An investigation of [delta]- and [mu]-opioid receptors: putative subtypes and interactions

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AN INVESTIGATION OF δ AND μ OPIOID RECEPTORS: PUTATIVE SUBTYPES AND INTERACTIONS

by

J. Elliott

A Doctoral thesis submitted in partial fulfilment of the requirements for the award of

Doctor of Philosophy of the Loughborough University of Technology

October 1994

Abstract

The possible existence of μ- and δ-opioid receptor subtypes, and interactions between μ- and δ-opioid receptors, has been examined in isolated tissue preparations, by radio-ligand binding in rodent brain homogenates and at the second messenger level using cell cultures of human neuroblastoma SH-SY5Y cells.

In the mouse vas deferens preparation, which contains both μ- and δ-opioid receptors, the potency of the μ-opioid agonists morphine (IC_{50} = 180 ± 13 nM) and [D-Ala^2,MePhe^{4}Gly(ol)^5]enkephalin (DAMGO) (IC_{50} = 15.3 ± 1.7 nM) was unaltered by the presence of either a sub-effective concentration or an IC_{50} concentration of the δ-opioid agonists [D-Pen^2,D-Pen^5]enkephalin (DPDPE) or [D-Ala^2,Glu^4]deltorphin (DELT II). A lack of interaction was also observed using the guinea-pig myenteric plexus-longitudinal muscle preparation. These results do not support the concept of interacting μ- and δ-opioid receptors and are discussed in terms of differences between opioid sensitive systems in the central nervous system and in the periphery.

Heterogeneity in the δ-opioid population was confirmed by the existence of stable and wash-sensitive components to the binding of the δ-opioid [^{3}H]DPDPE and the inability of [D-Ala^2]deltorphin I (DELT I) to displace approximately 20 % of [^{3}H]DPDPE binding in mouse brain homogenates. Attempts to show changes in δ-opioid receptor characteristics in rat brain induced by swim-stress or electroconvulsive shock treatment were unsuccessful.

To aid studies of δ-opioid receptors a series of novel synthetic δ-opioid ligands based on the δ-selective antagonist naltrindole were characterised by ligand-binding and isolated tissue bioassay. A highly selective antagonist was identified as were nonpeptide δ-ligands which showed full agonist properties in the mouse vas deferens.

Human neuroblastoma SH-SY5Y cells were shown to express μ- and δ-opioid receptors in the ratio of 2.5 : 1. The μ-opioid receptor binding sites on SH-SY5Y cells were not susceptible to the putative μ_1 irreversible antagonist naloxonazine but 60 % were blocked by the irreversible antagonist β-funaltrexamine. The insensitivity of a proportion of μ-opioid binding sites to β-funaltrexamine suggests some form of μ-opioid receptor heterogeneity.

In both SH-SY5Y cells and mouse neuroblastoma x rat glioma NG108-15 cells opioid agonists stimulated the binding of the GTP analogue [^{35}S]GTPγS to membranes. In SH-SY5Y cells the potency of morphine (IC_{50} = 63.9 ± 7.9 nM)

and DAMGO (IC_{50} = 30.4 ± 12 nM) to stimulate the binding of [^{35}S]GTPγS was unchanged in the presence of δ-selective concentrations of DPDPE and DELT II. However following chronic exposure of the cells to morphine (10 μM) the potency of morphine to stimulate [^{35}S]GTPγS binding was reduced by 2.4 - fold and the maximum stimulation was reduced by 33 %. This tolerance was accompanied by a 68 % loss of μ-opioid binding sites with no change in μ-opioid affinity for the remaining sites. Changes in agonist stimulated [^{35}S]GTPγS binding can be used as a measure of opioid tolerance in these cells.

Keywords: Opioid receptors, δ-opioid subtypes, μ-opioid subtypes, μ / δ interactions, tolerance, G proteins, [^{35}S]GTPγS binding, mouse vas deferens, guinea-pig ileum, SH-SY5Y cells

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Lastly, I cannot thank my family enough, the love and support has been immeasurable, this thesis is dedicated to them.

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ERRATA

page 35	line 24	[Sheehan *et al.*, 1986] should read [Sheehan *et al.*, 1986a]
41	6	Fig. 3.5 should read Fig. 3.4
72	28	should read (except those experiments conducted at St. John's [Yoburn *et al.*, 1991] or on Bristol rats)
80		figure should be labelled Fig. 4.28
82	7	froma should read from a
86	10	natrindole should read naltrindole
87	last line	Table 5.3 should read Table 5.2
94	8	relection should read reflection
95	13	[Rogers *et al.*, 1990] should read [Rogers *et al.*, 1990b]
139	13	relection should read reflection
	14	should read affording a greater level of tolerance
166	9	should read (1994a)
	12	should read (1994b)

In all cases Ke, the equilibrium dissociation constant, is taken as a measure of antagonist affinity.

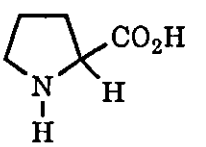
Abbreviations

amino acids	see overleaf
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
CNS	central nervous system
DADLE	[D-Ala^2,Leu^5]enkephalin
DALCE	[D-Ala^2,Leu^5,Cys^6]enkephalin
DAMGO	[D-Ala^2,MePhe^{4}Gly(ol)^5]enkephalin
DELT I	Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2
DELT II	Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2
DPDPE	[D-Pen^2,D-Pen^5]enkephalin
DSLET	[D-Ser^2,Leu^5,Thr^6]enkephalin
ECS	electroconvulsive shock
EDTA	ethylenediaminetetraacetic acid
GDP	guanosine diphosphate
GTP	guanosine triphosphate
GTPγS	guanosine 5'-[γ-thio]triphosphate
G protein	guanosine triphosphate binding protein
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
ICI 174,864	N,N-diallyl-Tyr-Aib-Aib-Phe-Leu (Aib = α-aminoisobutyric acid)
i.c.v.	intracerebroventricular
i.t.	intrathecal
[Leu^5]enkephalin	leucine enkephalin (Tyr-Gly-Gly-Phe-Leu)
[Met^5]enkephalin	methionine enkephalin (Tyr-Gly-Gly-Phe Met)
mRNA	messenger ribonucleic acid
PLC	phospholipase C
RX 8008M	16-Me cyprenorphine
s.c.	subcutaneous
TIPP	Tyr-Tic-Phe-Phe (Tic = tetrahydroisoquinoline-3-carboxylic acid)
Tris	tris-[hydroxymethyl]-aminomethane

Structure and coding of amino acids of the general structure: $H_2N\text{-}CH(R)\text{-}CO_2H$.

Amino acid	Three letter symbol	Single letter symbol	-R
Alanine	Ala	A	$-CH_3$
Arginine	Arg	R	$-(CH_2)_3\ NHC(=NH)NH_2$
Asparagine	Asn	N	$-CH_2CONH_2$
Aspartic acid	Asp	D	$-CH_2CO_2H$
Cysteine	Cys	C	$-CH_2SH$
Glutamine	Gln	Q	$-(CH_2)CONH_2$
Glutamic acid	Glu	E	$-(CH_2)CO_2H$
Glycine	Gly	G	-H
Histidine	His	H	$-CH_2$(4-imidazolyl)
Isoleucine	Ile	I	$-CH(CH_3)CH_2CH_3$
Leucine	Leu	L	$-CH_2CH(CH_3)_2$
Lysine	Lys	K	$-(CH_2)_4NH_2$
Methionine	Met	M	$-(CH_2)_2SCH_3$
Phenylalanine	Phy	F	$-CH_2Ph$
Proline*	Pro	P	
Serine	Ser	S	$-CH_2OH$
Threonine	Thr	T	$-CH(CH_3)OH$
Tryptophan	Trp	W	$-CH_2$(3-indolyl)
Tyrosine	Tyr	Y	$-CH_2$(4-hydroxyphenyl)
Valine	Val	V	$-CH(CH_3)_2$

*Proline is an imino acid of the stucture:



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CHAPTER 1
GENERAL INTRODUCTION

Thou hast the keys of Paradise, oh just, subtle, and mighty opium !

Thomas De Quincey (1785 - 1859)

1.1 Opioid Receptors

Pain can be described as a subjective, unpleasant sensory and emotional experience. While the relief of pain is always desirable, it must not be so effective as to hinder the treatment of the causitive factor(s). Severe pain is treated by utilising opiates or their derivatives, e.g., morphine and pethidine (Fig. 1.1). These types of drug are particularly useful because, rather than merely decreasing the perception of the painful stimulus itself, they relieve anxiety, tension and fear. Thus the patients, freed from suffering or distress, feel more comfortable and are able to tolerate pain even when they know it is still present.

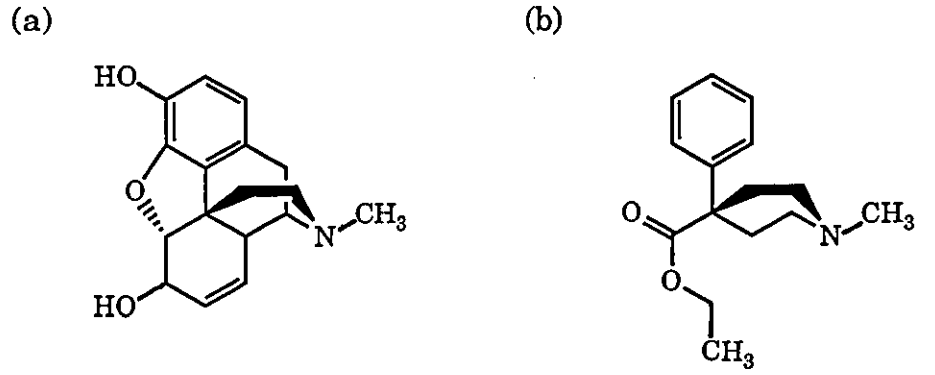


Figure 1.1 (a) morphine and (b) pethidine

Morphine is a naturally occurring alkaloid initially isolated from the opium poppy (*Papaver somniferum*) in 1803. Opium has been used for thousands of years but the Romans are credited for first realising its pain relieving properties and also its abuse potential. Unfortunately nowadays opiates are abused by an estimated 2.5 million drug users [Di Chiara & North, 1992].

