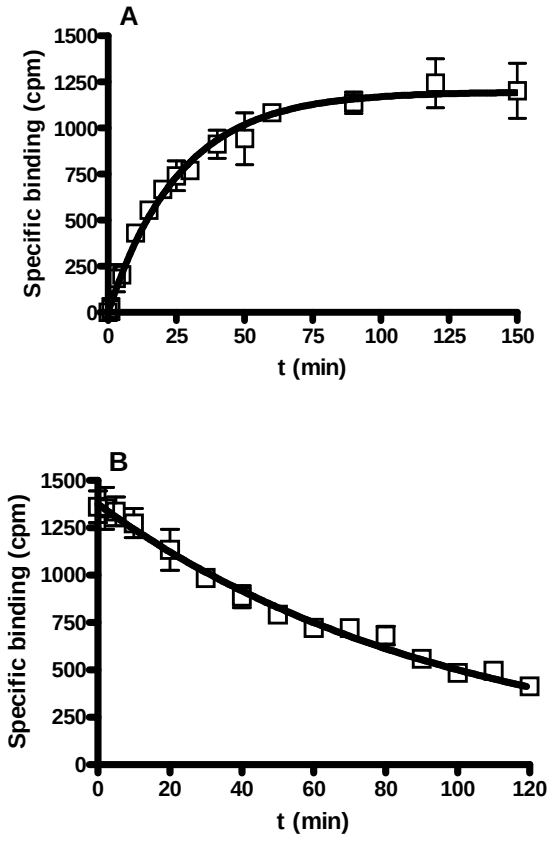


Figure 2. Time course of [^{3}H]Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH binding at 25 °C. A) 0.7 nM radioligand was incubated with rat brain membranes (200-300 μg) for the indicated times. B) Dissociation was initiated by the addition of 10 μM naloxone and measured for 120 minutes. The nonspecific binding was assessed with of 10 μM naloxone and subtracted from the total values to yield in specific binding (□). Means ± S.E.M., n=3, performed in duplicate. Non-visible S.E.M. is within the symbol.

Dissociation of the radioligand proceeded with first-order kinetics with a dissociation rate constant, $k_d = 0.010 \pm 0.001$ min^{-1}. This value and the observed pseudo-first-order rate constant, $k_{obs} = 0.039 \pm 0.003$ min^{-1}, were used to calculate the second-order association rate constant, $k_a = 0.016 \pm 0.002$ min^{-1} x nM^{-1}. The kinetically derived equilibrium dissociation constant K_D was calculated to be 0.64 nM (Table 2).

Table 2. Kinetic parameters of [^{3}H]Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH binding at 25 °C in rat brain membranes

Kinetic parameters	
k_{obs} (min^{-1}) k_d (min^{-1}) k_a (min^{-1} x nM^{-1})	0.039 ± 0.003 0.010 ± 0.001 0.016 ± 0.002
K_D (nM)	0.64

Where k_{obs} is the observed pseudo-first-order rate constant, k_d is the dissociation rate constant determined from the data shown in Figure 2 using GraphPad Prism computer program as described in Methods. The association rate constant, k_a was calculated with the following equation: $k_a = (k_{obs}-k_d)/[\text{radioligand}]$] where the concentration of the radioligand, [radioligand] was 0.7 nM. The equilibrium dissociation constant, K_D was calculated as follows: $K_D = k_d/k_a$. Data are means ± S.E.M. of 3 independent experiments performed in duplicate.

4.2. Equilibrium binding parameters of [^{3}H]Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH in various membranes

The specific binding of [^{3}H]Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH was saturable and of high affinity both in rat and wild type mouse brain, and hDOR-CHO membranes. Computer-assisted analysis of the binding hyperboles indicated that a single-site binding is preferred over a two-site model suggesting the existence of a single population of

binding sites with a K_D of 0.16 ± 0.005 nM and B_{max} of 85.9 ± 6.3 fmol x (mg protein)$^{-1}$ protein in rat, K_D= 0.18 ± 0.02 nM and B_{max}=102.7 ± 9.9 fmol x (mg protein)$^{-1}$ in wild type mouse and 0.57 ± 0.072 nM for the equilibrium dissociation constant and 3100 ± 163 fmol x (mg protein)$^{-1}$ for the receptor density in CHO cells transfected with the human delta opioid receptors (Table 3).

Table 3. Equilibrium binding parameters of [^{3}H]Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH in the absence and in the presence of NaCl in membranes of rat, mouse brains and hDOR-CHO cells

Membrane protein	**Condition**	**B_{max} (fmol x (mg protein)$^{-1}$)**	**K_D (nM)**
Rat brain	+ no addition	85.9 ± 6.3	0.16 ± 0.005
	+ NaCl	95.0 ± 3.8	0.04 ± 0.001*
Wt mouse brain	+ no addition	102.7 ± 9.9	0.18 ± 0.02
	+ NaCl	93.3 ± 1.5	0.024 ± 0.002*
DOR-KO mouse brain		Not detected	Not detected
hDOR-CHO	+ no addition	3100 ± 163	0.57 ± 0.072
	+ NaCl	3957 ± 135*	0.16 ± 0.007*

Receptor density, B_{max} and equilibrium dissociation constant, K_D values were calculated from the data of linear and non-linear regressions using GraphPad Prism computer program as described in Methods. There were no significant differences between the results of the two types of regressions. Data are means ± S.E.M. of at least 3 independent experiments each performed in duplicate. Statistically significant effects of '+ NaCl' (100 mM) versus '+ no addition' on the binding parameters in appropriate membranes were calculated using the Student t-test and indicated as * $p<0.05$.

