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Ph.D. Thesis

BIOCHEMICAL CHARACTERIZATION OF μ- AND δ-OPIOID RECEPTORS USING HIGHLY SELECTIVE OPIOID ANALOGUES

by

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We humans pride ourselves, rightly or not, on our intelligence and we are aware that the seat of that intelligence resides in that great gray structure we call ***the brain****.*

—ROBERT A. WALLACE (1991)
"Biology: The Science of Life"

To
my parents

CONTENTS

ABSTRACT

Opioid compounds include endogenous peptides and their synthetic analogues, alkaloids derived from opium, and semisynthetic alkaloids. They interact with specific cell-membrane receptors and modulate a variety of physiological processes, including pain perception, regulation of respiratory, gastrointestinal, cardiovascular, urinary and immune functions, regulation of body temperature and hormonal secretion. Development of tolerance and dependence to opioid compounds has also been thought to be mediated through opioid receptors. Pharmacological and biochemical studies have defined at least three major types of **opioid receptors, μ, δ and κ**, that differ in their ligand selectivity and anatomical distribution. Opioid receptors belong to the G-protein-coupled receptor superfamily with a seven transmembrane domains topology that are negatively linked to adenylyl cyclase.

Important tools to investigate opioid receptor multiplicity and functions have come from the **development of highly selective opioid drugs.** Most of the synthetic ligands mimic the structure of opioid peptides or natural alkaloids.

In this Ph.D. work we investigate the opioid receptor binding characteristics of several newly synthesized ***peptide radioligands***, as follow: the dermorphin tetrapeptide analogue [^{3}H]Tyr-D-Ala-Phe-Phe-NH_2 ([^{3}H]TAPP), the deltorphin analogues [^{3}H]S-Atc^3,Ile^{5,6}deltorphin I and [^{3}H]R-Atc^3,Ile^{5,6}deltorphin II; and the ***alkaloid ligands***: benzofuran derivatives of naloxone, oxycodone and oxymorphone.

Characteristic binding parameters (affinity and selectivity) were determined and compared with those of other well-known compounds labeling opioid receptors.

Ligand-receptor interaction was characterized by the use of **radioligand binding assays**. In addition to rat brain membrane preparations, Chinese hamster ovary (CHO-μ/1) cell line transfected with the cloned rat μ-opioid receptor has also been used for biochemical characterization of [^{3}H]TAPP.

(I). Interaction of a newly synthesized **dermorphin tetrapeptide analogue [^{3}H]TAPP** with the μ-opioid receptor was characterized and compared in membrane preparations from rat brain and from CHO-μ/1 cells. In rat brain, [^{3}H]TAPP specifically labeled a single class of opioid sites with a dissociation constant (K_d) of 0.31 nM and maximal number of binding sites (B_{max}) of 119 fmol/mg protein. In CHO-μ/1 cell membranes, the K_d and B_{max} values were 0.78 nM and 1807 fmol/mg protein, respectively. Competition binding studies indicated that in rat brain membranes

[^{3}H]TAPP labeled a receptor site with pharmacological properties similar to those exhibited by the μ-opioid receptors heterologously expressed on CHO cells. [^{3}H]TAPP binding was stereospecific, and potentially inhibited by the selective μ-ligands. Specific binding of [^{3}H]TAPP was significantly inhibited by Na^+ ions and guanine nucleotides, in agreement with the agonist character of the peptide. Moreover, the decrease of specific [^{3}H]TAPP binding in the presence of non-hydrolizable GTP analogue, Gpp(NH)p, also indicated the functional coupling of the μ-opioid receptor labeled by this ligand to a G-protein regulated signal transduction system in both rat brain and CHO-μ/1 cells.

It was demonstrated that in rat brain membranes, the specific, saturable binding of [^{3}H]TAPP is pharmacologically identical to the rat μ-opioid receptor expressed in CHO cells. Compared with the best known μ-selective agonist radioligand peptides, [^{3}H]TAPP showed the ***highest affinity and excellent selectivity for the μ-opioid receptor.***

(II). New deltorphin I and II analogues with altered hydrophobic and stereoelectronic properties were obtained through the substitution of Phe^3 in the "message domain" with a conformationally restricted amino acid, 2-aminotetralin-2-carboxylic acid (Atc); Val residues in the "address domain at positions 5 and 6 were replaced with the more lipophilic amino acid, Ile. The resulted compounds **[^{3}H]S-Atc^3,Ile^{5,6}deltorphin I** and **[^{3}H]R-Atc,Ile^{5,6}deltorphin II** specifically labeled a single class of opioid sites with high affinity (K_d ~ 0.3 nM), B_{max} values of 130 fmol/mg protein in rat brain membranes, and a very low non-specific binding (<15%) was observed. Their binding was saturable, stereospecific and inhibited by δ-selective ligands with high potency. These radioligands were able to discriminate between the δ_1- and δ_2-receptor subtypes. Both Na^+ ions and guanine nucleotides decreased radioligand binding confirming the agonist character of these peptides. The reduction of specific binding observed in the presence of Gpp(NH)p, also suggested that in brain the δ-opioid receptors labeled by these ligands are G-protein coupled.

The new radiolabeled Atc-deltorphin analogues showed a marked δ-selectivity and the highest δ-receptor affinity compared with than their parent compounds, deltorphin I and deltorphin II and with other δ-selective peptide radioligands. Binding studies demonstrated that the new deltorphin analogues, **[^{3}H]S-Atc^3,Ile^{5,6}deltorphin I and [^{3}H]R-Atc^3,Ile^{5,6}deltorphin II** ***are the most potent and δ-selective radioligands available.***

(III). Using the *"message-address" concept*, new **non-peptide ligands** obtained by the addition of benzofuran moiety to the non-selective opioid antagonist, naloxone, and to the μ-

selective agonists, oxymorphone and oxycodone. Structure-activity relationship was examined for these new heterocyclic compounds.

Determination of opioid receptor binding profiles showed that the addition of benzofuran moiety to these fused ring opioids conferred ***δ-selectivity and changed the pharmacological properties of the parent compound.*** The Na^+ indices suggested a partial agonist character for oxymorphone- and oxycodone-benzofuran, and an antagonist character for naloxone-benzofuran. All three compounds were capable of irreversible inhibition of the opioid binding sites in a dose dependent manner.

In **conclusion**, all the newly characterized opioid analogues, both peptides and non-peptides, were found to be highly selective ligands either for μ- or δ-opioid receptors. Therefore, they can represent potentially useful tools to study the cellular and molecular mechanisms involved in the actions of opioid drugs and endogenous opioid peptides.

ABBREVIATIONS

Aib	aminobutiric acid
Atc	2-aminotetralin-2-carboxylic acid
B_{max}	maximal number of binding sites, receptor density
BSA	bovine serum albumin
cAMP	adenosine 3',5'- cyclic monophospate
cDNA	complementary deoxyribonucleic acid
CHO	Chinese hamster ovary
CNS	central nervous system
cpm	counts per minute
CTAP	D-Pen-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2
DADLE	[D-Ala^2,Leu^5]enkephalin
DAMGO	[D-Ala^2,$MePhe^4$,Gly-ol^5]enkephalin
DTLET	[D-Thr^2,Leu^5,Thr^6]enkephalin
DMEM	Dulbeco's modified Eagle's medium
DPDPE	[D-Pen^2,D-Pen^5]enkephalin
DSLET	[D-Ser^2,Leu^5,Thr^6]enkephalin
GPI	guinea pig ileum
Gpp(NH)p	5'-guanylyl-imidophosphate
G-protein	guanine nucleotide-binding regulatory protein
G_i-protein	inhibitory G-protein
GTP	guanosine 5'-triphosphate
Hyp	hydroxyproline
ICI174,864	N,N,-diallyl-Tyr-Aib-Aib-Phe-Phe-Leu-OH

IC197,067	(2S)-N-[2-(N-methyl-3,4-dichlorophenilacetamid)-3-methylbutyl] pirrolidin hydroxycloride
k_{+1}	association rate constant
k_{-1}	dissociation rate constant
K_d	equilibrium dissociation constant
K_i	inhibition constant
mRNA	messenger ribonucleic acid
MVD	mouse vas deferens
n_H	Hill coefficient
NG108-15	mouse neuroblastoma *x* rat glioma hybrid cells
nor-BNI	nor-binaltorphimine
PBS	phosphate-buffered saline
*p*Cl-DPDPE	[D-Pen^2,*p*Cl-Phe^4,D-Pen^5]enkephalin
PEI	polyethyleneimine
Pen	penicillamine
PMSF	phenylmethylsulfonyl fluoride
PNS	peripheral nervous system
POMC	proopiomelanocortin
PL017	Tyr-Pro-MePhe-D-Pro-NH_2
PTX	pertussis-toxin
SEM	standard error of the mean
SKF10,047	N-allyl-normetazocine
TAPP	Tyr-D-Ala-Phe-Phe-NH_2
Tic	tetrahydroisoquinoline-3-carboxylic acid
TIPP	Tyr-Tic-Phe-Phe-OH
TIPP[Ψ]	Tyr-TicΨ[CH_2-NH]-Phe-Phe-OH
TM	transmembrane
Tris	tris-(hydroxymethyl)-aminomethane
U50,488H	*trans*-3,4-dichloro-N-methyl-1-[2-(1-pyrrolidinyl)-cycohexyl] benzeneacetamide
U69,593	5α,7α,8β-(-)-N-methyl-N[7-1-pyrrolidinyl)-1-oxaspiro(4-5)dec-8-yl]benzeneacetamide

1. INTRODUCTION

1.1. Where It All Started

It started with *the need for pain relief* and an inexplicable attraction for a *plant alkaloid-morphine*.

The highly subjective nature of **pain** makes it difficult to define and to treat it clinically. Pain is a perception of an aversive or unpleasant sensation that originates from a specific region of the body that can be modulated by a wide range of behavioral experiences. *Acute pain* is associated with a negligible tissue damage and is thought to serve as a physiological warning to guard the integrity of the organism. *Chronic pain* is associated with a prolonged tissue damage and injuries to the peripheral (PNS) and central nervous system (CNS) resulted from a number of complex changes in nociceptive pathways (for a review, see Dray and Urban, 1996; Fig 1).

Opium (from "opos" the Greek word for juice), obtained from the unripe seed capsule of poppy plant *Papaver somniferum*, has been used in medicine for more than 5,000 years (for a review, see Benyhe, 1994a). The analgesic and anti-diarrheal properties of opium were already recognized by the Sumerians and the early dynastic Egyptians, and the therapeutic use of opium was discussed by Hippocrates, Dioscorides and Galen. The nature of the mood changes also produced by opium has been the basis for its non-medicinal use (and abuse). Opium eating and smoking replaced the consumption of alcoholic drinks in Islamic countries, such as Arabia, Turkey and Iran. Opium was also consumed as a favorite substance of pleasure in India and China.

In 1805, a German pharmacist, Friedrich Sertüner, isolated the active component of opium and named it **morphine** after "Morpheus", the Greek god of dreams. Unfortunately, morphine has just as much potential for abuse as opium does. This promoted medicinal chemists to attempt to develop safer and more efficacious compounds, with the goal of providing analgesia with reduced abuse potential and reduced side effects, such as respiratory depression. In 1898, heroine was claimed to be more potent than morphine and free from abuse liability.

Since the 1970s, large efforts in research have been devoted to understand how morphine and related alkaloids work in the CNS to produce analgesia. Pharmacological, biochemical and

localization studies led to (1) *the identification of opioid receptors*, and (2) *the isolation and characterization of endogenous opioid peptides.*

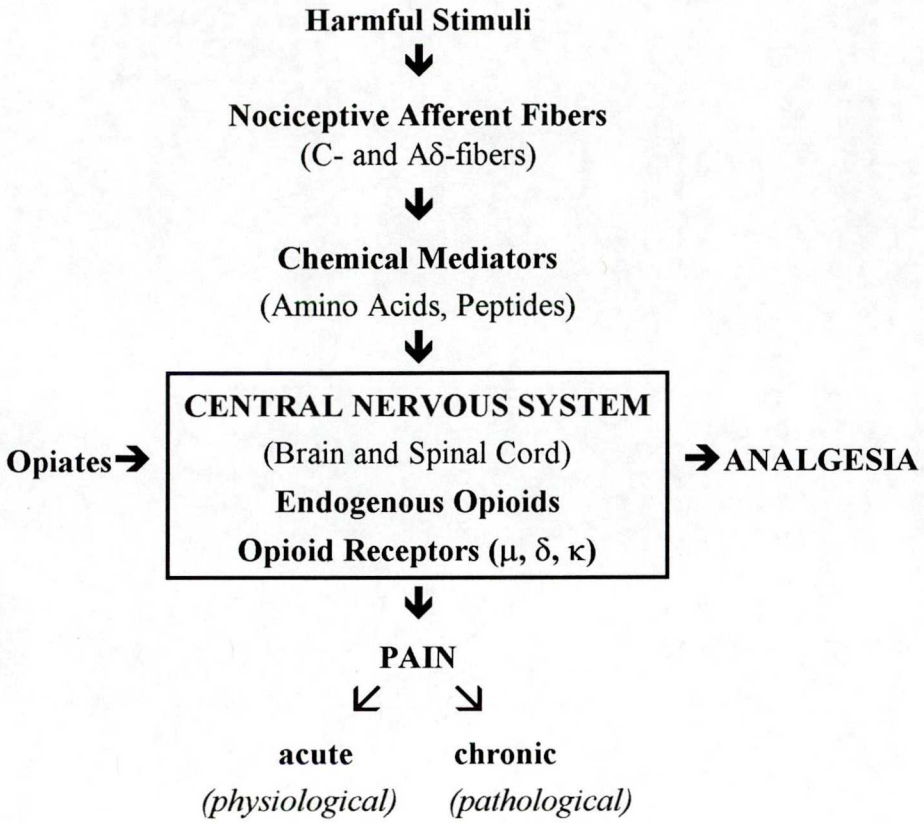


Fig. 1. *PAIN AND ANALGESIA.* Harmful stimuli (thermal, mechanical, chemical) applied to the skin, joints or muscle, activate nociceptive afferent fibers that provide information to the CNS about tissue damage. Signals are conducted to the spinal cord and higher centers in the brain where further processing occurs, resulting in pain awareness. Endogenous opioid system (endogenous opioids and opioid receptors) is located at key point in the pain modulatory pathways.

Opioid drugs exert their physiological actions by interacting with specific cell-membrane receptors, and at least three major **types of opioid receptors**, μ, δ and κ, were defined. **Endogenous opioid peptides** are represented by small peptides that occur naturally in the mammalian brain and amphibian skin (for a review, see Dhawan *et al.*, 1996).

To understand the structure and function of opioid receptors, it required **isolation and purification** from membranes of a variety of tissues (for a review, see Borsodi, 1991). Their **distribution** is distinct and species-specific (for a review, see Mansour *et al.*, 1988).

A new era was opened in opioid receptors research after the first **cloning** experiments in 1992 (for a review, see Knapp *et al.*, 1995). The availability of the cloned opioid receptors allowed studies of individual receptor types with regard to pharmacological profile, structure-function

analysis, cellular effector coupling and regulation of expression (for a review, see Kieffer, 1995). Manipulation of opioid receptors by *site-directed mutagenesis*, *deletions* and *chimera constructions* was providing information on which domains of the protein may be important for ligand binding and receptor function (for a review, see Zaki *et al.*, 1996).

Cellular mechanisms of opioid activity have been widely studied to understand how opioid substances act in the CNS and PNS to produce their effects, which are classified as "acute" and "chronic". The **acute effects** of opioids include analgesia, respiratory depression, constipation, sensation of well-being, etc. The **chronic effects** are represented by tolerance and dependence (for reviews, see Pasternak, 1993; Olson *et al.*, 1995).

The **development of new opioid analgesics** with reduced side effects has been a primary aim of opioid pharmacology in the last decades (for reviews, see Schiller, 1991; Takemori and Portoghese, 1992; Borsodi and Tóth, 1995). Since the elucidation of morphine structure and the analysis of naturally occurring peptides, chemical synthesis has provided a wide diversity of compounds with high affinity for opioid receptors, variable degree of selectivity towards the different receptor types with agonist, antagonist, or mixed agonist/antagonist properties.

1.2. Endogenous Opioid System

1.2.1. Opioid Receptor Types and Subtypes

Opioid receptors were first hypothesized in 1954 (Becket and Casy, 1954). In 1973, three groups demonstrated the existence of specific receptors for opioids (Pert and Snyder, 1973; Simon *et al.*, 1973; Terenius, 1973).

One of the earliest finding to suggest *multiplicity of opioid receptors* was provided by Martin *et al.* (1976), that different classes of opioid drugs produced distinct behavioral syndromes in chronic spinal dogs. They proposed the existence of three **types** of opioid receptors, named after the drugs used in the studies: μ (**mu**, for morphine-like compounds), κ (**kappa**, for ketocyclazocine-like drugs), and σ (**sigma**, for drugs such as SKF10,047). Further support for this idea came from bioassays carried out in peripheral tissues. Lord *et al.* (1977) suggested the existence of yet another receptor type, δ (**delta**), named from the mouse vas deferens (MVD) bioassay, where enkephalins were found to be particularly potent (Table I).

These early pharmacological observations were later confirmed using molecular biology approaches, demonstrating μ-, δ- and κ-receptors as distinct opioid binding sites (for a review, see Knapp *et al.*, 1995), with σ-receptors being non-opioid in nature (Zukin and Zukin, 1988). Additional receptor types may also be parts of the opioid receptor system: ε (**epsilon**) receptor (Wüster *et al.*, 1979), ζ (**zeta**) receptor (Zagon *et al.*, 1991), and λ (**lambda**) site (Grevel *et al.*, 1985). Among these putative opioid receptors, the ε-receptor has been studied in greater detail in rat vas deferens (RVD), where β-endorphin showed high affinity (Wüster *et al.*, 1979). Later, this ε-site has been described as a possible subtype of the κ-receptor (Nock *et al.*, 1990).

Pert and Snyder (1973) were the first to show the existence of *stereoselective opioid binding sites* in fragments from rat brain, offering the first biochemical approach to opioid receptor studies.

TABLE I. *Heterogeneity of Opioid Receptors*

Receptor	μ	δ	κ
Prototype ligand	Morphine	Enkephalins	Ketocyclazocine
Endogenous ligand	β-Endorphin Endomorphins Dermorphins	[Met^5]Enkephalin [Leu^5]Enkephalin Deltorphins	Dynorphin A
Selective agonists	Dihydromorphine DAMGO PL017	DADLE DSLET DPDPE D-Ala^{2}Deltorphin analogues	U50,488H U69,593 ICI197,067 Ethylketocyclazocine Bremazocine
Selective antagonists	CTAP Cyprodime β-FNA	ICI174,864 TIPP Naltrindole	Nor-BNI

The heterogeneity of opioid receptors is now generally accepted (Table I). Furthermore, the existence of **subtypes** of these receptors has also been suggested (for a review, see Pasternak, 1993; Table II).

Based primarily on differences in their affinities for opioid peptides and alkaloids, **μ-receptors** have been subdivided in two **subtypes**, μ_1 and μ_2, respectively. (Pasternak *et al.*, 1980; Lutz *et al.*, 1985; Pasternak and Wood, 1986; Pick *et al.*, 1991). The μ_1-receptor displays a high affinity for both opioid peptides and alkaloids. It has been proposed that their activation mediate analgesia (Table II). Among the opioid peptides which bind to the μ_1-subtype with high affinity

are the enkephalins, [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin (DAMGO), and some of their δ-receptor preferring analogues, such as [D-Ala^2,Leu^5]enkephalin (DADLE), [D-Ser^2,Leu^5,Thr^6]enkephalin (DSLET). The μ_2-subtype only display a high affinity for alkaloid derivatives of morphine and low affinity for DAMGO, and is thought to mediate opioid-induced depression of the respiratory system and gastrointestinal dysfunction (Pasternak and Wood, 1986; Table II).

The development of highly selective antagonists also helps to distinguish between μ-opioid receptor subtypes. β-Funaltrexamine (β-FNA) irreversibly inactivates both μ_1- and μ_2-receptors equally well (Recht and Pasternak, 1987). Naloxonazine and naloxazone can selectively antagonize μ_1-receptors (Pick *et al.*, 1991).

TABLE II. *Central and Peripheral Actions Mediated by Opioid Receptor Types and Their Subtypes*

Receptor	Analgesia	Other Effects
μ		
μ_1	Supraspinal	Prolactin release Feeding Acetylcholine release in the brain
μ_2	Spinal	Respiratory depression Inhibition of gastrointestinal transit Dopamine turnover Feeding Cardiovascular effects
δ		
δ_1	Supraspinal	Dopamine turnover
δ_2	Spinal and supraspinal	
κ		
κ_1	Spinal	Diuresis Feeding
κ_2	Unknown	
κ_3	Supraspinal	

from Pasternak, 1993.

The evidence for the existence of two **δ-receptor subtypes**, δ_1 and δ_2, is based on functional *in vivo* studies (Jiang *et al.*, 1991; Mattia *et al.*, 1991; Sofouglu *et al.*, 1991, Crook *et al.*, 1992). Several δ-receptor selective agonists and antagonists have been used in these studies (Table I). [D-Pen^2,D-Pen^5]enkephalin (DPDPE) is thought to be agonist at the δ_1-subtype (Mosberg *et al.*,

1983) while D-Ala^{2}deltorphin II and DSLET are highly selective δ_2-agonists. Experiments with antagonists led to the conclusion of [D-Ala^2,Leu^5,Cys^6]enkephalin (DALCE) (Bowen *et al.*, 1987) and 7-benzylidenenaltrexone (Sofouglu *et al.*, 1993), being selective blockers of the δ_1-binding site. Naltrindole 5'-isothiocyanate (Jiang *et al.*, 1991) and naltriben, the benzofuran analogue of naltrindole (Sofouglu *et al.*, 1991), showed selectivity for the δ_2-receptor subtype.

Some evidence from binding (Negri *et al.*, 1991; Sofouglu *et al.*, 1992; Chakrabarti *et al.*, 1993) and second messenger studies (Búzás *et al.*, 1994) also suggested δ-receptor heterogeneity.

Both δ_1- and δ_2-receptor subtypes appear to mediate antinociception in mice at the supraspinal level while the δ_2-receptor is also involved in antinociception at the spinal level (Jing *et al.*, 1991; Mattia *et al.*, 1992; Table II).

The existence of two **κ-receptor subtypes**, κ_1 and κ_2, was originally suggested, but later two more subtypes, κ_3 and κ_4, were also described (for a review, see Wollemann *et al.*, 1993). κ_1-Receptors differ from the κ_2-subtypes in their ligand selectivity. Benzeneacetamide derivatives U50,488H, U69,593 and ICI197,067, bind to the κ_1-receptor, whereas benzomorphans ethycyclazocine, nor-binaltorphimine (nor-BNI) and bremazocine, bind to both κ-subtypes (Kim *et al.*, 1996).

The most important analgesic actions of κ-opioid receptor agonists are localized in the spinal cord (Table II). Whereas κ_1 analgesia, in the mouse, is predominantly localized to the spinal cord, κ_3 analgesia is mediated supraspinally (for reviews, see Pasternak, 1993; Wollemann *et al.*, 1993). The pharmacology of the κ_2-subtype is still unknown.

1.2.2. Endogenous Opioid Peptides

Historically, the discovery of opioid receptors preceded the isolation and characterization of **opioid peptides**. After the identification of opioid receptors in 1973, the search for their endogenous opioids was very intense.

In 1975, Hughes *et al.* successfully isolated and identified the structure of two endogenous opioid pentapeptides from pig brains, and named ***enkephalins***. Both Tyr-Gly-Gly-Phe-Met ([Met^5]enkephalin) and Tyr-Gly-Gly-Phe-Leu [(Leu^5]enkephalin) have central opioid action. Simantov and Snyder (1976) isolated the same two peptides from calf brain, confirming the findings of Hughes *et al.* (1975). Since than other endogenous opioid peptides, *e.g.* *β*-

endorphin (Cox *et al.*, 1976) and ***dynorphin*** (Goldstein *et al.*, 1979) have been characterized. They all contain the same sequence, Tyr-Gly-Gly-Phe at the N-terminal end (Table III).

Each of the opioid peptides is the product of a larger *precursor protein*. In **mammals** , there are three such precursors: (a) *proenkephalin* contains four copies of [Met^5]enkephalin, one copy of [Leu^5]enkephalin and several extended enkephalins, including [Met^5]enkephalin-Arg^6-Gly^7-Leu^8, [Met^5]enkephalin-Arg^6-Phe^7, and peptides E, F (Noda *et al.*, 1982); (b) *prodynorphin* gives rise to dynorphins and neoendorphins (Goldstein *et al.*, 1979, 1981); (c) *proopiomelanocotin* (POMC) contains β-lipotropin, and gives rise to α-, β- and τ-endorphins, and also to the adrenocorticotropic (ACTH) and melanocyte stimulating (α, β and γ-MSH) hormones (Nakanishi *et al.*, 1979). Some of the endogenous opioid peptides and their precursors are shown in Table III.

Opioid peptides identified in mammalian brain are considered to be endogenous agonists for the δ- (enkephalins) and κ- (dynorphins) receptors, but none of the identified endogenous opioids has absolute pharmacological specificity for a given receptor type (Mansour *et al.*, 1995b). Recently, Zadina *et al.* (1997) isolated from bovine frontal cortex two tetrapeptides, named ***endomorphins***, Tyr-Pro-Trp-Phe-NH_2 (endomorphin-1) and Tyr-Pro-Phe-Phe-NH_2 (endomorphin-2). These peptides exhibited the highest specificity and affinity for the μ-receptor among all endogenous opioids so far discovered in the mammalian nervous system.

After the characterization of enkephalins, endorphins and dynorphins on mammals, a fourth family of highly potent opioid peptides has been discovered in the **amphibian** skin. Frogs belonging to the genus *Phyllomedusa* produce several peptides, named ***dermorphins*** and ***deltorphins*** (Erspamer *et al.*, 1989; Mor *et al.*, 1989; Mignogna *et al.*, 1992; Negri *et al.*, 1992; Barra *et al.*, 1994; Table III).

All amphibian opioids have an amino acid with the rare (in a mammalian cortex) D-enantiomer instead of the normal L-isomer. They also contain a common N-terminal sequence Tyr-D-Xaa-Phe, in which D-Xaa is either D-Ala, D-Met or D-Leu (Table III).

Regarding their selectivities, dermorphins preferentially bind to the μ-opioid receptor (see Chap. 1.7.1; Mignogna *et al.*, 1992) while deltorphins show high affinity and selectivity for the δ-opioid receptor (see Chap. 1.7.2.1; Erspamer *et al.*, 1989).

Amphibian peptides are also processed from precursor proteins that derive from different genes (Richter *et al.*, 1990).

Opioid peptides have also been isolated in **invertebrates**. Enkephalin peptides were found in mollusks (Leung and Stefano, 1984) and in annelids (Salzet *et al.*, 1995).