The mechanisms by which morphine, and opiates in general, exert their myriad of pharmacological effects remained a mystery until the 1970's. In 1973 several laboratories published results showing that opiates bind to stereospecific receptors (opioid receptors) in neuronal membranes [Pert & Snyder, 1973; Simon *et al.*, 1973; Wong & Hong, 1973]. These findings

intensified the search for an opioid ligand endogenous to the mammalian central nervous system.

This research culminated in 1975 when Hughes and Kosterlitz isolated and characterised two pentapeptides from porcine brain [Hughes, 1975a; Hughes *et al.* 1975b,c]. These peptides were found to differ only in the carboxy-terminal amino acid and were named methionine enkephalin ([Met^5]enkephalin) and leucine enkephalin ([Leu^5]enkephalin) (Fig. 1.2). The enkephalins were found to mimic the pharmacological actions of morphine in *in vitro* bioassays, in which opioids inhibit electrically stimulated contractions of smooth muscle.

(a) Tyr-Gly-Gly-Phe-Met (b) Tyr-Gly-Gly-Phe-Leu

Figure 1.2 (a) [Met^5]enkephalin and (b) [Leu^5]enkephalin

It was at about this time that Martin and co-workers established the existence of multiple opioid receptors [Martin *et al.*, 1976; Gilbert & Martin, 1976]. They examined various alkaloids and found that they did not substitute for each other in the prevention of withdrawal symptoms in the tolerant chronic spinal dog. Thus the existence of mu (μ; at which morphine was the prototype agonist), kappa (κ; at which ketocyclazocine was the prototype agonist) and sigma (σ; at which N-allynormetazocine was the prototype agonist) receptors was proposed.

Although the enkephalins are pharmacologically very similar to the classical alkaloids there are some discrepancies. Kosterlitz's group found that the rank order of potency of morphine and the enkephalins was not the same in the guinea-pig ileum as in the mouse vas deferens preparation. In addition, the sensitivity of these drugs to antagonism by naloxone (Fig. 1.3) varied greatly. These findings lead to the proposal of the existence of the enkephalin-selective delta (δ) opioid receptor [Lord *et al.*, 1977].

Sigma (σ) receptors are no longer classified as opioid receptors since they are insensitive to the antagonist naloxone and it is generally accepted that there are three opioid receptor types, namely, μ, δ and κ. Furthermore endogenous ligands for each of these receptor types have been elucidated, i.e., the enkephalins and the extended [Met^5]enkephalin β-endorphin [Lord *et al.*,

1977] (Fig. 1.4) which act at μ- and δ-opioid receptors and the extended [Leu^5]enkephalins, the dynorphins which mediate their effects *via* κ-opioid receptors [Chavkin *et al.*, 1982]. However, none of the peptides show absolute specificity and some cross-over of activity is seen. Chemical synthesis has led to the development of receptor selective ligands, such as [D-Ala^2,MePhe^4,Gly(ol)^5]enkephalin (DAMGO) for μ, [D-Pen^2,D-Pen^5]-enkephalin (DPDPE) for δ and U69593 ((5α,7α,8β)-(+)-N-methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]benzeneacetamide) for κ. Furthermore some ligands are thought to differentiate opioid receptors into opioid receptor subtypes, as discussed later. A list of those ligands discussed in this thesis is given in Table 1.1.

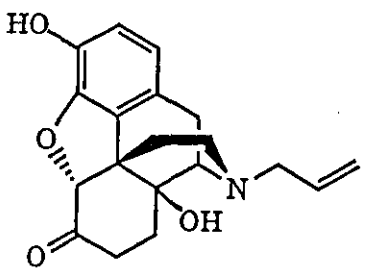


Figure 1.3 Naloxone

Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr
-Leu-Phe-Lys-Asn-Ala-Ile-Val-Lys-Asn-Ala-His-Lys-Lys-Gly-Gln

Figure 1.4 β-endorphin (human)

Table 1.1 Opioid ligand specificity (explanations of any abbreviations and / or subtype specificity are discussed where appropriate in the text)

μ-agonists	μ-antagonists
DAMGO	β-funaltrexamine (irreversible)
fentanyl	CTOP
morphiceptin	CTP
morphine	cyprodime
normorphine	naloxonazine (irreversible)
	naloxone
δ-agonists	**δ-antagonists**
CP-OH	BNTX
DADLE	DALCE (long term, irreversible)
DALCE (short term)	FIT (irreversible)
DELT I & DELT II	ICI 174,864
DPDPE	naltriben
DSLET	naltrindole
[Leu^5]enkephalin	5'NTII (irreversible)
[Met^5]enkephalin	TIPP
κ-agonists	**κ-antagonists**
CI977	nor-binaltorphimine
U69593	
mixed agonists	**mixed antagonists**
β-endorphin	diprenorphine
etorphine	16-Me cyprenorphine (RX 8008M)
pentazocine (partial agonist)	

1.2 Molecular Biology of the Opioid Receptors

1.2.1 Cloning of the μ-, δ- and κ-opioid receptors

The existence of the three opioid receptor types, μ, δ and κ has recently been confirmed by molecular characterisation. In 1992 two groups independently published the sequence (372 amino acids) of the δ-opioid receptor clone isolated from neuroblastoma x glioma NG108-15 hybrid cells, [Evans *et al.*, 1992; Kieffer *et al.*, 1992] which express opioid receptors exclusively of the δ type [Chang & Cuatrecasas, 1979]. Briefly, a cDNA library was prepared from NG108-15 cells which was then transfected into COS cells. Using the δ-opioid selective peptide [^{125}I][D-Ala^2,D-Leu^5]enkephalin (DADLE) the transfected cells were screened and a cluster of positive cells identified. The DNA of these cells was twice extracted, purified and retransfected before an individual cDNA (DOR-1) was isolated. The opioid binding characteristics of COS cells expressing DOR-1 were examined and found to be indicative of the presence of a δ-opioid receptor. In addition, forskolin-stimulated cyclic adenosine monophosphate (cAMP) accumulation in DOR-1 transfected cells was inhibited by both the nonselective alkaloid etorphine and the δ-selective peptide [D-Pen^2,D-Pen^5]enkephalin (DPDPE). This latter result illustrates functional coupling of the receptor to the effector enzyme adenylate cyclase as present in NG108-15 cells [Law *et al.*, 1983].

In the following year, 1993, molecular cloning and functional expression of a μ-opioid receptor from rat brain [Chen *et al.*, 1993] and a κ-opioid receptor from mouse brain [Yasuda, *et al.*, 1993] was achieved. The μ- and κ-opioid receptors consist of 398 and 380 amino acids respectively. The amino acid sequences of the three opioid receptor types are given in Fig. 1.5., where an amino acid common to two or more receptors is underlined. The single amino acid code has been used and this is explained in the abbreviations at the beginning of the thesis. More recently the amino acid sequences of the human μ- and δ-opioid receptor clones have been published [Wang *et al.*, 1994a; Knapp *et al.*, 1994].

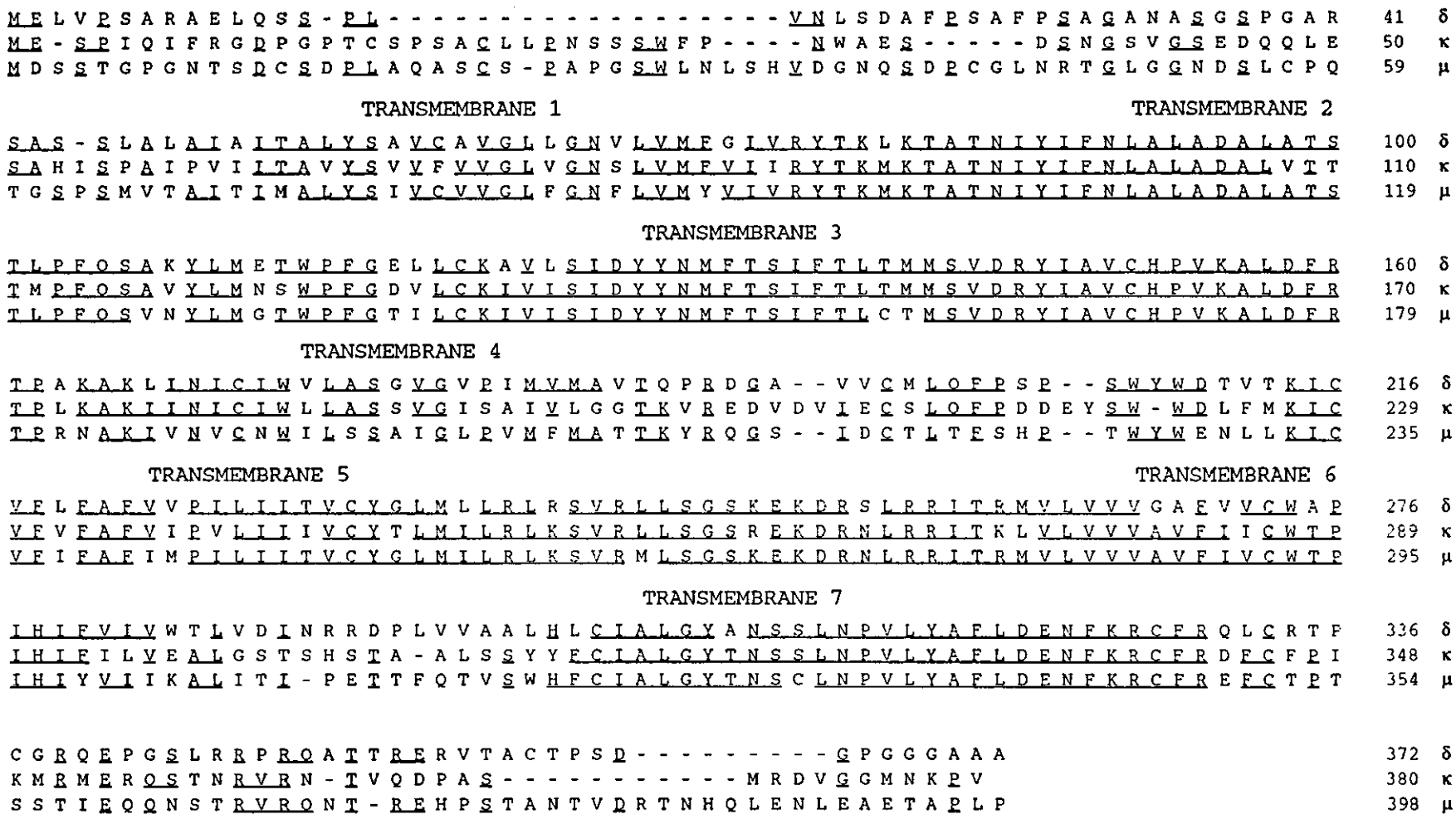


Figure 1.5 Alignment of the primary sequences for the opioid receptors (mouse δ [Evans *et al.*, 1992], mouse κ [Yasuda *et al.*, 1993] and rat μ [Chen *et al.*, 1993], using the single amino acid code. Underlined regions indicate conserved amino acids between the receptors. (Adapted from Reisine & Bell, 1993)

All three opioid receptor types, μ, δ and κ, belong to the superfamily of 7-transmembrane domain G protein coupled receptors (see section 1.3). Thus their general stucture displays certain characteristics, i.e., an extracellular amino terminus, 7 transmembrane regions, 3 extracellular loops, 3 intracellular loops and an intracellular carboxy acid terminus, as outlined in the diagram below (Fig. 1.6).