Notably, no detectable binding was observed in DOR-KO mouse brain membranes showing that deletion of DOR-1 gene results in complete elimination of [^{3}H]Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH binding sites. The antagonist feature of the new radioligand was assessed using the well-known effect of Na^+-ions on opioid binding (Pert and Snyder, 1974). The K_D of the binding sites labeled with [^{3}H]Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH significantly decreased in the presence of 100 mM NaCl in rat and wild type mouse brain and hDOR-CHO membranes, respectively (Table 3). Receptor densities also significantly increased to 3957 ± 135 fmol x (mg protein)$^{-1}$ in the presence of NaCl in the latter. These results indicate that the new radioligand behaves as an antagonist in binding assays.

The specificity of [^{3}H]Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH binding was studied with increasing concentrations of various unlabeled site-specific opioid ligands in displacement experiments. The K_i value derived from a single-site binding for the unlabeled ligand together with that of other opioid ligands is listed in Table 4. The δ-selective ligands showed the highest affinities in the sub- and low nanomolar range for [^{3}H]Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH binding, with a rank order of potency: naltrindole > Tyr-Tyc-(2*S*,3*R*)-β-MePhe-Phe-OH > Ile^{5,6}-deltorphin II >TIPP > DPDPE both in rat and mouse brain membranes. It was found that the unlabeled Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH was 3- and 7-fold more potent than the parent compound, TIPP (Table 4) in mouse and rat brain membranes, respectively.

Among all the ligands tested, naltriben (putative δ_2-selective antagonist) showed the highest affinity and BNTX (prototypic δ_1 antagonist) was 175 times less potent (Table 5). It should be noted that the δ_2-specific ligands, agonists and antagonists alike, were slightly more potent than δ_1-ligands in mouse brain membranes (Table 5). The universal opioid antagonist, naloxone displayed a K_i value of 52.6 ± 8.0 or 11.1 ± 1.6 nM in rat or mouse brain, which is in a good agreement with literature data showing that it labels μ-receptors with 700–fold higher affinity than δ-sites (Akiyama *et al*., 1985). Low potency was seen in the case of μ- and κ-ligands both in rat and mouse brain membranes (Table 4). Thereby, Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH is a highly specific antagonist of the δ-opioid receptors. These results indicate that both δ_1- and δ_2-specific ligands have high

affinity, with a tendency of slightly higher one of the latter, to compete for the binding sites of Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH in radioligand binding experiments *in vitro.*

Table 4. K_i values of site-specific opioid ligands for the binding sites of [^{3}H]Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH in rat and mouse brain membranes

LIGAND	**K_i (nM)**	
	Rat brain membrane	Mouse brain membrane
Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH	0.68 ± 0.08	0.86 ± 0.09
TIPP (δ)	4.85 ± 0.51	2.37 ± 0.28
Naltrindole (δ)	0.24 ± 0.02	0.20 ± 0.02
DPDPE (δ)	7.34 ± 0.87	2.94 ± 0.26
Ile^{5,6}-deltorphin II (δ)	1.85 ± 0.36	1.97 ± 0.20
DAMGO (μ)	618 ± 97	201 ± 23
Endomorphin-2 (μ)	>10000	NM
U50,488 (κ)	>1000	60 ± 9
D-Ala^2-dynorphin-NH_2 (κ)	820 ± 139	201 ± 23
Naloxone (μ >> κ > δ)	52.6 ± 8.0	11.1 ± 1.6

Brain membranes (150-250 μg) were incubated with 0.7-1.5 nM [^{3}H]Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH in the presence of 10^{-12}-10^{-5} M of unlabeled ligands for 90 min at 25 °C. K_i values were calculated by fitting the displacement curves using GraphPad Prism program nonlinear least-squares algorithm. Specific binding in the absence of opioids was 67± 2.4 fmol x (mg protein)$^{-1}$ and 64 ± 6.1 fmol x (mg protein)$^{-1}$ in rat and mouse brain membranes. Means ± S.E.M. of n ≥ 3, all performed in duplicate. NM means not measured.

Table 5. K_i values of the putative δ-opioid subtype selective ligands for the binding sites of [^{3}H]Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH

LIGAND	**K_i (nM)**
Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH	0.86 ± 0.09
DPDPE (δ_1)	2.94 ± 0.26
BNTX (δ_1)	3.21 ± 0.22
Ile^{5,6}-deltorphin II (δ_2)	1.97 ± 0.20
Naltriben (δ_2)	0.0183 ± 0.0009

Mouse (≈150 ug protein) brain membranes were incubated with 0.7-1.5 nM [^{3}H]Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH in the presence of 10^{-12}-10^{-5} M of unlabeled ligands for 90 min at 25 °C. K_i values were calculated by fitting the displacement curves using GraphPad Prism program nonlinear least-squares algorithm. Specific binding in the absence of opioids was around 64 ± 6.1 fmol x (mg protein)$^{-1}$. Means ± S.E.M. of n ≥ 3, all performed in duplicate.

4.3. Probing the subtype specificity of Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH in antinociception assay in mice

The *in vivo* specificity of Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH was investigated by measuring its ability to antagonize the analgesic effect of putative subtype selective agonists at maximally effective doses as determined earlier (Zhu *et al.*, 1999). It was found that Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH (10 µg, 13 nmol) significantly inhibited by about 60% the effect of nearly equimolar dose of DPDPE, the putative δ_1-selective agonist (Figure 3A). The same concentration of the antagonist had no significant effect on the antinociceptive effect of the putative δ_2-selective agonist, Ile^{5,6}-deltorphin II (15 µg, 19 nmol) and the µ-specific agonist, DAMGO (Figure 3B, C). These results suggest that Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH may behave as a putative δ_1-specific antagonist in the tail-flick analgesic assay.