TABLE III. *Endogenous Opioid Peptides*

Precursor	Opioid Peptide	Structure	Selectivity
Proopioimelanocortin (POMC)	β-endorphin	*Tyr-Gly-Gly-Phe*-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu	μ>δ>>κ
Proenkephalin	[Leu^5]enkephalin	*Tyr-Gly-Gly-Phe*-Leu	δ>μ>>κ
	[Met^5]enkephalin	*Tyr-Gly-Gly-Phe*-Met	μ~δ>>κ
	[Met^5]enkephalin-Arg^6-Phe^7	*Tyr-Gly-Gly-Phe*-Met-Arg-Phe	κ_2
Prodynorphin	Dynorphin $A_{(1-8)}$	*Tyr-Gly-Gly-Phe*-Leu-Arg-Arg-Ile	κ>>μ>δ
	Dynorphin $A_{(1-13)}$	*Tyr-Gly-Gly-Phe*-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys	κ>δ~μ
	Dynorphin $A_{(1-17)}$	*Tyr-Gly-Gly-Phe*-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln	κ>δ~μ
	α-Neoendorphin	*Tyr-Gly-Gly-Phe*-Leu-Arg-Lys-Tyr-Pro-Lys	
	β-Neoendorphin	*Tyr-Gly-Gly-Phe*-Leu-Arg-Lys-Tyr-Pro	
Others			
β-Casein derivatives	Morphiceptin	Tyr-Pro-Phe-Pro-NH_2	μ
	β-Casomorphin	Tyr-Pro-Phe-Pro-Gly-Pro-Ile	μ
Frog skin peptides	Dermorphin	Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2	μ
		Tyr-D-Ala-Phe-Gly-Tyr-Hyp-Ser-NH_2	μ
		Tyr-D-Ala-Phe-Gly-Tyr-Hyp-Ser-OH	μ
		Tyr-D-Ala-Phe-Gly-Tyr-Pro-Lys-OH	μ
		Tyr-D-Ala-Phe-Trp-Tyr-Pro-Lys-OH	μ
		Tyr-D-Ala-Phe-Trp-Asn-OH	μ
		Tyr-D-Ala-Phe-Trp-Tyr-Pro-Asn-OH	μ
		Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-Gly-Glu-Ala-OH	μ
		Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-Gly-Glu-Ala-Lys-Lys-Ile-OH	μ
	Deltorphin	Tyr-D-Met-Phe-His-Leu-Met-Asp-NH_2	δ
	D-Ala^2,Asp^{4}Deltorphin I	Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2	δ
	D-Ala^2,Glu^{4}Deltorphin II	Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2	δ
	D-Leu^{2}Deltorphin	Tyr-D-Leu-Phe-Ala-Asp-Val-Ala-Ser-Thr-Ile-Gly-Asp-Phe-Phe-His-Ser-Ile-NH_2	δ

1.3. Other Opioid-Related, Receptor-Like Recombinant Proteins

A new member of the opioid receptor family and another opioid peptide have been discovered in the past few years (for a review, see Civelli *et al.*, 1997).

The novel receptor designed as **opioid receptor-like** ($\mathbf{ORL_1}$) has high (approximately 65%) homology and shares many of the structural features of the opioid receptors, but does not bind specifically any of the known opioid peptides or alkaloids with high affinity (Bunzow *et al.*, 1994; Mollereau *et al.*, 1994; Wick *et al.*, 1994).

A heptadecapeptide has been isolated from rat (Meunier *et al.*, 1995), from porcine (Reinscheid *et al.*, 1995) and from bovine brain (Okuda-Ashitaka *et al.*, 1996), and named **nociceptin** or **orphanin FQ**. This peptide structurally resembles dynorphin $A_{(1-17)}$, and behaves as a potent endogenous ligand to the ORL_1 receptor.

The wide distribution of ORL_1 mRNA and nociceptin precursor in the CNS of rodents, particularly in the limbic system, suggested their involvement in pain perception (Houtani *et al.*, 1996; Mollereau *et al.*, 1996; Nothacker *et al.*, 1996; Okuda-Ashitaka *et al.*, 1996).

Nociceptin plays a role in decreasing motor activity (Devin *et al.*, 1996; Noble and Roques, 1997), in reducing neuromuscular tone of mice (Reinscheid *et al.*, 1995), in regulation of systemic blood pressure and regional blood flow (Gumusel *et al.*, 1997).

Many functions of the orphan receptor and the novel opioid peptide are presently unclear, but their characterization may provide clues as to the structural requirements of the opioid receptors, and to their common evolution (Ma *et al.*, 1997).

1.4. Distribution and Function of Opioid Receptors

Opioid receptors are widely distributed throughout the brain and peripheral tissues of all animals, although patterns of distribution of μ-, δ- and κ-receptors, and the quantity of receptors varies between species and various major anatomical regions.

The relative abundance of opioid binding sites from the **species** varies dramatically (Table IV).

The ontogeny of different types of receptors is quite distinct. In rat, μ- and κ-receptors appear earlier in development than δ-receptor does (Kornblume *et al.*, 1987).

TABLE IV. *Relative Proportions of Opioid Receptor Types in Several Species*

Species	μ (%)	δ (%)	κ (%)	References
Rat	46	42	12	Schiller, 1991
Guinea-pig	24	32	44	Schiller, 1991
Mouse	25	62	13	Mansour *et al.*, 1988
Frog	18	10	72	Benyhe *et al.*, 1994b
Pigeon	14	10	76	Mansour *et al.*, 1988
Human	29	34	37	Mansour *et al.*, 1988

The values represent binding data in human frontal cortex and forebrain tissue of the rat, guinea-pig, mouse, frog and pigeon.

Opioid receptor distribution varies between **anatomical regions**. In general, the laminar patterns distribution are distinctive (Mansour e*t al.*, 1988; Hiller and Fan, 1996).

In the **CNS**, the μ-opioid receptors have been localized by autoradiographical studies in the caudate puntamen, neocortex, thalamus, nucleus accumbens, hippocampus, amygdala, hypothalamus, periaquenductal gray, raphe nuclei, globus pallidus and spinal cord. The δ-opioid receptors have a more restricted distribution in the CNS than other opioid receptors. They are more dense in olfactory bulb, caudate puntamen, nucleus accumbens and neocortex. The κ-receptors are particularly enriched in the cerebral cortex, striatum, substantia nigra and hypothalamus (Mansour e*t al.*, 1988).

In the **periphery**, opioid receptors are found in myenteric plexus and in certain smooth muscles, such as guinea pig ileum (GPI), mouse vas deferens (MVD), rabbit vas deferens (LVD), hamster vas deferens (HVD) and rat vas deferens (RVD). In GPI, the μ- and κ-receptors exist, but MVD contain in addition to the predominant δ-receptor, also the μ- and κ-receptors. Peripheral tissues are extremely useful as *in vitro* bioassay systems for opioids and their receptors (for a review, see Leslie, 1987).

Opioid receptors are present in many neurally derived **cell lines**. δ-Receptors are found in mouse neuroblastoma *x* rat glioma hybrid cells (NG108-15), in human neuroblastoma SK-N-SH cells and in PC12h rat pheochromocytoma cells. μ-Receptors are present in human neuroblastoma SH-SY5Y cells and in 7315c cell line which is derived from a rat pituitary tumor (for a review, see Leslie, 1987).

Cloning of opioid receptors in 1992, made possible the application of new techniques to study their distribution. Receptor mRNA could be visualized using *in situ* hybridization techniques. Antibodies could be raised to the amino acid sequences, and the receptor proteins could be

localized using immunohistochemical methods (for a review, see Mansour *et al.*, 1995a). Within the spinal cord, δ-receptors are mainly restricted to axons, whereas μ-and κ-receptors are present on both primary afferent axons and the cell bodies, and dendrites of a population of neurons in the superficial dorsal horn. Enkephalin-containing terminals are often found in close proximity to membranes containing either the δ- or μ-opioid receptors, whereas dynorphin-containing terminals are often found in the proximity of κ-receptors (Elde *et al.*, 1995).

Localization of opioid receptors and opioid peptides can explain many of the pharmacological actions of opioids. Besides the substantial evidence of overlapping between the functions of different opioid receptor types, there is also conclusive evidence for their specialization (for a review, see Pasternak, 1993; Table II).

The μ-receptors seem to be important in feeding behavior, regulation of respiratory, cardiovascular and gastrointestinal functions, thermoregulation and hormone secretion (Pasternak and Wood, 1986). The κ-receptors may be involved in water metabolism. Consumatory behavior seems also to be, in part, regulated via the κ-receptors. The κ-agonists appear to be important in the gut motility, temperature control and various endocrine functions (Iyengar *et al.*, 1986; Wollemann *et al.*, 1993). The δ-receptors play a role in motor integration, gastrointestinal motility, olfaction, respiration, cognitive function and mood driven behavior. They are also involved in stress-induced analgesia (Kitchen *et al.*, 1995). The role of opioid receptors in mental illness, memory and learning behavior has also been reported (for a review, see Olson, 1995). In addition, recent evidence suggested that opioid receptors also participate in the control of immune system (Roy and Loh, 1996).

Genetic approaches are available to study *in vivo* the role of mammalian opioid systems in regulating many physiological functions, including pain perception and analgesia, responses to stress, aggression and dominance (Matthes *et al.*, 1996; König *et al.*, 1996).

Investigation of the behavioral effects of morphine in mice revealed that the lack of μ-receptors abolish the analgesic effects of morphine, place-preference activity and physical dependence (Matthes *et al.*, 1996). It has been suggested that δ- and κ-receptors do not mediate, even partially, any of the major biological actions of morphine in the absence of the μ-receptor. This raises the important issue of *cooperativity between the opioid receptors* (Traynor and Elliot, 1993) which might take place at the molecular level through receptor allosteric interactions or second messenger systems, or occur at a functional level on separate neurons.

1.5. Molecular Biology of Opioid Receptors

Due to the importance of opioid receptors in opioid pharmacology and physiology, many groups attempted to isolate and purify these proteins with the aim of ultimately describing: (1) *the molecular structure responsible for ligand-binding properties of the receptors*, and (2) *the molecular mechanisms involved in transducing ligand binding into physiologically relevant signals*.

The first successful attempts for **solubilization** and **purification** of active opioid receptors were described in 1980 (for a review, see Borsodi, 1991). The solubilized species consists of protein and lipids, the last ones have been shown to play, in some cases, a crucial role in the reconstitution of opioid binding sites (Gomathi and Sharma, 1993).

The next step in the functional characterization of opioid receptors was their **molecular cloning** (for a review, see Knapp *et al.*, 1995). Research on opioid receptors was entering a new era, when the receptors could be examined not only in terms of their ligand-binding properties, but also regarding their gene structure and mRNA expression.

The genes encoding opioid receptors have been characterized in mouse and human. The existence of introns in their structure raises the possibility that these genes can be alternately spliced and give rise to different variants of the receptors (for a review, see Zaki *et al.*, 1996).

Kieffer *et al.* (1992) and Evans *et al.* (1992) independently reported the isolation and pharmacological characterization of the first high affinity opioid receptor, the δ-type, using a mouse cDNA prepared from NG108-15 cells. The rat δ-receptor was first cloned by Fukuda *et al.* (1993) from a rat cerebral cortex cDNA library. Cloning of other members of opioid receptor family was also reported: the rat μ- (Chen *et al.*, 1993a; Fukuda *et al.*, 1993; Wang *et al.*, 1993; Zastawny *et al.*, 1994; Bunzow *et al.*, 1995) and κ-opioid receptors (Chen *et al.*, 1993b; Minami *et al.*, 1993); the mouse κ-receptor has been isolated as a member of the related somatostatin receptor (Yasuda *et al.*, 1993), and the guinea pig κ-receptor (Xie *et al.*, 1994).

Because human opioid receptors are the ultimate targets of therapeutic opioid drugs, it was particularly important to have clones of these receptors (Knapp *et al.*, 1994; Mansson *et al.*, 1994; Wang *et al.*, 1994).

It was also reported that the cloned opioid receptors correspond to μ_1-, δ_2- and κ_1-opioid receptor subtypes (Knapp *et al.*, 1994; Lai *et al.*, 1994; Raynor *et al.*, 1994).

Molecular properties of the cloned opioid receptors are summarized in Table V.

TABLE V. *Characteristics of the Cloned Opioid Receptors*

	μ	δ	κ
Gene family	7TM G protein-coupled	7TM G protein-coupled	7TM G protein-coupled
Gene organization	intronic	intronic	intronic
mRNA size	10-16 kb	4.5 kb 11.0 kb	5.2 kb
Amino acid length	400 aa human 398 aa mouse 372 aa rat	372 aa human 372 aa mouse 398 aa rat	380 aa human 380 aa mouse 380 aa rat
Number of glycosylation sites	5	2	2
Number of phosphorylation sites	3-4	4-7	5-7

TM-transmembrane domain; aa-amino acid.

The cloning efforts have clearly identified opioid receptors as members of the **G-protein-coupled receptors** superfamily. Deduced amino acid sequences predicted the seven transmembrane domains (Fig. 2). Opioid receptors are homologous with the receptors for somatostatin, angiotensin, interleukin-8 neuropeptide Y and histamine (Evans *et al.*, 1992; Kieffer *et al.*, 1992; Chen *et al.*, 1993b; Li *et al.*, 1993).

A striking structural homology is observed among the opioid receptors at both nucleic acid and amino acid levels (overall around 60%). These similarities are highly conserved across species. The highest homology between opioid receptor proteins is found in the putative transmembrane domains, the intracellular loops and a portion of the C-terminal tail adjacent to the seventh transmembrane (TMVII) domain. The most pronounced differences are found in the second and third extracellular loops, as well as in the N- and C-terminal domains (Chen *et al.*, 1993b; Fig. 2). The N-terminal domain contains glycosylation sites that are thought to play role in receptor trafficking (Wang *et al.*, 1993; Surrat *et al.*, 1994). A palmitoylation site is present within the C-terminal domain, and a disulfide bond is existing between the first and second extracellular loops similar to many G-protein-coupled receptors. The third intracellular loop is involved in coupling of G-proteins (Merkouris *et al.*, 1996). This region has consensus sequences for interaction with phosphorylation, which might be involved in receptor desensitization, being the sites for interaction with protein kinase A and C.

The cloning of opioid receptors provided a unique opportunity to examine the issues of receptor structure and ligand specificity. Chimeric analysis of the μ-, δ and κ-opioid receptors suggested

that there are distinct molecular interaction for each of the ligand classes of non-peptide agonist, non-peptide antagonists, and peptide agonists (for a review, see Zaki *et al.*, 1997).

Deletions and formation of chimeric receptors have indicated that the entire C- and N-terminal domains are not involved in the binding of antagonists to either δ- or μ-receptors (Kong *et al.*, 1994; Surratt *et al.*, 1994; Zhu *et al.*, 1997), but that the first and the third extracellular loops are responsible for binding selectivity of μ-agonists (Onogi *et al.*, 1995; Xue *et al.*, 1995). Chimeric μ/κ-receptors provide evidence that the extracellular loop II and the C-terminus portion of TMIV domain of κ-receptors are involved in dynorphin binding and are able to differentiate between peptide and non-peptide ligands (Xue *et al.*, 1994). Data on μ/δ-chimeras indicated that the high affinity δ-receptor binding involves TMV-VII domains and the intervening extracellular loop regions II and III, while the μ-receptor binding involves extracelluar and the N-terminus half of TMIII domain. μ-Alkaloids interact with a region spanned by TMV-VII domains (Fukuda *et al.*, 1995). In human δ-receptor, the third extracelullar loop is important in the determination of the selectivity of the δ-peptide and non-peptide agonists, but has no effect on the binding of the μ-selective peptide and non-peptide agonists (Varga *et al.*, 1996).

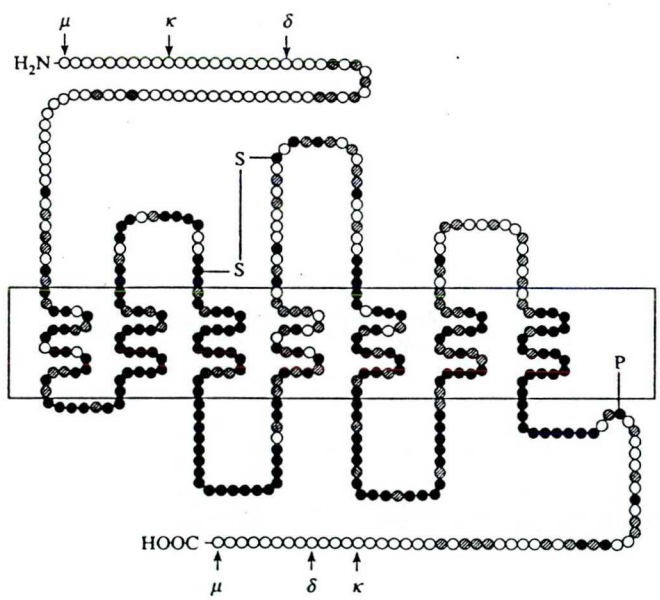


Fig. 2. *MEMBRANE TOPOLOGY AND SIMILARITY DISTRIBUTION AMONG OPIOID RECEPTORS.* The N-terminus is located extracellularly and the C-terminus is located intracellularly. The N- and C-termini for each opioid receptor are marked by arrows and the corresponding receptor name. The putative disulfide bond is depicted as (-S-S-) and the palmitoylation site is denoted as P. Membrane lipid bilayer is shown as an open box. ●-identical amino acids among all three receptors; ⊘-identical in two out of three receptors; ○-unique for each receptor (from Chen *et al.*, 1993b).

One target for **mutation studies** has been the negatively charged Asp^{128} residue in the TMIII domain. Studies on the mouse δ-opioid receptor showed that this negative charge residue is not critical for ligand binding but contributes to stabilization of the spatial conformation of the binding pocket (Befort *et al.*, 1996). Substitution of Asp^{95} in the TMII domain of mouse δ-opioid receptor with Asn significantly reduces binding of δ-selective agonists, but not binding of antagonists or selective agonists (Kong *et al.*, 1993).

The cloned receptors have been expressed in various heterologous host cells, including epithelial cells, COS (monkey fibroblast) and CHO (Chinese hamster ovary), or embryonic HEK 293 cells. Excitable cells, such as the pituitary cell line GH_3, the pheochromocytoma PC12 cell line or *Xenopus* oocytes has also been used to investigate pharmacological properties of cloned opioid receptors (for reviews, see Kieffer, 1995; Piros *et al.*, 1996a). The binding affinities of opioid ligands generally agree with those reported for native receptors in brain homogenates.

The cloning of opioid receptors has profoundly affected the understanding of opioid receptor expression, regulation and function.

1.6. Cellular Mechanisms of Opioid Activity

The biological effects of opioids begin with the agonist binding to the cell-surface receptor and ends with a series of cellular responses. Transduction mechanisms for opioid receptors have been widely studied in different tissues, cell types or neuron preparations.

Opioid receptors are *coupled to guanine nucleotide-binding regulatory proteins* (**G-proteins**), which in turn modulate intracellular effectors.

G-proteins are heterotrimers consisting of α, β and γ-subunits. In the cycle of G-protein activation, the receptor interacts with the G-protein and decreases the affinity of the guanosine diphosphate (GDP)-bound α-subunit for GDP and increases its affinity for guanosine triphosphate (GTP) (for a review, see Fraser *et al.*, 1994).

The functional coupling of the three opioid receptors with G-proteins was established on the bases that guanine nucleotides, such as GTP, GDP and 5'-guanylyl-imidophosphate Gpp(NH)p, decrease the specific binding of agonists, and that the latter compounds stimulate GTPase activity (for a review, see Childers, 1991).

Interaction of ligands with opioid receptors is differentially affected by cations. For example, Na^+ ions reduce the affinity of opioid receptor for agonists but not for antagonists. Different mechanisms have been proposed to explained this phenomenon. It has been hypothesized the Na^+ ions allosterically transforms opioid receptor sites from conformations which bind agonists more readily to conformations which bind antagonists more readily (Pert and Snyder, 1974). To investigate the mechanisms through which Na^+ modulates opioid receptor properties. Kong *et al.* (1993) mutated the Asp^{95} residue, in the second transmembrane region of the protein molecule, to Asn in the cloned δ-opioid receptor and found that Na^+ regulation of agonist binding was lost. This residue seems to play a role in the Na^+ regulation of agonist binding rather than being directly involved in agonist ligand recognition.

Prior to their cloning, it was known that all three opioid receptor types interact with a variety of **effectors**, including (1) *decreasing cAMP concentration by inhibition of adenylyl cyclase*, (2) *regulation of Ca^{2+} channel activity*, (3) *activation of K^+ conductance*, (4) *regulation of phospholipase C activity* (for a review, see Childers, 1991).

The availability of cloned receptors allows the examination of the basic cellular events involved in receptor-effector coupling for each individual opioid receptor type (for reviews, see Kieffer *et al.*, 1995; Piros *et al.*, 1996a).

Opioid receptors from brain, as well as those expressed in a clonal cell line, are negatively coupled to **adenylyl cyclase** through pertussis toxin (PTX)-sensitive G-proteins, G_i/G_o (McKenzie and Milligan, 1990; Offerman *et al.*, 1991; Kieffer *et al.*, 1992; Carter and Medzihradsky, 1993; Befort *et al.*, 1996; Murthy *et al.*, 1996).

A variety of electrophysiological evidence suggested that effects of agonists in opening **K^+ channels** and in closing **Ca^{2+} channels**, are not mediated through second messenger system, but through direct interaction between G-protein and ion channel.

Coupling of opioid receptors to inward K^+ rectifying channels was described in the *Xenopus* oocytes (Henry *et al.*, 1995; Ma *et al.*, 1995). Activation of μ- or δ-receptors can lead to an increase in K^+ conductance that cause hyperpolarization and inhibition of firing, and it is likely that this is responsible for most of the acute effects of systematically administrated opioids (for a review, see DiChiara and North, 1992). All three types of opioid receptors are functionally coupled to a variety of voltage-dependent Ca^{2+} channels (North, 1991). Ca^{2+} channels, N and L-type, appear to be involved in the reduction of neuronal Ca^{2+} current, and their coupling to opioid receptors is probably mediated by the βγ-subunit of the G_i/G_o-protein (Piros *et al.*, 1996b).

G_i/G_o proteins also activate, via βγ-subunits, **phospholipase C** which catalyzes the formation of the cellular messengers, inositol triphosphate (IP_3) and diacylglycerol (Murthy *et al.*, 1996). Subsequently, IP_3 facilitates the release of Ca^{2+} from intracellular stores resulting in increase free intracellular Ca^{2+} levels (Heagy *et al.*, 1992; Smart *et al.*, 1994, 1995).

Activation of the cloned opioid receptors was shown to elicit an increase in arachidonate release, suggesting opioid receptor-mediated activation of **phospholipase A**. PTX-sensitive G-proteins, G_i/G_o, seem to be involved in these responses (Fukuda *et al.*, 1996).

Coupling of opioid receptors with cholera toxin (CTX)-sensitive G-protein, G_s, responsible for excitatory effects of opioid agonists, has also been hypothesized (Crain and Shen, 1990). These so-called "excitatory" opioid receptors would be activated by lower (<nM) concentration of opioids than the "inhibitory" receptors coupled to G_i/G_o proteins (for a review, see Smart and Lambert, 1996). The increase of cAMP production, attributable to opioid receptor stimulation, is suggested to be due to the activation of type II adenylyl cyclase via the βγ-subunits of G-proteins (Chan *et al.*, 1995).

Experimental data indicated that the same receptors can evoke both stimulatory and inhibitory processes, suggesting conformational alteration of the opioid receptor from a form coupled primarily to inhibitory G_i/G_o-proteins to one also capable of interacting with stimulatory G_s-proteins (Wu *et al.*, 1997).

One of the final physiological end-points for opioids is *modulation of neurotransmitters release*, such as noradrenaline (Matsumoto *et al.*, 1994), dopamine (Manzanares *et al.*, 1991; Schad *et al.*, 1996; Feigenbaum *et al.*, 1996), acetylcholine (Mulder *et al.*, 1984), substance P (Mudge *et al.*, 1979), serotonine (Yoshioka *et al.*, 1993), as well as various hormons, such as vasopressin (Iversen *et al.*, 1980), somatostatin (Ipp *et al.*, 1978), insulin and glucagon. It was suggested that opioid regulation of neurotransmitter release is related to changes in the intracellular Ca^{2+} concentration (Xu and Gintzler, 1992; Smart *et al.*, 1994).

Shortening of the action potential by opioids has generally been considered to be a useful model of their inhibition of Ca^{2+} influx and transmitter release at presynaptic terminals of primary afferent nociceptive neurons, thereby accounting for *opioid-induced analgesia*. Stimulatory effects on neurotransmission, including the increase of the rate of neuronal firing and prolongation of the action potential by the increase in Ca^{2+} influx and neurotransmitter release at presynaptic terminals, may play a role in the *development of tolerance* (for a review, see Smart and Lambert, 1996).

1.7. Development of Receptor Selective Opioid Analogues

Most of the naturally occurring opioids do not have high selectivity for opioid receptor types and subtypes. Development of potent and stable agonists and antagonists with increased selectivity for each opioid receptor types continue to be an important goal in opioid pharmacology even though substantial progress has been made in the last decades (for reviews, see Schiller, 1991; Takemori and Portoghese, 1992; Borsodi and Tóth, 1995).

Initial studies were primarily concerned with stabilizing the opioid peptides against enzymatic degradation or enhancing their ability to cross the blood-brain barrier. Today, the design of new ligands is based an the combination of the classical medical chemistry approach of systematic modification of lead structure, with extensive use of biophysical studies, and with application of combination with computer-assisted modeling, molecular mechanics and molecular dynamics studies. A major hypothesis is that each receptor type and subtype has specific and different stereostructural and conformational requirements for the given ligands. The most increasing attention has been focused on peptide and non-peptide opioids acting at the μ- and δ-receptors. Most of the synthetic ligands mimic the structure of opioid peptides or of natural alkaloids.

Several *approaches* can be applied to develop receptor-selective **opioid peptide analogues,** including:

- *substitution, deletion or addition of natural or synthetic amino acids;*
- *conformational restriction through appropriate peptide cyclization;*
- *peptide bond replacement.*
- *bivalent ligands containing two opioid moieties separated by a spacer;*

The "message-address" concept proposed by Schwyzer (1977) for endogenous agonists, has been used by Portoghese as a successful strategy to design **peptidomimetic opioid antagonists** (for a review, see Takemori and Portoghese, 1992). According to this concept, specific regions of opioid ligands are responsible for receptor transduction process, that leads to a specific biological effect ("message"), while other regions are considered to be responsible for receptor binding, without contributing to the transduction process ("address").

Antagonists have two major advantages (1) *they can be employed to evaluate the selectivity of new agonists,* and (2) *they can be used to study the interaction of endogenous opioid peptides with opioid receptor types and subtypes.*

All the newly designed ligands are evaluated for the biological activities including potency, selectivity, efficacy and agonist/antagonist properties. Some of them are already used as

therapeutic agents because they provide the ultimate treatment for pain, but their use is complicated by many other effects.

1.7.1. μ-Receptor Selective Ligands

Among the three main opioid receptor types, the μ-receptors are perhaps of the greatest clinical importance because of their involvement in drug addiction and modulation of pain perception (Pasternak *et al.*, 1993).

Morphine and other opioid alkaloids, as well as some of the endogenous opioid peptides bind to the μ-receptor. A number of linear analogues of enkephalins, β-casomorphins and dermorphins with μ-selectivity have been developed, some of them also in radiolabeled form (for a review, see Schiller *et al.*, 1991). Among them are: DAMGO (Handa *et al.*, 1981), Tyr-Pro-MePhe-D-Pro-NH_2 (PL017) (Chang *et al.*, 1983; Blanchard *et al.*, 1987), Tyr-D-Ala-Gly-$NH(CH_2)_2$-$CH(CH_3)_2$ (TRIMU 5) (Gacel *et al.*, 1988), and Tyr-D-Arg-Phe-Lys-NH_2 (DALDA) (Schiller *et al.*, 1989).

Since natural **enkephalins** are rapidly degraded by various peptidases, initial efforts were aimed at making the peptide molecules more resistant to enzymatic degradation. This goal was achieved through introduction of D-Ala in position 2 of the peptide sequence and through amidation of the C-terminal carboxyl group. The D-amino acid substituted in the second position was found to be vital for receptor binding and biological activity (Misicka *et al.*, 1995).

Unlike in the case of enkephalins, the L-configuration of Pro^2 residue in **β-casomorphin** analogues is required for opioid activity (Chang *et al.*, 1981). Deletion of the C-terminal tripeptide in β-casomorphin resulted in morphiceptin (Tyr-Pro-Phe-Pro-NH_2), which display higher μ-receptor selectivity than the parent heptapeptide. The linear β-casomorphin analogue, PL017, showed higher preference for μ-receptors over δ-receptors (Chang *et al.*, 1983).

In contrast to mammalian opioid peptides, those isolated from amphibian skin, including **dermorphin** (Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2) and dermorphin-related peptides (see Chap. 1.2.2 and Table III), contain a D-Ala residue in position 2 and a carboxyamide group at the C-terminal end. They were shown to be highly potent and selective μ-opioid agonists (Richter *et al.*, 1990; Mignogna *et al.*, 1992; Negri *et al.*, 1992).