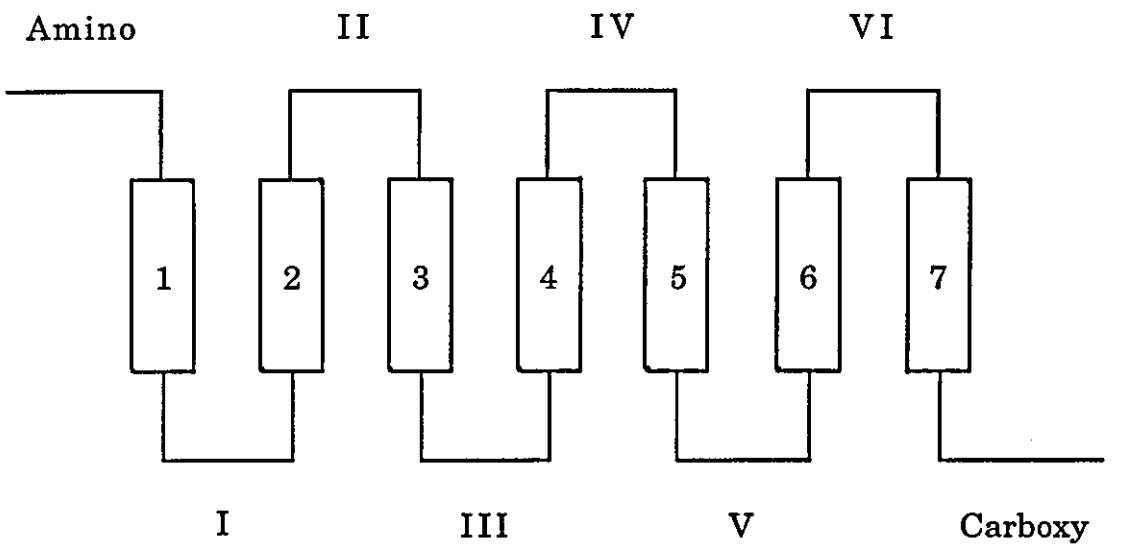


Figure 1.6 Structure of G protein-coupled receptors as deduced from hydropathy plots of the cloned receptors. The receptor protein consists of seven transmembrane α helices (1 - 7), joined by intra- and extra-cellular loops (I - VI), and connected to an extracellular amino acid terminus and an intracellular carboxy terminus. Adapted from Birnbaumer *et al.*, 1990.

By analysis of the amino acid sequences of the opioid receptors it is readily determined that 57 % of the amino acid sequences of the three types of opioid receptors are identical. The greatest homology is seen in transmembrane 2 (84 %) > 3 (82 %) > 5 = 7 (71 %) > 6 (50 %) > 1 (45 %) > 4 (30 %). The sequences of the intracellular loops are highly conserved; loop I shows 90 %, loop III shows 91 % and loop V shows 78 % sequence homology respectively. Whereas the extracellular loops are generally much more divergent; loop II shows 67 %, loop IV shows 24 % and loop VI shows 7 % sequence homology respectively.

There is very little sequence or size homology at either the amino or carboxy termini. The divergent extracellular regions of the receptors may prove to be vital for ligand binding and therefore explain ligand selectivity profiles.

Compared to other classes of receptors the opioid receptors most closely resemble somatostatin receptors [Reisine & Bell, 1993]. Indeed it has been demonstrated that somatostatin analogues exhibit high affinity for opioid receptors, e.g., CTP [D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-NH_2 [Pelton *et al.*, 1985].

1.2.2 Possible uses of the opioid clones

This breakthrough in opioid molecular biology should lead to rapid advances in opioid pharmacology. For instance, whereas most tissues express more than one opioid receptor type, individual receptor types can now be investigated since cell lines can be transfected with a selective cDNA [Abood *et al.*, 1994]. Alternatively opioid receptor function could be examined by generating mice (for example) deficient in a particular receptor by gene deletion.

Using autoradiographic techniques the availability of the cDNAs for the three opioid receptor types will enable the anatomical distribution of the mRNAs to be determined and therefore provide knowledge of the opioid receptor expression pattern in the central nervous system [Miotto *et al.*, 1994].

In addition, knowledge of the amino acid sequence and therefore the structure of the opioid receptors should also lead to a better understanding of their function. Several groups are currently investigating chimeric receptors [e.g., Meng *et al.*, 1994; Xue *et al.*, 1994], in which for example a sequence from the μ-opioid receptor replaces a specific sequence in a κ-opioid receptor. The binding and function of this chimeric receptor is then compared to the original receptors to elucidate which regions of the receptor may be responsible for determining ligand specificity and / or coupling to intracellular effectors. Alternatively mutagenesis or deletion of certain amino acids can be tested [Hong *et al.*, 1994]. In the long-term, these kinds of studies should provide information useful for the rational design of more opioids.

Finally, characterisation of cDNAs amino acid sequences from human opioid receptors, μ [Wang *et al.*, 1994a], δ [Knapp *et al.*, 1994] and κ (yet to be determined), should provide an excellent screen for testing new opioids.

1.3 G Proteins

The binding of an agonist to a receptor is only the first stage in a cascade of events that leads to a release of chemical second messengers that in turn initiate characteristic changes within the cell. Intracellular events are often activated by effector proteins such as enzymes or ion channels. G proteins provide a link between cell-surface receptors and effector proteins at the plasma membrane. Each of the three opioid receptor types is known to be G protein coupled and able to inhibit adenylate cyclase and Ca^{2+} and K^+ ion channels [Law *et al.*, 1983; Bhoola & Pay, 1986; North, 1986].

G proteins are heterotrimers consisting of 3 subunits (Fig. 1.7): the α-subunit has a single high affinity binding site for guanine nucleotides (GDP and GTP) and is unique to the particular type of G protein, while the β- and γ-subunits are bound together acting as a complex which can be shared among various α-subunits. Not all G proteins are the same with most variability found in the α-subunit. For example different forms of G protein regulate adenylate cyclase. Thus G_s and G_q can stimulate adenylate cyclase activity while G_i inhibits this enzyme. Two-way regulation of calcium channels can also occur in a similar manner [Hepler & Gilman, 1992]. At present, G proteins are classified on the basis of their amino acid sequences into four major families, namely, G_s, G_i, G_q and G_{12}.

G proteins act as switches turning the transmembrane signal on and off. They are able to achieve this function by a cycle of events known as the G protein turnover cycle (Fig. 1.7). The resting form of the G protein consists of the GDP-bound form of the α-subunit bound to the βγ-complex. When an agonist activates the receptor a conformational change of the G protein is induced releasing the bound GDP which is exchanged for GTP. The GTP-bound form of the α-subunit undergoes another conformational change resulting in dissociation of the G protein from the receptor and also dissociation of the α-subunit from the βγ-complex, yielding the form of the G protein active in stimulating downstream events. Both the GTP-bound α-subunit [Birnbaumer *et al.*, 1990] and the βγ-complex are able to activate effector proteins [Sternweiss, 1994]. The α-subunits possess intrinsic GTPase activity which causes hydrolysis of the terminal phosphate of bound GTP and the formation of the GDP-bound form of α-subunit with release of free inorganic phosphate, and subsequently deactivation of the active species. The