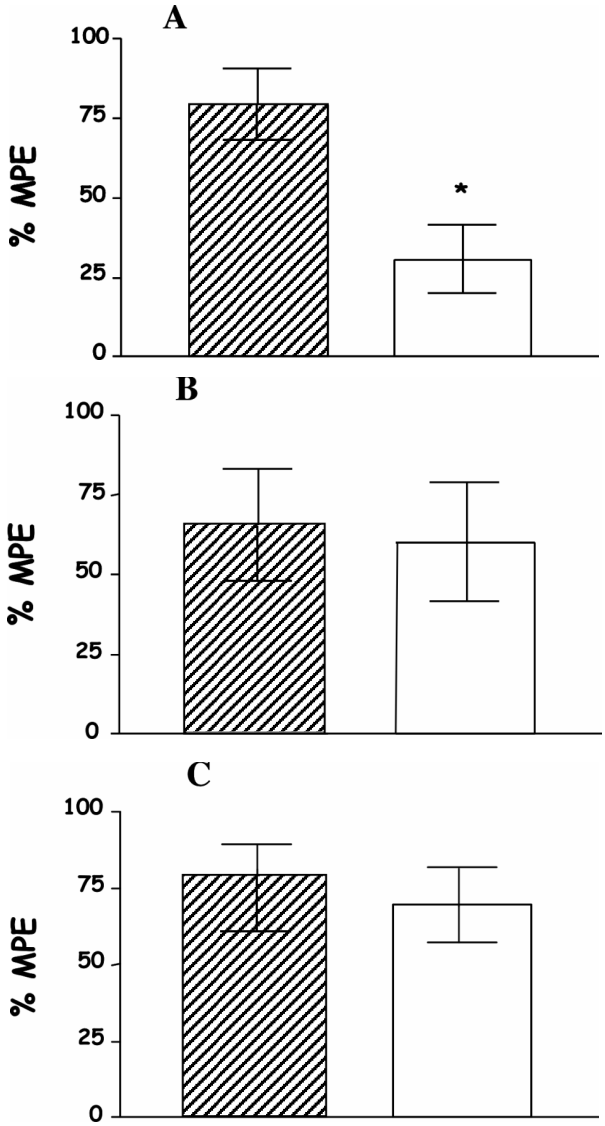


Figure 3. Mice were injected *intrathecally* with 5 µl saline (striped boxes) or Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH (10 µg, 13 nmol, empty boxes) followed by 2 µl of either A) DPDPE (8 µg, 12 nmol, hypothetical δ_1-agonist), B) Ile^{5,6}-deltorphin II (15 µg, 19 nmol, hypothetical δ_2-agonist) or C) DAMGO (6 ng, 12 pmol, µ-agonist). The radiant heat tail-flick analgesic assay utilized a light intensity that produced baseline latencies ranging from 2-3 s. A 10 sec cut-off was imposed to minimize tissue damage. % MPE was calculated as described in Methods. Group comparisons were performed by Student's *t*-test analysis. Significant antagonist effect is shown as * $p < 0.01$, $n \geq 7$.

4.4. Probing the subtype specificity in [^{35}S]GTPγS functional assays using putative δ_1- and δ_2-opioid selective ligands in various membranes

To examine the *in vitro* pharmacology of the new TIPP-derivative in various systems, we have also performed ligand-stimulated [^{35}S]GTPγS functional assay. It is a widely applied method, which shows G-protein activation due to agonist action, the first step in the functioning of any G-protein coupled receptor, such as the opioid receptors.

Using agonists of various specificity, at various concentrations, it was found that DPDPE resulted in 32.9 ± 1.9 % stimulation with an ED_{50} of 2009 ± 67 nM, while 38.0 ± 1.1 % stimulation and 584 ± 13 nM ED_{50} value was determined for deltorphin II in wild type mouse membranes (Figure 4, Table 6). It can be seen that Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH dose-dependently inhibited DPDPE-stimulation of G-protein activation and completely eliminated at 100 nM (Figure 4A). Nanomolar concentrations of the antagonist decreased the potency, without influencing the efficacy, of the agonist. Thereby, this concentration was used for further studies in determining its potency on putative δ-subtypes, see below.

The antagonist potency (K_e) of Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH was determined and compared to that of known subtype-specific antagonists using a fixed concentration of the antagonists against various concentrations of δ_1- and δ_2-agonists (Table 6-7). The optimal concentration of each antagonist was determined in preliminary experiments so that it should increase the ED_{50} without affecting the E_{max} value of the agonist. K_e values of the TIPP-derivative were calculated as described in Methods and compared to those of proposed δ_1- (BNTX) and δ_2-selective (naltriben) antagonists (Table 6-7). The antagonist potency of Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH was 2.49 ± 0.06 and 0.30 ± 0.01 nM against DPDPE and deltorphin II in mouse brain membranes, respectively. These values agree well with the affinity of the ligand in binding experiments. Although the δ_2-antagonist naltriben was more potent (lower K_e) against the δ_2-agonist and BNTX was equipotent against DPDPE and deltorphin II, the rank order of the antagonist potencies was similar in the case of the two agonists: naltriben > Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH > TIPP > BNTX in mouse brain homogenate (Table 6).