In the search for more potent μ-selective ligands, Schiller *et al.* (1989) designed dermorphin tetrapeptide analogues carrying a net positive charge in the peptide chain. Substitution of $D\text{-}Ala^2$

with D-Arg, resulted in a compound DALDA showing extremly high preference for μ-receptors over δ-receptors.

Dermorphin-related tetrapeptideamide containing a D-Ala residue in position 2 and the aromatic residue, Phe, in both the 3- and the 4-position of the peptide sequence was also synthesized. Possible intramolecular interactions between the two aromatic rings in the isolated molecule or during the process of binding to the opioid receptors have been suggested to have interesting effects on receptor selectivity. The resulted opioid analogue Tyr-D-Ala-Phe-Phe-NH_2 (TAPP) showed high μ-receptor affinity and excellent μ-selectivity (Schiller *et al.*, 1989).

1.7.2. δ-Receptor Selective Ligands

1.7.2.1. δ-Selective Agonists

Natural enkephalins display slight preference for δ-receptors over μ-receptors. Efforts have been made to develop more δ-selective **enkephalin** analogues (for a review, see Schiller, 1991).

Substitution of D-Ala in position 2 of [Leu^5]enkephalin and inversion of the configuration in position 5 led to a compound, [D-Ala^2,Leu^5]enkephalin (DADLE), with only slightly improved δ-receptor selectivity. Another improvement in δ-selectivity was achieved through substitution of D-Ser residue in position 2 of [Leu^5]enkephalin and extension of the peptide chain with a Thr residue at the C-terminus (Gacel *et al.*, 1980). The resulting hexapeptide [D-Ser^2,Leu^5,Thr^6]enkephalin (DSLET), showed quite high δ-receptor affinity, but also μ-receptor affinity. In comparison with DSLET, an analogue containing D-Thr residue in place of D-Ser^2, [D-Thr^2,Leu^5,Thr^6]enkephalin (DTLET) (Zajac *et al.*, 1983) was about 3-times more δ-selective. The new compounds, [D-Ser^2(O-*tert*-butyl),Leu^5,Thr^6]enkephalin (DSTBULET), and [D-Ser^2(O-*tert*-butyl),Leu^5,Thr^6(O-*tert*-butyl)]enkephalin (BUBU) showed to be highly potent and selective full agonists at the δ-receptor (Delay-Goyet *et al.*, 1988; Gacel *et al.*, 1988).

One way for enhancing δ-selectivity of enkephalins was the incorporation of *conformational constraints* in the molecule through *peptide cyclization*. Analogues containing Pen (penicillamine) residue in positions 2 and 5, connected with a dithio bridge, [D-Pen^2,L-Pen^5]enkephalin (DPLPE), ([D-Pen^2,D-Pen^5]enkephalin (DPDPE), and [D-Pen^2,*p*Cl-Phe^4,D-

Pen[5]]enkephalin (*p*Cl-DPDPE) showed markedly improved δ-selectivity (Mosberg *et al.*, 1983; Tóth *et al.*, 1990).

Among the opioid peptides known in the literature, **deltorphins**, isolated from skin extracts of frogs, are the most potent and δ-selective agonists. Structure-activity studies were undertaken to establish molecular determinants which contribute to this high δ-affinity and δ-selectivity (Salvadori *et al.*, 1991; Sasaki *et al.*, 1991, 1995; Lazarus *et al.*, 1992, 1993).

Modification in the side-chains of individual amino acids in deltorphins influenced significantly the receptor binding properties. The aromatic side chains of Tyr^1 and Phe^3 in the "message" domain, and the aryl side chain of Leu^5 in the "address" domain, were found to play essential roles in conferring the high δ-affinity and selectivity. The crucial role of D-enantiomer at position 2 was evident, following the change in the stereocenter to the L-conformer which resulted in losses in δ-affinity by several orders of magnitude (Lazarus *et al.*, 1992). Enhancing the hydrophobicity at positions 5 and 6, increases in δ-affinity and selectivity were observed, suggesting that these positions are important hydrophobic cores in deltorphins (Salvadori *et al.*, 1991; Sasaki *et al.*, 1991, 1995; Lazarus *et al.*, 1993; Nevin *et al.*, 1994).

Conformationally restricted Phe^3-substituted deltorphin I and II *analogues* were designed in an attempt to enhance δ-receptor affinity and/or selectivity of deltorphins (Schiller *et al.*, 1992b; Tóth *et al.*, 1997).

Tóth et al. (1997) have applied two approaches to develop very active and highly δ-selective deltorphin analogues, with altered hydrophobic and stereoelectronic properties: **(a)** substitution of Val residues at positions 5 and 6 in the "address" domain, with the more lipophilic amino acid, Ile, and **(b)** conformational restriction of the Phe^3 residue in the "message" domain.

Thus, substitution of Phe^3 residue in deltorphins, with a conformationally restricted amino acid, 2-aminotetralin-2-carboxylic acid (Atc) (Fig. 3), gave rise to analogues with extraordinary δ-receptor affinity and δ-selectivity.

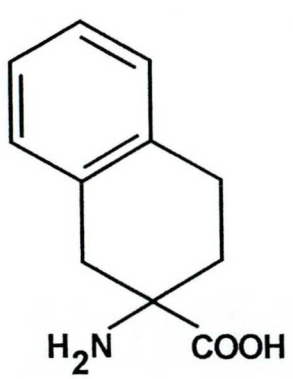


Fig. 3. *STRUCTURE OF 2-AMINOTETRALIN-2-CARBOXYLIC ACID (ATC).*

1.7.2.2. δ-Selective Antagonists

Non-peptide opioid antagonists are preferred as pharmacological tools, because they can generally penetrate the CNS, and are less subjected to metabolic inactivation than peptides and their analogues.

Naloxone and naltrexone are the most widely employed opioid antagonists to analyze the effects mediated by specific receptors, but they have a limited use due to their moderate receptor type selectivity.

Following the guidelines of the *"message-address" theory* (Schwyzer, 1977), Portoghese (1988) developed highly selective non-peptide opioid ligands with potent δ-antagonist activity. The design of such compounds was exploring using a naltrexone-derived structure ("message component) that is joint to a benzene moiety which was consider to be the key δ "address" component.

One of the first compounds showing δ-antagonist potency was **naltrindole** (Portoghese *et al.*, 1988). The δ-antagonist activity was maintained when the indole system was replaced by other heterocycles that contain a benzene moiety, like benzofuran in **naltriben**. The heterocyclic portion of these ring systems simulates Phe^4 residue of enkephalins but in a conformationally rigid setting, and functions mainly as a rigid spacer to hold the benzene moiety and that (Portoghese *et al.*, 1990, 1991).

Several **opioid peptide-derived δ-antagonists** that contain 1,2,3,4-tetrahydroisoquinoline-3-carboxilic acid (Tic) residue in the 2-position of the peptide sequence were also designed (Schiller *et al.*, 1993).

The tetrapeptide Tyr-Tic-Phe-Phe-OH (TIPP) and its analogue containing a reduced peptide bound Tyr-Tic$\Psi(CH_2NH)$-Phe-Phe-OH (TIPP[Ψ]), prepared in tritiated form, displayed high δ-receptor affinity and selectivity, high potency as δ-antagonists (Nevin *et al.*, 1993, 1995).

Stable peptide and non-peptide ligands, both agonists and antagonists, with exceptionally high affinity towards the opioid receptors are very useful for understanding the mechanism of opioid action at the level of endogenous systems, in neurochemical processes of various mental diseases and pain states.

2. AIM OF THE STUDIES

More detailed knowledge of the molecular basis of opioid receptor multiplicity and function requires the rational design of new opioid drugs. Many ligands based on the structure of opioid peptides and of natural alkaloids with high selectivities and affinities for each type of opioid receptors have been synthesized. The development of highly selective μ- and δ-opioid ligands with agonist or antagonist properties represents a particular challenge.

The aim of this thesis is the *study of opioid receptor binding characteristics* of several newly synthesized:

- ***peptide radioligands***:

  -dermorphin tetrapeptide analogue -**[^{3}H]Tyr-D-Ala-Phe-Phe-NH$_2$ ([^{3}H]TAPP)**;

  -deltorphin analogues -**[^{3}H]S-Atc^3,Ile^{5,6}deltorphin I**;

  -**[^{3}H]R-Atc^3,Ile^{5,6}deltorphin II;**

- ***alkaloid ligands***:

  -benzofuran derivatives of -naloxone;

  -oxycodone;

  -oxymorphone.

Ligand-receptor interaction is characterized by the use of ***radioligand binding assays.*** Membrane preparations from r**at brain** were used in this study because they represent a good source for both μ- and δ-receptors being more than 40% of the total opioid receptor population. Chinese hamster ovary (**CHO-μ/1**) cell line transfected with the cloned rat μ-receptor was also used to study the interaction of [^{3}H]TAPP with the μ-opioid receptor.

The major goals were:

- *to have novel opioid ligands with improved stability and specificity;*
- *to establish structural requirements of the new ligands for high affinity and selectivity to opioid receptors;*
- *to classify the agonist/antagonist character of the novel synthesized ligands;*
- *to measure their opioid activity in kinetic, equilibrium and competition binding studies;*
- *to compare their opioid binding properties with those of other well-known compounds labeling opioid receptors.*

3. MATERIALS AND METHODS

3.1. Chemicals

Opioid peptides:

-TAPP, TIPP, TIPP[Ψ] were synthesized by Dr. P. W. Schiller (Laboratory of Chemical Biology and Peptide Research, Clinical Research Institute, Montreal, Canada) (Schiller *et al.*, 1989; Schiller *et al.*, 1993);

-DAMGO and DSLET were purchased from Bachem Feinbiochemica (Bubendorf, Switzerland);

-deltorphin II, Ile^{5,6}deltorphin II, S-Atc^{3},Ile^{5,6}deltorphin I and R-Atc^{3},Ile^{5,6}deltorphin II were synthesized by Dr. G. Tóth (Isotope Laboratory of the Biological Research Center, Szeged, Hungary) (Sasaki *et al.*, 1991; Tóth *et al.*, 1997);

-DPDPE was kindly provided by Dr. K. Medzihradszky (Central Research Institute for Chemistry, Budapest, Hungary).

Synthetic alkaloids:

-U69,593 was obtained from Upjohn Company (Kalamazoo, MI, USA);

-cyprodime was synthesized by Dr. H. Schmidhammer (Institute of Organic and Pharmaceutical Chemistry, University of Innsbruck, Innsbruck, Austria) (Schmidhammer *et al.*, 1989);

-dihydromorphine was synthesized by Dr. G. Tóth (Isotope Laboratory of the Biological Research Center, Szeged, Hungary);

-nor-BNI was kindly provided by Dr. P. S. Portoghese (Department of Chemistry, University of Minnesota, Minneapolis, MN, USA);

-naltrexone, naloxone, naltrindole, naltriben, naloxone (R_2)OCH_3 and benzofuran derivatives of naloxone, oxycodone and oxymorphone were prepared by Dr. S. Hosztafi (Alkaloida Chemical Company, Tiszavasvari, Hungary) (Portoghese *et al.*, 1988, 1991);

-dextrorphan and levorphanol were provided from Hoffman-La Roche (Nutley, NJ, USA).

The following **radioligands** were prepared by Dr. G. Tóth (Isotope Laboratory of the Biological Research Center, Szeged, Hungary), according to the published protocols: [^{3}H]TAPP (**I**); [^{3}H]S-Atc^{3},Ile^{5,6}deltorphin I and [^{3}H]R-Atc^{3},Ile^{5,6}deltorphin II (Darula *et al.*, 1997; **II**);

[^{3}H]naloxone (Tóth *et al.*, 1982); [^{3}H]Ile^{5,6}deltorphin II (Nevin *et al.*, 1994); [^{3}H]TIPP[ψ] (Nevin *et al.*, 1995) and [^{3}H]naltrindole (Yamamura *et al.*, 1992).

Other **radioligands** used: [^{3}H]DAMGO, [^{3}H]U69,593 and [^{3}H]*p*Cl-DPDPE were purchased from Du Pont-New England Nuclear (Boston MA, USA).

Characteristics of the radioligands used in this study are summarized in Table VI.

TABLE VI. *Radioligands Used in This Study*

Radioligand	Structure	Selectivity	Molar activity (Ci/mmol)
*[^{3}H]TAPP	tetrapeptide	μ-agonist	56.8
*[^{3}H]S-Atc^3,Ile^{5,6}deltorphin I	heptapeptide	δ-agonist	34.5
*[^{3}H]R-Atc^3,Ile^{5,6}deltorphin II	heptapeptide	δ-agonist	36
[^{3}H]Naloxone	14-OH-morphinan	antagonist	71.9
[^{3}H]DAMGO	pentapeptide	μ-agonist	59
[^{3}H]Ile^{5,6}deltorphin II	heptapeptide	δ-agonist	49.5
[^{3}H]*p*Cl-DPDPE	pentapeptide	δ-agonist	49
[^{3}H]TIPP[Ψ]	tetrapeptide	δ-antagonist	47.9
[^{3}H]Naltrindole	indolomorphinan	δ-antagonist	46.1
[^{3}H]U69,593	benzeneacetamide	κ-agonist	47

*[^{3}H]TAPP, [^{3}H]S-Atc^3,Ile^{5,6}deltorphin I and [^{3}H]R-Atc^3,Ile^{5,6}deltorphin II are characterized in this study. Data from all other radioligands is from the literature.

Tris-(hydroxymethyl)-aminomethane (Tris), bestatin, phenylmethylsulphonyl fluoride (PMSF), bacitracin, captopril, bovine serum albumin (BSA), polyethyleneimine (PEI), Gpp(NH)p, Dulbecco's modified Eagle's medium (DMEM) and phosphate-buffered saline (PBS) were purchased from Sigma Chemicals (St. Louis, MO, USA). Fetal calf serum was obtained from Jaques BOYS (Remise, France). Geneticin (G418) and trypsin were provided from GIBCO (Grand Island, NY, USA). All other reagents used were of analytical grade.

3.2. Animals

Wistar rats (250-300 g body weight) were obtained from the Animal House of the Biological Research Center (Szeged, Hungary). Rats were housed in groups of four, maintained on a 12/12 h light/dark cycle and allowed free access to food and water until the time of sacrifice for binding studies.

3.3. Cell Culture

The CHO-μ/1 cell line stably transfected with the rat μ-opioid receptor was a kind gift from Dr. Z. Vogel (Department of Neurobiology, The Weizmann Institute of Science, Rehovot, Israel). Cells were grown in DMEM with 10% (vol/vol) fetal calf serum and 400 μg/ml geneticin (G418), in 100-mm glass culture dishes. Cells were maintained in a humidified atmosphere of 95% air/5% CO_2 at 37°C. When the cells achieved 80% confluence, they were splited 1:2 (approximately every three day). Single cells were prepared by trypsin treatment with the use of 0.1% trypsin in PBS, followed by washing with medium. Cells were resuspended in fresh medium at concentrations of 1 x 10^6 cells/ml.

3.4. Membrane Preparation

3.4.1. Rat Brain Membrane Preparation

Crude membrane fractions were prepared from Wistar rat brains, according to the method described by Simon *et al.* (1986).

The rats were decapitated, brains minus cerebella were rapidly removed, and washed with ice-cold 50 mM Tris-HCl buffer (pH 7.4). The brains were homogenized in 5 volumes (vol/wt) of buffer using a Teflon glass homogenizer, diluted in 30 volumes (vol/wt) of buffer and filtrated through four layers of gauze. After centrifugation at 40,000 x g for 20 min at 4°C, in a Sorvall RC5C centrifuge using a SS-34 rotor, the pellets were resuspended in 30 volumes (vol/wt) fresh Tris-HCl buffer and incubated for 30 min at 37°C to remove any endogenous opioids. The centrifugation step was repeated and the pellets were resuspended in 5 volumes (vol/wt) of 50 mM Tris-HCl containing 0.32 M sucrose (pH 7.4) to give a final protein concentration of 3-4 mg/ml. Aliquots of 5 ml from this preparation were stored at -70°C until use.

3.4.2. CHO-μ/1 Cell Membrane Preparation

Membranes from 1-2 x 10^8 CHO-μ/1 cells were prepared according to the method described by Fukuda *et al.* (1993).

DMEM containing 10% (vol/vol) FCS was removed from cell monolayers and washed twice with 5 ml PBS buffer. Cells were detached by scraping and centrifuged at 1,000 *x g* for 10 min. Pellets were suspended in 50 mM Tris-HCl buffer (pH 7.4) at a concentration of 1 *x* 10^7 cells/ml, and homogenized using a Teflon glass homogenizer. A crude membrane fraction was isolated by centrifugation at 20,000 *x g* for 25 min at 4°C. The pellets were resuspended in 10 ml fresh Tris-HCl buffer (pH 7.4) to give a final protein concentration of 1-2 mg/ml. Aliquots of 2 ml from this preparation were stored at -70°C for further use.

3.5. Radioligand Binding Assays

Prior to performing the binding assays, frozen rat brain membranes were thawed and separated from the Tris-HCl/sucrose medium by centrifugation at 40,000 *x g* for 20 min at 4°C. Experiments were carried out in plastic test tubes for the tritiated peptides or glass test tubes for the tritiated alkaloid ligands. Binding experiments were usually performed in 50 mM Tris-HCl buffer (pH 7.4). Modifications to this protocol are shown below:

-[^{3}H]S-Atc^3,Ile^{5,6}deltorphin I, [^{3}H]R-Atc^3,Ile^{5,6}deltorphin II and [^{3}H]naltrindole binding were determined in an assay buffer consisting of 50 mM Tris-HCl, 1 mg/ml BSA, 50 μg/ml bacitracin, 10 μM captopril, 30 μg/ml bestatin and 0.1 mM PMSF (pH 7.4) (**II**; Yamamura *et al.*, 1992);

-[^{3}H]*p*Cl-DPDPE binding was measured in 50 mM Tris-HCl buffer (pH 7.4) supplemented with 5 mM $MgCl_2$, 1 mg/ml BSA and 0.1 mM PMSF (Vaughn *et al.*, 1989).

The final volume of the reaction mixture was 1 ml consisting of:

-800 μl membranes (400-600 μg protein, in the case of rat brain membrane homogenates or 100-250 μg protein in the case of CHO-μ/1 cell membrane preparations);

-100 μl radioligand;

-100 μl Tris-HCl buffer (to determine total binding) or alternatively, 100 μl of 10 μM naloxone (to determine non-specific binding) or 100 μl of opioid ligand at various concentrations (for competition experiments).

Reactions were started with the addition of membrane suspension. Assay tubes were kept in water-bath shaker at proper temperature for a period of time, as it is indicated in Table VII. Incubations were terminated by rapid filtration under vacuum through Whatman glass fiber

filters (GF/B or C), followed by washing with 3 *x* 5 ml of ice-cold 50 mM Tris-HCl buffer (pH 7.4) using a Brandel Cell Harvester. Filters were dried and the bound radioactivity was measured in scintillation vials containing 5 ml of toluene-based scintillation cocktail using a Beckman LS 5000TD Scintillation Counter (40-50% counting efficiency for tritium).

TABLE VII. *Binding Assay Conditions Used in This Study*

Radioligand	Concentration (nM)	Incubation temperature (°C)	Incubation time (min)	Filter
*[^{3}H]TAPP	0.5	25	45	C
*[^{3}H]S-Atc^3,Ile^{5,6}deltorphin I	0.5	35	90	C
*[^{3}H]R-Atc^3,Ile^{5,6}deltorphin II	0.5	35	90	C
[^{3}H]Naloxone	1.0	0	60	B
[^{3}H]DAMGO	0.5	35	45	C
[^{3}H]Ile^{5,6}deltorphin II	0.5	35	45	C
[^{3}H]*p*Cl-DPDPE	0.5	25	270	B/PEI[a]
[^{3}H]TIPP[Ψ]	0.5	25	30	C
[^{3}H]Naltrindole	0.5	25	90	B/PEI[a]
[^{3}H]U69,593	1.0	30	30	B/PEI[a]

*Binding conditions for [^{3}H]TAPP, [^{3}H]S-Atc^3,Ile^{5,6}deltorphin I and [^{3}H]R-Atc^3,Ile^{5,6}deltorphin II are determined in this study. Data from all other radioligands is from the literature.
[a]Whatman GF/B filter papers were socked in 0.1% PEI at pH 10 for 60 min.

All assays were performed in duplicate, and repeated at least three times. The given values represent the means (± SEM). Protein concentration was measured according to the method of Bradford (1976) using BSA as a standard.

A. The time course for radioligand ***association*** was determined by incubation of the membrane suspension for various periods of time in the absence or in the presence of 10 μM unlabeled naloxone to define total and non-specific binding, respectively. The reactions were terminated by immediate filtration at the specified times. The specific binding was calculated by substraction of the non-specific binding from the total binding.

In the ***dissociation experiments***, radioligand was incubated with the membrane preparation, and dissociation was initiated by the addition of an excess concentration of unlabeled naloxone (10 μM), once the steady-state had been reached. The residual binding was measured, following immediate filtration, at various time points.

B. Saturation binding experiments were performed with increasing concentrations of radioligands: -[^{3}H]TAPP (0.01-3.5 nM);

-[^{3}H]S-Atc^3,Ile^{5,6}deltorphin I (0.06-6 nM);

-[^{3}H]R-Atc^3,Ile^{5,6}deltorphin II (0.03-5.5 nM).

C. Competition binding experiments were carried out by protein incubation with radioligands in the presence of 9-11 concentrations of competing ligands. Concentration, incubation temperature and incubation time for the used radioligands are listed in Table VII.

D. To asses ***the effect of Na^+ ions and Gpp(NH)p,*** membranes were incubated in the presence of increasing concentrations of NaCl (5-100 mM) or Gpp(NH)p (10-200 μM).

E. For ***Na^+ index determinations***, binding experiments were performed with [^{3}H]naloxone in the absence or in the presence of 100 mM NaCl.

D. Determination of wash-resistant binding. Washing experiments were performed as previously described (Krizsán *et al.*, 1991). Membrane suspensions (2-3 mg) were preincubated for 30 min at room temperature with three different concentrations (1, 10 and 100 μM) of the studied ligand in a final volume of 1 ml. After incubation, the samples were diluted to 30 ml with 50 mM Tris-HCl buffer (pH 7.4) and centrifuged at 31,000 *x* g for 10 min at 4°C. The pellets were resuspended in 30 ml fresh Tris-HCl buffer, incubated for 10 min on ice and recentrifuged. The washing step was repeated four times. After the last centrifugation, the final pellets were resuspended in 5 ml Tris-HCl buffer, and the total and non-specific binding were determined with 1 nM [^{3}H]naloxone. Control values represent the specific binding of [^{3}H]naloxone to membranes preincubated only with buffer and treated in the same way.

3.6. Analysis of Binding Data

The simplest model describing the interaction of a receptor, R, with a ligand, L, to form a complex, RL, is the bimolecular reaction:

$$R + L \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} RL \qquad (1)$$

According to the *Principle of Mass Action*, at equilibrium:

$$K_d = ([R][L])/[[RL] = k_{-1}/k_{+1} \qquad (2)$$

where: K_d-equilibrium dissociation constant;
[R]-concentration of receptor;
[L]-concentration of ligand;
[RL]-concentration of receptor-ligand complex, also referred to as the amount bound, [B];
k_{+1}, k_{-1}-association and dissociation rate constants of ligand L, respectively.

Two *principal parameters characterize the ligand-receptor interaction* (for a review, see Leslie, 1987). **(1) Affinity** is a measure of the ability of a ligand to bind to a specific receptor, and is represented by equilibrium dissociation constant (K_d). The K_d of a radioligand may be determined by: (a) *kinetic experiments* or (b) *saturation experiments.*

(2) Selectivity can be defined as the ratio of the dissociation constants at primary and secondary sites, obtained in *competition binding experiments.*

3.6.1. Kinetic Studies

Kinetic data are analyzed according to the method described by Weiland and Molinoff (1981). The rate of association of a radioligand with a receptor is determined by measuring the amount of bound ligand [B], as a function of time. If the total concentration of radioligand is much higher than the amount specifically bound at equilibrium, it means that most of the ligand remains free, and only a small fraction of ligand is bound even at equilibrium (pseudo-first order conditions).

The **observed association rate constant**, $\mathbf{k_{obs}}$, is calculated as the slope of the plot $\ln[B_e/(B_e-B_t)]$ versus time, according to the equation:

$$B_t = B_e * [1 - e^{(-k_{obs} * t)}] \quad (3)$$

where: B_t-the amount of radioligand specifically bound at time t;
B_e-the amount of radioligand specifically bound at equilibrium.

The second order **association rate constant**, $\mathbf{k_{+1}}$, can be derived by the equation:

$$k_{+1} = (k_{obs} - k_{-1})/[L] \quad (4)$$

The **dissociation rate constant**, $\mathbf{k_{-1}}$, is determined by stopping the association of the radioligand and the receptor, and measuring the amount of radioligand that remains bound as a function of time. According to the equation:

$$B_t = B_o * e^{(-k_{-1} * t)} \quad (5)$$

k_{-1} is the slope of the plot $\ln(B_t/B_o)$ versus time.

Once the association and dissociation rate constants have been determined, their ratio can be calculated to provide the kinetically determined **equilibrium dissociation constant**, $\mathbf{K_d}$:

$$K_d = k_{-1}/k_{+1} \quad (6)$$

3.6.2. Saturation Studies

Two methods are used for analyzing of equilibrium binding data, the *Scatchard plot* and the *Hill plot* (for a review, see Weiland and Molinoff, 1981). They are both transformations of the data obtained, when increasing amounts of radioligand are added to a fixed concentration of receptors, and the amount of radioligand bound, [B], is measured as a function of the concentration of radioligand.

Equation (2) can be rearranged to give the *Scatchard equation*:

$$[B]/[F] = (B_{max}/K_d) - ([B]/K_d) \quad (7)$$

where: [B]=[RL]-concentration of specifically bound ligand;
[F]=[L]-total free concentration of ligand;
$B_{max}=R_t$-maximal number of binding sites; $R_t = [R] + [RL]$; (8)

A plot of the ratio [B]/[F] versus the concentration of bound ligand has a slope equal to the negative reciprocal of the dissociation constant, $-1/K_d$, and the intercept on the abscissa provides a measure of the **maximal number of binding sites**, $\mathbf{B_{max}}$.

A linear Scatchard plot reflects interaction of a ligand in a simple bimolecular manner with a single class of binding sites, or with multiple classes of binding sites with equal affinity. Non-linear Scatchard plots may reflect more complex models, including cooperative interactions between binding sites or the presence of multiple classes of binding sites for which the radioligand has different affinities.

If the B_{max} is known, then the saturation curve can be plotted as a *Hill plot*:

$$\log([B]/([B_{max}] - [B])) = n_H * \log[L] - n_H * \log IC_{50} \quad (9)$$

where: $\mathbf{n_H}$-Hill coefficient;
$\mathbf{IC_{50}}$-concentration of ligand at which 50% of the binding sites are occupied.

A plot of $\log([B]/([B_{max}]-[B]))$ versus log[L] has a slope value of n_H, and the intercept on the abscissa of $\log IC_{50}$.

When the reaction follows the *Principle of Mass Action* at equilibrium, the Hill coefficient will be equal to 1. A Hill coefficient significantly different from 1 indicates a more complex ligand-receptor interaction than described by equation (1). This may result from a heterogeneity of binding sites, negatively or positive cooperativity between sites, or two/three component binding system (for a review see, Leslie, 1987).

3.6.3. Competition Studies

If kinetic and saturation binding studies are used to measure the direct interaction of a radioligand with a receptor, the competitive inhibition of the binding of a radioligand by unlabeled compounds can be used to indirectly characterize this interaction. Indirect binding assays are essential to characterized completely a population of receptors.