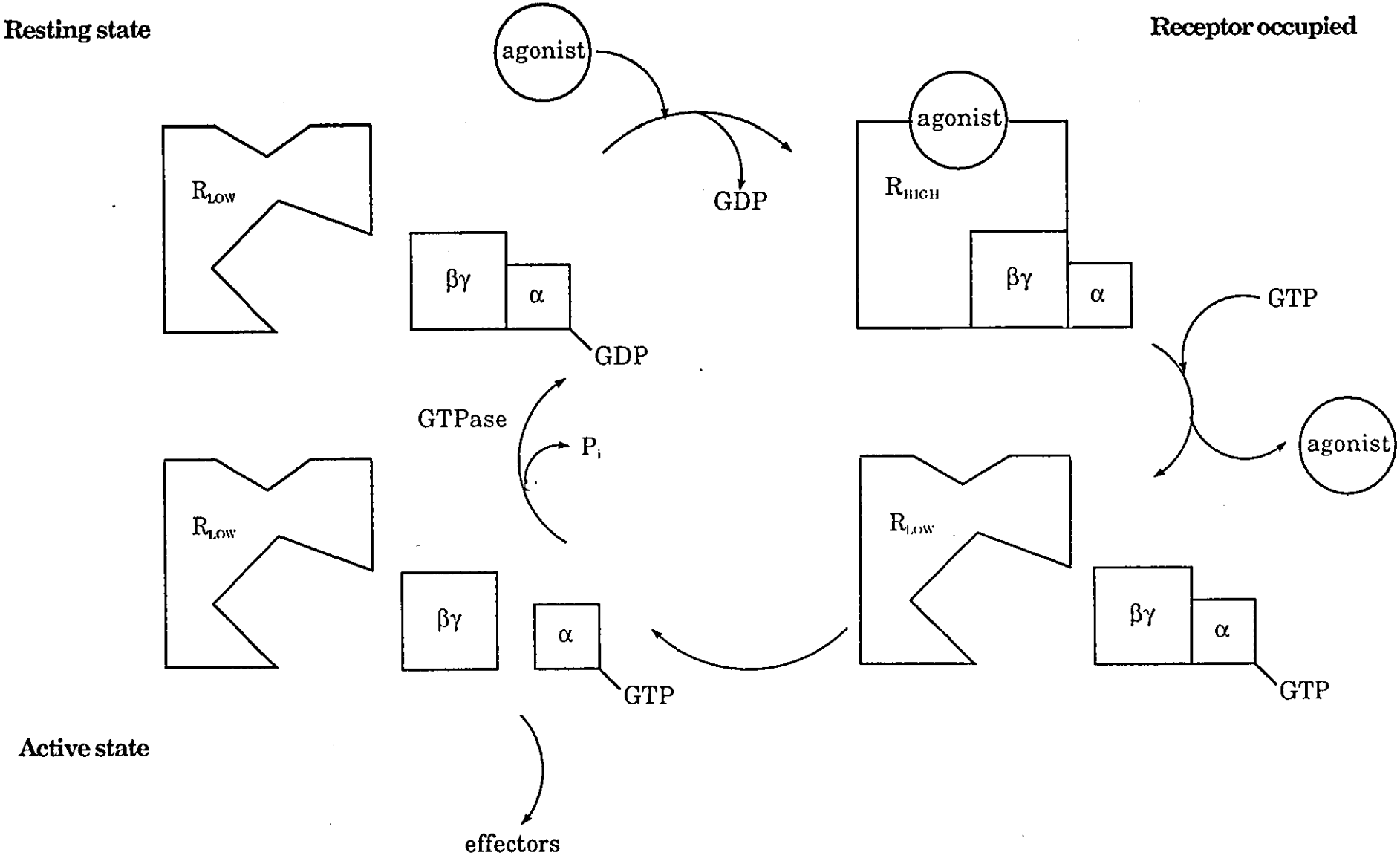


Figure 1.7 Receptor interactions with G proteins, a detailed explanation is given in the text. (Adpated from Taylor, 1990).

GDP-bound form of the α-subunit has high affinity for the βγ-complex and so reassociation occurs and the cycle is complete. Even at this initial stage signal amplification may occur because the binding of agonist to receptor can activate many equivalent identical G proteins, which in turn may activate effector proteins through both the GTP-bound α-subunit and the βγ complex [Simon *et al.*, 1991].

In the absence of an agonist the rate of dissociation of GDP from the α-subunit is much lower than the rate of GTPase hydrolysis of the bound GTP to GDP. Therefore, under normal conditions the amount of G protein in the active form of dissociated βγ-complex and GTP-bound α-subunit is low. However, in the presence of agonist and Mg^{2+} ions the rate of guanine nucleotide exchange is increased while the rate of GTPase hydrolysis is unaffected. Thus the proportion of G protein in the active form is increased. The receptor only has high affinity (R_{HIGH}) for the G protein when the guanine nucleotide-binding site of the α-subunit is empty and when the α-subunit is bound to the βγ-complex. This interaction is only short-lived because GTP quickly binds to the α-subunit, thus reducing the affinity of the receptor for the G protein [Taylor, 1990].

The α-subunits of the majority of G proteins are substrates for bacterial toxins. In the presence of GTP, cholera toxin, isolated from *Vibrio cholera*, catalyses the transfer of the adenosine diphosphate- (ADP) ribose moiety of nicotinamide adenine dinucleotide (NAD) to a specific arginine residue of the G_s protein α-subunit. This modification inhibits the intrinsic GTPase activity of the α-subunit and thus constitutively activates these G proteins, i.e., the lifetime of the active state is increased. The toxin of *Bordetella pertussis* , pertussis toxin, in the presence of ATP is able to ADP-ribosylate a specific cysteine residue of the G_i protein α-subunit. This treatment inhibits receptor-mediated G_i protein activation [Birnbaumer *et al.*, 1990]. Thus, for example, the inhibition of adenylate cyclase by agonists acting *via* pertussis-toxin sensitive G_i proteins would be blocked. Not all G proteins are cholera toxin- or pertussis toxin-sensitive [Hepler & Gilman, 1992]. G proteins insensitive to either of these toxins are thought to regulate receptor-mediated activation of phospholipase C (PLC) (either G_q and / or G_{12}). The effector enzyme PLC catalyses the formation of the second messengers inositol 1,4,5-triphosphate [$Ins(1,4,5)P_3$] and diacylglycerol from phosphatidylinositol 4,5-bisphosphate [$PtdIns(4,5)P_2$].

Physiological effects result from agonist-induced activation of effector systems, such as phospholipase A_2, cyclic guanylate cyclase, transporters for Mg^{2+} and glucose, and also Ca^{2+}, K^+ and Na^+ ion channels, activating second messengers [Taylor, 1990]. Alterations in second messenger levels may induce one of a variety of intracellular changes, such as gene transcription, selective protein phosphorylation, secretion, membrane depolaristion and cytoskeleton reorganisation.

1.4 Receptor Subtypes and Cross-Talk

Opioids are used clinically for the management of pain but unfortunately they also display unwanted side-effects including sedation, respiratory depression, constipation, nausea and vomiting. Additionally their misuse can lead to dependence and addiction [Rang & Dale, 1991]. Since therapeutic effects and side-effects may be mediated *via* different receptor types and or subtypes, the development of highly selective ligands is very important.

The receptor selectivity profile of opioids is generally determined using radioligand receptor binding assays as well as isolated tissue preparations. Many of these pharmacological studies have produced results suggesting further subdivisions of the receptor types μ, δ and κ. Evidence for some of these classifications is discussed below. However, the existence of receptor subtypes has not to date been proven by molecular cloning. Even though several laboratories have independently cloned each of the opioid receptor types μ, δ and κ, the cDNAs of each receptor type are apparently products of identical genes in several species [Uhl *et al.*, 1994]. Thus, perhaps subtypes arise from varying interactions with effector systems, in particular differential coupling of the receptor to G protein(s) or post-translational processing of some kind.

1.4.1 μ- / δ-opioid interactions

The ability of δ-opioid agonists to modulate the *in vivo* actions of μ-opioid agonists to promote or decrease their activity has been demonstrated (for review see Traynor & Elliott, 1993). The interactions have been reported for antinociceptive tests in which drugs were administered intracerebroventricularly (i.c.v.) and analagesia tested *via* the tail-flick hot water test [Vaught & Takemori, 1979; Lee *et al.*, 1980; Jiang *et al*, 1990a] and also in experiments where opioids were given intrathecally (i.t.) and the paw-withdrawal test was employed to measure the mechanical nociception threshold [Miaskowski *et al.*, 1992; Sutters *et al.*, 1990]. Indeed, peripherally administered δ-opioid agonists have been demonstrated to modulate the centrally-mediated antinociceptive effects of μ-opioid agonists [Porreca *et al.*, 1990]. These effects have also been shown to extend to other activities, such as bladder contraction [Sheldon *et al*, 1989], gut propulsion [Heyman, 1987], EEG and EEG spectral power [Stamidis & Young, 1992a,b] and antitussive activity [Kamei *et al.*, 1993; 1991].

Interactions are observed following the administration of a sub-effective dose of certain δ-opioid agonists. Thus, for example, the δ-opioid agonist DPDPE affords an increase in the potency of μ-opioid ligands whilst the δ-opioid agonist [Met^5]enkephalin shifts dose-response curves for the μ-opioid agonists to the right. Both the positive and negative modulatory actions of δ-opioid agonists are prevented by pretreatment with the selective δ-opioid antagonist ICI 174,864 (N,N-diallyl-Tyr-Aib-Aib-Phe-Leu-OH) [Jiang *et al.*, 1990a]. Of note is the observation that unlike δ-receptor selective peptides, subeffective doses of some μ-opioid receptor peptides are unable to modulate morphine analgesia [Barrett & Vaught, 1982].

Only some δ-opioid antagonists are able to block the modulatory actions of δ-opioid agonists. [D-Ala^2,Leu^5,Cys^6]enkephalin (DALCE) is an irreversible δ-opioid antagonist. When administered i.c.v. it is able to selectively antagonise direct δ-opioid mediated actions. However, DALCE is not able to prevent the modulatory actions of DPDPE or [Met^5]enkephalin on morphine-induced antinociception. Hence DALCE is able to discriminate between δ-opioid receptors that, when activated, cause modulation of morphine-mediated antinociception and those which lead to direct antinociceptive responses when activated by δ-opioid agonists [Jiang *et al.*, 1990a]. Furthermore, the conformationally constrained enkephalin [D-Ala^2,(2R,3S)∇^EPhe^4,Leu^5]-enkephalin (CP-OH) exhibits opposing effects in that it is able to modulate the actions of morphine but is unable to produce analgesia [Shimohigashi *et al.*, 1988]. Modulation of μ-opioid agonists by δ-opioid agonists has also been studied following removal of a proportion of the μ-opioid receptor population using the μ-selective irreversible antagonists β-funaltrexamine and naloxonazine. It was found that while β-funaltrexamine pre-treatment had no effect on the analgesic effects of the δ-opioid agonists, their modulatory actions were inhibited. In contrast, neither the modulatory or the direct effects of the δ-opioid agonists were inhibited by naloxonazine pre-treatment [Heyman *et al.*, 1989a].