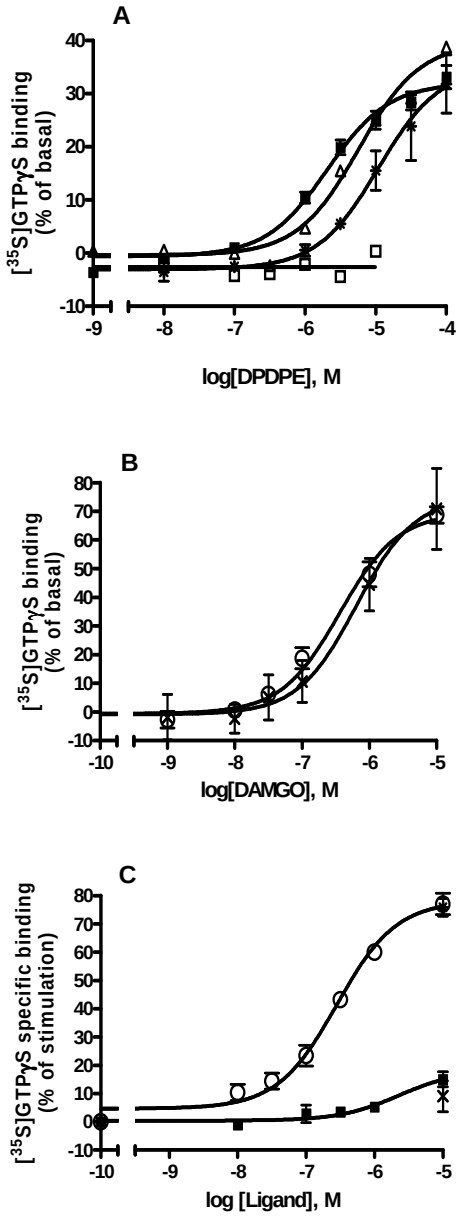


Figure 4. Effect of Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH on agonist-stimulated [^{35}S]GTPγS binding in wt (A, B) and DOR-KO (C) mouse brain membranes. A) DPDPE (■) at 10^{-8}-10^{-4} M was co-incubated with Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH at 5 nM (△), 10 nM (*) or 100 nM (□). DAMGO (○) at 10^{-9}-10^{-5} M was co-incubated with Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH at 100 nM (X). Basal activities (80.5 ± 15.5 and 63.4 ± 1.4 fmol x (mg protein)$^{-1}$ in wild type and DOR-KO mouse brain, respectively) were assessed in the absence of opioids and defined as 0%. Means ± S.E.M., n ≥ 3, performed in triplicate. Non-visible S.E.M. is within the symbol.

Table 6. Antagonist potency (K_e) of Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH and putative δ-opioid receptor subtype specific antagonists in mouse brain membranes

Wild type mouse	**E_{max} (% stimulation)**	**ED_{50} (nM)**	**K_e (nM)**
DPDPE (δ_1)	32.9 ± 1.9	2009 ± 67	
+ Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH (10 nM)	35.9 ± 6.1	10806 ± 93*	2.49 ± 0.06$^{\#}$
+ TIPP (10 nM)	40.5 ± 2.9	9924 ± 164*	2.66 ± 0.15$^{\#}$
+ BNTX (10 nM)	27.3 ± 3.7	9894 ± 338*	2.65 ± 0.02$^{\#}$
+ naltriben (4 nM)	34.3 ± 2.0	12245 ± 396*	0.77 ± 0.05^{+}
Deltorphin II (δ_2)	38.0 ± 1.1	584 ± 13	
+ Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH (5 nM)	43.9 ± 2.2	10177 ± 327*	0.30 ± 0.01^{+}
+ TIPP (5 nM)	44.0 ± 5.3	4133 ± 482*	0.86 ± 0.10$^{+,\#}$
+ BNTX (10 nM)	34.3 ± 0.7	3245 ± 100*	2.24 ± 0.13$^{\#}$
+ naltriben (1 nM)	39.9 ± 3.0	3775 ± 517*	0.20 ± 0.03^{+}

Increasing concentrations (10^{-10}-10^{-4} M) of DPDPE or deltorphin II were incubated alone or in the presence of constant concentrations of Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH, TIPP, BNTX and naltriben as indicated. Basal activities assessed in the absence of opioids were defined as 0 %. E_{max} and ED_{50} values were fitted using GraphPad Prism software. K_e values were calculated as described in Methods. The data represent means ± S.E.M., n ≥ 3, all performed in triplicate. Significant differences were determined by one-way-ANOVA and set at $p < 0.05$ as follows: binding parameters of DPDPE or Deltorphin II in the absence and presence of antagonists *, antagonist potency of BNTX vs. other antagonists $^{+}$, and antagonist potency of naltriben vs. antagonists $^{\#}$.

Since the mouse brain is very heterogeneous and the presence of μ-opioid receptors has been shown to influence the pharmacology of the ligands (Traynor and Elliot, 1993; Zaki *et al*., 1996; Scherrer *et al*., 2004), the existence of δ-subtypes and their blockade by antagonists were also investigated in CHO cells transfected with the human δ_2-opioid receptors (Malatynska *et al*., 1995). DPDPE gave 237.9 ± 13.1% stimulation with an

ED_{50} of 24.4 ± 0.3 nM and Ile^{5,6}-deltorphin II gave 152.9 ± 4.0% stimulation over basal activity and its ED_{50} value was 2.0 ± 0.1 nM in hDOR-CHO membranes (Table 7).

Table 7. Antagonist potency (K_e) of Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH and putative δ-opioid receptor subtype specific antagonists in hDOR-CHO cell membranes

hDOR-CHO	**E_{max} (% stimulation)**	**ED_{50} (nM)**	**K_e (nM)**
DPDPE (δ_1)	237.9 ± 13.1	24.4 ± 0.3	
+ Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH (10 nM)	239.2 ± 20.4	508.1 ± 18.6*	0.51 ± 0.02^{+}
+ TIPP (10 nM)	250.8 ± 10.9	319.7 ± 43.9*	0.85 ± 0.11^{+}
+ BNTX (10 nM)	238.2 ± 23.1	112.6 ± 12.7*	2.86 ± 0.36$^{\#}$
+ naltriben (1 nM)	230.0 ± 29.9	636.5 ± 31.7*	0.04 ± 0.004^{+}
Ile^{5,6}-deltorphin II (δ_2)	152.9 ± 4.0	2.0 ± 0.1	
+ Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH (10 nM)	179.0 ± 5.2	146.7 ± 13.1*	0.15 ± 0.02^{+}
+ TIPP (10 nM)	168.9 ± 8.5	95.9 ± 8.0*	0.22 ± 0.02$_{+,\#}$
+ BNTX (10 nM)	152.6 ± 6.6	49.0 ± 3.9*	0.44 ± 0.05$^{\#}$
+ naltriben (1 nM)	152.5 ± 6.4	150.3 ± 12.9*	0.01 ± 0.001^{+}

Increasing concentrations (10^{-10}-10^{-4} M) of DPDPE or Ile^{5,6}-deltorphin II were incubated alone or in the presence of constant concentrations of Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH, TIPP, BNTX and naltriben as indicated. Basal activities assessed in the absence of opioids were defined as 0 %. E_{max} and ED_{50} values were fitted using GraphPad Prism software. K_e values were calculated as described in Methods. The data represent means ± S.E.M., n ≥ 3, all performed in triplicate. Significant differences were determined by one-way-ANOVA and set at $p < 0.05$ as follows: binding parameters of DPDPE or Ile^{5,6}-deltorphin II in the absence and presence of antagonists *, antagonist potency of BNTX vs. other antagonists $^{+}$, and antagonist potency of naltriben vs. antagonists $^{\#}$.