The simplest model describing the interaction of a radioligand, L, and a competitive inhibitor, I, with a receptor, R, is:

$$R + L \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} RL \qquad (10)$$

$$R + I \underset{k_{-1i}}{\overset{k_{+1i}}{\rightleftharpoons}} RI \qquad (11)$$

$$K_i = ([R][I])/[[RI] \qquad (12)$$

where: [I]-concentration of competitive inhibitor;
[RI]-concentration of receptor-inhibitor complex;
k_{+1i}, k_{-1i}-association and dissociation rate constants of competitive inhibitor, I;
$\mathbf{K_i}$-equilibrium dissociation constant for competitive inhibitor (**inhibition constant**).

The concentration of a competing ligand that inhibits specific binding of radioligand in concentration [L] by 50% at equilibrium (IC_{50}) is related to the equilibrium dissociation constant (K_d) of the compound. This relationship is described by the *equation of Cheng and Prusoff* (Cheng and Prusoff, 1973):

$$K_i = IC_{50}/(1 + [L]/K_d) \qquad (13)$$

If assay conditions are such that $[L]/K_d << 1$, then $K_i \approx IC_{50}$. IC_{50} values can be estimated by construction of standard dose-response semilogaritmic plots.

Competition binding data could be analyzed by the non-linear least squares fitting **program LIGAND** (Munson and Rodbard, 1980). Unlike the Cheng and Prusoff estimation, which assume that both labeled and unlabeled ligands interact with homogenous population of binding sites, this program is used for analysis of displacement of radioligand binding to heterogeneous sites.

4. RESULTS AND DISCUSSION

I. BINDING CHARACTERISTICS OF THE AGONIST PEPTIDE [^{3}H]TYR-D-ALA-PHE-PHE-NH_2 IN RAT BRAIN AND CHO-μ/1 CELL MEMBRANES

In an effort to develop opioid peptides with enhanced affinity and selectivity towards the μ-opioid receptors, a dermorphin tetrapeptide analogue containing a D-Ala residue in position 2, two Phe residues in positions 3 and 4 and a carboxyamide group at the C-terminal was synthesized (see Chap. 1.7.1). The resulted ligand **Tyr-D-Ala-Phe-Phe-NH_2 (TAPP)** shows structural relationship to the recently discovered highly selective endogenous peptide endomorphin (see Chap. 1.2.2).

TAPP was prepared in tritium-labeled form, with a high specific radioactivity (56.8 Ci/mmol) and its binding properties were determined and compared in membrane preparations from rat brain and from a CHO-μ/1 cell line stably transfected with the rat μ-opioid receptor (**I**).

4.1. Effect of the Temperature on Specific [^{3}H]TAPP Binding

To determine appropriate conditions of [^{3}H]TAPP binding, the effect of incubation temperature was initially investigated in rat brain membranes (Table VIII). Results of these experiments indicated that there are changes in [^{3}H]TAPP specific binding in function of temperature with the maximal binding at 25°C. Therefore, all the subsequent assays were carried out at this temperature.

TABLE VIII. *Effect of the Temperature on Specific [^{3}H]TAPP Binding*

Temperature (°C)	Specific Binding[a]	
	cpm	fmol/mg
0	372	15.37
25	1004	41.47
35	718	29.66

[a]Rat brain membranes were incubated at the given temperature, for 45 min, in the presence or in the absence of 10 μM naloxone.

4.2. Association and Dissociation Kinetics of [^{3}H]TAPP

Binding of [^{3}H]TAPP to membranes from rat brain and CHO-μ/1 cells gradually increased with time and reached the steady state after 40 min incubation at 25°C. Dissociation of specifically bound radioligand was initiated by the addition of 10 μM unlabeled naloxone at the steady-state, and showed that the binding process was reversible. Representative plots of experiments for both the time course of [^{3}H]TAPP association to rat brain and to CHO-μ/1 cell membranes, and its dissociation from the same membranes are shown in Fig. 4.

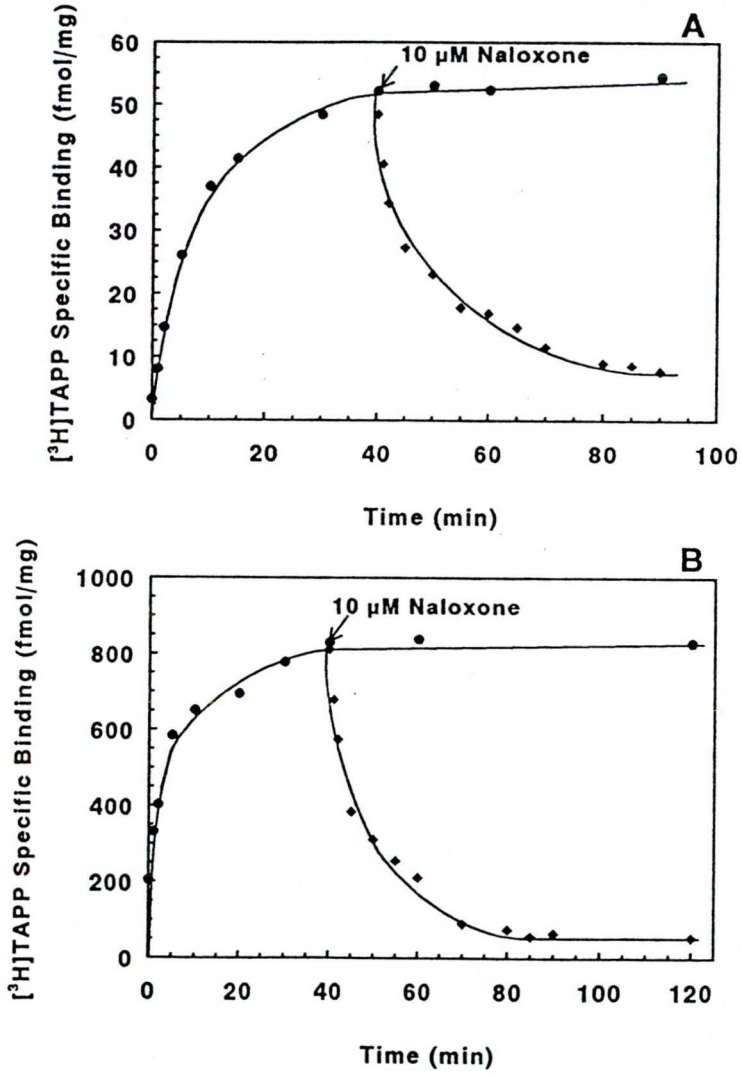


Fig. 4. *ASSOCIATION AND DISSOCIATION OF [^{3}H]TAPP BINDING.*
Membranes from rat brain **(A)** or CHO-μ/1 cells **(B)** were incubated with 0.5 nM [^{3}H]TAPP for various periods of time at 25°C as described in 'Materials and Methods'. Radioligand dissociation was initiated by addition of 10 μM naloxone (↓) at the steady state.

TABLE IX. *Kinetic Parameters of [^{3}H]TAPP Binding to Rat Brain and CHO-μ/1 Cell Membranes at 25 °C*

Tissue	k_{+1} (sec^{-1} M^{-1})	k_{-1} (sec^{-1})	K_d (nM)
Rat Brain	$1.96 \pm 0.66 \times 10^6$	$9.29 \pm 2.49 \times 10^{-4}$	0.47
CHO-μ/1 Cells	$2.34 \pm 1.03 \times 10^6$	$17.03 \pm 5.30 \times 10^{-4}$	0.73

Kinetic studies revealed rapid, monophasic association and dissociation of [^{3}H]TAPP binding from opioid receptors in membrane preparations used.

Association (k_{+1}) and dissociation (k_{-1}) rate constants were determined as described in 'Materials and Methods' (see Chap. 3.6.1). The estimated values are given in Table IX. From these rate constants, the kinetically derived equilibrium dissociation constants, K_d, of 0.47 and 0.73 nM were calculated for [^{3}H]TAPP binding to rat brain and CHO-μ/1 cell membrane preparations, respectively.

4.3. Saturation Studies of [^{3}H]TAPP Binding

Saturation experiments were performed on membranes from rat brain and from CHO-μ/1 cells (Fig. 5). The binding of [^{3}H]TAPP at 25°C was saturable at the concentration range used (0.01-3.5 nM).

The K_d and the maximal number of binding site (B_{max}) values were calculated by linear regression analysis of saturation isotherms from Scatchard plots, as described in 'Materials and Methods' (see Chap. 3.6.2) (Table X).

TABLE X. *Equilibrium Parameters of [^{3}H]TAPP Binding to Rat Brain and CHO-μ/1 Cell Membranes at 25 °C*

Tissue	K_d (nM)	B_{max} (fmol/mg)	n_H
Rat Brain	0.31 ± 0.02	119.13 ± 8.20	1.01 ± 0.02
CHO-μ/1 Cells	0.78 ± 0.09	1806.15 ± 138.35	1.04 ± 0.07

In rat brain, [^{3}H]TAPP labeled a single class of binding sites, with a K_d value of 0.31 nM and maximal binding of 119 fmol/mg protein (Fig. 5A). Similarly, the results of saturation studies with transfected CHO-μ/1 cell membranes indicated that [^{3}H]TAPP bound with a K_d value of 0.78 nM and a B_{max} value of 1806 fmol/mg protein (Fig. 5B).

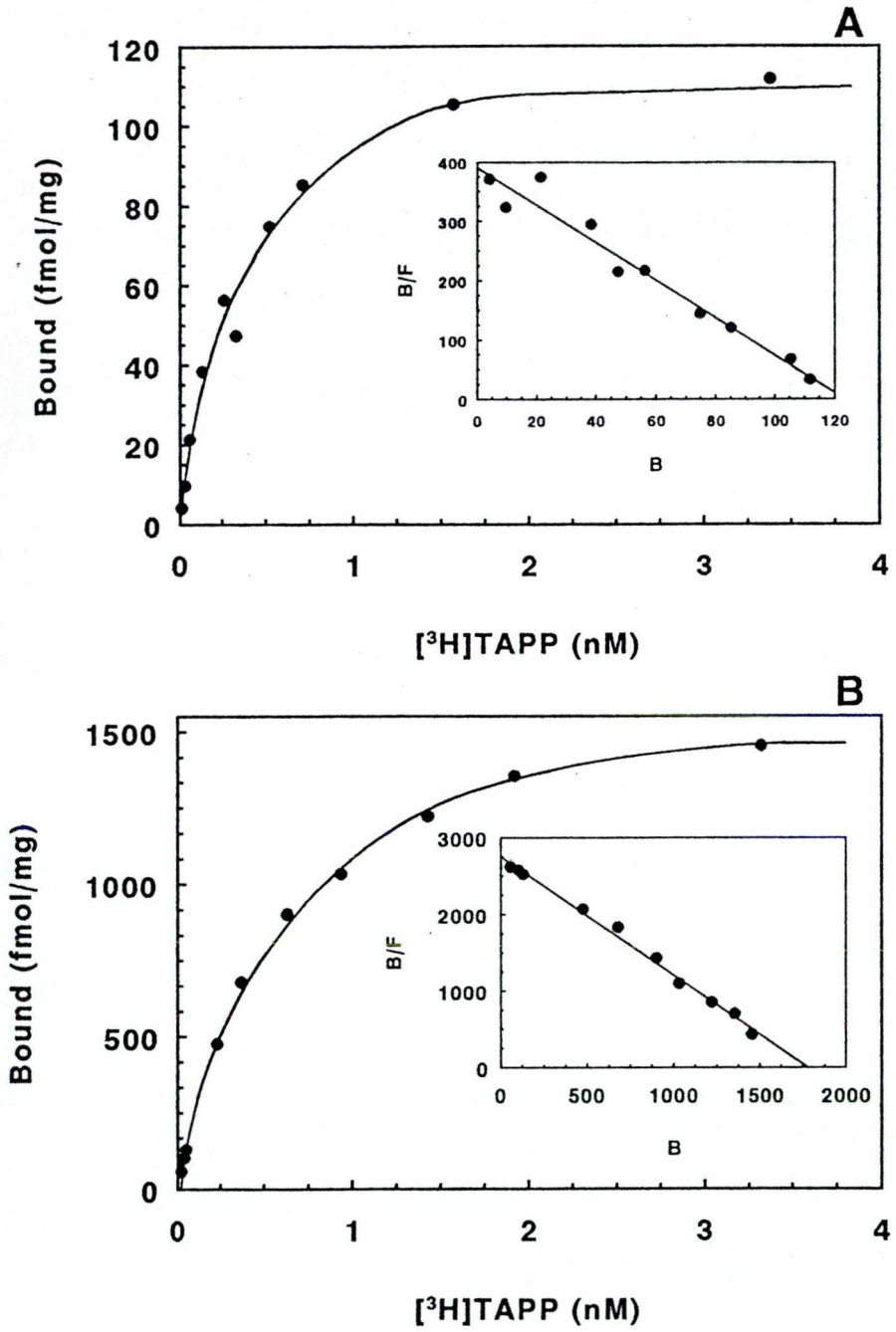


Fig. 5. *SATURATION OF [^{3}H]TAPP BINDING.*
Membranes from rat brain **(A)** or CHO-μ/1 cells **(B)** were incubated with increasing concentrations of [^{3}H]TAPP in the absence or in the presence of 10 μM naloxone for 45 min at 25°C. insert: Scatchard plots.

The Hill coefficient (n_H) values for [^{3}H]TAPP binding were calculated and their values were found to be close to the unity, also suggesting radioligand binding to a single population of opioid receptors and the non-cooperative nature of the binding process (Fig. 6; Table X).

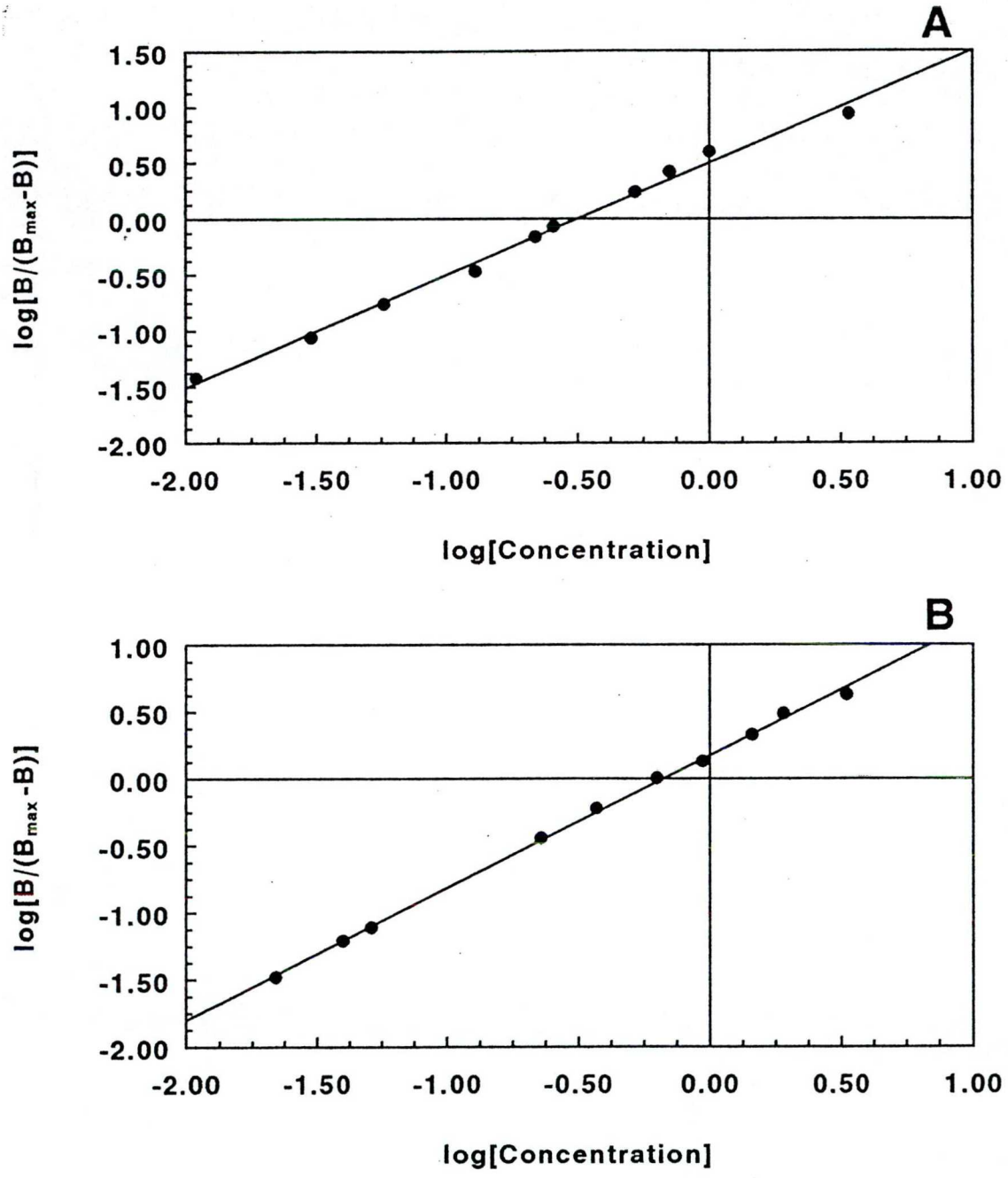


Fig. 6. *REPRESENTATIVE HILL PLOTS FOR [^{3}H]TAPP BINDING TO MEMBRANES FROM RAT BRAIN* ***(A)*** *AND CHO-μ/1 CELLS* ***(B)***.

The obtained B_{max} value, of 119 fmol/mg protein, for [^{3}H]TAPP binding to rat brain membranes was in agreement with those reported for μ-receptor density of other μ-opioid peptide radioligands, including [^{3}H]dermorphin (Amiche *et al.*, 1988), the enkephalin analogue [^{3}H]DAMGO (Delay-Goyet *et al.*, 1987) and the β-casomorphin analogue [^{3}H]PL017 (Blanchard *et al.*, 1987).

In membranes from CHO-μ/1 cells tansfected with the rat μ-opioid receptor, the calculated B_{max} value, 1800 fmol/mg protein, was similar with that observed for [^{3}H]DAMGO binding to the same type of cells (Avidor-Reiss *et al.*, 1995; Bunzow *et al.*, 1995).

[^{3}H]TAPP showed high affinity in both preparations (K_d values under nanomolar range), but the number of labeled binding sites in CHO-μ/1 cell membranes was significantly higher than that in rat brain homogenates, indicating a higher receptor expression in recombinant cells.

Besides, it is well established that the brain contains multiple opioid binding sites, whereas CHO-μ/1 cells contain a homogeneous population of μ-opioid receptors (Avidor-Reiss *et al.*, 1995; Bunzow *et al.*, 1995).

The K_d values for [^{3}H]TAPP binding determined from saturation studies were found to be in good agreement with those derived from kinetic studies (Table IX). The higher μ-receptor affinity of [^{3}H]TAPP in rat brain may be due to differences in the membrane environment of CHO-μ/1 cells versus brain homogenates.

The non-specific ratio of radioligand binding to rat brain preparations was <30% of total binding at a radioligand concentration equal to the K_d value, whereas in transfected CHO-μ/1 cell membranes it was much lower, about 10% of total binding under the same conditions.

The performed saturation binding studies, revealed that [^{3}H]TAPP specifically labeled a single population of opioid binding sites with high affinity in both membrane preparations.

4.4. Stereoselectivity of [^{3}H]TAPP Binding

The stereoselectivity of [^{3}H]TAPP binding to rat brain and CHO-μ/1 cell membranes was indicated by the high affinity of the opioid agonist, levorphanol, and the low affinity of a pharmacologically inactive enantiomer, dextrorphan, as it has been determined in [^{3}H]TAPP binding displacement experiments (Fig. 7; Table XI).

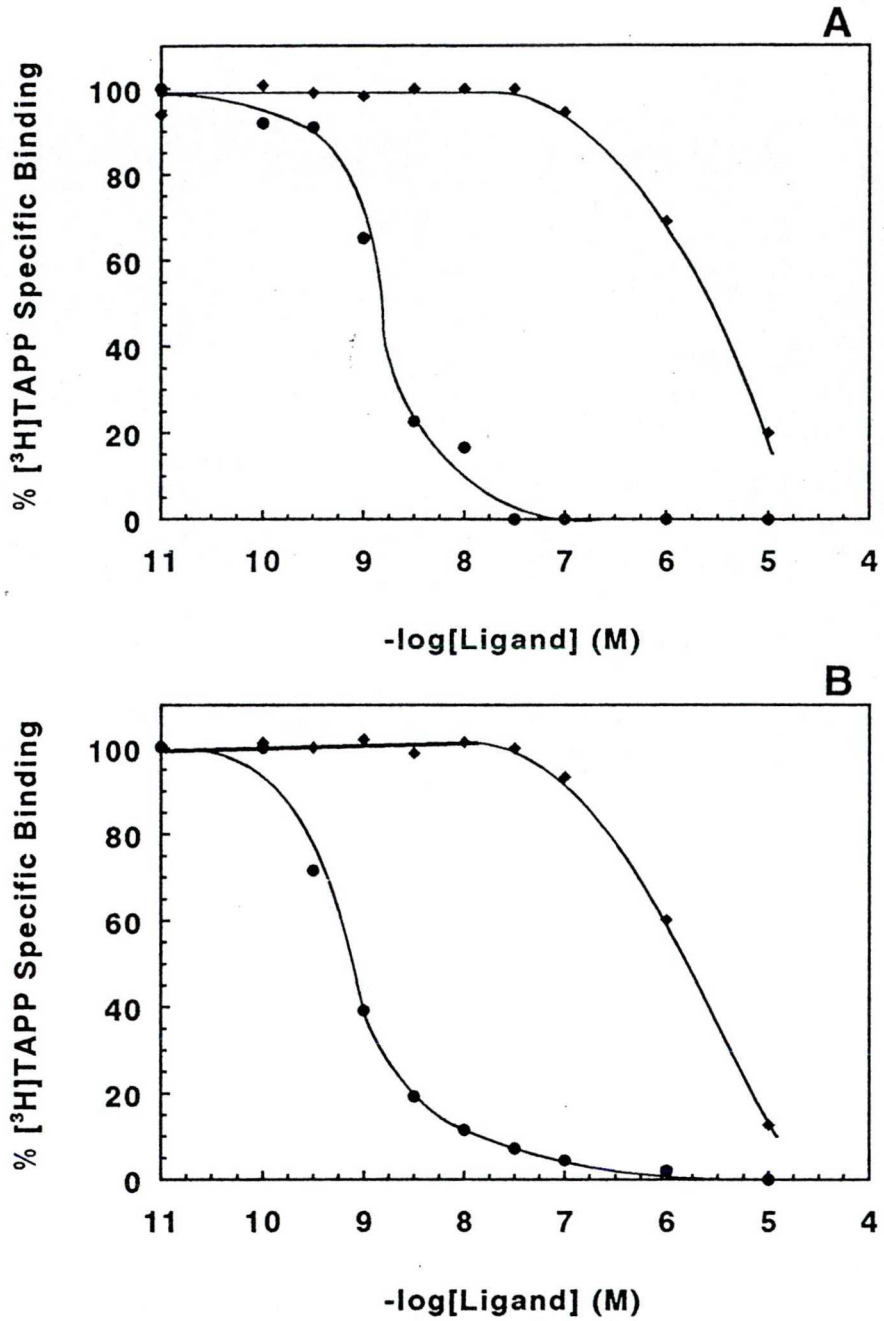


Fig. 7. *STEREOSELECTIVITY OF [^{3}H]TAPP BINDING.*
Membranes from rat brain **(A)** or CHO-μ/1 cells **(B)** were incubated with 0.5 nM [^{3}H]TAPP in the presence of increasing concentrations of two enantiomers, levorphanol (●) and dextrorphan (◆), for 45 min at 25°C.

4.5. Competition Studies of [^{3}H]TAPP Binding

To further characterize the properties of [^{3}H]TAPP, the abilities of various type-selective opioid ligands to displace its binding from rat brain and in CHO-μ/1 cell membranes were assessed (Fig. 8). The data calculated as binding inhibition constant (K_i) values are shown in Table XI.

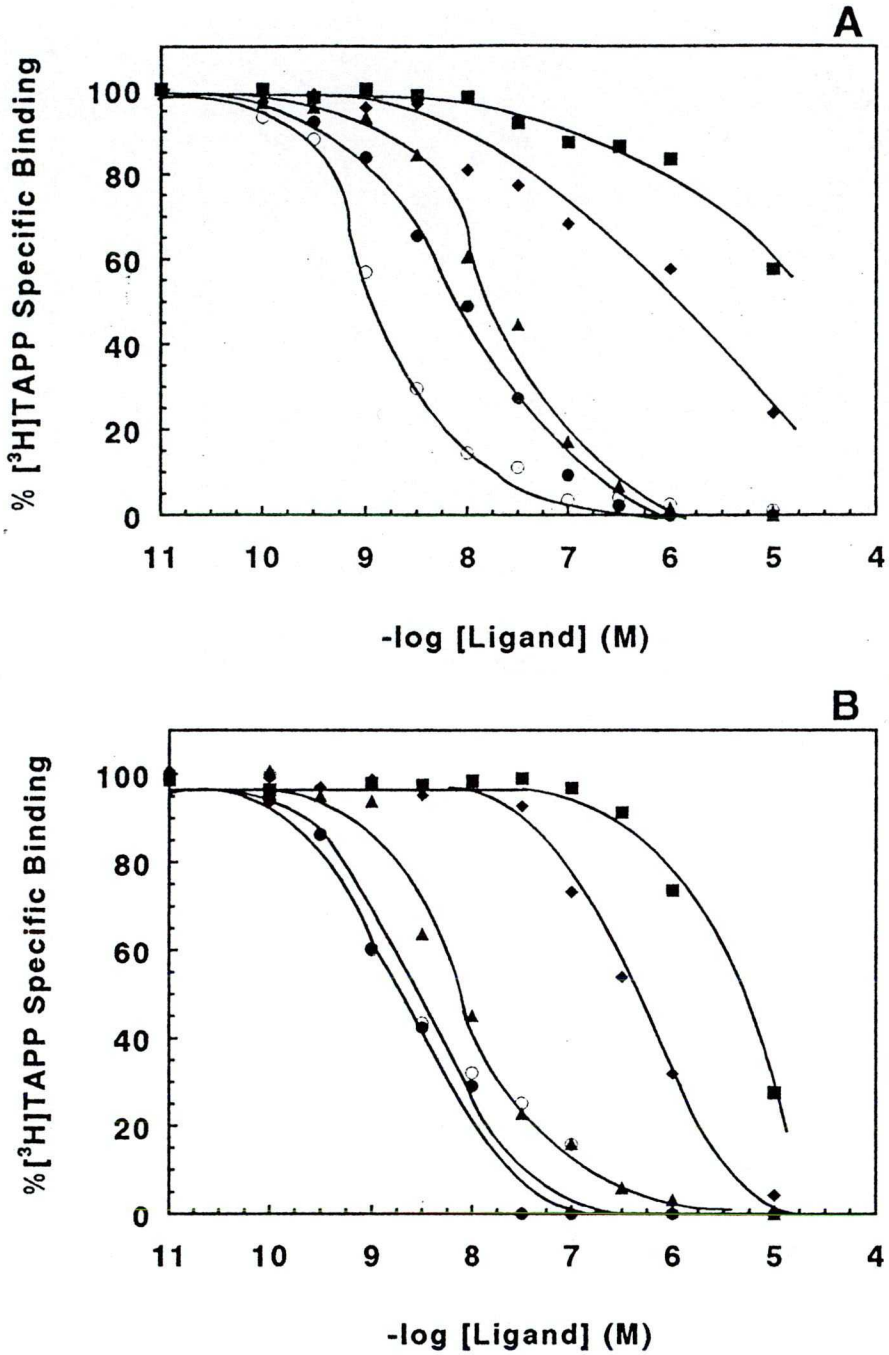


Fig. 8. *COMPETITION CURVES FOR [^{3}H]TAPP BINDING SITE BY OPIOID LIGANDS.* Membranes from rat brain **(A)** or from CHO-μ/1 cells **(B)** were incubated with 0.5 nM [^{3}H]TAPP in the presence of increasing concentrations of DAMGO (●), dihydromorphine (○), cyprodime (▲), U69,593 (◆), and Ile^{5,6}deltorphin II (■), for 45 min at 25°C.