Interestingly not all μ-opioid ligands are responsive to modulation. Thus DPDPE, in the mouse tail-flick assay, administered either i.c.v. or subcutaneously (s.c.), increases the potency of morphine, normorphine, levorphanol, methadone and codeine but not the generally more efficacious μ-opioid agonists [D-Ala^2,MePhe^4,Gly(ol)^5]enkephalin (DAMGO), [NMePhe^3,D-Pro^4]morphiceptin (PLO17), phenazocine, sufentanil or etorphine [Vaught & Takemori 1979; Heyman *et al.*, 1989b]. On the other hand, potentiation of the

activity of the μ-opioid agonists PLO17 and DAMGO at higher (ED_{50}) levels of δ-opioid ligands, administered intrathecally, has been reported in the rat hot plate test [Malmberg & Yaksh, 1992]. In addition, i.t. administered DAMGO was potentiated by DPDPE as measured in the paw-withdrawal test [Miaskowski *et al.*, 1992]. Indeed specific ratios of μ- to δ-opioid agonists may be required in order for synergism to be exhibited [Horan *et al.*, 1992; Adams *et al.*, 1993]. The reasons for this apparent discrepancy between observations is unknown but may be related to species differences and / or the route of drug administration employed.

The possible modulatory actions of δ-opioid agonists on μ-opioid agonists are supported by both physiological and biochemical studies which suggest close association of μ- and δ-opioid receptors. For example it is known that μ- and δ-opioid receptors exist on the same neuron in the mouse hypogastric ganglion [Rogers & Henderson, 1990], on myenteric neurones from guinea-pig ileum [Egan & North, 1981], in the dorsal horn [Zieglgansberger *et al.*, 1982] and dorsal root [Fields *et al.*, 1980] of the rat spinal cord and in the rat neostriatum [Schoffelmeer *et al.*, 1988]. Indeed in the latter tissue it has been proposed that μ- and δ-opioid receptors share the same inhibitory G protein.

The concept of interacting μ- and δ-opioid systems has received support from mathematical analyses of ligand binding assays [Rothman *et al.*, 1988; Demoliou-Mason & Barnard, 1986; Barrett & Vaught, 1983]. Following these studies δ-opioid receptors have been divided into so-called μ-complexed (δ_{cx}) and μ-non-complexed (δ_{ncx}) types. Biochemical evidence has been provided by the ability of both μ- and δ-opioid ligands to inhibit the covalent linking of β-endorphin to rat striatal membranes [Schoffelmeer *et al.*, 1990]. In addition, recent work with newly developed antagonists lends support to the presence of a heterogeneous δ-opioid receptor population, leading to a division into so-called δ_1 and δ_2 subtypes [Jiang *et al.*, 1991]. However, the relationship between the two definitions of δ-opioid subtypes is unclear [Traynor & Elliott, 1993]. A detailed discussion of both μ- and δ-opioid receptor subtypes is given below.

1.4.2 δ-Opioid receptor subtypes

In addition to the division of δ-opioid receptors into μ-complexed (δ_{cx}) and μ-non-complexed (δ_{ncx}) subtypes there is an alternative classification of putative δ-opioid receptor subtypes based on results of *in vivo* studies using the mouse tail-flick hot water test of antinociception and the use of selective antagonists, in particular those based on naltrindole [Portoghese *et al.*, 1988] (Fig. 1.8). Thus the peptides DPDPE and DADLE are thought to activate δ_1-opioid receptors, and are antagonised by the irreversibly acting peptide DALCE and 7-benzylidenenaltrexone (BNTX) (Fig. 1.8). Conversely, the highly δ-selective peptide [D-Ala^2,Glu^4]deltorphin (DELT II) (Fig. 1.9) and [D-Ser^2,Leu^5,Thr^6]-enkephalin (DSLET) are agonists at the δ_2-opioid receptor subtype, while the irreversible ligand naltrindole-5'-isothiocyanate (5'NTII) and naltriben (the benzofuran analogue of naltrindole) are antagonists [e.g. Sofuoglu *et al.*, 1991; Jiang *et al.*, 1991]. In agreement with this subdivision DPDPE displays a lack of cross-tolerance with DELT II or DSLET [Sofuoglu *et al.*, 1991; Mattia *et al.*, 1991]. Furthermore, the antinociceptive actions of DPDPE and DELT II can be differentially prevented by potassium channel blockers, i.e. DPDPE is inhibited by glybenclamide, while DELT II is sensitive to tetraethylammonium [Wild *et al.*, 1991].

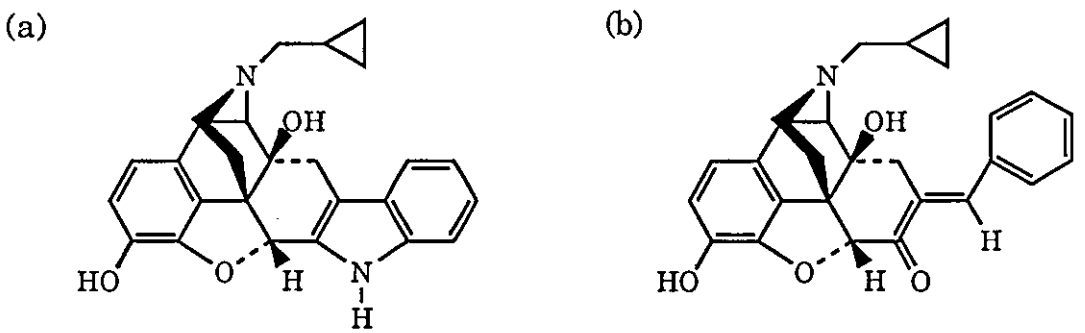


Figure 1.8 (a) naltrindole and (b) 7-benzylidenenaltrexone

(a) Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2
(b) Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2

Figure 1.9 (a) DELT I and (b) DELT II

The distribution of δ-opioid subtypes may not be homogeneous within the central nervous system (CNS). For example, naltriben is three times more effective at antagonising DSLET in the mouse spinal cord than the brain [Sofuoglu *et al.*, 1991]. When administered i.c.v. β-endorphin causes spinal antinociception *via* release of [Met^5]enkephalin and subsequent stimulation of δ-opioid receptors in the spinal cord. NTB administered i.t. attenuates the analgesic effects of i.c.v. β-endorphin whereas BNTX does not, suggesting involvement of δ_2- but not δ_1-opioid receptors [Tseng *et al.*, 1993; Takemori & Portoghese, 1993]. In addition, δ-opioid agonists are more susceptible to antagonism by naltrindole 5'-isothiocyanate than DALCE [Mattia *et al.*, 1992] suggesting the δ-opioid receptors of the mouse spinal cord may be of the δ_2 subtype. This latter proposal is not supported by another study using the putative δ_1-opioid agonist DPDPE, the putative δ_2-opioid receptor agonist DELT II and the selective δ_1-opioid antagonist BNTX and the δ_2-opioid receptor preferring antagonist NTB, which concludes that both δ_1- and δ_2-opioid receptors are involved in antinociceptive effects in the mouse spinal cord [Sofuoglu *et al.*, 1993]. Furthermore, both δ-opioid receptor subtypes are thought to be involved in antinociception in the rat spinal cord [Sewart & Hammond, 1993]. The antinociceptive effects of the two endogenous enkephalins i.e. [Met^5]- and [Leu^5]enkephalin, are reported to be mediated *via* δ_2-opioid receptors in the spinal cord. Conversely, i.c.v. administered [Met^5]- and [Leu^5]enkephalin are inhibited by BNTX but not NTB, suggesting involvement of δ_1-opioid receptors in the brain [Takemori & Portoghese, 1993].

The existence of multiple δ-opioid subtypes also helps to explain the findings of ligand binding assays. In rat brain homogenates the displacement of [^{3}H][D-Ala^2]deltorphin I (DELT I) by DPDPE is biphasic suggesting δ-opioid receptor heterogeneity [Negri *et al.*, 1991]. Studies in mouse brain have shown that the level of [^{3}H]DSLET binding sites (in the presence of the μ-selective peptide DAMGO to block μ-receptors) is higher than the level of [^{3}H]DPDPE sites, suggesting δ_2-opioid receptors predominate in this tissue [Sofuoglu *et al.*, 1992]. The δ_1-opioid receptor antagonist BNTX is also able to discriminate between δ-opioid receptor subtypes in guinea-pig brain, it is able to compete for [^{3}H]DPDPE binding sites with 100 - fold higher potency than for [^{3}H]DSLET sites [Portoghese *et al.*, 1992]. Finally, [^{3}H]naltrindole, a ligand nonselective for δ-opioid receptor subtypes, recognises multiple binding sites in mouse brain but only a single binding site in mouse vas deferens when displaced by [4'-Cl-Phe^4]DPDPE or DELT II [Fang *et al.*, 1994].

In addition to the study using [^{3}H]naltrindole in mouse brain and vas deferens, there have been other studies suggesting that δ-opioid receptors in the brain and the periphery are different. Using antagonists selective for the putative subtypes no variation in actions is observed in the mouse vas deferens, suggesting an homogeneous δ-opioid receptor population exists [Wild *et al.*, 1993a]. Furthermore while BNTX is able to discriminate δ-opioid receptors in the brain this selectivity does not extend to the mouse vas deferens preparation where it is equally potent at inhibiting the actions of DPDPE and DSLET [Portoghese *et al.*, 1992].

The two classifications of δ-opioid subtypes, δ_{cx} / δ_{ncx} and δ_1 / δ_2, may be complementary since DALCE is known to block both the δ_{ncx}-opioid site and the δ_1-opioid site [Rothman *et al.*, 1992]. This implies that the δ_{cx}-opioid site corresponds to the δ_2-opioid site, and therefore the δ_{cx}-opioid agonist CP-OH and the δ_2-opioid antagonist NTB should act at the same receptor. However, in the mouse vas deferens preparation NTB is an antagonist but CP-OH is inactive as either an agonist or an antagonist. Thus further work is required to establish whether the two δ-opioid receptor subtype classifications are superimposable or, for example, whether peripheral δ-opioid receptors need a separate classification.