The antagonist potencies of the tested ligand displayed the same rank order, i.e. naltriben > TIPP ~ Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH > BNTX both against DPDPE and Ile^{5,6}-deltorphin II. These results imply that δ-opioid receptor subtypes could not be

distinguished by the ligand-stimulated [^{35}S]GTPγS assay neither in a cell line expressing a homogenous population of δ-opioid receptors nor in wild type mouse brain membranes.

4.5. Autoradiographic distributions of [^{3}H]Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH and [^{3}H]Ile^{5,6}-deltorphin II

The specificity of the binding sites in mosue brain of [^{3}H]Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH was studied by receptor autoradiography and compared to that of [^{3}H]Ile^{5,6}-deltorphin II (putative δ_2-subtype specific ligand).

Table 8. Autoradiographic signal intensities of [^{3}H]Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH and [^{3}H]Ile^{5,6}-deltorphin II binding in representative regions of wild type mouse brain

Brain regions	[^{3}H]Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH binding	[^{3}H]Ile^{5,6}-deltorphin II binding
Olfactory bulb (total)	+++	+++
External plexiform layer	++++	++++
Olfactory tubercle	++	++
Primary motor cortex	++	++
Occipital cortex	+	+
Caudate putamen	++	++
Medial septal nucleus	+	+
Hippocampus	+	+
Thalamus	+	+
Hypothalamus	+	+
Cerebellum	+	+
Corpus callosum	+	+

The sections were incubated with [^{3}H]Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH (2 nM) or [^{3}H]Ile^{5,6}-deltorphin II (1.5 nM) as described in Methods. The regions of interest of *coronal* sections were outlined on the computer screen and their signal intensities were measured. Grey scale values between 0-33, 33-66, 66-90 and 90-100% were considered as brain areas with low (+), medium (++), high (+++) and extremely high (++++) receptor densities, respectively.

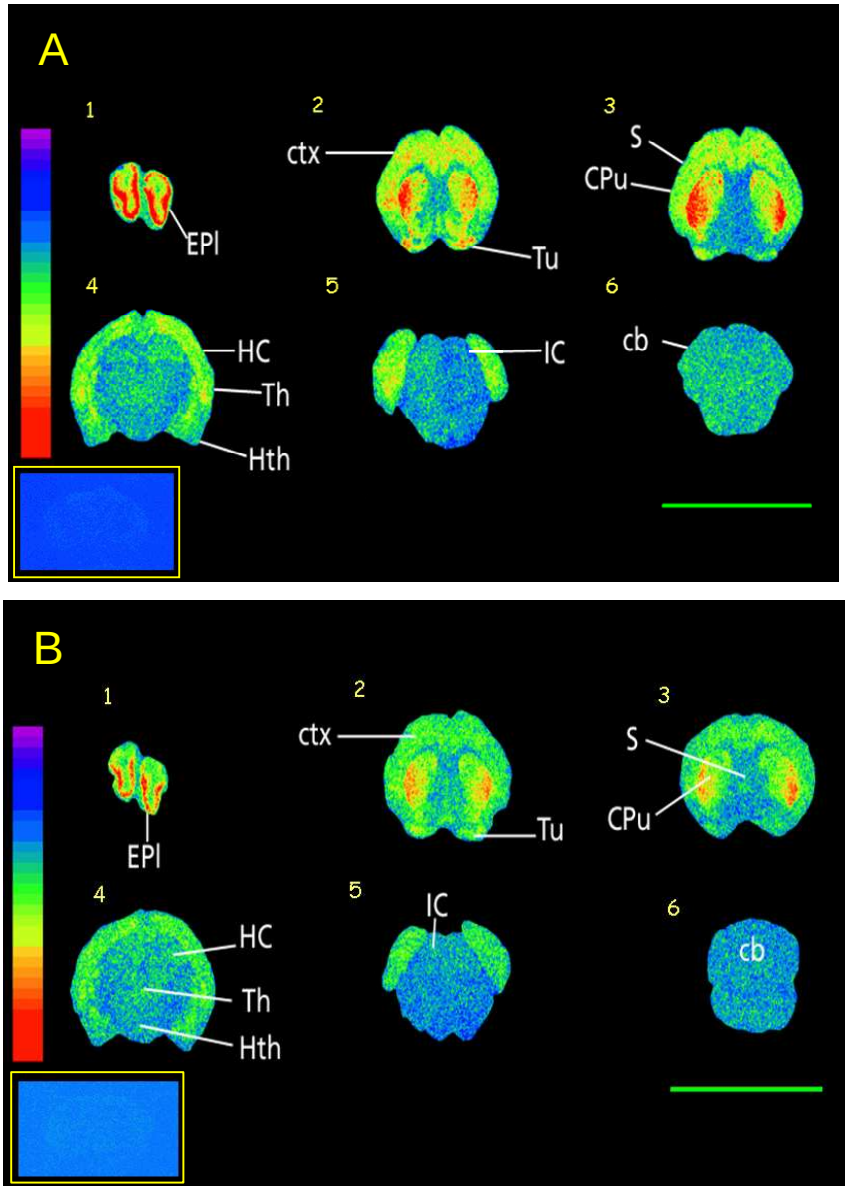


Figure 5. False-colored autoradiograms of the binding sites labeled with 2 nM [^{3}H]Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH (A) and 1.5 nM [^{3}H]Ile^{5,6}-deltorphin II (B). Representative *coronal* sections are shown at the following approximate Bregma values: 3.56 (1), 1.10 (2), 0.14 (3), -1.58 (4), -4.84 (5) and -6.84 (6) mm outlined according to Franklin and Paxinos (Franklin and Paxinos 2004). CPu: caudate-putamen (striatum), cb: cerebellum, ctx: cerebral cortex, EPI: external plexiform layer of olfactory bulb, HC: hippocampus, Hth: hypothalamus, IC: inferior colliculus, S: septum, Th: thalamus, Tu: olfactory tubercle. Scale bar = 1 cm is shown in green at the lower right hand corner. The color bar was computer-generated and designed to give a measure of relative densities within an autoradiogram. Red, yellow and blue colors represent the highest, medium and lowest levels of binding, respectively. Insert in the left bottom corner: representative false-colored autoradiogram of the nonspecific binding measured in the presence of 1 μM naloxone.