Examination of competition curves against [^{3}H]TAPP showed that the data were best fitted by a one-site model (Fig. 8). This finding indicated again that [^{3}H]TAPP does not label multiple affinity states.

Several tested μ-receptor selective ligands, including dihydromorphine, DAMGO, levorphanol and cyprodime, competed for [^{3}H]TAPP binding sites in both rat brain and CHO-μ/1 cell membranes with much higher affinities than the κ-selective U69,593, and δ-selective peptide ligands Ile^{5,6}deltorphin II and TIPP.

In rat brain membranes, the μ-selective ligands display K_i values between 0.3-7.2 nM. About the same range of affinities was observed in CHO-μ/1 cell membranes (Table XI).

The relatively low K_i values observed with the δ-selective ligands, DSLET (Gacel *et al.*, 1988) and naltrindole (Portoghese *et al.*, 1988), is in agreement with the previously established fact that they are not among the most selective δ-ligands.

From the inhibition constants, the selectivity ratios, K_i^{δ}/K_i^{μ} and K_i^{κ}/K_i^{μ}, were calculated and were found to be 2200 and 510, respectively, in rat brain homogenates. In transfected CHO-μ/1 cell membranes, the obtained values were 1300 and 200, respectively.

TABLE XI. Inhibition of [^{3}H]TAPP Binding to Rat Brain and CHO-μ/1 Cell Membranes by Type-Selective Opioid Ligands

Ligand	K_i (nM)	
	Rat Brain	**CHO-μ/1 Cells**
μ-selective		
Dihydromorphine	0.28 ± 0.02	0.63 ± 0.11
DAMGO	1.16 ± 0.29	1.29 ± 0.28
Cyprodime	7.23 ± 1.36	4.59 ± 1.69
κ-selective		
U69,593	591 ± 23	253 ± 37
δ-selective		
DSLET	8.71 ± 1.63	7.34 ± 0.72
Naltrindole	25.7 ± 1.4	10.8 ± 0.8
TIPP	1704 ± 333	9153 ± 765
Ile^{5,6}deltorphin II	2518 ± 832	1717 ± 142
Levorphanol	0.51 ± 0.13	0.38 ± 0.06
Dextrorphan	779 ± 123	539 ± 217

Membranes were incubated with 0.5 nM [^{3}H]TAPP in the presence of increasing concentrations of competing opioid ligands, for 45 min at 25°C.

The results from competition binding studies indicated that in rat brain membranes [^{3}H]TAPP labeled the μ-receptor site with pharmacological properties similar to those exhibited by the μ-opioid receptors heterologously expressed in CHO cell membranes (Avidor-Reiss *et al.*, 1995).

4.6. Effect of Na^+ Ions and Gpp(NH)p on Specific [^{3}H]TAPP Binding

The interaction of [^{3}H]TAPP with the native and cloned μ-opioid receptor has also been characterized in terms of its modulation by Na^+ ions and guanine nucleotides.

The effect of Na^+ ions on specific [^{3}H]TAPP binding to rat brain and CHO-μ/1 cell membranes was investigated by the addition of variable concentrations of NaCl, from 5 to 100 mM (Fig. 9), as described in 'Materials and Methods' (see Chap. 3.5).

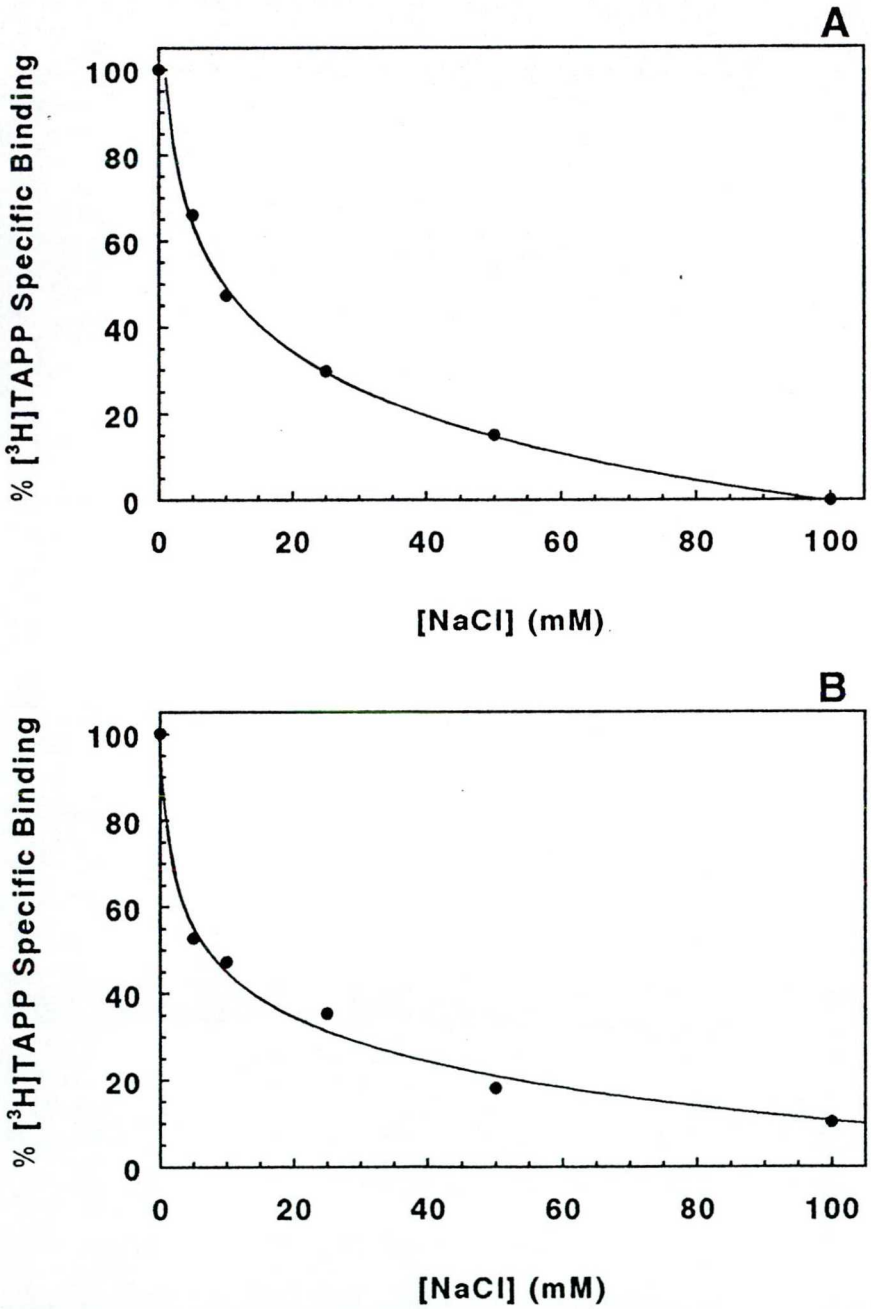


Fig. 9. *EFFECT OF Na^+ IONS ON [^{3}H]TAPP SPECIFIC BINDING*
Membranes from rat brain **(A)** or CHO-μ/1 cell **(B)** were incubated with 0.5 nM [^{3}H]TAPP in the presence of increasing concentrations of NaCl, for 45 min at 25°C.

The presence of Na^+ ions greatly decreased the specific binding of [^{3}H]TAPP to μ-opioid receptor with half maximal inhibition at 10-20 mM salt concentration. In rat brain membranes, [^{3}H]TAPP binding was almost completely abolished in the presence of 50 mM NaCl.

The effect of the non-hydrolysable analogue of GTP, Gpp(NH)p, on specific [^{3}H]TAPP binding was also investigated by the addition of increasing concentrations of nucleotide, from 10 to 200 μM (Fig. 10). It has been observed that Gpp(NH)p produced about 80% reduction in specific [^{3}H]TAPP binding at a concentration of 100 μM.

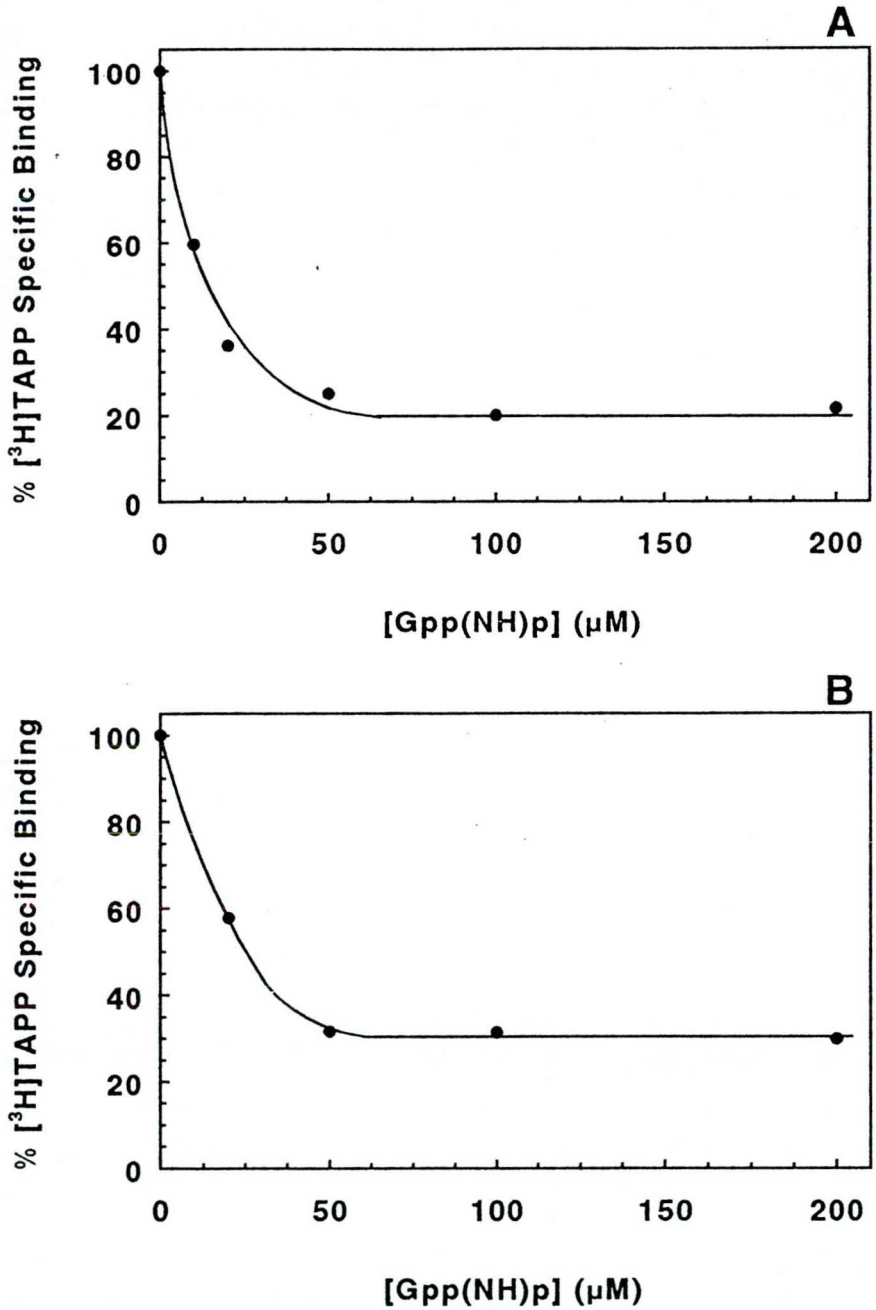


Fig. 10. *EFFECT OF Gpp(NH)p ON [^{3}H]TAPP SPECIFIC BINDING.*
Membranes from rat brain **(A)** or CHO-μ/1 cell **(B)** were incubated with 0.5 nM [^{3}H]TAPP in the presence of increasing concentrations of Gpp(NH)p, for 45 min at 25°C.

The effect of Na^+ ions and Gpp(NH)p on decreasing the specific binding of [^{3}H]TAPP is consistent with the agonist nature of the peptide.

It is well established, that receptor binding of the tritiated opioid antagonists is enhanced by Na^+ ions, while binding of the tritiated agonists is diminished. It has been hypothesized that the binding of Na^+ induces a conformational change which renders the opioid receptor site less likely to bind agonists and more likely to bind antagonists (Pert and Snyder, 1974).

Mutagenesis studies on the cloned opioid receptors suggested that the site for Na^+ modulation of ligand binding is a conserved Asp^{95} in the second transmembrane region of the protein molecule (see Chaps. 1.5; 1.6; Kong *et al.*, 1993) but the molecular mechanism of this action remains to be elucidated.

The reduction of specific binding of [^{3}H]TAPP in the presence of exogenous nucleotide, such as Gpp(NH)p, also indicated the functional coupling of the μ-opioid receptor to a G-protein regulated signal transduction system in rat brain, as well as in the transfected CHO-μ/1 cells (Childers, 1991).

4.7. Comparison of [^{3}H]TAPP binding Properties with Those of Other μ-Selective Agonist Peptide Radioligands

Opioid receptor binding properties in rat brain membranes of the newly synthesized and characterized opioid peptide analogue [^{3}H]TAPP were compared with those of the best-known μ-selective agonist peptide radioligands, such as [^{3}H]dermorphin (Amiche *et al.*, 1988), the enkephalin analogue [^{3}H]DAMGO (Handa *et al.*, 1981) and the β-casomorphin analogue [^{3}H]PL017 (Blanchard *et al.*, 1987) (Table XII).

TABLE XII. *Binding Characteristics of μ-Selective Agonist Peptide Radioligands in Rat Brain Membranes*

Compound	Sequence	K_d (nM)	B_{max} (fmol/mg)	K_i^{δ}/K_i^{μ} [a]
Dermorphin[1]	Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2	0.46	95	1100
TAPP	**Tyr-D-Ala-Phe-Phe-NH_2**	**0.31**	**119**	**2200**[b]
DAMGO	Tyr-D-Ala-Gly-MePhe-Gly-ol	3.9[2]	150[2]	2600[b,3,4]
PL017[5]	Tyr-Pro-MePhe-D-Pro-NH_2	6.0	160	260

[a] K_i^{δ}(DPDPE or [b]$Ile^{5,6}$ deltorphin II)/K_i^{μ} (DAMGO)

[1]Amiche *et al.*, 1988; [2]Delay-Goyet *et al.*, 1987; [3]Schiller, 1991; [4]Tóth *et al.*, 1997; [5]Blanchard *et al.*, 1987.

Among these compounds, the newly characterized radioligand showed the highest affinity and very good μ-receptor selectivity.

This radioligand showed 2-times higher selectivity for the μ-opioid receptor than that of its parent compound, dermorphin, and about the same binding affinity. Compared to one of the most used μ-receptor selective radioligands, [^{3}H]DAMGO, [^{3}H]TAPP exhibits a marked increase in affinity and comparable μ-receptor selectivity.

The results obtained in the present study indicate that the **new dermorphin tetrapeptide analogue [^{3}H]TAPP** interacted with high affinity with the μ-opioid receptor in both membrane preparations (**I**).

A very good correlation was observed between [^{3}H]TAPP binding in rat brain homogenates versus that in CHO cells transfected with the μ-opioid receptor. In rat brain membranes, [^{3}H]TAPP specifically labeled an opioid receptor site with pharmacological properties identical to those exhibited by the cloned rat μ-receptor expressed in CHO cells.

In addition, the binding of [^{3}H]TAPP was found to be modulated by Na^+ ions and guanine nucleotides indicating the agonist character of the ligand, and also that the native and cloned μ-opioid receptor, labeled by this radioligand, is functionally coupled to G-proteins.

This tetrapeptide [^{3}H]TAPP exhibited the highest μ-receptor affinity and excellent selectivity among the μ-selective agonist peptide radioligands.

Importantly, this radioligand fulfilled the criteria of steroselectivity, saturability, reversibility, and low non-specific binding necessary for useful radioligands.

II. BINDING CHARACTERISTICS OF [^{3}H]S-ATC^3,ILE^{5,6} DELTORPHIN I AND [^{3}H]R-ATC^3,ILE^{5,6} DELTORPHIN II IN RAT BRAIN MEMBRANES

The most potent and δ-selective agonists are the deltorphins which have been isolated from frog skin (see Chap.1.2.2). They are structurally flexible molecules and modification in the side-chains of the individual amino acids significantly changes their receptor binding properties (see Chap. 1.7.2.1).

Deltorphins contain two distinct regions which confer specific attributes to the molecule, a N-terminal "message" domain that defines biological responsiveness and a C-terminal "address" domain that influences binding affinities for a specific receptor type.

Deltorphin analogues were developed involving the modifications in the side chains at positions 3, 5 and 6, which cause changes in the hydrophobic and stereoelectronic properties. The new peptides were obtained by substitution of Phe^3 in the "message domain", with a conformationally restricted amino acid Atc (see Fig 3); Val residues at positions 5 and 6 in the "address domain" were replaced with the more lipophilic amino acid, Ile (see Chap. 1.7.2.1). The most highly δ-selective deltorphin analogues, S-Act^3,$Ile^{5,6}$deltorphin I and R-Atc^3,$Ile^{5,6}$deltorphin II, were prepared in tritium-labeled form, with 34.5 and 36 Ci/mmol specific radioactivity, respectively, and their opioid binding sites specific were characterized in rat brain membrane fractions (**II**).

4.8. Effect of the Temperature on Specific [^{3}H]S-Atc^3,$Ile^{5,6}$Deltorphin I and [^{3}H]R-Atc^3,$Ile^{5,6}$Deltorphin II Binding

The effect of incubation temperature on specific binding of [^{3}H]S-Atc^3,$Ile^{5,6}$deltorphin I and [^{3}H]R-Atc^3,$Ile^{5,6}$deltorphin II, was examined using the rat brain membrane preparations (Table XIII). As the incubation temperature was increased, specific binding also increased, and the maximal binding was obtained at 35°C in the temperature range used. Therefore, all the subsequent binding experiments were performed at this temperature.

TABLE XIII. *Effect of the Temperature on [^{3}H]S-Atc^3,$Ile^{5,6}$Deltorphin I and [^{3}H]R-Atc^3,$Ile^{5,6}$Deltorphin II Specific Binding*

Temperature (°C)	Specific Binding[a]			
	[^{3}H]S-Atc^3,$Ile^{5,6}$deltorphin I		[^{3}H]R-Atc^3,$Ile^{5,6}$deltorphin II	
	cpm	fmol/mg	cpm	fmol/mg
0	280	13.06	443	31.78
25	1605	74.84	1294	92.74
35	1984	92.52	1573	112.74

[a]Rat brain membranes were incubated at the given temperature, for 90, min in the presence or in the absence of 10 μM naloxone.

4.9. Association and Dissociation of [^{3}H]S-Atc^3,Ile^{5,6}Deltorphin I and [^{3}H]R-Atc^3,Ile^{5,6}Deltorphin II

Kinetic studies revealed slow, monophasic association and dissociation of [^{3}H]S-Atc^3,Ile^{5,6}deltorphin I and [^{3}H]R-Atc^3,Ile^{5,6}deltorphin II binding in rat brain membrane preparations (Fig. 11 and Fig. 12). Specific binding of these radioligands reached the steady state after 60 min and was stable up to 3 hr at 35°C (Fig. 11). Therefore an incubation time of 90 min was chosen for subsequent experiments to assure that equilibrium conditions had been reached.

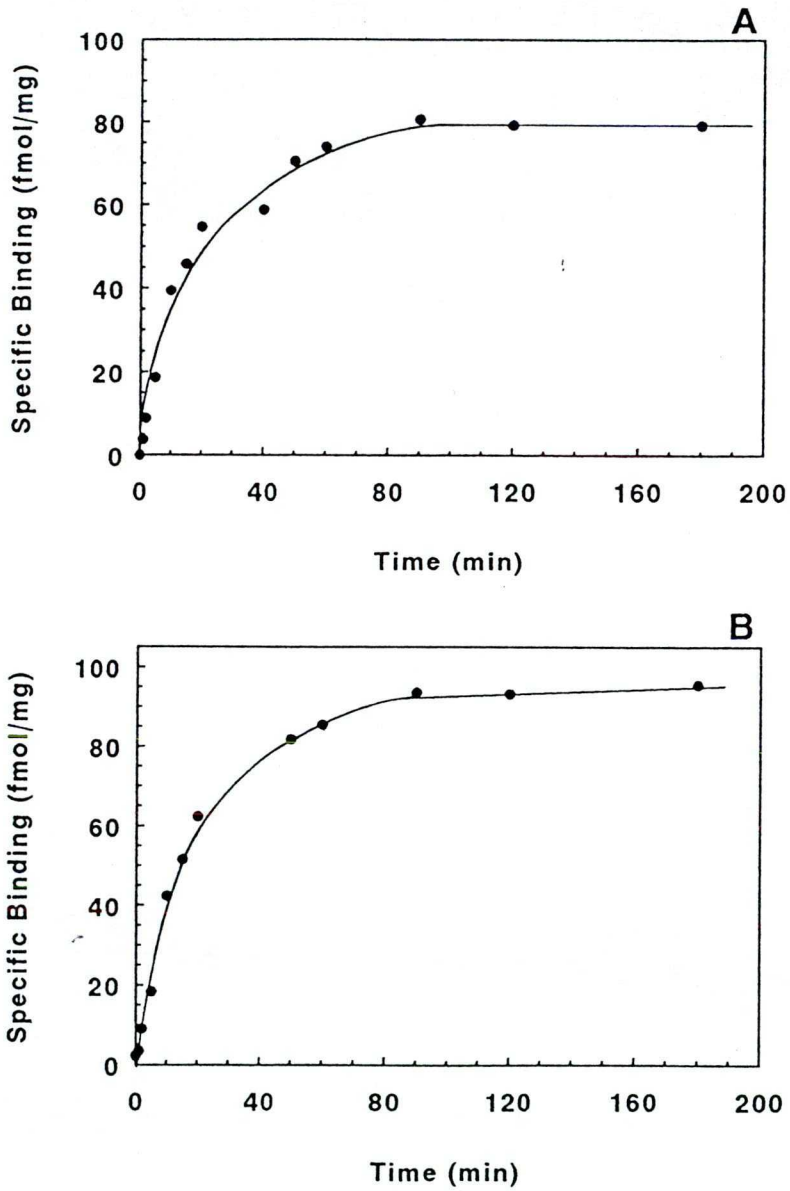


Fig. 11. *ASSOCIATION OF [^{3}H]S-ATC^3,ILE^{5,6}DELTORPHIN I (A) AND [^{3}H]R-ATC^3,ILE^{5,6}DELTORPHIN II (B) BINDING.*
Rat brain membranes were incubated with 0.5 nM of appropriate radioligand for various periods of time at 35°C as described in 'Materials and Methods'.

Dissociation of specifically bound radioligands from rat brain membranes was initiated by the addition of unlabeled naloxone (10 μM) after 2 hr of incubation and showed that the binding process is reversible and occurs with very low rate (Fig. 12).

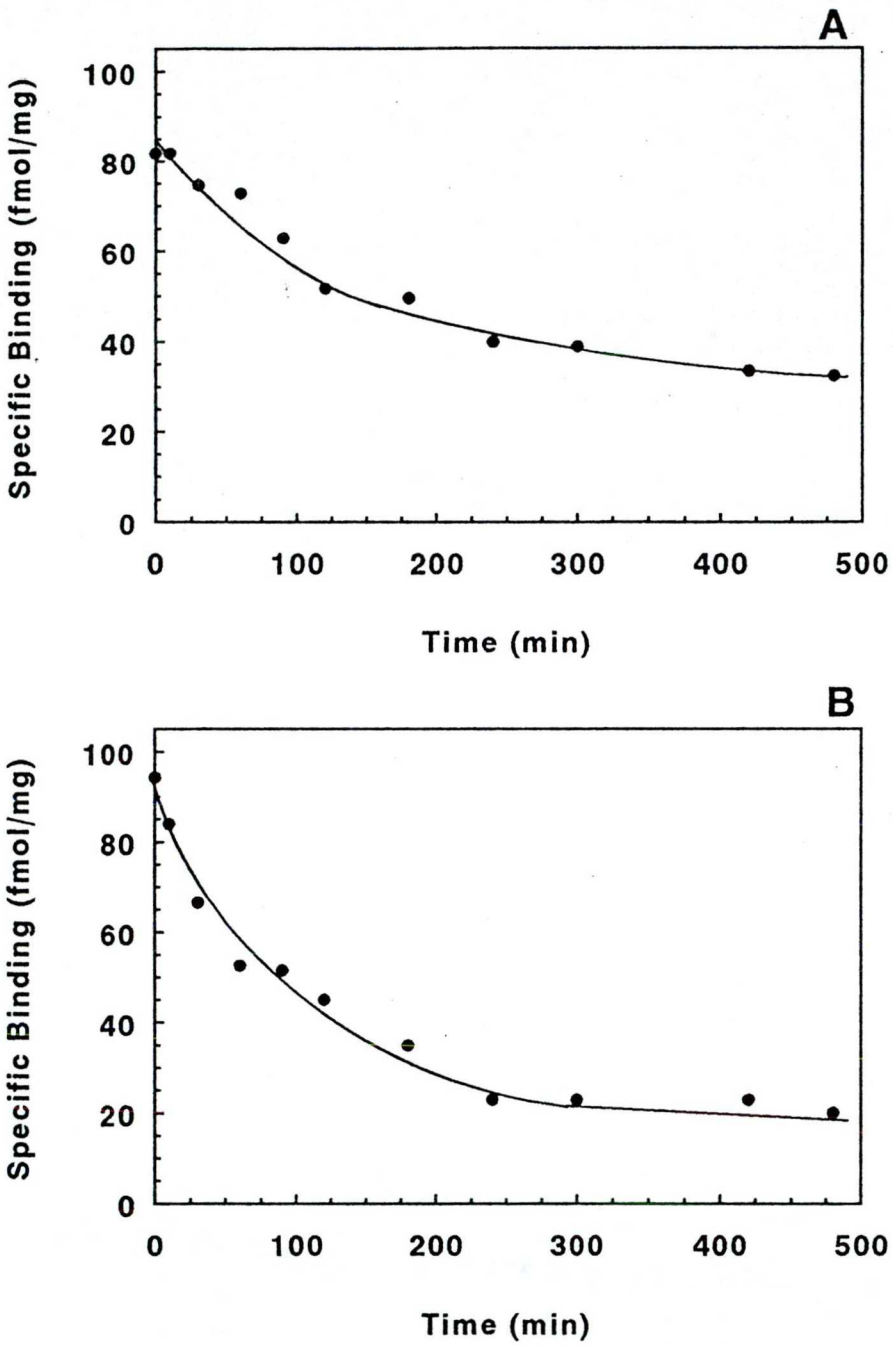


Fig. 12. *DISSOCIATION OF [^{3}H]S-ATC^3,ILE^{5,6}DELTORPHIN I* ***(A)*** *AND [^{3}H]R-ATC^3,ILE^{5,6}DELTORPHIN II* ***(B)*** *BINDING.*
Radioligands (0.5 nM) were incubated with rat brain membranes for 2 hr to reach the steady state and then dissociation was initiated by addition of 10 μM naloxone as described in 'Materials and Methods'.

Using k_{+1} and k_{-1} rate constants, determined as describes in 'Materials and Methods" (see Chap. 3.6.1), the kinetically derived equilibrium dissociation constants, K_d, of [^{3}H]S-Atc^3,Ile^{5,6}deltorphin I and [^{3}H]R-Atc^3,Ile^{5,6}deltorphin II were calculated and found to be 0.037 and 0.069 nM, respectively (Table XIV).