1.4.3 μ-Opioid receptor subtypes

It has been proposed that μ-opioid receptors should be divided into the subtypes μ_1 and μ_2 [Pasternak & Wood, 1986]. The μ_2-opioid receptor represents the classical morphine receptor whereas the μ_1-opioid receptor is able to bind opiates and enkephalins equally well and with very high affinity (K_D values < 1 nM). Thus under this classification enkephalins bind to δ- and μ_1-opioid receptors [Itzhak & Pasternak, 1986].

Naloxonazine (Fig. 1.10) binds irreversibly to the μ_1-opioid receptor subtype and reversibly to the μ_2-opioid receptor subtype [Hahn *et al.*, 1982]. Binding studies with [^{3}H]naloxonazine suggest that about 40 % of μ-opioid receptors in rat brain are of the μ_1-opioid receptor subtype [Johnson & Pasternak, 1984] and further studies indicate that naloxonazine possesses approximately 10 - fold selectivity for the μ_1-site [Cruciani *et al.*, 1987]. Use of naloxonazine *in vivo* suggests the μ_1-and μ_2-opioid receptor subtypes appear to have different physiological roles, with the μ_1-opioid receptor mediating analgesia and the μ_2-opioid receptor mediating respiratory depression [Ling *et al.*, 1985].

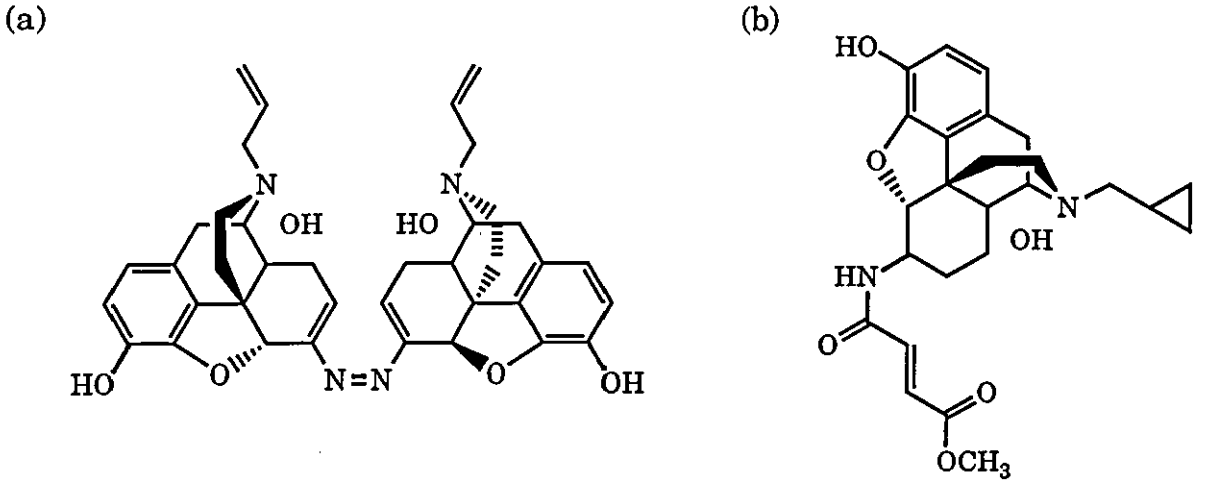


Figure 1.10 (a) naloxonazine and (b) β-funaltrexamine

The irreversible μ-opioid receptor antagonist β-funaltrexamine (Fig. 1.10) blocks μ-opioid mediated responses both *in vivo* and *in vitro* [Takemori & Portoghese, 1985]. Use of this ligand has implied μ-opioid receptor heterogeneity since several studies have demonstrated μ-opioid receptors to be either β-funaltrexamine sensitive or β-funaltrexamine insensitive, the latter representing 30 % of μ-opioid receptor sites in the brain [Rothman *et al.*, 1983; 1987]. Furthermore, in competition experiments μ-opioid receptor ligands are unable to differentiate between β-funaltrexamine sensitive and β-funaltrexamine insensitive sites, confirming that the β-funaltrexamine insensitive sites are a form of μ-opioid receptor [Franklin & Traynor, 1991].

1.5 General Aims

The preceeding discussion suggests heterogeneity of both μ- and δ-opioid receptors based on several pieces of evidence. In particular the differential activity of either μ- or δ-opioid selective ligands and μ / δ interactions. The aim of this work is to study both μ- and δ-opioid ligands in a variety of tissues and under various conditions in order to provide evidence for subtypes and / or agonist interactions as follows:-

a) study μ- and δ-opioid agonists in both the mouse vas deferens and guinea-pig myenteric plexus-longitudinal muscle preparations,
b) examine δ-opioid ligand binding in mouse brain homogenates and novel δ-opioid ligands in both ligand binding assays and isolated tissue preparations,
c) evaluate μ- and δ-opioid receptors in human neuroblastoma SH-SY5Y cells using ligand binding assays and putative subtype selective ligands,
d) examine the ability of ligands to activate G proteins in SH-SY5Y cells as measured by increased incorporation of the labelled nucleotide [^{35}S]GTPγS.

CHAPTER 2
MATERIALS AND METHODS

Science is one thing, wisdom is another. Science is an edged tool with which men play like children, and cut their own fingers.

Sir Arthur Eddington (1882 - 1944)

2.1 Materials

2.1.1 Radiochemicals

[^{3}H]CI977 (5R-(5α,7α,8β)-N-methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]-4-benzofuranacetamide) (0.78 TBq / mmol; 21.1 Ci / mmol), was a gift from Dr. J.C. Hunter, Parke Davis Neuroscience Research Centre, Cambridge.

[^{3}H]DAMGO ([D-Ala^2,MePhe^4,Gly(ol)^5]enkephalin) (2.22 TBq / mmol; 60 Ci / mmol), was from Amersham International plc., Aylesbury.

[^{3}H]Diprenorphine (1.74 TBq / mmol; 47 Ci / mmol), was bought from Amersham International plc., Aylesbury.

[^{3}H]DPDPE ([D-Pen^2,D-Pen^5]enkephalin) (1.27 TBq / mmol; 34.3 Ci / mmol), was from Dupont, NEN® Research Products, Stevenage.

[^{35}S]GTPγS (Guanosine 5'-[γ-thio]triphosphate) (46.1 TBq / mmol; 1245 Ci / mmol), was purchased from Dupont, NEN® Research Products, Stevenage.

[^{3}H]Naltrindole, (1.10 TBq / mmol; 29.7 Ci / mmol), was from Dupont, NEN® Research Products, Stevenage.

[^{3}H]TIPP (Tyr-Tic-Phe-Phe) (Tic = tetrahydroisoquinoline-3-carboxylic acid), (2.02 TBq / mmol; 54.5 Ci / mmol), was bought from Institute of Isotopes, Budapest, Hungary.

[^{3}H] U69593 ((5α,7α,8β)-(+)-N-methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]benzeneacetamide) (2.00 TBq / mmol; 54 Ci / mmol), was bought from Amersham International plc., Aylesbury.

Structures of the radiochemicals are given in Fig. 2.1.

2.1.2 Chemicals

Ecoscint scintillation fluid was from National Diagnostics.

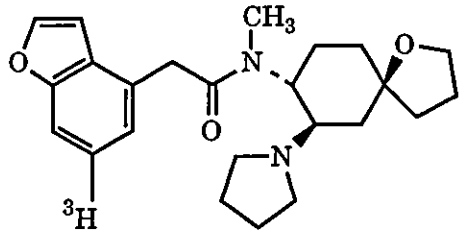
HEPES (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]) , Guanosine diphosphate (GDP), pertussis toxin, Sigmacote® and Trizma base (Tris[hydroxymethyl]aminomethane) were purchased from Sigma, Poole.

$CaCl_2.2H_2O$, ethylenediaminetetraacetic acid (EDTA), Glucose, KCl, KH_2PO_4, $MgSO_4.7H_20$, NaCl and $NaHCO_3$ were bought from Fisons, Loughborough.

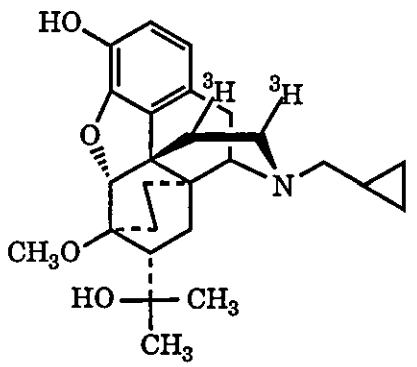
(b)

[3,5,^{3}H]Tyr-D-Ala-Gly-MePhe-NH$(CH_2)_2$-OH

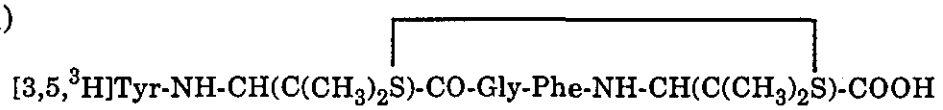
(a)



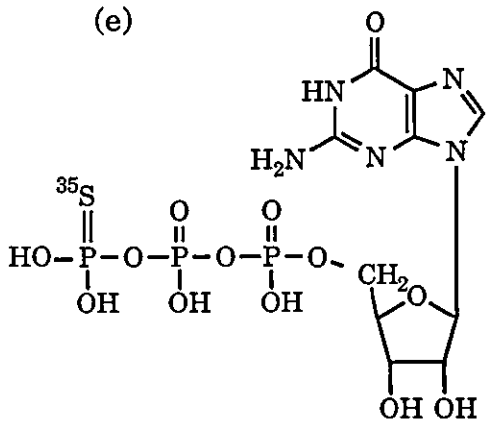
(c)



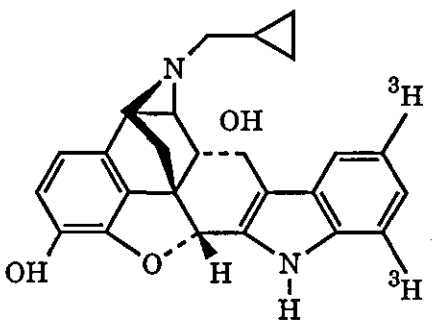
(d)



(e)



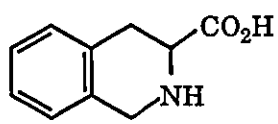
(f)



(g)

[3,5,^{3}H]Tyr-Tic-Phe-Phe-OH

Tic =



(h)

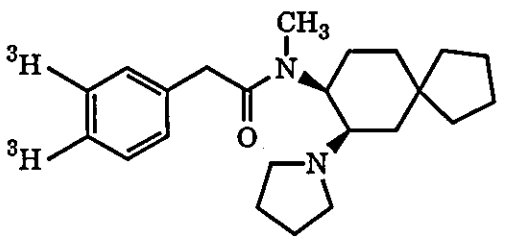


Fig. 2.1 (a) [^{3}H]CI977, (b) [^{3}H]DAMGO, (c) [^{3}H]diprenorphine, (d) [^{3}H]DPDPE, (e) [^{35}S]GTPγS, (f) [^{3}H]naltrindole, (g) [^{3}H]TIPP and (h) [^{3}H]U69593

2.1.3 Drugs

β-Funaltrexamine was donated by Dr. A. Hayes, Glaxo, Ware.