There were no significant differences between the distributions of the binding sites of the two radioligands (Figure 5, Table 8). High levels were detected in the olfactory bulb, extremely high in the external plexiform layer of the olfactory bulb, medium levels were shown in the olfactory tubercle, nucleus of the caudate putamen and the primary motor cortex. Low signals were observed in the hippocampus, thalamus, hypothalamus and cerebellum (Table 8). These results are in good agreement with the distribution of [^{3}H]Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH in rat brain (data not shown, see Birkas *et al.*, 2008).

Thereby, the regional distributions of the two radioligands did not reveal significant differences and agree well with that reported for [^{3}H]TIPP (Bakota *et al*., 1998) and other δ-opioid ligands (Mansour *et al*., 1993; Bausch *et al*., 1995) in mouse brain. No specific labeling was detected with either [^{3}H]Ile^{5,6}-deltorphin II or [^{3}H]Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH in DOR-KO mouse brain sections (data not shown).

5. Discussion

Here we report on the detailed pharmacological characterization of a new, highly potent, δ-opioid antagonists, [^{3}H]Tyr-Tic-(*2S,3R*)-β-MePhe-Phe-OH. The novelty of the ligand is that Phe^3 was substituted by L-*threo* (*2S,3R*)- β-MePhe^3 in the prototype of a new class of highly potent and selective δ-opioid antagonists, TIPP. It was selected for radiolabeling followed by detailed receptor binding characterization based on previous studies, showing that β-methyl substitutions in different configurations had profound effects on the potency, selectivity and agonist/antagonist character of TIPP derived peptides (Tourwe *et al.,* 1998).

Association and dissociation experiments showed that the formation of the ligand-receptor complex proceeded with second-order kinetics and was reversible. Saturation experiments were performed in membranes of rat and mouse brains, and in hDOR-CHO cells. In all of these systems, the equilibrium saturation experiments revealed a single population of high affinity binding sites (Table 3). It should be noted that the receptor density was slightly higher in mouse than in rat brain. K_D values obtained in equilibrium binding studies are in good agreement with the results of the kinetic experiments (Table 2). Also, the low nanomolar affinities agree well with previous literature data using the parent compound or its analogs (Nevin *et al.,* 1993; Ioja *et al.,* 2007) as well as other δ-selective ligands (Mosberg *et al*., 1983; Portoghese *et al*., 1988; Sasaki *et al*., 1991).

We also investigated the K_D values of the TIPP-analog in the presence of NaCl, since it is well known from the literature that Na^+ increases the affinity of the opioid antagonists (Simon *et al*., 1975). We found that the K_D value of [^{3}H]Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH decreased by about 4-fold in rat brain and hDOR-CHO and about 7-fold in mouse brain membranes showing increased affinity of the ligand in the presence of NaCl. These results suggest that the TIPP-analog retained the antagonistic character of the parent ligand (Table 3). No specific binding was detected in DOR-KO mouse brain membranes showing that the new ligand is specific for δ-opioid receptors.

Competition binding experiments were performed and K_i values were defined in rat and mouse brain membranes (Table 4). These results show that all the tested δ-opioid ligands displaced the [^{3}H]Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH with K_i-s in nanomolar range. Low potencies were shown in the case of μ- and κ-opioid ligands confirming the

δ-opioid specificity of the TIPP-analog. It was also shown, that Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH was about 7-fold and 3-fold more potent compared to the parent ligand in rat and mouse brain membranes, respectively (Table 4). These results confirm and extend the previous data obtained with the unlabeled new TIPP analog competing for [^{3}H]DPDPE binding in rat brain (Tourwe *et al*., 1998).

Interestingly, we found that Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH displaced more binding of [^{3}H]Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH than the nonselective δ-antagonist, naltrindole or any other tested δ-opioid ligand in mouse brain membrane in competition binding assay. These results suggest that the investigated TIPP-analog may also bind to another receptor population, such as other subtypes of the δ-opioid receptors, than the tested ligands. Therefore, we tried to recognize the putative δ-opioid receptor subtypes, and investigate the possible δ_1-δ_2-selectivity of the TIPP-analog by *in vivo* and *in vitro* methods.

Although there are strong *in vivo* pharmacological evidences to support the existence of the δ-opioid receptor subtypes (Jiang *et al*., 1991; Mattia *et al*., 1991; Sofuoglu *et al*., 1991; van Rijn and Whistler, 2009; Zaki *et al*., 1996), that is still not clear *in vitro*. Therefore, firstly we have examined the antagonist effect of Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH in mouse tail-flick analgesic test using the putative δ_1-specific agonist, DPDPE, the δ_2-selective agonisty Ile^{5,6}-deltorphin II and the μ-specific agonist DAMGO (Figure 3). We found that the TIPP-derivative inhibited by about 60% the antinociceptive effect of the hypothetical δ_1-selective DPDPE, without significantly changing the effect of Ile^{5,6}-deltorphin II or DAMGO. These results suggest that the Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH behaves as a δ_1-selective antagonist *in vivo*.