TABLE XIV. *Kinetic Parameters of [^{3}H]S-Atc^3,Ile^{5,6}Deltorphin I and [^{3}H]R-Atc^3,Ile^{5,6}Deltorphin II Binding to Rat Brain Membranes at 35 °C*

Tissue	k_{+1} (sec^{-1} M^{-1})	k_{-1} (sec^{-1})	K_d (nM)
[^{3}H]S-Atc^3,Ile^{5,6}Deltorphin I	$1.27 \pm 0.15 \times 10^6$	$4.66 \pm 0.76 \times 10^{-5}$	0.037
[^{3}H]R-Atc^3,Ile^{5,6}Deltorphin II	$1.06 \pm 0.16 \times 10^6$	$7.33 \pm 1.41 \times 10^{-5}$	0.069

The binding of the new deltorphin analogues occurred with lower association and dissociation rates than those of [^{3}H]deltorphin II (Búzás *et al.*, 1992) and [^{3}H]Ile^{5,6} deltorphin II (Nevin *et al.*, 1995). Slow association and dissociation kinetics were also observed for the conformationally constrained enkephalin analogues [^{3}H]DPDPE (Akiyama *et al.*, 1985) and [^{3}H]*p*Cl-DPDPE (Vaughn *et al.*, 1989).

It can be suggested that the conformational restriction of Phe residue from position 3 in the peptide sequence in [^{3}H]S-Atc^3,Ile^{5,6}deltorphin I and [^{3}H]R-Atc^3,Ile^{5,6}deltorphin II can play an important role in the binding process, by reducing the accessibility of the ligand molecule to the binding site.

Since radioligands binding requires long incubation time period (90 min) and high incubation temperature (35°C), several protease inhibitors (BSA, captorpil, bacitacin, bestatin and PMSF) were added to the incubation mixture to prevent the possible degradation of the receptor protein.

4.10. Saturation Studies of [^{3}H]S-Atc^3,Ile^{5,6}Deltorphin I and [^{3}H]R-Atc^3,Ile^{5,6}Deltorphin II Binding

The specific binding of [^{3}H]S-Act^3,Ile^{5,6}deltorphin I and [^{3}H]R-Atc^3,Ile^{5,6}deltorphin II to rat brain membranes was saturable at 35°C at the concentration range used (0.06-6 nM and 0.03-5.5 nM, respectively) (Fig. 13).

The linearity of the Scatchard plots suggested that [^{3}H]S-Atc^3,Ile^{5,6}deltorphin I and [^{3}H]R-Atc^3,Ile^{5,6}deltorphin II specifically labeled a homogeneous population of opioid binding sites with high affinity (Fig. 13), as it was indicated by the K_d values of 0.28 and 0.25 nM, respectively (Table XV).

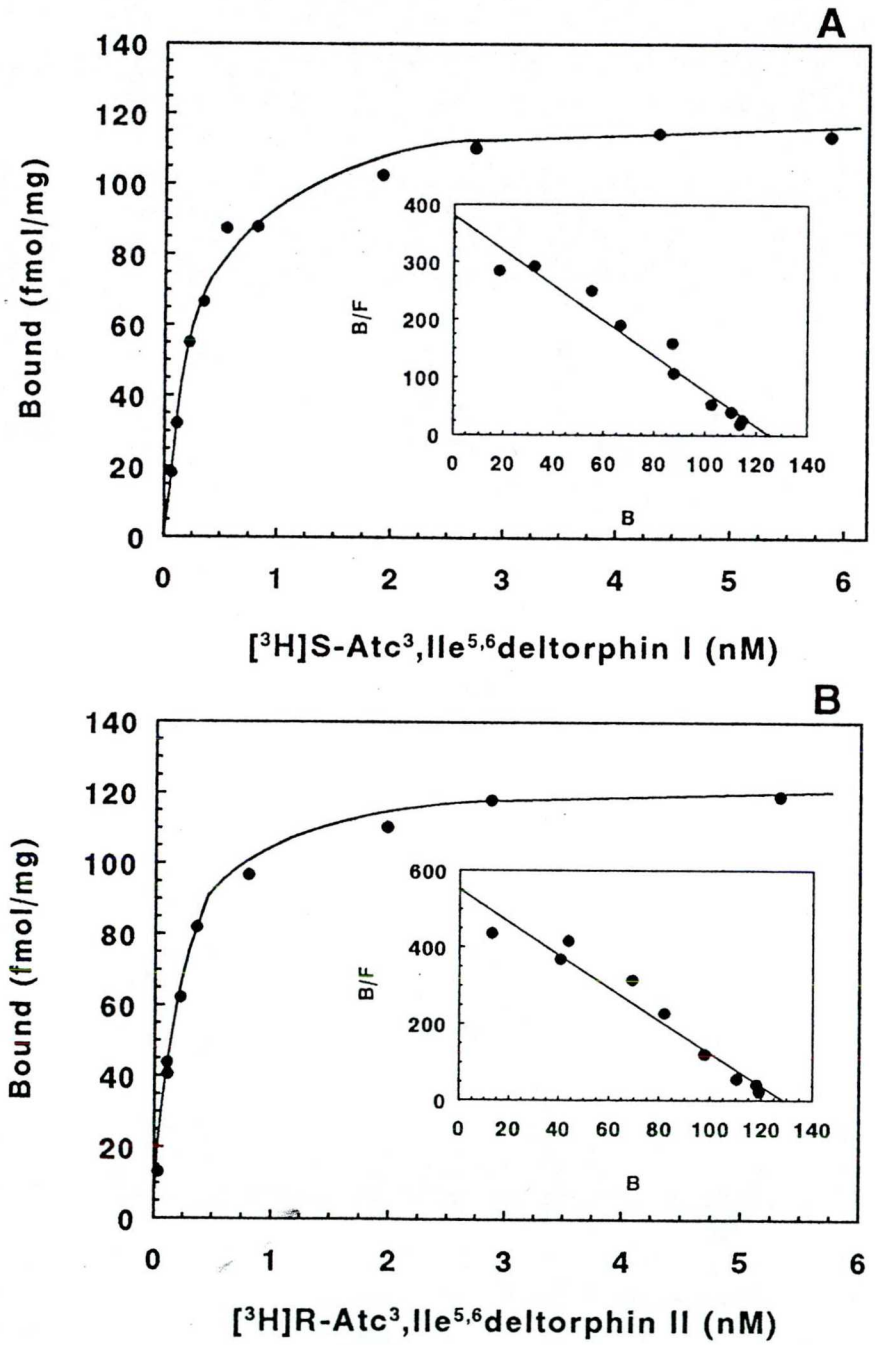


Fig. 13. *SATURATION OF [^{3}H]S-ATC^3,ILE^{5,6}DELTORPHIN I* ***(A)*** *AND [^{3}H]R-ATC^3,ILE^{5,6}DELTORPHIN II* ***(B)*** *BINDING.*
Rat brain membranes were incubated with increasing concentrations of radioligand in the absence or in the presence of 10 μM unlabeled naloxone, for 90 min at 35°C. insert: Scatchard plots.

TABLE XV. *Equilibrium Parameters of [^{3}H]S-Atc^3,Ile^{5,6}Deltorphin I and [^{3}H]R-Atc^3,Ile^{5,6}Deltorphin II Binding to Rat Brain Membranes at 35°C*

Ligand	K_d (nM)	B_{max} (fmol/mg)	n_H
[^{3}H]S-Atc^3,Ile^{5,6}Deltorphin I	0.28 ± 0.06	129.69 ± 5.37	0.94 ± 0.03
[^{3}H]R-Atc^3,Ile^{5,6}Deltorphin II	0.25 ± 0.03	131.01 ± 4.96	0.97 ± 0.08

The calculated Hill coefficients (n_H) for this site were close to the unity, also suggesting that the radioligands bind to one site in a non-cooperative binding process (Fig. 14; Table XV).

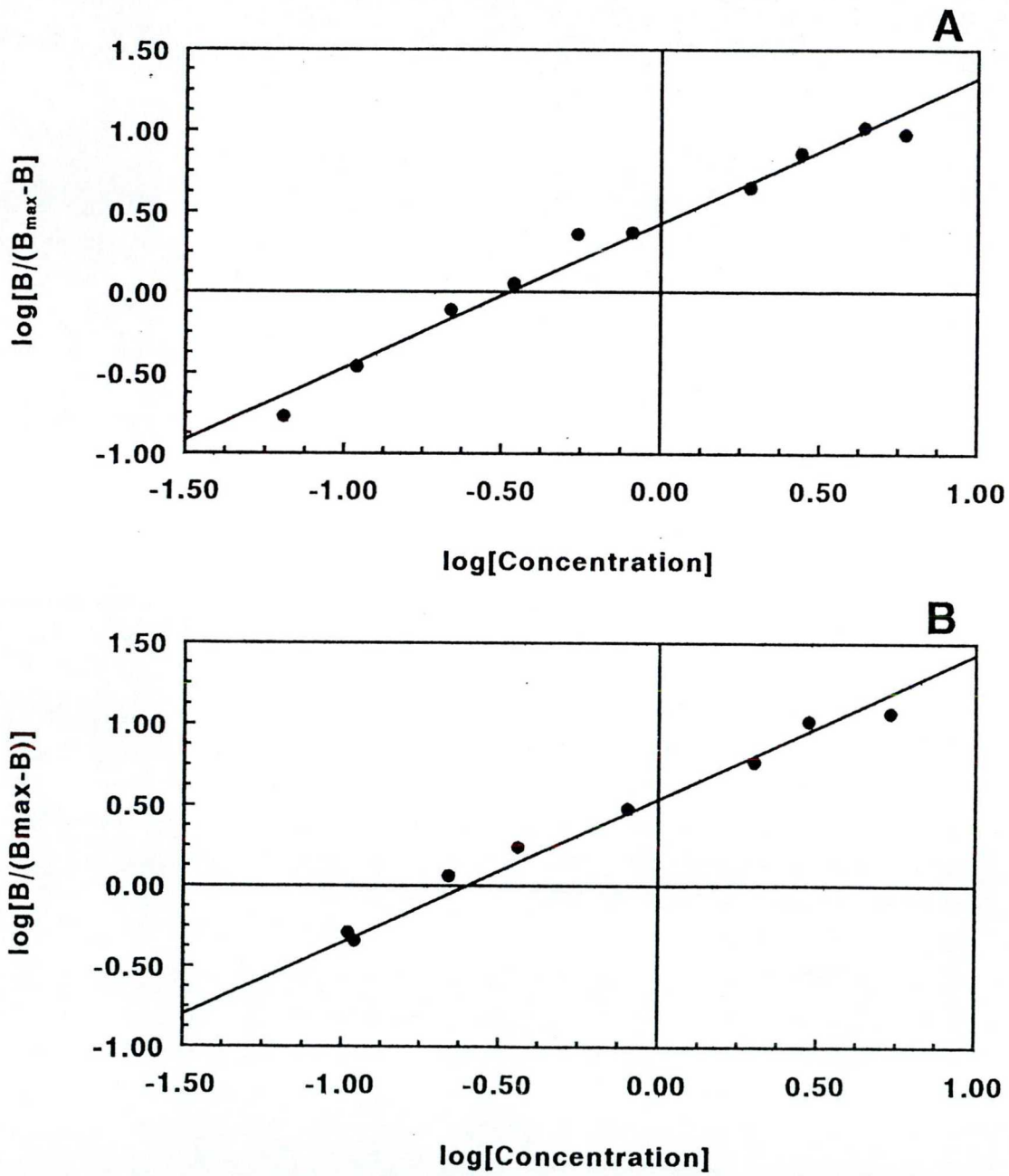


Fig. 14. *REPRESENTATIVE HILL PLOTS FOR [^{3}H]S-ATC^3,ILE^{5,6}DELTORPHIN I **(A)** AND [^{3}H]R-ATC^3,ILE^{5,6}DELTORPHIN II **(B)** BINDING.*

The non-specific binding ratios of each radioligand was <15% of total binding at a radioligand concentration equal to the K_d values.

The B_{max} values were calculated from Scatchard plots, as described in 'Materials and Methods' (see Chaps. 3.6.2), and the obtained values, of 130 fmol/mg protein, were found to be in agreement with those reported for δ-receptor density of other δ-opioid peptide radioligands, including [^{3}H]Ile^{5,6} deltorphin II (Nevin *et al.*, 1994), [^{3}H]DPDPE, [^{3}H]DSLET and [^{3}H]DLTET (Delay-Goyet *et al.*, 1985).

The K_d values obtained from Scatchard analysis of saturation binding data were higher that those calculated from the kinetic experiments (Table XIV). Such discrepancies between equilibrium and kinetics derived K_d values were also reported for [^{3}H]DADLE (Pryhuber *et al.*, 1982), [^{3}H]DTLET (Zajac *et al.*, 1983) and [^{3}H]DSBULET (Deley-Goyet *et al.*, 1988).

These results may be explained by the formation of a slow-dissociating, high affinity agonist conformation of the δ-opioid receptor.

4.11. Stereoselectivity of [^{3}H]S-Atc^3,Ile^{5,6}Deltorphin I and [^{3}H]R-Atc^3,Ile^{5,6}Deltorphin II Binding

The high affinity of the opioid agonist, levorphanol, and the low affinity (K_i >10,000 nM) of the pharmacologically inactive isomer, dextrorphan, indicated the stereoselectivity of opioid binding sites labeled by [^{3}H]S-Atc^3,Ile^{5,6}deltorphin I and [^{3}H]R-Atc^3,Ile^{5,6}deltorphin II in rat brain preparations, as it has been determined in binding displacement experiments (Fig. 15; Table XVI).

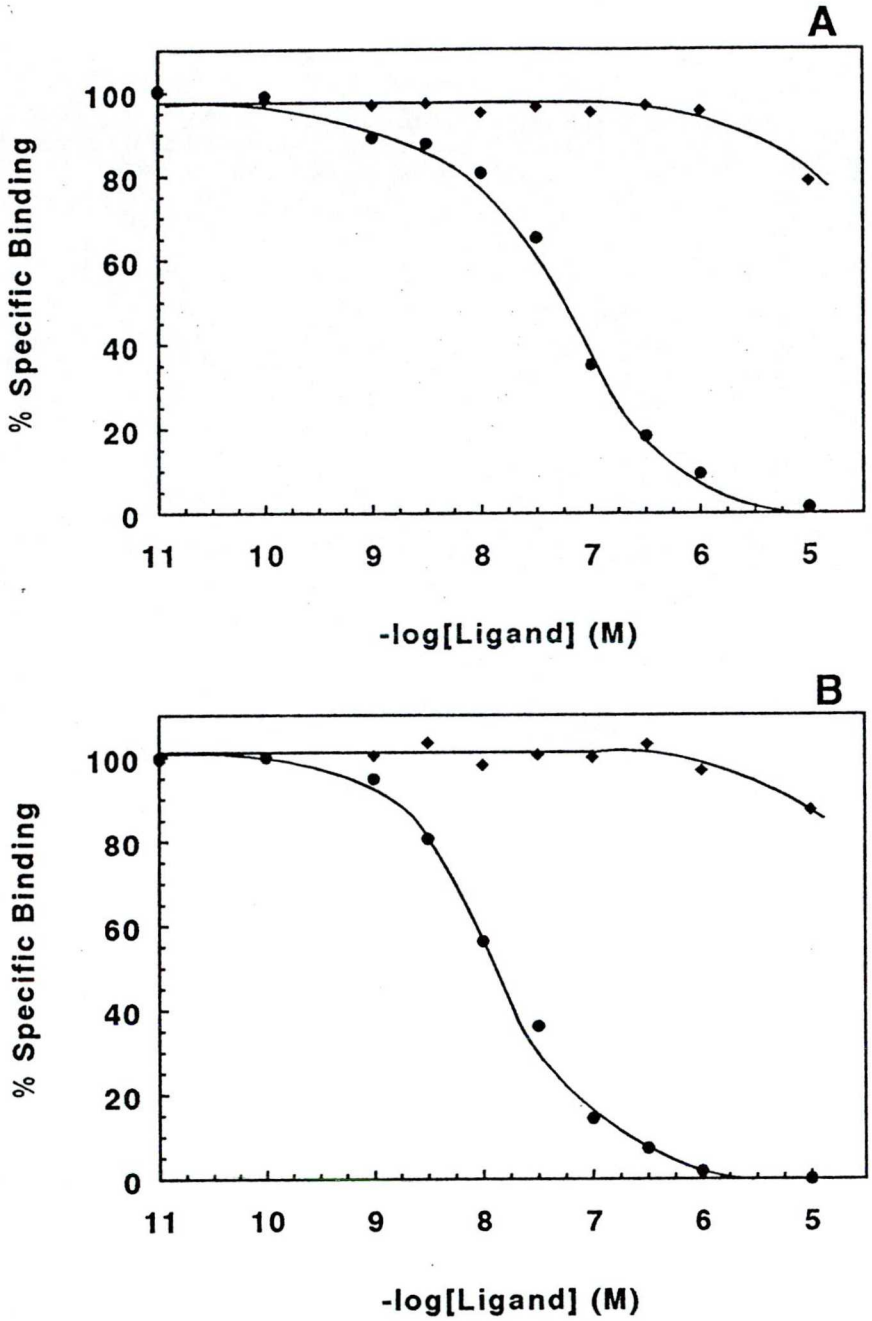


Fig. 15. *STEREOSELECTIVITY OF [^{3}H]S-ATC^3,ILE^{5,6}DELTORPHIN I* ***(A)*** *AND [^{3}H]R-ATC^3,ILE^{5,6}DELTORPHIN II* ***(B)*** *BINDING.*
Rat brain membranes were incubated with 0.5 nM radioligand in the presence of increasing concentrations of two enantiomers, levorphanol (●) and dextrorphan (◆), for 90 min at 35°C.

4.12. Competition Studies of [^{3}H]S-Atc^3,Ile^{5,6}Deltorphin I and [^{3}H]R-Atc^3,Ile^{5,6}Deltorphin II Binding

The binding of [^{3}H]S-Atc^3,Ile^{5,6}deltorphin I and [^{3}H]R-Atc^3,Ile^{5,6}deltorphin II to rat brain membranes can be displaced by a range of compounds pharmacologically active on opioid receptors (Fig. 16).

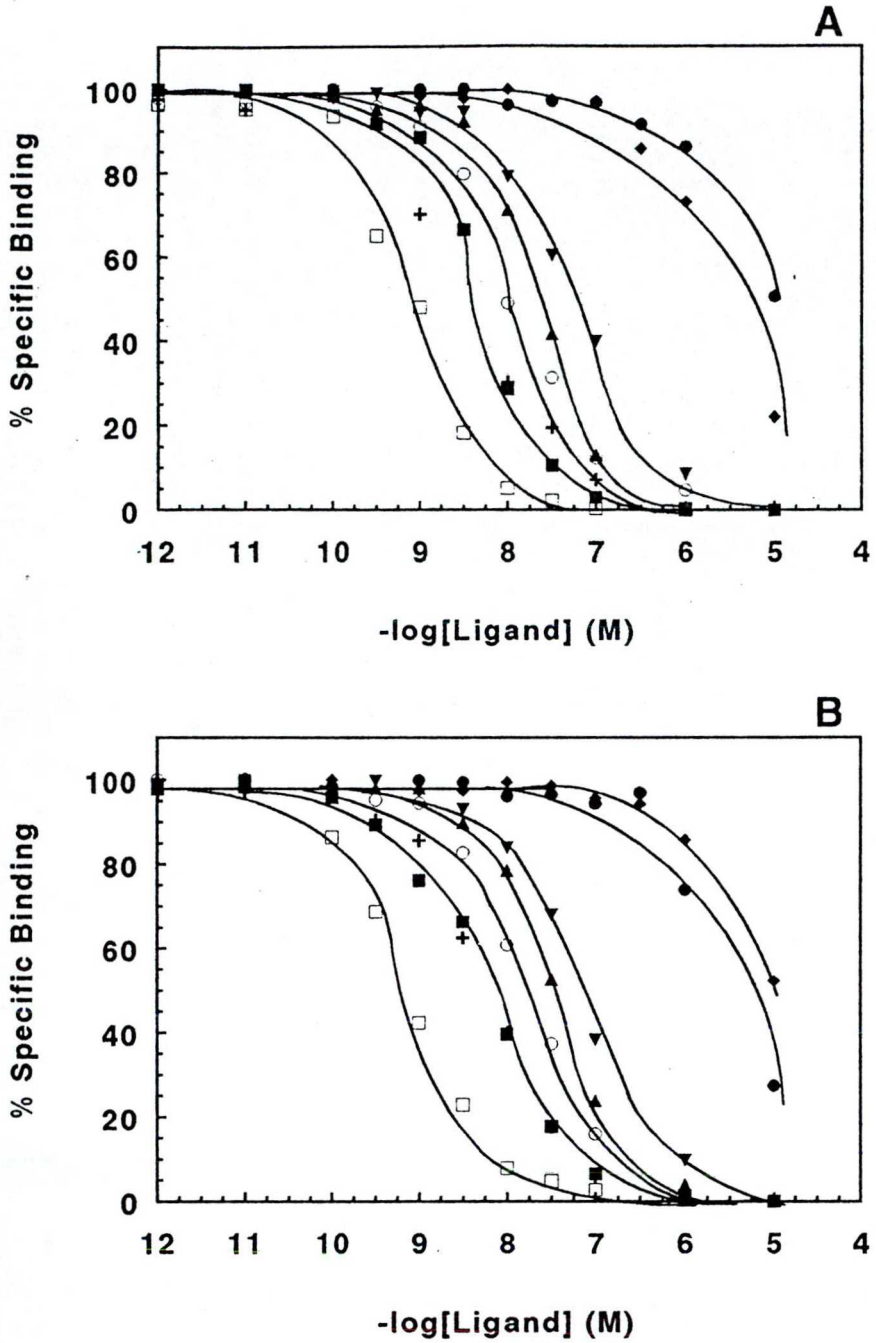


Fig. 16. *COMPETITION CURVES FOR [^{3}H]S-ATC^3,ILE^{5,6}DELTORPHIN I* ***(A)*** *AND [^{3}H]R-ATC^3,ILE^{5,6}DELTORPHIN II* ***(B)*** *BINDING SITES BY OPIOID LIGANDS.* Rat brain membranes were incubated with 0.5 nM radioligand in the presence of increasing concentrations of DAMGO (●), U69,593 (◆), Ile^{5,6}deltorphin II (■), naltrindole (❑), TIPP[Ψ] (▲), DSLET (○), deltorphin II (✚) and DPDPE (▼), for 90 min at 35°C.

Only the ligands selective for δ-receptors, such as deltorphin II, Ile^{5,6}deltorphin II, naltrindole TIPP[Ψ], DSLET and DPDPE, were found to be very potent inhibitors of the binding . They exhibit K_i values between 0.2-25 nM. Ligands with preferential affinities for μ- (DAMGO) or κ- (U69,593) opioid receptors were much less effective, as it is indicated by the higher K_i values (Table XVI).

TABLE XVI. *Inhibition of [^{3}H]S-Atc^3,Ile^{5,6}Deltorphin I and [^{3}H]R-Atc^3,Ile^{5,6}Deltorphin II Binding to Rat Brain Membranes by Type-Selective Opioid Ligands*

Ligand	K_i (nM)	
	[^{3}H]S-Atc^3,Ile^{5,6}deltorphin I	**[^{3}H]R-Atc^3,Ile^{5,6}deltorphin II**
δ-selective		
Deltorphin II	2.33 ± 0.16	1.85 ± 0.55
Ile^{5,6}deltorphin II	1.34 ± 0.07	2.02 ± 0.21
Naltrindole	0.24 ± 0.07	0.26 ± 0.02
TIPP[Ψ]	5.59 ± 0.81	7.79 ± 0.95
DSLET	3.31 ± 0.29	5.51 ± 0.83
DPDPE	25.1 ± 3.1	21.1 ± 1.1
μ-selective		
DAMGO	924 ± 91	1423 ± 160
κ-selective		
Nor-BNI	31.6 ± 2.9	42.9 ± 6.6
U69,593	743 ± 104	1624 ± 470
Levorphanol	19.2 ± 1.9	5.66 ± 0.52
Dextrorphan	>10,000	>10,000

Membranes were incubated with 0.5 nM [^{3}H]S-Atc^3,Ile^{5,6}deltorphin I or [^{3}H]R-Atc^3,Ile^{5,6}deltorphin II in the presence of increasing concentrations of competing opioid ligands, for 90 min at 35°C.

From the inhibition constants, the selectivity ratios, K_i^{μ}/K_i^{δ} and $K_i^{\kappa}/K_i^{\delta}$, were calculated and found to be 3900 and 3100, respectively, for [^{3}H]S-Atc^3,Ile^{5,6}deltorphin I, and 5500 and 6300, respectively, for [^{3}H]R-Atc^3,Ile^{5,6}deltorphin II.

Pharmacological studies suggested the existence of δ-receptor subtypes, based on the individual selectivities of several opioid ligands (see Chap. 1.2.1). DPDPE which is thought to be agonist at the δ_1-receptor subtype, and the selective agonists at the δ_2-subtype, DSLET and deltorphin II, showed one order of magnitude difference in inhibiting binding of the new radioligands (Table XVI). Therefore, these results indicate that [^{3}H]S-Atc^3,Ile^{5,6}deltorphin I and [^{3}H]R-Atc^3,Ile^{5,6}deltorphin II could recognize better the δ_1- than δ_2-receptor subtype. Previous results with other deltorphin analogue [^{3}H]Ile^{5,6}deltorphin II showed that in the case of this ligand there was no distinction between the abilities of DPDPE and DSLET to inhibit the binding (Nevin *et al.*, 1995). It can be suggested that the conformational restriction of the Phe residue from position 3 in deltorphins may play a role in determination of δ-subtype selectivity.

Competition binding studies demonstrated that both deltorphin analogues, [^{3}H]S-Atc^3,Ile^{5,6}deltorphin I and [^{3}H]R-Atc^3,Ile^{5,6}deltorphin II, potentially and selectively labeled δ-opioid receptors in rat brain homogenates.

4.13. Effect of Na^+ Ions and Gpp(NH)p on Specific [^{3}H]S-Atc^3,Ile^{5,6}Deltorphin I and [^{3}H]R-Atc^3,Ile^{5,6}Deltorphin II Binding

Binding of [^{3}H]S-Atc^3,Ile^{5,6}deltorphin I and [^{3}H]R-Atc^3,Ile^{5,6}deltorphin II to opioid receptors was regulated by Na^+ ions (Fig. 17) and Gpp(NH)p (Fig. 18).

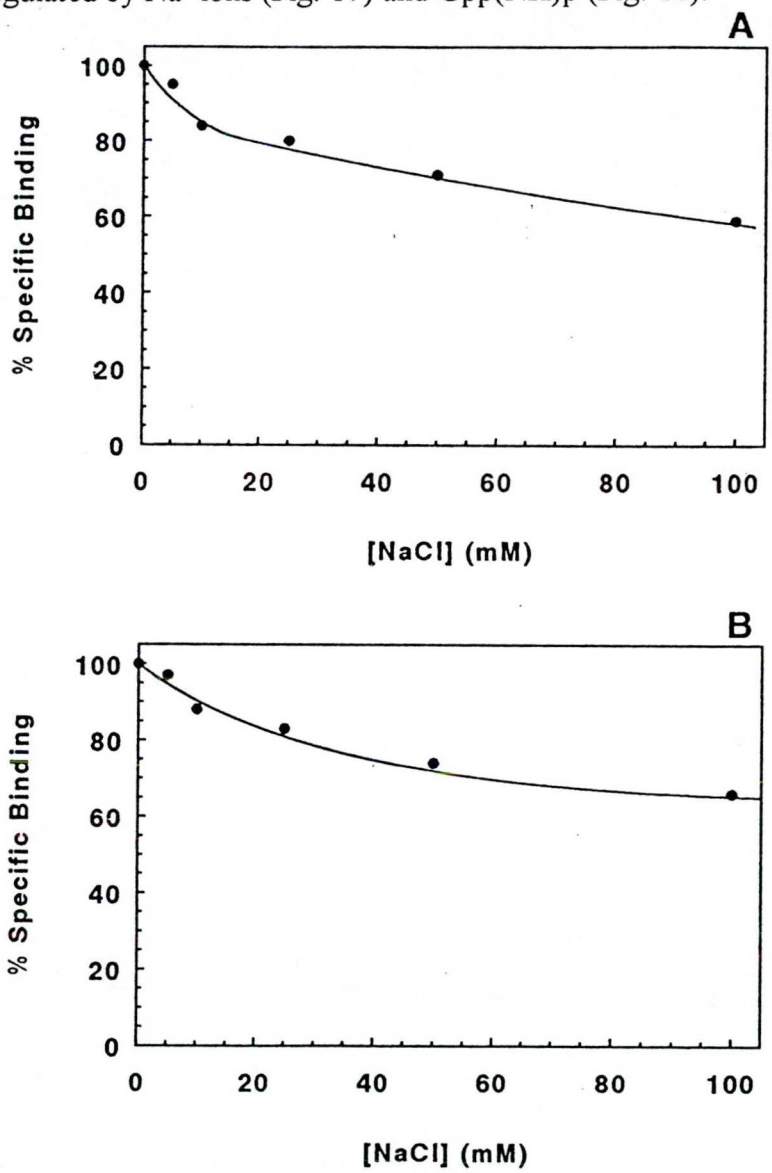


Fig. 17. *EFFECT OF NA^+ IONS ON S-ATC^3,ILE^{5,6}DELTORPHIN I (A) AND [^{3}H]R-ATC^3,ILE^{5,6}DELTORPHIN II (B) SPECIFIC BINDING.*
Rat brain membranes were incubated with 0.5 nM of the appropriate radioligand in the presence of increasing concentrations of NaCl, for 90 min at 35°C.