BNTX (7-benzylidenenaltrexone), cyprodime and naltrindole.HCl.5/2H_2O, were purchased from Research Biochemicals Inc., SEMAT, St. Albans.

Etorphine and RX8008M (16-methyl cyprenorphine) were donated by Reckitt & Colman, Hull.

Fentanyl citrate, morphine and naloxone were bought from Sigma, Poole.

H7 (1-(5-isoquinolinesulfonyl)-2-methylpiperazine) was purchased from Calbiochem - Novabiochem (UK) Ltd., Nottingham.

Naltrindole analogues were synthesised by Dr. H. Schmidhammer, Innsbruck University, Austria.

Suramin (8,8'-[carbonylbis[imino-3,1-phenylenecarbonyl-imino(4-methyl-3,1-phenylene)-carbonyl-imino]]bis-1,3,5-naphthalenetrisulfonic acid) was a gift from Prof. S. Nahorski, Leicester University.

2.1.4 Peptides

β-Endorphin (human), [D-Ala^2,MePhe^4,Gly(ol)^5]enkephalin (DAMGO), [D-Pen^2,D-Pen^5]enkephalin (DPDPE), [Leu^5]- and [Met^5]enkephalin were purchased from Sigma, Poole.

[D-Ser^2,Leu^5,Thr^6]enkephalin (DSLET) and [D-Ala^2,D-Leu^5]enkephalin (DADLE) were bought from Research Biochemicals Inc., SEMAT, St. Albans.

[D-Ala^2]deltorphin I (DELT I), and [D-Ala^2, Glu^4]deltorphin II (DELT II) were obtained from Peninsula, St. Helens.

Tyr-Tic-Phe-Phe (Tic = tetrahydroisoquinoline-3-carboxylic acid) (TIPP) was kindly donated by Dr. S.J. Paterson, St. Thomas's hospital, London.

2.1.5 Cell culture media

Dulbecco's HAT medium with L-glutamine, foetal calf serum, Fungizone, L-glutamine, Minimum Essential Medium, penicillin / streptomycin and trypsin / EDTA were bought from Gibco Laboratories, Paisley.

2.1.6 Buffers

The composition of Krebs solution used in isolated tissue studies (mM): NaCl (118), $NaHCO_3$ (29), KCl (4.7), $CaCl_2$ (2.5), $MgSO_4.7H_2O$ (4.0) KH_2PO_4 (1.2) and Glucose (11.1). For mouse and hamster vasa deferentia $MgSO_4.7H_2O$ was omitted.

The Krebs / HEPES buffer used in whole cell ligand binding studies contained (mM):
NaCl (118), $NaHCO_3$ (25), KCl (4.7), $CaCl_2$ (2.5), $MgSO_4.7H_2O$ (1.2) KH_2PO_4 (1.2), Glucose (11.7) and HEPES (10), pH 7.4.

The buffer used in [^{35}S]GTPγS binding studies was (mM):
NaCl (100), $MgCl_2.6H_20$ (10) and HEPES (20), pH 7.4.

The buffer used in membrane binding studies was Tris (50 mM) acidified with HCl to pH 7.4.

2.1.7 Animals

Male Dunkin-Hartley guinea-pigs (250-500 g), were bought from David Hall, Newchurch, Burton-on-Trent.

Male CSI mice (25-30 g) and male Syrian hamsters (140-200 g), were from Nottingham University Medical School.

Male rats (Wistar, Hooded Lister and Sprague Dawley) (150-250 g), were purchased either from Nottingham University Medical School or B & K Universal, Hull.

Animals were fed on a standard laboratory diet and kept on a 12 h light / dark cycle at a temperature of 20°C.

2.1.8 Equipment

Isolated tissue assays

Grass S88 Stimulator, Grass Medical Instruments, Quincy, Mass., U.S.A.

Harvard Universal Oscillograph and transducers, Harvard Apparatus Limited, Edenbridge, Kent.

Ligand binding studies

Brandel Cell Harvester M-48R, Gaithersburg, U.S.A.
Liquid scintillation counter, MINAXI TRI-CARB® 4000 series, United Technologies Packard, Pangbourne.
Polytron PT 10-35 Homogeniser, Kinematica GmbH, Littau, Switzerland.
Tissue Tearor, Biospec products, Bartlesville, OK, U.S.A.
Ultracentrifuge, either LKB Bromma 2330 Ultrospin 55 or Optima TL100, Beckman, High Wycombe.
Whatman GF/B filter strips, Whatman, Maidstone.

Cell culture

Centrifuge, FP510, Labsystems Oy, Finland.
Class II Microbiological Safety Cabinet, Walker Safety Cabinets Ltd, Glossop, Derbyshire.
Incubator, GC4, Grant instruments, Cambridge.
Powerpette, Jencons Scientific Ltd, Leighton Buzzard.
Tissue culture plastics, Gibco laboratories, Paisley.

2.2 Methods

Introduction

The actions of opioids can be examined by a variety of methods. For example, *in vivo* antinociceptive properties of opioids are commonly characterised using the hot water tail-flick test. Alternatively electrophysiology studies investigate the action of opioids on single cells and can provide information about ion channels. The distribution of opioid receptors is elucidated from autoradiographic techniques. However, in order to determine through which receptors a particular opioid mediates its actions, the simplest methods are isolated tissue preparations and ligand binding assays. Since the majority of work that follows is concerned with receptor interactions and subtypes a brief outline of these latter two methods is given below.

Isolated tissue preparations

Many mammalian tissues contain opioid receptors e.g., the vasa deferentia of the rat, hamster and rabbit, and the mouse ileum. However, the tissues most commonly studied are the guinea-pig ileum and the mouse vas deferens preparations. This is because of their durability and also their lack of rhythmic spontaneous contractions.

Electrical stimulation of isolated segments of the guinea-pig ileum (suspended in oxygenated Krebs buffer) produces longitudinal muscle contractions which can be measured either isometrically or isotonically. These contractions can be inhibited by opioid agonists, through inhibition of presynaptic acetylcholine release from the myenteric plexus [Paton, 1957; Cowie *et al.*, 1968]. Importantly the potency of inhibition of contractions by opioids correlates well with analgesic potencies [Kosterlitz & Waterfield, 1975]. Moreover the guinea-pig ileum myenteric plexus-longitudinal muscle preparation is known to contain both functional μ- and κ-opioid receptors [Chavkin *et al.*, 1982]. Thus this tissue is routinely used to screen μ- and κ-opioid agonists and antagonists.

The δ-opioid receptor actions of opioids are usually examined in the mouse vas deferens. Electrical stimulation of mouse vas deferens (bathed in oxygenated Krebs buffer minus magnesium ions) also produces longitudinal

muscle contractions. Opioids inhibit these contractions by prevention of norepinephrine release [Hughes *et al.*, 1975d]. This tissue possesses all three types of opioid receptors, μ, δ and κ, with δ-opioid receptors predominant. Thus, although this tissue is not quite as robust as the guinea-pig ileum myenteric plexus-longitudinal preparation, it is useful when investigating the δ-opioid mediated effects of drugs.

Ligand binding assays

Ligand binding assays usually involve a radioactive form (either ^{125}I or ^{3}H) of opioid agonist or antagonist binding to a membrane preparation of target tissue. The tissue is often a homogenate prepared from rodent brain or cells grown in culture. For a reversible ligand-receptor interaction, ligand molecules bind to receptors to form a ligand / receptor complex. There is a maximum number of specific receptor sites per unit of tissue, $B_{max.}$ At equilibrium, the rate of association equals the rate of dissociation of this complex. The association binding constant, K_A, and the dissociation binding constant, K_D, can be determined under steady-state conditions. Radiolabelled ligand competes with the unlabelled version and in order to determine the amount of radioligand bound and the amount free a suitable separation technique is required. For the majority of tissue preparations rapid filtration is used.

Unfortunately radiolabelled ligands often bind non-specifically to both biological and non-biological matter, such as non-receptor tissue, filters, test tubes etc. A measure of the specific binding is given by the difference between total binding and the binding that occurs in the presence of a large excess of unlabelled ligand which recognises the same specific receptor site. The unlabelled competitive ligand should have high affinity for the radioligand binding site and is better if it is chemically very different to the radioligand to avoid competition at non-receptor sites. Naloxone is commonly used for ligand binding studies of opioids.

Nowadays highly selective tritiated ligands are commercially available and allow the binding of a drug to a particular receptor type to be examined.

The more selective the radioligand the less complex the receptor interactions in a tissue co-expressing more than one opioid receptor type.

Experimental conditions

2.2.1 In vitro preparations

Mouse and hamster vasa deferentia

Vasa deferentia were quickly removed from CSI mice or Syrian hamsters and prepared for field stimulation in 3 ml siliconised organ baths (using Sigmacote) containing Krebs, minus $MgSO_4.7H_20$, buffer solution at 37°C (aerated with 5 % CO_2 in 95 % O_2). An electrical pulse train of 750 ms duration, with a 250 ms delay, 3 peaks / s and a supramaximal voltage, was used to stimulate the tissues *via* platinum ring electrodes. The contractions were recorded isotonically.