The *in vitro* binding selectivity of [^{3}H]Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH was studied by binding experiments and receptor-autoradiography. We compared the potency of our TIPP-analog with ligands of well-known δ-opioid receptor subtype selectivities in competitive experiments. We found that the δ_2-ligands, agonists and antagonists alike, were slightly more potent than δ_1-ligands in displacing [^{3}H]Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH from its binding sites (Table 5).

W also examined the distribution of the binding sites of the [^{3}H]Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH by receptor autoradiography and compared to that of the hypothetical δ_2-

selective [^{3}H]Ile^{5,6}-deltorphin II in mouse brain slides. We detected high levels of the binding sites in the olfactory bulb, especially in the external plexiform layer of it (Table 8). Medium levels were shown in the olfactory tubercle, the caudate putamen and the primary motor cortex, while low densities were found in the hippocampus, thalamus, hypothalamus and cerebellum (Figure 5). These results are also in a good agreement with the distributions of the binding sites of [^{3}H]Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH in rat brain coronal sections (data not shown, see Birkas *et al.*, 2008). The distributions of [^{3}H]Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH and [^{3}H]Ile^{5,6}-deltorphin II agree well with the known patterns of the δ-opioid receptors (Bausch *et al*., 1995; Mansour *et al*., 1993) and in particular with the parent ligand TIPP (Bakota *et al*., 1998).

Contrary to our results, Hiller *et al*. (1996) found significant differences between the localization of the binding sites labeled with [^{3}H]DPDPE or [^{3}H]DSLET in the case of some single anatomical structures. No specific labeling was detectable with either [^{3}H]Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH or [^{3}H]Ile^{5,6}-deltorphin II in DOR-KO mouse brains (data not shown), which agrees well with our results of saturation binding and the results of Zhu *et al*. (1999). They found that the δ_2-opioid gene (DOR-1) encodes both the hypothetical δ_1- and δ_2-opioid receptors, and the deletion of this gene eliminates any δ-opioid binding (Zhu *et al*., 1999).

We also studied the receptor functionality by the ligand-stimulated [^{35}S]GTPγS assays using putative δ_1- (DPDPE, BNTX) and δ_2- (deltorphin II, Ile^{5,6}-deltorphin II, naltriben) opioid ligands. We determined the K_e values of the antagonists and we compared the selectivity of the Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH to them in wild type mouse and hDOR-CHO cell membranes. DPDPE and deltorphin II were similarly potent and efficacious agonists in wild type mouse, resulting 33% and 38% stimulation of [^{35}S]GTPγS binding over basal activities, which agree well with the result of Parkhill and Bidlack (2002). The K_e value of the TIPP-analog against DPDPE was closer to that of the putative δ_1-selective BNTX, while it was closer to that of the δ_2-selective naltriben against deltorphin II (Table 6). These results suggest that the antagonist potency of the Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH was affected by the δ-selectivity of the agonists. However, the δ_2-selective naltriben was much more effective than the putative δ_1-selective BNTX against DPDPE and deltorphin II too and both of these two antagonist

were much more active in blocking the effect of the hypothetical δ_2-selective deltorphin II than that of the δ_1-selective DPDPE (Table 6). These results agree well with the result of Parkhill and Bidlack (2002) suggesting that the the δ-opioid receptor subtypes were indistinguishable in the *in vitro* functional test in mouse brain and hDOR-CHO cell membranes with those ligands available up to now.

As it is known from the literature, the functionality of the δ-opioid receptors may be altered in the presence of the μ-opioid receptors (Traynor and Elliot, 1993; Zaki *et al.*, 1996, and references therein). Therefore, we were interested to see whether different results would be obtained in homogenous system or not, such as in hDOR-CHO cell membranes expressing only the δ-opioid receptors. We found that the putative δ_2-selective naltriben was more potent than the δ_1-selective BNTX against DPDPE and Ile^{5,6}-deltorphin II in hDOR-CHO cells, which is in good agreement with our results in mouse brain membranes (Table 7). The antagonist potencies of the tested ligand displayed the same rank order suggesting that δ-opioid receptor subtypes could not be distinguished in hDOR-CHO cells transfected with the DOR-1 gene. We also found that the putative δ_1-selective DPDPE stimulated the G-protein activation with an E_{max} value 237.9 ± 13.1 %, while the δ_2-selective Ile^{5,6}-deltorphin II gave an E_{max} value 152.9 ± 4.0 % in hDOR-CHO membranes (Table 7). Since only δ-opioid receptors are expressed in hDOR-CHO membranes (Malatynska et al., 1995), receptors could form just δ-opioid receptors homomers or perchance δ_1-δ_2 heteromers. There is no data in the literature, whether the agonists, DPDPE and Ile^{5,6}-deltorphin II activate the same receptor population in hDOR-CHO. As a conclusion of our results we can summarize that the δ_1-selective profile of the Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH seen *in vivo* could not be detected *in vitro*.

We also found that DPDPE stimulated [^{35}S]GTPγS binding was blocked dose-dependent manner by the TIPP-analog in wild type mouse brain (Figure 4A), and no significant stimulation by DPDPE could be detected in DOR-KO mouse brain membranes (Figure 4C). We also demonstrated that DAMGO stimulated [^{35}S]GTPγS binding was not changed in DOR-KO mouse brain membranes compared to that in wild type mouse brain suggesting that deletion of the DOR-1 gene does not result in compensatory changes in the μ-opioid system (Figure 4B,C).

Although the putative δ-opioid receptor subtypes could not be distinguished *in vitro*, it does not exclude the possibility of the existence of these subtypes. One possible explanation of the subtypes is that different ligands have different pharmacokinetics, and as a consequence, different receptor populations are accessible to different ligands. Also selectivity of a ligand *in vitro* may not correspond to its specificity *in vivo* and *vice versa*. Several studies suggest that if multiple opioid-receptor subtypes exist, they could be derived from a single gene, and multiple mechanisms might account for the observed distinct pharmacologic profiles. Alternative splicing of receptor mRNA could be one possible mechanism. Previous data have shown that an Asp95Asn substitution in the transmembrane II receptor domain impaired the binding of delta agonists, although it did not appear to be important for the binding of δ-antagonists (Kong *et al*., 1993). Recently, another (A107V) polymorph of δ-opioid receptor has been described (Sacharczuk *et al*., 2010).