Increasing the concentration of NaCl from 5 mM to 100 mM produced a 40-50% reduction in the specific binding of [^{3}H]S-Atc^3,Ile^{5,6}deltorphin I and [^{3}H]R-Atc^3,Ile^{5,6}deltorphin II to rat brain membranes (Fig. 17).

The effect of Gpp(NH)p on specific binding of [^{3}H]S-Atc^3,Ile^{5,6}deltorphin I and [^{3}H]R-Atc^3,Ile^{5,6}deltorphin II is shown in Fig. 18.

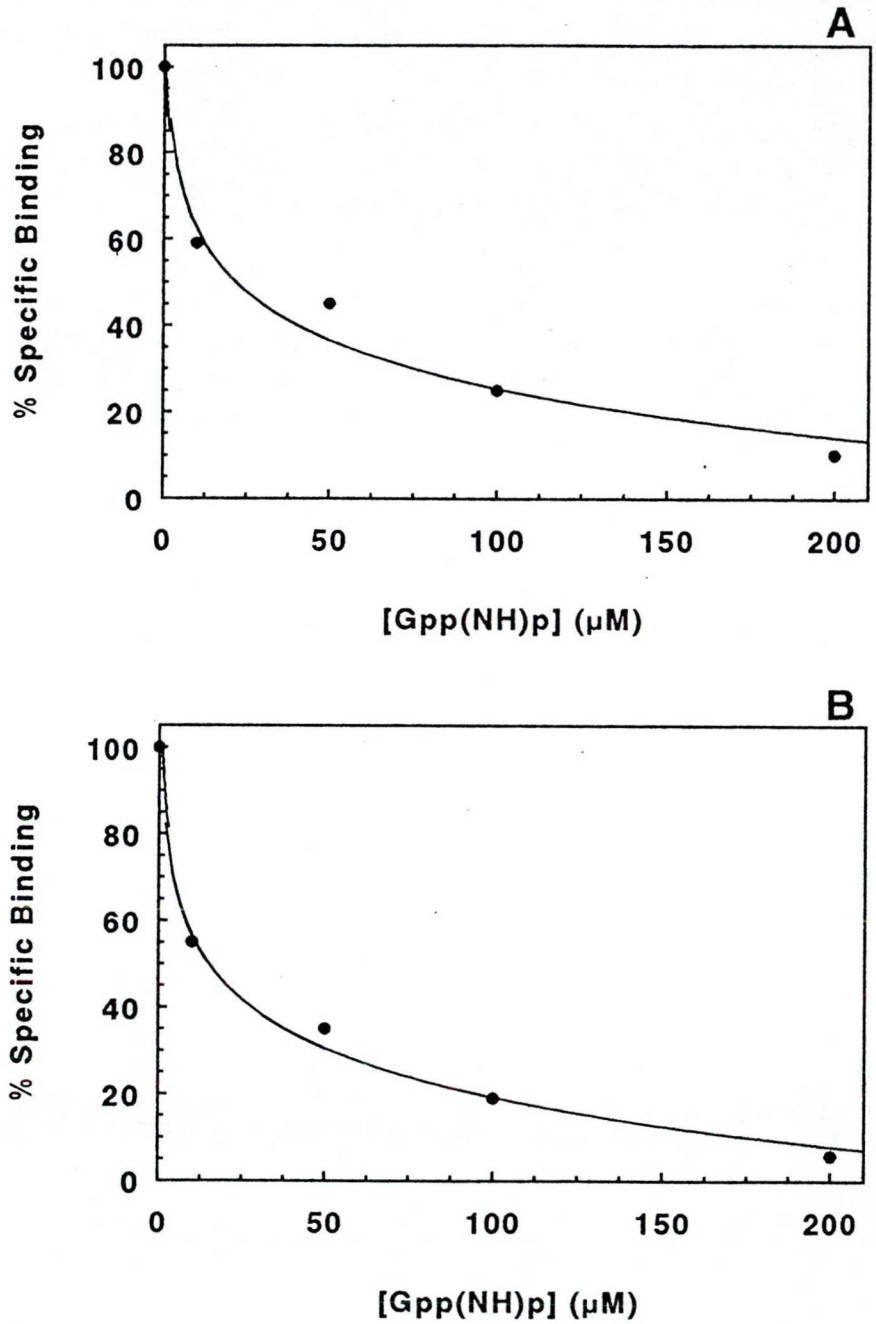


Fig. 18. *EFFECT OF Gpp(NH)p ON [^{3}H]S-ATC^3,ILE^{5,6}DELTORPHIN I* ***(A)*** *AND [^{3}H]R-ATC^3,ILE^{5,6}DELTORPHIN II* ***(B)*** *SPECIFIC BINDING*
Rat brain membranes were incubated with 0.5 nM of the appropriate radioligand in the presence of increasing concentrations of Gpp(NH)p, for 90 min at 35°C.

Gpp(NH)p (10-200 μM) produced a concentration-dependent inhibition of specific binding of radioligands up to a concentration of 200 μM at which a 20% inhibition was achieved.

The effect of Na^+ ions and guanine nucleotides in decreasing [^{3}H]S-Atc^3,Ile^{5,6}deltorphin I and [^{3}H]R-Atc^3,Ile^{5,6}deltorphin II specific binding to rat brain membranes was similar to that observed for the other opioid peptide radioligand [^{3}H]TAPP which was describe in this study (see Chap. 4.6). These results showed that all these compounds exhibit agonist properties.

The reduction of specific binding of deltorphin analogues in the presence of Gpp(NH)p, also indicated the brain δ-opioid receptors, labeled by these ligands, are G-protein coupled (Childers, 1991).

4.14. Comparison of [^{3}H]S-Atc^3,Ile^{5,6}Deltorphin I and [^{3}H]R-Atc^3,Ile^{5,6}Deltorphin II Binding Properties with Those of Other Peptide Radioligands Labeling δ-Opioid Receptor

Opioid receptor binding properties in rat brain membranes of the novel synthesized and characterized deltophin analogues [^{3}H]S-Atc^3,Ile^{5,6}deltorphin I and [^{3}H]R-Atc^3,Ile^{5,6}deltorphin II were compared with those of the best-known δ-selective peptide radioligands (Table XVII).

The new deltorphin I and II analogues characterized in this study showed better affinities and much higher selectivities than their parent compounds, deltorphin I and deltorphin II, respectively (Erspamer *et al.*, 1989; Salvadori *et al.*, 1991; Búzás *et al.*, 1992; Table XVII).

Increasing the lipophilicity at positions 5 and 6 in the "address" domain of deltorphins resulted in a analogue, Ile^{5,6}deltorphin II, with improved binding characteristics (Sasaki *et al.*, 1992; Nevin *et al.*, 1994). Furthermore, replacement of Phe at position 3 in the "message" domain with a conformationally restricted amino acid, Atc, led to more active and δ-selective agonist deltorphin analogues (Tóth *et al.*, 1997; **II**).

Compared to other δ-selective radioligand peptides, including the enkephalin analogues, [^{3}H]DPDPE, [^{3}H]DSLET and [^{3}H]DTLET (Delay-Goyet *et al.*, 1985), and the conformationally restricted tetrapeptides, [^{3}H]TIPP (Nevin *et al.*, 1993) and [^{3}H]TIPP[Ψ] (Nevin *et al.*, 1995), the investigated deltorphin analogues showed a marked δ-selectivity and the highest δ-receptor affinity in rat brain membranes (Table XVII).

TABLE XVII. *Binding Characteristics of δ-Receptor Selective Peptide Radioligands in Rat Brain Membranes*

Compound	Sequence	K_d (nM)	B_{max} (fmol/mg)	K_i^{μ} /K_i^{δ}[a]
Deltorphin I[1]	Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2	0.10	61	1200[b,d]
Deltorphin II[2]	Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2	1.90	92	900[b,c]
Ile[5,6]deltorphin II[3]	Tyr-D-Ala-Phe-Glu-Ile-Ile-Gly-NH_2	0.40	121	2500[b,f]
S-Atc[3],Ile[5,6]deltorphin I	**Tyr-S-Atc-Phe-Asp-Ile-Ile-Gly-NH_2**	**0.28**	**130**	**3900[b,f]**
R-Atc[3],Ile[5,6]deltorphin II	**Tyr-R-Atc-Phe-Glu-Ile-Ile-Gly-NH_2**	**0.25**	**131**	**5500[b,f]**
DPDPE[4]	Tyr-D-Pen-Gly-Phe-D-Pen-OH	10.5	118	35[b,g]
*p*Cl-DPDPE[5]	Tyr-D-Pen-Gly-*p*Cl-Phe-D-Pen-OH	0.33	87	120[b,g]
DSLET[4]	Tyr-D-Ser-Gly-Phe-Leu-Thr-OH	3.5	141	75[b,e]
DLTET[4]	Tyr-D-Thr-Gly-Phe-Leu-Thr-OH	1.19	103	270[c,d]
TIPP[6]	Tyr-Tic-Phe-Phe-OH	0.64	82	600[b,f]
TIPP[Ψ][7]	Tyr-TicΨ(CH_2NH)-Phe-Phe-OH	0.98	105	3300[b,f]

[a] K_i^{μ} ([b]DAMGO or [c]Dermorphin)/K_i^{δ}([d]Deltorphin I, [e]DSLET, [f]Naltrindole or [g]DPDPE) (for a review, see Schiller, 1991).

[1]Erspamer *et al.*, 1989; [2]Búzás *et al.*, 1992; [3]Nevin *et al.*, 1994; [4]Delay-Goyet *et al.*, 1985; [5]Vaughn *et al.*, 1989; [6]Nevin *et al.*, 1993; [7]Nevin *et al.*, 1995.

The obtained results in the binding studies with the new **radiolabeled Atc-deltorphin analogues** indicated that these heptapeptides specifically labeled with high affinity the δ-opioid receptor in rat brain membranes preparations and no changes in binding characteristics were observed with the different conformers (**II**). The binding of these radioligands was temperature- and time-dependent, saturable, stereoselective and with very low non-specific (<15%) binding ratios.

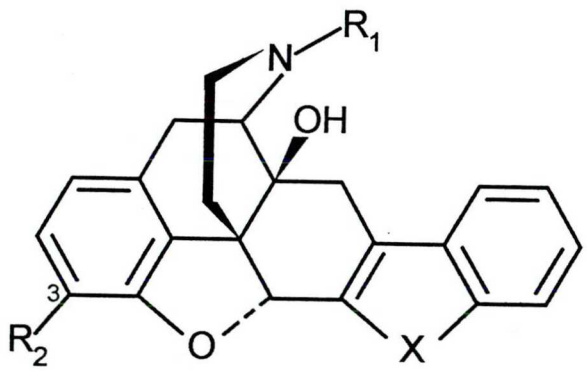
In addition, the binding of [^{3}H]S-Atc[3],Ile[5,6]deltorphin I and [^{3}H]R-Atc[3],Ile[5,6]deltorphin II, was found to be regulated by Na^+ ions and guanine nucleotides, in agreement with the agonist character of these opioid peptides, and also suggesting that in rat brain the δ-opioid receptor is functionally coupled to G-protein.

The new deltorphin analogues, [^{3}H]S-Atc[3],Ile[5,6]deltorphin I and [^{3}H]R-Atc[3],Ile[5,6]deltorphin II, were found to exhibit the highest affinities and selectivities for the δ-opioid receptors among the best-known δ-receptor selective radioligands.

III. BINDING CHARACTERISTICS OF BENZOFURAN DERIVATIVES OF NON-PEPTIDE OPIOIDS IN RAT BRAIN MEMBRANES

Highly selective heterocyclic opioid ligands with potent δ-antagonist activity have been developed on the basis of the *"message-address" concept.* Based on the naltrexone structure, the addition of receptor type specific "address" element, such as pyrole or furan, resulted in two δ-selective antagonist compounds, naltrindole and naltriben, respectively (see Chap. 1.7.2.2).

Using this strategy, new **heterocyclic compounds** were obtained by the addition of benzofuran moiety to the opioid antagonist, naloxone, and to the μ-selective agonists, oxymorphone and oxycodone. Their structures are shown in Fig. 19. Structure-activity relationship was examined for these compounds (**III**).



Compound	X	R_1	R_2
Naltrindole	NH	CH_2-$CH(CH_2)_2$	OH
Naltriben	O	CH_2-$CH(CH_2)_2$	OH
Naloxone-benzofuran	O	CH_2-$CH{=}CH_2$	OH
Oxymorphone-benzofuran	O	CH_3	OH
Oxycodone-benzofuran	O	CH_3	OCH_3

Fig. 19. *STRUCTURES OF THE STUDIED COMPOUNDS.*

4.15. Binding Affinities and Selectivities of Benzofuran Derivatives at μ-, δ- and κ-Opioid Receptors

The newly synthesized benzofuran derivatives have been characterized in terms of binding affinities and selectivities to opioid receptors, by displacement of highly receptor type-selective radioligands from rat brain membranes (Table XVIII).

Binding to the μ-opioid binding site was evaluated with the agonist peptide [^{3}H]DAMGO (Handa *et al.*, 1981) (Fig. 20). Binding to the δ-opioid receptor was assessed with several compounds which have been reported as highly δ-selective radioligands, including the agonist peptides [^{3}H]Ile^{5,6}deltorphin II (Nevin *et al.*, 1994) (Fig. 21A) and [^{3}H]*p*Cl-DPDPE (Vaughn *et al.*, 1989), the antagonist peptide [^{3}H]TIPP[Ψ] (Nevin *et al.*, 1995), and the antagonist alkaloid derivative [^{3}H]naltrindole (Yamamura *et al.*, 1992; Borsodi *et al.*, 1993) (Fig. 21B). The agonist radioligand [^{3}H]U69,593 was employed to evaluate the binding to the κ-binding site (Lahti *et al.*, 1985).

In addition to the novel synthesized ligands, for comparison five more compounds have been studied in receptor binding assays: naltrexone, naloxone, naloxone (R_2)OCH_3, naltrindole and its benzofuran analogue naltriben. The binding data expressed as K_i values are shown in Table XVIII.

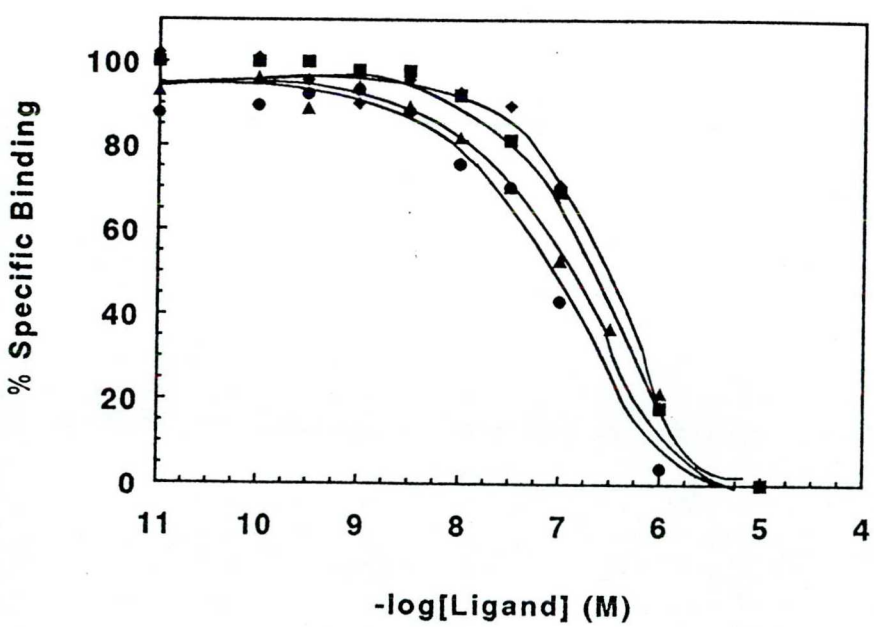


Fig. 20. *COMPETITION CURVES FOR [^{3}H]DAMGO BINDING SITE BY THE STUDIED COMPOUNDS.*
Rat brain membranes were incubated with 0.5 nM [^{3}H]DAMGO in the presence of increasing concentrations of naltriben (●), naloxone-benzofuran (◆), oxymorphone-benzofuran (■), and oxycodone-benzofuran (▲), for 45 min at 35°C.

TABLE XVIII. *Binding Affinities of the Studied Compounds at μ-, δ- and κ-Receptors*

Compound	K_i (nM)						$K_i\mu/K_i\delta$[a]
	[^{3}H]DAMGO	[^{3}H]Ile^{5,6}deltorphin II	[^{3}H]*p*Cl-DPDPE	[^{3}H]TIPP[Ψ]	[^{3}H]Naltrindole	[^{3}H]U69,593	
Naltrexone	0.41 ± 0.07	4.13 ± 0.12	4.96 ± 0.01	3.14 ± 0.17	24.15 ± 2.88	1.12 ± 0.75	0.10
Naloxone	2.46 ± 0.06	41.2 ± 9.4	22.3 ± 9.8	23.9 ± 2.9	90.9 ± 1.4	13.4 ± 4.9	0.06
Naloxone (R_2)OCH_3	42.1 ± 6.2	968 ± 184	743 ± 74.3	267 ± 32	211 ± 66	>10,000	0.04
Naltrindole	53.1 ± 5.4	0.16 ± 0.05	0.33 ± 0.11	0.24 ± 0.06	0.63 ± 0.06	13.4 ± 8.5	332
Naltriben	80.8 ± 2.3	0.54 ± 0.09	0.43 ± 0.08	0.64 ± 0.32	0.99 ± 0.30	>10,000	150
Naloxone-benzofuran	307 ± 49	1.21 ± 0.33	1.40 ± 0.35	0.47 ± 0.03	2.06 ± 0.70	>10,000	254
Oxymorphone-benzofuran	204 ± 22	3.09 ± 0.09	2.00 ± 0.48	0.60 ± 0.07	4.28 ± 1.85	>10,000	66
Oxycodone-benzofuran	140 ± 24	45.5 ± 17.2	33.5 ± 8.7	40.8 ± 11.5	49.1 ± 22.8	>10,000	3.1

Rat brain membranes were incubated either with the μ-selective radioligand [^{3}H]DAMGO, with the δ-selective radioligands, [^{3}H]naltrindole, [^{3}H]Ile^{5,6}deltorphin II, [^{3}H]TIPP [Ψ] or [^{3}H]*p*Cl-DPDPE, or with [^{3}H]U69,563 as κ-selective radioligand, in the presence of increasing concentration of ligands as described in 'Materials and Methods' (Chap. 3.5). [a] $K_i\mu$([^{3}H]DAMGO)/$K_i\delta$([^{3}H]Ile^{5,6}deltorphin II).

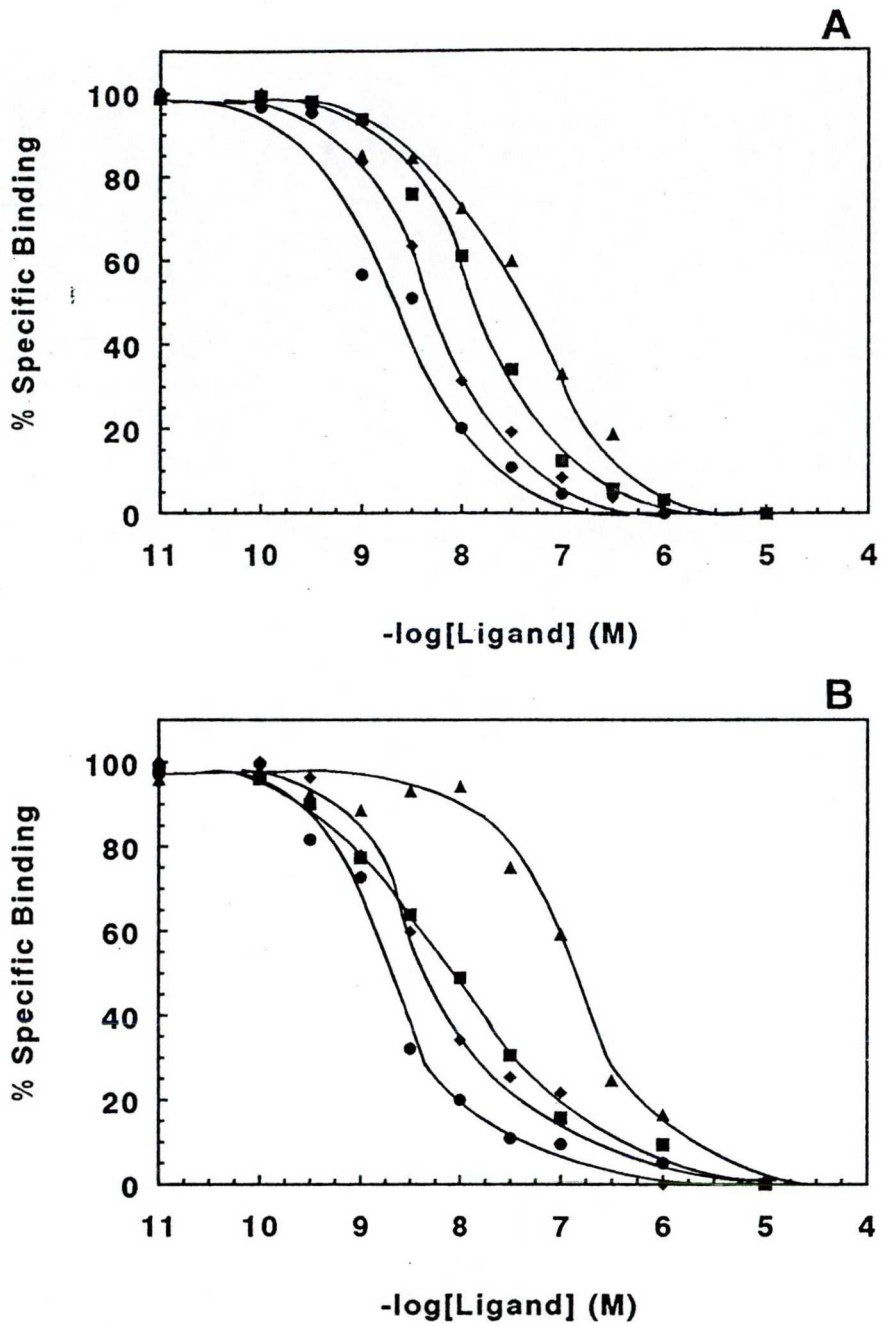


Fig. 21. *COMPETITION CURVES FOR [^{3}HILE^{5,6}DELTORPHIN II* ***(A)*** *AND [^{3}H]NALTRINDOLE* ***(B)*** *BINDING SITES BY THE STUDIED COMPOUNDS.*
Rat brain membranes were incubated with 0.5 nM [^{3}H]Ile^{5,6}deltorphin II or 0.5 nM [^{3}H]naltrindole in the presence of increasing concentrations of naltriben (●), naloxone-benzofuran (◆), oxymorphone-benzofuran (■), and oxycodone-benzofuran (▲).

All three newly synthesized benzofuran derivatives displayed the highest affinities for the δ-opioid receptor, much less potency toward the μ-binding site and were the least effective at the κ-site. Data were best fitted to a one-site model for each receptor type.

Naltrexone and naloxone exhibited high affinity for all three types of opioid with slight preferences for the μ-site. Naltrindole and naltriben were, as expected, highly potent and

selective ligands for the δ-receptor, as was the benzofuran derivative corresponding to naloxone (Table XVIII).

The μ-selectivities of oxymorphone and oxycodone has been previously established (Krizsán *et al.*, 1991; Fürst *et al.*, 1994). Our studies revealed that benzofuran derivative of oxymorphone showed high affinity at δ-receptor, whereas the oxycodone-benzofuran was moderately potent at this site (**III**) (Table XVII).

The (R_2)O-methyl derivative of naloxone was very weak δ-selective and moderately potent at μ-receptor. Methylation of the hydroxyl group in 3-position in oxycodone-benzofuran and naloxone $(R_2)OCH_3$ significantly reduces the affinity of the ligands for both μ- and δ-sites. This confirms previous findings that the free hydroxyl group from position 3 is an essential requirement for high affinity binding to opioid receptors (Oguri *et al.*, 1987; Cheng *et al.*, 1991). This phenomenon has also been described for some of the morphine metabolites having the hydroxyl group in position 3 methylated or glucuronated (Mignat *et al.*, 1995). The importance of this group was supported by the observation that morphine-3-glucuronide, a major metabolite of morphine, has poor affinity to opioid receptors and lacks analgesic activity (Pasternak *et al.*, 1987; Oguri *et al.*, 1987).

From the calculated selectivity ratios, K_i^{μ}/K_i^{δ}, has been observed that naloxone-benzofuran showed higher values than naltriben, 254 and 150, respectively (Table XVIII).

Table XIX. *Relative Affinities of the Studied Compounds*

Compound	**% Relative Affinity**[a]		
	μ	δ	κ
Naltrexone	68.3	6.8	24.9
Naloxone	80.4	4.8	14.8
Naloxone $(R_2)OCH_3$	95.5	4.1	0.4
Naltrindole	0.3	98.5	1.2
Naltriben	0.6	99.3	0.1
Naloxone-benzofuran	0.4	99.5	0.1
Oxymorphone-benzofuran	1.4	98.5	0.3
Oxycodone-benzofuran	24.4	75.3	0.3

[a]Relative affinities were calculated according to Kosterlitz and Paterson (1980), using the following equation: Relative affinity = $K_{a,x}/(K_{a,\mu} + K_{a,\delta} + K_{a,\kappa})$, where $K_a = 1/K_i$; μ-radioligand [^{3}H]DAMGO, δ-radioligand [^{3}H]Ile^{5,6}deltorphin II, and κ-radioligand [^{3}H]U69,593.

The δ-selectivity of the benzofuran derivatives corresponding to naloxone, oxymorphone and oxycodone, was also demonstrated by the relative affinity constants introduced by Kosterlitz and Paterson (1980). The obtained values (98-99%) indicated that they were bound almost exclusively to the δ-site (Table XIX). Naltrexone, naloxone and naloxone $(R_2)OCH_3$ showed preference for the μ-receptor. The lowest value for the δ-site was obtained for oxycodone-benzofuran, while the highest value were obtained for naloxone-benzofuran, which suggested that the last compound has a high δ-receptor selectivity and exhibit considerable preference for the δ-receptor over other opioid receptors. The κ-receptor selectivity of each of the three compounds was negligible (Table XIX).

The addition of the benzofuran moiety transformed the non-selective ligand, naloxone, and the μ-receptor selective compounds, oxymorphone and oxycodone, into δ-selective analogues.

4.16. Agonist/Antagonist Character of Benzofuran Derivatives

Another goal of this study was to see, if the modification in the structure of the parent compounds change their pharmacological profile. Sodium index (Na^+ index) has been used to classify the agonist/antagonist properties.

Affinities of benzofuran derivatives to [^{3}H]naloxone binding site were measured in the absence or in the presence of 100 mM NaCl (Fig. 22). It has been observed that all the studied ligands exhibited low affinities for [^{3}H]naloxone binding sites. Results are shown in Table XX.

TABLE XX. *Affinities of the Tested Compounds to [^{3}H]Naloxone Binding Sites in the Absence or in the Presence of NaCl*

Compound	K_i (nM)		Na^+ Index[a]	Character
	$-Na^+$	$+Na^+$		
Naltriben	44	44	1	antagonist
Naloxone-benzofuran	97	61	0.6	antagonist
Oxymorphone-benzofuran	520	1397	2.7	mixed
Oxycodone-benzofuran	1164	4700	4	mixed

[a] Na^+ index is calculated as a ratio $K_i(+Na^+)/K_i(-Na^+)$

When NaCl was added in the incubation medium, the binding of naltriben and naloxone-benzofuran to the [^{3}H]naloxone binding sites were not affected, whereas the affinities of

oxymorphone-benzofuran and oxycodone-benzofuran were slightly decreased (Fig. 22). It is well established that binding to opioid receptors of agonists, but not antagonists, is decreased in the presence of Na^+ ions (see Chap. 1.6; Pert and Snyder, 1974).

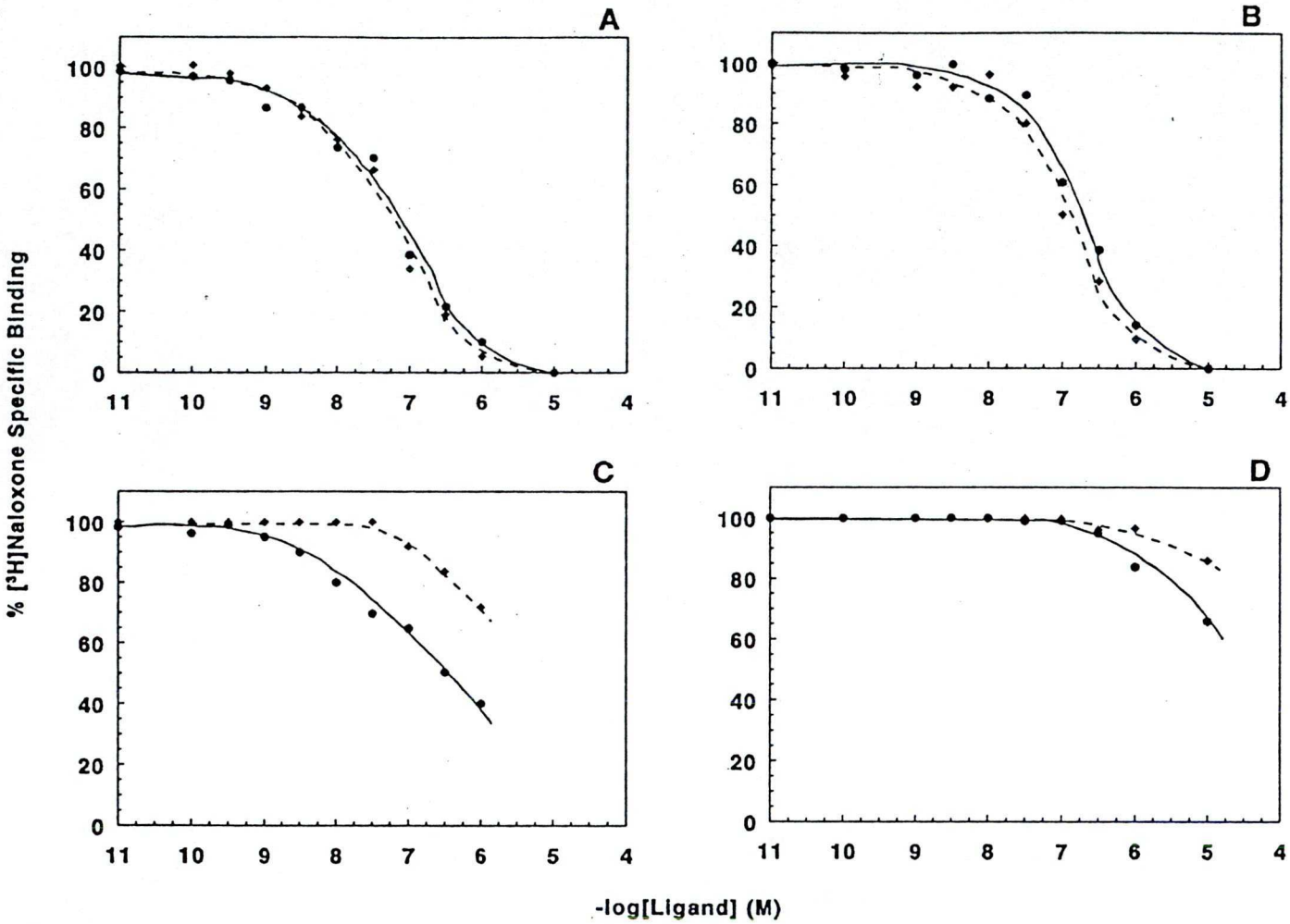


Fig. 19. *COMPETITION CURVES FOR [^{3}H]NALOXONE BINDING SITE BY THE STUDIED COMPOUNDS IN THE ABSENCE (●) OR IN THE PRESENCE (◆) OF 100 mM NaCl.*
Rat brain membranes were incubated with 1 nM [^{3}H]naloxone in the presence of increasing concentrations of ligands, for 60 min at 0°C. **A**: naltriben; **B**: naloxone-benzofuran; **C**: oxymorphone-benzofuran; **D**: oxycodone-benzofuran.

Na^+ indices were calculated, and the low values (≤1) obtained for naltriben and naloxone-benzofuran indicated the antagonist properties of these compounds. Oxymorphone-benzofuran and oxycodone-benzofuran showed higher (>1) values for Na^+ indices, but still in a lower range, suggesting their partial agonist character (Table XX).

Pharmacological properties of the investigated compounds correlated very well with their structure. Like morphine, the μ-selective agonists oxymorphone and oxycodone, posses a methyl group at the 17-N position, whereas the antagonists, naltrexone and naloxone, have more bulky substituents, such as allyl or cyclopropylmethyl, at this position (see Fig. 19).

Addition of the benzofuran moiety does not change the antagonist character of naloxone, but induces a slight change in the character of the μ-selective opioid agonists oxymorphone and oxycodone.

4.17. Determination of Wash-Resistant Binding of Benzofuran Derivatives

The ability of benzofuran derivatives to block irreversibly any population of opioid binding sites has been examined (Fig. 23). Rat brain membranes were preincubated with different concentrations (1, 10, 100 nM) of the studied compounds as previously described (Krizsán *et al.*, 1991). After extensive washing steps performed as described in 'Materials and Methods' (see Chap. 3.5), the remaining [^{3}H]naloxone specific binding was measured.

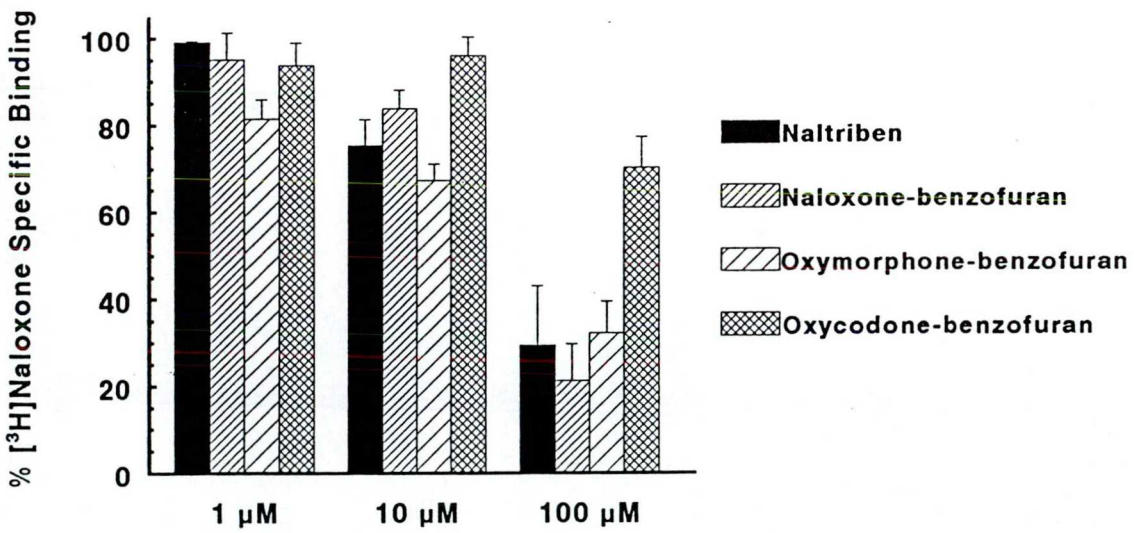


Fig. 23. *IRREVERSIBLE INHIBITION OF [^{3}H]NALOXONE SPECIFIC BINDING BY BENZOFURAN DERIVATIVES.*
Rat brain membranes were incubated with three concentration of the indicated compounds for 30 min at room temperature, and extensively washed. Control values represent the specific binding of [^{3}H]naloxone to membranes preincubated with buffer and treated in the same way, as described in the 'Materials and Methods'. Results are shown as the remaining [^{3}H]naloxone specific binding in percentage of control.

All three compounds were capable of inhibiting irreversible the specific binding of [^{3}H]naloxone in a dose dependent manner. The most intensive inhibition (~ 70%) was detected with naltriben and the benzofuran derivatives of naloxone and oxymorphone at a concentration of 100 μM, whereas oxycodone-benzofuran showed only 30% inhibition of [^{3}H]naloxone binding at the same concentration.

The fact of dose-dependent prolonged blockade observed for the tested benzofuran derivatives compounds suggests a specific interaction between the ligand and the receptor binding site.

The obtained results are consistent with *the "message-address" concept* used by Portoghese (1988) to design highly selective and potent non-peptide δ-receptors antagonists. According to this model, the benzene moiety in the newly developed benzofuran derivatives is viewed as the δ "address" component that is responsible for the enhanced effects at δ-receptors and the low binding and potency at the other opioid sites (Portoghese *et al.*, 1990). The two parts, "message" and "address", are connected through a rigid spacer which can be indole as in the case of naltrindole, or furan as in the case of naltriben and in the novel compounds characterized in this study. Conformational rigidity is an important requirement because a rigid "address" moiety might confer greater selectivity for the target receptor by precluding possible conformational adaptation in the binding to other opioid receptor types (Portoghese *et al.*, 1990; 1991).

Examination of the chemical structure, binding affinities, selectivities and pharmacological properties of the benzofuran derivatives corresponding to naloxone, oxycodone and oxymorphone make possible certain predictions about structure-activity relationships for the investigated compounds.

The addition of benzofuran moiety to the non-peptide opioids naloxone, oxymorphone and oxycodone, change their properties from non-selective or μ-selective compounds into highly δ-receptor selective ligands.

The chemical group at position 3 in morphinan structure is a very important determinant of binding to opioid receptors. The hydroxyl group at this position confers the highest potency of naltrexone, naloxone, naltrindole, naloxone-benzofuran and oxymorphone-benzofuran to opioid receptors. Oxycodone-benzofuran and naloxone (R_2)OCH_3, with a methoxy group, showed decreased binding.

The N-methyl benzofuran derivatives of oxymorphone and oxycodone have partial agonistcharacter, whereas the N-allyl (naloxone) benzofuran derivative showed no change in antagonist property of the parent compound.

The newly designed **benzofuran derivatives of naloxone, oxymorphone and oxycodone** characterized in this study showed high δ-selectivity. These compounds were able to block partially the opioid binding sites. All conclusions in the receptor type preference drawn from the rat opioid receptor binding assays (**III**) were fully confirmed by pharmacological studies in isolated organ, GPI and MVD, preparations (Rónai *et al.*, 1997).

5. Conclusions

The most important findings in this study can be summarized as follows:

The binding properties of a radiolabeled dermorphin tetrapeptide analogue ***[^{3}H]Tyr-D-Ala-Phe-Phe-NH$_2$ ([^{3}H]TAPP)*** *were determined and compared in membrane preparations from rat brain and from CHO-μ/1 cells stably transfected with the cloned rat μ-opioid receptor.*

- *In rat brain, [^{3}H]TAPP specifically labeled a single class of opioid sites with K_d value of 0.31 nM and B_{max} value of 119 fmol/mg protein. In transfected CHO-μ/1 cell membranes, the K_d and B_{max} values were 0.78 nM and 1807 fmol/mg protein.*
- *Binding of [^{3}H]TAPP to rat brain was pharmacologically identical to that observed in CHO-μ/1 cell membranes.*
- *Specific binding of [^{3}H]TAPP was stereoselective and significantly inhibited by Na^+ ions and guanine nucleotides indicating the agonist character of the ligand, and the functional coupling of μ-opioid receptor to G-protein in both preparations.*
- *[^{3}H]TAPP showed the highest affinity and excellent selectivity for the μ-opioid receptor among the best-known μ-selective agonist radioligand peptides.*

Opioid binding characteristics of two conformationally restricted deltorphin analogues, ***[^{3}H]S-Atc^3,Ile^{5,6}deltorphin I*** *and* ***[^{3}H]R-Atc^3,Ile^{5,6}deltorphin II,*** *were determined in rat brain membrane preparations.*

- *The two new radioligand peptides specifically labeled a single class of binding sites with high affinity ($K_d \approx 0.3$ nM, B_{max} values of 130 fmol/mg protein) and a very low (<15) non-specific binding.*
- *The binding of radiolabeled Atc-deltorphin analogues was saturable, stereospecific and no change in binding characteristics were observed with the different conformers.*

- *Both Na^+ ions and guanine nucleotides decreased the specific binding of these radioligands, indicating the agonist nature character of these peptides, and coupling of the δ-opioid receptor to G-protein in rat brain.*
- *[^{3}H]S-Atc^3,Ile^{5,6}deltorphin I and [^{3}H]R-Atc^3,Ile^{5,6}deltorphin II, are two of the most potent and δ-selective radioligands available. Moreover, they are able to distinguish between the δ_1- and δ_2-receptor subtypes.*

Structure-activity relationship was investigated for several ***new heterocyclic compounds*** *obtained by the addition of* ***benzofuran moiety*** *to the opioid antagonist naloxone, and to the μ-selective agonists oxycodone and oxymorphone.*

- *The presence of benzofuran moiety was found to confer δ-receptor selectivity of the ligand*
- *The phenolic OH group in position 3 of the morphinan skeleton is essential for a high affinity binding to opioid receptor.*
- *The addition of benzofuran moiety to the structure of naloxone conserves the antagonist character, but in the case of oxymorphone and oxycodone, the change into their structure induces a mixed agonist-antagonist character.*
- *These compounds were capable to block partially the opioid binding sites.*

All the novel developed and characterized opioid analogues, both peptides and non-peptides, were found to be potent and highly selective either for the μ- or δ-opioid receptors. They can be represent potentially useful tools to study the role of the μ- or δ-opioid receptors in a variety of biochemical, pharmacological and physiological processes, to understand the molecular events underlying pain control and drug addiction.

Characterization of New Opioid Analogues

Ligand	Structure	Affinity	Specificity	Character
[^{3}H]TAPP	peptide	high	μ	agonist
[^{3}H]S-Atc^3,Ile^{5,6}deltorphin I	peptide	high	δ	agonist
[^{3}H]R-Atc^3,Ile^{5,6}deltorphin II	peptide	high	δ	agonist
Naloxone-benzofuran	heterocyclic	high	δ	antagonist
Oxymorphone-benzofuran	heterocyclic	high	δ	mixed
Oxycodone-benzofuran	heterocyclic	moderate	δ	mixed

ÖSSZEFOGLALÁS

Az **opioid vegyületek** családjába az endogén peptideken kívül szintetikus analógjaik, az ópiumból nyert alkaloidok, és félszintetikus alkaloidok tartoznak. Ezek specifikus sejtfelszíni receptorokon keresztül közvetítik hatásaikat, és számos élettani folyamatot modulálnak, így a fájdalomérzékelést, a légzésszabályozást, a gyomor-bélrendszer, a szív- és érrendszer, a kiválasztórendszer és az immunrendszer mûködését, a testhômérséklet-szabályozást, és egyes hormonok szekrécióját. Mai ismereteink szerint az opioid származékokhoz való hozzászokás és a velük kapcsolatban kialakuló függôség létrejöttében is valószínûleg szerepet játszanak az opioid receptorok.

A farmakológiai és biokémiai kísérletek alapján legalább három **opioid receptor típus** különíthetô el (μ, δ, κ), amelyek ligandszelektivitásukban és neuroanatómiai eloszlásukban is különböznek. Az **endogén opioid peptidek** közül a δ-receptor az enkafalinokat és a deltorfinokat, a μ-receptor a dermorfinokat és az újonnan felfedezett endomorfinokat, a κ-receptor a dinorfinokat köti nagy affinitással. A morfin és az alkaloid származékok elsôsorban a μ-receptorhoz kötôdnek.

Az opioid receptorok közelmúltban történt klónozása felderítette, hogy mindhárom receptor típus a hét transzmembrán régióval rendelkezô, G-fehérjéhez kapcsolt receptorok családjába tartozik. Közösek abban is, hogy a sejtmembránban gátolják az adenilil cikláz enzimaktivitását, serkentik a K^+-áramot, és pertussis toxin érzékeny G-fehérjéken keresztül Ca^{2+}-csatorna záró hatásuk van.

Bár az opioid receptorok farmakológiai és funkcionális tulajdonságait régóta intenzíven kutatják, a szerkezet és a kiváltott hatások összefüggése még ma sem ismert minden részletében. Az opioid receptorok funkciójának kutatásának igen fontos eszközei az e receptorok iránt nagy affinitással és szelektivitással rendelkezô **új peptid és alkaloid opioid analógok.**

Doktori munkámban néhány újonnan szintetizált, tríciummal jelzett peptid és alkaloid ligand kötési tulajdonságainak jellemzését végeztem el. A peptidek a következôk voltak: a dermorfin tetrapeptid analóg [^{3}H]Tyr-D-Ala-Phe-Phe-NH_2 ([^{3}H]TAPP), valamint a deltorfin analóg [^{3}H]S-Atc^3,Ile^{5,6}deltorfin I és [^{3}H]R-Atc^3,Ile^{5,6}deltorfin II. Az alkaloidok közül a naloxon benzofurán származékait, az oxikodont és az oximorfont vizsgáltam.

Meghatároztam a receptorkötési paramétereket (affinitás és szelektivitás), és azokat más, jól ismert vegyületéhez hasonlítottam.

A receptor-ligand kölcsönhatást radioligand kötési kísérletekben jellemeztük. A [^{3}H]TAPP esetében a patkányagy membránpreparátum mellett klónozott μ-opioid receptorral transzfektált tengerimalac ovárium (CHO) sejtvonalon is dolgoztam a biokémiai karakterizálás során.

(I). A **dermorfin tetrapeptid analóg [^{3}H]TAPP** kötôdésének vizsgálata a μ-receptorhoz, és összehasonlítása patkányagy membránpreparátumban és CHO-μ/1 sejteken.

Patkányagy membránpreparátumban a [^{3}H]TAPP szelektíven jelölte az opioid kötôhelyek egy csoportját, a disszociációs konstans (K_d) 0.31 nM, a kötôhelyek maximális száma (B_{max}) 119 fmol/mg protein volt. A CHO-μ/1 sejt membránban a K_d és a B_{max} értéke 0.78 nM, illetve 1807 fmol/mg volt. A radioligand nemspecifikus kötése patkányagyban a teljes kötés 30%-a alatt maradt, míg a transzfektált CHO-μ/1 sejtvonalon igen kedvezô, mindössze 10% körüli értéket mutatott.

Különbözô típusszelektív ligandokkal végzett kompetíciós kíserleteink azt mutatták, hogy a [^{3}H]TAPP kötés farmakológiailag hasonló volt patkányagyban és a transzfektált CHO-μ/1 sejtvonalon. A kötés sztereospecifikus, és μ-ligandokkal hatékonyan gátolható volt. A δ- és κ-receptorra szelektív ligandok sokkal kevésbé gátolták a [^{3}H]TAPP kötést mindkét rendszer esetében.

A specifikus [^{3}H]TAPP kötést Na^+-ionok és guanin nukleotidok szignifikánsan gátolták, ami a ligand agonista karakterét mutatja. Sôt, a nemhidrolizáló GTP analóg Gpp(NH)p jelenléte és a specifikus [^{3}H]TAPP kötés csökkenése közötti összefüggés a μ-receptor és G-fehérje szignál transzdukciós rendszer kapcsolatára utal.

A legjobban ismert μ-szelektív peptid agonista radioligandokkal, így a [^{3}H]dermorfinnal, az enkefalin analóg [^{3}H]DAMGO-val és a β-kazomorfin analóg [^{3}H]PL017-tel való összehasonlításban a [^{3}H]TAPP kiváló affinitást és szelektivitást mutatott a μ-receptorok felé.

Kísérleteinkben a specifikus, telíthetô [^{3}H]TAPP kötés farmakológiailag azonosnak bizonyult patkány agyban és a patkány μ-opioid receptorokat expresszáló CHO sejtekben. A radioligand megfelelt a sztereoszelektivitás, telíthetôség, reverzibilitás és alacsony nemspecifikus kötés követelményeinek.

(II). A lineáris peptidek, mint pl. a deltorfinok, szerkezetileg flexibilis molekulák, konformációsan gátolt analógjaik azonban nagyobb affnitást és szelektivitást mutattak a δ-opioid receptor felé. A "message" domén Phe^3 aminosavának a konformációsan gátolt 2-

aminotetralin-2-karbonsavval (Atc) történô szubsztitúciójával módosított hidrofób és sztereoelektronikus tulajdonságokat mutató új deltorfin analógokat kaptunk. Az "address" domén 5-ös és 6-os helyzetû Val oldalláncait a lipofilebb Ile aminosavval szubsztituáltuk, majd az így kapott származékokat radioaktív izotóppal jelzett formában is elôállítottuk, és patkány agy membránpreparátumban vizsgáltuk.

A **[^{3}H]S-Atc^3,Ile^{5,6}deltorfin I** és a **[^{3}H]R-Atc^3,Ile^{5,6}deltorfin II** 35°C-on az opioid kötôhelyek egy csoportját jelölte nagy affinitással (K_d ~ 0.3 nM), 130 fmol/mg-os B_{max} értékkel, a konformerek kötési tulajdonságaiban nem volt különbség. A nemspecifikus kötés aránya mindkét radioligand esetében 15% alatt volt. A kötés telíthetô volt, sztereospecifkus, és az opioid receptorokon ható vegyületek széles körével gátolható. Csak a δ-szelektív ligandok gátolták nagy affinitással a [^{3}H]S-Atc^3,Ile^{5,6}deltorfin I és a [^{3}H]R-Atc^3,Ile^{5,6}deltorfin II kötést. Egyes altípusszelektív δ-ligandok, mint a DSLET (δ_2) és a DPDPE (δ_1) eltérô mértékû gátló hatása miatt feltételezhetô, hogy a vizsgált radioligandok különbséget tesznek a δ_1 és a δ_2 receptor alosztály között.

Na^+-ionok és guanil nukleotidok egyaránt csökkentették a [^{3}H]S-Atc^3,Ile^{5,6}deltorfin I és a [^{3}H]R-Atc^3,Ile^{5,6}deltorfin II kötést patkány agy membránhoz, további bizonyítékot szolgáltatva a ligandok agonista karakterére. Gpp(NH)p jelenlétében a specifikus kötés csökkent, ami az agyi δ-receptorok G-fehérje kapcsoltságára utal.

Az új deltorfin I és II analógok a kiindulási vegyületeknél jóval nagyobb affinitással és szelektivitással rendelkeznek. Más δ-szelektív peptidekhez viszonyítva jó δ-szelektivitást, és a legnagyobb δ-receptor affinitást mutatták. A kötési kísérletek alapján a két új, konformációsan gátolt deltorfin analóg, a [^{3}H]S-Atc^3,Ile^{5,6}deltorfin I és a [^{3}H]R-Atc^3,Ile^{5,6}deltorfin II a ma ismert legpotensebb és δ-szelektívebb radioligand.

(III). Szerkezet-hatás vizsgálatokat végeztünk néhány új **nem-peptid analóg**gal, melyeket az opioid antagonista naloxon, illetve a μ-szelektív agonista oximorfon és oxikodon benzofurán csoporttal történt addíciójával kaptunk. A szintézis célja a benzofurán csoport δ-receptor felismerésben való szerepének felderítése volt. Receptorkötési vizsgálatokat és agonista/antagonista karakter meghatározást végeztünk kompetíciós kísérletekben patkány agy membránpreparátumban.

A kapott eredmények összhangban állnak a "message-address" elmélettel. A modell szerint az új benzofurán származékokban a benzol csoport felel meg a δ-receptor felismerését végzô "address"-nek, ezáltal jó δ-szelektivitást, a többi opioid receptor típuson pedig igen alacsony

affinitást kaptunk. A molekula két funkcionálisan elkülönülô részét egy viszonylag merev térkitöltô csoport köti össze, ami a naltrindol esetében indol csoport, a naltribennél és a most vizsgált új vegyületeknél pedig furán.

Az új benzofurán analógok kémiai szerkezetének, kötési és farmakológiai tulajdonságainak összehasonlítása a naloxonéval, oxikodonéval és oximorfonéval lehetôséget nyújt szerkezet-hatás összefüggések felderítésére. A benzofurán csoport addíciója az elôbb felsorolt vegyületekhez nem szelektív vagy μ-szelektív anyagokból igen jó δ-szelektivitású ligandok kialakulását eredményezi.

A morfinváz 3-as helyzetû csoportja kiemelkedôen fontos az opioid receptorokhoz történô kötôdés szempontjából. Ebben a helyzetben a legnagyobb affinitást a hidroxil csoport biztosította a naltrexon, naloxon, naltrindol, naloxon-benzofurán és oximorfon-benzofurán vegyületekben. A metoxi csoportot tartalmazó oxikodon-benzofurán és naloxonszármazék kisebb affinitast mutatott a kötési tesztekben.

A vizsgált vegyületek farmakológiai tulajdonságai jól megfeleltethetôk voltak a szerkezetüknek. Az oximorfon és az oxikodon N-metil benzofurán származéka részleges agonista tulajdonságokat mutatott, míg a naloxon N-allil benzofurán származékai megôrizték a kiindulási vegyület antagonista jellegét. Mindhárom nempeptid analóg koncentrációfüggô módon irreverzibilisen blokkolta az opioid kötôhelyeket.

Összefoglalásul, a kifejlesztett új opioid peptid és nempeptid ligandok jó szelektivitást mutattak a μ- vagy a δ-opioid receptorokhoz. Tulajdonságaik alapján e ligandok az opioid receptorok biokémiai, farmakológiai és fiziológiai tanulmányozásának, esetleg a fájdalomcsillapítás és a drogfüggôség mechanizmusának megismerésének hatékony eszközei lehetnek.

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Szeged, December , 1997

LIST OF PUBLICATIONS

This thesis is based on the following publications:

I. SPETEA, M., Ötvös, F., Tóth, G., Nguyen, T. M. -D., Schiller, P. W., Vogel, Z., and Borsodi, A.

Interaction of agonist peptide [^{3}H]Tyr-D-Ala-Phe-Phe-NH_2 with μ-opioid receptor in rat brain and CHO-μ/1 cell line.

Peptides, accepted for publication, 1997.

II. SPETEA, M., Darula, Zs., Tóth, G., and Borsodi, A.

Synthesis and binding characteristics of highly selective radiolabelled deltorphin analogues containing 2-aminotetralin-2-carboxylic-acid in position 3.

Neuropeptides 31: 483-488, 1997.

III. SPETEA, M., Nevin, S. T., Hosztafi, S., Rónai, A. Z., Tóth, G., and Borsodi, A.

Affinity profiles of novel δ-receptor selective benzofuran derivatives of non-peptide opioids.

Neurochem. Res., submitted, 1997.

These publications are refereed to in the text by the above roman numerical. Other publications are mentions in the 'References' list.

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