Growing number of data have shown that G-protein coupled receptors, among them the opioid receptors, are able to form homo-and heterooligomers, which may change the pharmacology of the receptors and provide an explanation for the existence of receptor subtypes (George *et al*., 2000; Jordan and Devi, 1999; Levac *et al*., 2002). The contribution of the δ-opioid receptor oligomerization to the existence of receptor subtypes with different pharmacology is still contradictory. It has been suggested that the δ_1-opioid receptor is a result of heterodimerization between the δ- and κ-opioid receptors (Portoghese *et al*., 2003). Others proposed that while the δ_1-opioid receptor is a δ-μ heterodimer, the δ_2-opioid receptor is a δ-δ homodimer (van Rijn and Whistler, 2009) Deltorphin II was suggested to be a full agonist for δ–μ-receptor heterodimer (Fan *et al.*, 2005) DAMGO, DPDPE, morphine, endomorphin-1, endomorphin-2, etc were able to activate the heterodimer (George *et al.*, 2000). It is an intriguing observation of the present work that δ_2-like features manifest both in the cell line expressing recombinant δ-receptors and mouse brain, a conclusion also reached by others (Parkhill and Bidlack, 2002; Sofuoglu *et al.*, 1991). Since only δ_2-receptors exist as functional monomers, the appearance of δ_1-like behaviors would be expected, due to the presence of μ-and κ-sites, which may form heterooligomers with the δ-receptors in the latter.

Hypothetically, the different cellular localization, thereby distinct cellular milieu of the δ-opioid receptor protein could manifest in different pharmacological profiles (*Ho et al.*, 1997). It has been documented that the majority of δ-opioid receptors is localized in the cytoplasm, and only the minority of the δ-opioid receptors is located in the plasma membrane under normal homeostatic conditions (Arvidsson *et al.*, 1995; Cahill *et al.*, 2001; Zhang *et al.*, 1998). Distribution of the receptors is dynamically regulated, thus it is possible that subtypes of the δ-opioid receptor could be selectively expressed on the surface only under certain physiological conditions**,** such as drug exposure. The receptor protein primarily partitions into membrane lipid raft microdomains in brain membranes, NG108-15 cells and CHO cells. Huang *et al*. (2007) found that the treatment with full agonists shifts a part of the δ-opioid receptor out of lipid rafts, which may undergo internalization. Several GPCRs and their downstream effectors have been shown to be regulated by lipid rafts/caveolae (Chini and Parenti, 2004; Ostrom and Insel, 2004; Pike, 2003).

This question will need further investigations in the future when methods will be available to resolve the issue under physiological conditions.

6. Summary

- We characterized the conformationally constrained, tritiated peptide, [^{3}H]Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH by *in vitro* binding experiments and receptor autoradiography:
    - association and dissociation experiments showed that the formation of the ligand-receptor complex proceeded with a second-order kinetics and was reversible;
    - saturation experiments revealed a single population of high affinity binding sites and the antagonist character of the ligand;
    - it was also shown, that the new radioantagonist was more potent than the parent ligand;
    - the detected distribution of the [^{3}H]Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH agreed well with the well-known distribution of the δ-opioid receptors;
    - it was proved that deletion of the DOR-1 gene eliminates any Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH binding without any compensatory changes of the μ-opioid receptors in DOR-KO mouse brains , showing that it is a δ-selective ligand.
- We showed that Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH is a potent δ-opioid antagonist in the tail-flick analgesic assay. Moreover, it behaved as a δ_1-selective antagonist against putative subtype selective agonists.
- Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH also behaved as a potent δ-antagonist in the in *vitro* functional test. However, the hypothetical δ-opioid receptor subtypes were indistinguishable *in vitro*.

As a conclusion, we can summarize that the new antagonist may be a valuable pharmacological tool for various applications, including studies on binding to receptors, intracellular and tissue distribution. In addition, since it is a topographically constrained ligand, it may contribute to the understanding of the structural and topographic requirements of peptide binding to δ-opioid receptors.

7. References

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Acknowledgements

Firstly, I would like to thank my supervisor, ***Dr. Mária Szűcs***, for giving me the chance to work in her group, for giving me useful advices during my work.

I am thankful for ***Dr. Géza Tóth and Dr. Csaba Tömböly***, for giving us many of the ligands, which were necessary for my Ph.D. work.

I would like to thank ***Dr. John Pintar and Dr. Ting Wen*** for the *in vivo* tail-flick experiments and for providing us DOR-KO mice brains.

Many thanks are due to ***Dr. Károly Gulya*** for giving me the possibility to work in his laboratory and for giving me many useful advices.

I am grateful to ***Dr. Lidia Bakota***, who taught me for processing receptor-autoradiography and helped me during these experiments. I also thank her for her friendship and her heartening words.

I am grateful to ***Ildikó Némethné*** for her excellent technical assistance and for her friendship. I will never forget her nice words, and she will always be a part of my life and I will always look for her as a kind member of my family.

I also wish to thank ***Dr. Resat Cinar and Orsolya Kékesi*** for their useful advices and for their friendships.

I also would like to thank ***Dr. Attila Keresztes*** for his useful advices and for ***Éva Tóthné*** for her excellent technical help.

I am grateful to all of ***my colleagues in the Opioid Research Group and in the Isotope Laboratory***.

I would like to thank for ***the leaders of the University of Szeged*** for giving me the possibility to wade in Ph.D. program and for financial supporting me. I am also thankful for ***the leaders of the Institute of Biochemistry of the Biological Research Centre*** for supporting me and for giving me the chance to finish my Ph.D. work.

Last, but not least, I would like to thank my family, especially ***my husband and my parents*** for supporting me and for always having faith in me.