Electrical stimulation was initiated after an equilibrium period of 90 min. Firstly the supramaximal voltage was determined. Dose-response curves of agonists were constructed for inhibition of contractions by cumulative dosing; when the maximal response to one dose had been achieved, the next dose was added and so on until approximately 80 % inhibition was attained. The tissue was then washed by overflow and allowed to recover to its control twitch height, it was allowed to rest for approximately 15 min before being tested again.

Guinea-pig ileum myenteric plexus-longitudinal muscle bioassay

Lengths (5 cm) of myenteric plexus-longitudinal muscle were prepared from the small intestine of guinea-pigs (Dunkin-Hartley) in Krebs solution at a temperature of 37°C. The tissues were stimulated using square wave pulses of supramaximal voltage, of 0.4 ms duration and a frequency of 0.16 Hz. The remainder of the procedure is the same as for vasa deferentia, described above.

Calculation of antagonist affinity values

The tissue was incubated with the antagonist for 10 min (unless otherwise stated) before the dose-response curve to the agonist was performed. Antagonist affinities were determined as equilibrium dissociation constants (K_e values). Unless otherwise stated K_e values were determined with a single dose of antagonist using the equation:

$$K_e = [\text{antagonist}] / (DR-1)$$

where DR = IC_{50} in presence of antagonist / IC_{50} in absence of antagonist [Kenakin, 1984].

Alternatively a Schild plot (Fig. 2.2) was performed [Arunlakshana & Schild, 1959]; the tissue was treated in the same manner except that three doses of the antagonist were tested. A graph of log(DR-1) versus log[antagonist] was plotted, and the K_e was determined from the x-intercept.

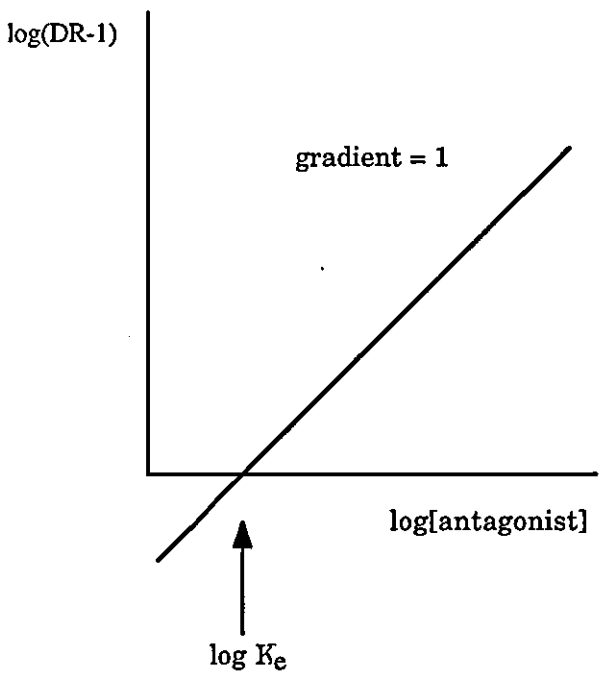


Fig. 2.2 Schild plot

2.2.2 Ligand binding assays

The general method for guinea-pigs, mice, and rats is as follows:
Animals were decapitated and the brain (minus cerebellum) was suspended in 8 volumes of 50 mM Tris-HCl buffer (pH 7.4). The tissue was homogenised using a Polytron PT 10-35 (setting 7, 5 s). The homogenate was centrifuged at 50,000 g for 20 min, the supernatant discarded, and the pellet resuspended in Tris-HCl buffer. The membrane suspension was incubated at 37°C for 30 min (to help dissociation of endogenous opioids) and then recentrifuged as before. The supernatant was once more discarded and the resulting pellet was resuspended by homogenisation in Tris-HCl buffer to give a known concentration in the range of 1:50 - 1:100 w / w (original weight of wet tissue).

The membrane homogenates were incubated for a set time with the suitable tritiated ligand (and competing drugs if required), either in duplicate or triplicate. In order to separate bound and free ligand the incubation mixture was rapidly vacuum-filtered and washed twice with 4 mls of ice-cold Tris-HCl buffer. The filter papers were placed in scintillation vials to which Ecoscint was added and the amount of bound tritiated ligand was determined by use of a liquid scintillation counter.

Non-specific binding was determined as the binding remaining with 10 μM naloxone present, while specific binding was calculated by subtracting the non-specific binding from the total binding. The IC_{50} was determined from concentration effect curves of the data (either by hand or from a Hill plot [Hill, 1910]) and by applying the equation of Cheng and Prusoff [1973] equilibrium inhibition constants (K_i values) were calculated:

$$K_i = (IC_{50} \times K_D) / (K_D + [L^*])$$

Washing experiments

The general procedure outlined above was followed but after the initial centrifugations and incubation the tissue was resuspended and the resultant homogenate was divided in half. One half was kept on ice as a control and the other half was washed four times. The centrifugation procedure was as per normal and washings between centrifugations were for only 10 min at 25°C, this was to minimise degradation of the tissue. The supernatants were saved

and then spun at 100,000 g. The resulting pellets were combined and then resuspended in the same volume of Tris-HCl buffer as the control and washed pellets. Binding was analysed as above.

Determination of binding characteristics by Scatchard analysis

Membrane homogenates (from brain tissue diluted 1:70 w / w containing approximately 1 mg / ml protein) were incubated at 25°C for 40 min with varying concentrations of tritiated ligand (0.005 - 20 nM) in the presence of either water (control) or 10 μM naloxone to define non-specific binding.

Binding capacities (B_{max}) and equilibrium dissociation constants (K_D values) were calculated either from Scatchard transformation of the specific binding data using the 'EBDA' program [McPherson, 1985] or fitted by-eye. The protein content of the tissue was determined using the Lowry method [Lowry, 1951].

2.2.3 Swim-stress and ECS treatment of rats

This section of experiments was performed in conjunction with Dr. H. Jackson, Reckitt and Colman Psychopharmacology Unit, School of Medical Sciences, University Walk, Bristol, BS8 1TD.

Male Wistar rats (B & K Universal, Hull) (150-200 g) were used in these experiments. The rats were subjected to either (a) 3 min swim-stress at 25°C as previously described [Jackson *et al.*, 1989a], or (b) electroconvulsive treatment of 100 mA for 0.5 s [Jackson & Nutt, 1991]. The rats were sacrificed at various time intervals after these treatments, the brains being rapidly removed and frozen over liquid nitrogen, for later determination of δ-opioid binding.

2.2.4 Cell culture

SH-SY5Y human neuroblastoma cells

The SH-SY5Y cell line was kindly donated by Dr. D. Lambert, Department of Anaesthesia, Leicester University. The cells were cultured in Minimum Essential Medium (MEM) supplemented with 10 % foetal calf serum, fungizone® (2.5 μg / ml amphotericin), L-glutamine (584 μg / ml), penicillin (50 units / ml) and streptomycin (50 μg / ml) at 37°C in a humidified 5 % CO_2 atmosphere. Cells were passaged when confluent (using trypsin (500 μg / ml) and EDTA (200 μg / ml) in a physiological solution) and fed thrice weekly. The cells were rinsed twice and then harvested in HEPES (20 mM, pH 7.4) buffered saline containing 1 mM EDTA and dispersed by agitation. A cell pellet was obtained by centrifugation at 250 g, the pellet was twice washed in Krebs / HEPES buffer and was then used in whole cell experiments.

If membranes were needed for ligand binding assays then the cell pellet was washed in MEM minus supplements before being suspended in Tris-HCl (pH 7.4). The pellet was homogenised using a tissue tearer. The centrifugation and incubation procedure was as described above, in section 2.2.2. The resulting pellet was resuspended in Tris-HCl buffer. Unless otherwise stated 100-200 μg of cell protein were added per ml of assay. The ligand binding assay was conducted in the same manner as described previously (section 2.2.2).

For alkylation experiments whole cell suspensions were incubated in Krebs / HEPES buffer (pH 7.4) in the presence of β-funaltrexamine 1-1000 nM for 1 h at 37°C. Both treated and control cells were then collected by centrifugation (250 g) and washed 4 times to remove any unbound drug. Control experiments showed this gave maximal washout of the non-covalently bound affinity ligand. Either whole cells or membranes were used in standard ligand binding assays.

Neuroblastoma x glioma NG108-15 hybrid cells

NG108-15 cells were kindly donated by Dr. M. Keen, Dept. of Pharmacology, University of Birmingham. The cells were cultured in Dulbecco's HAT (hypoxanthine (13.6 μg / ml), aminopterin (0.176 μg / ml) and thymidine (3.88 μg / ml)) medium supplemented with 5 % foetal calf serum and L-glutamine (584 μg / ml), at 37°C in a humidified 5 % CO_2 atmosphere. Cells were grown as monolayers, passaged when confluent and fed on alternate days. The cells were harvested in Dulbecco's Modified Essential Medium (DMEM) minus supplements by mechanical agitation. A cell pellet was obtained in the same manner as described previously for SH-SY5Y cells. Unless otherwise stated 100-200 μg of cell protein were used per ml of assay.

2.2.5 [^{35}S]GTPγS binding

Cells were harvested into [^{35}S]GTPγS binding buffer ((mM) NaCl (100), $MgCl_2.6H_20$ (10) and HEPES (20)) and homogenised with a tissue tearer, then centrifuged at 50,000 g for 15 min. The supernatant was discarded and the pellet rehomogenised and once more centrifuged as before. The resulting pellet was resuspended to give 100-200 μg of protein per ml of assay. The membrane homogenates were incubated for 1 h at 30°C in the presence of 100 pM [^{35}S]GTPγS, 3 μM GDP and varying concentrations of opioid. The tissue was then rapidly vacuum-filtered and washed twice with 4 mls of ice-cold [^{35}S]GTPγS binding buffer. The filter papers were placed in scintillation vials to which Ecoscint was added and the amount of bound [^{35}S]GTPγS was determined by counting in the carbon-14 channel of a liquid scintillation counter.

The amount of stimulated [^{35}S]GTPγS binding is given by the difference in dpm values in the presence and absence of opioid. The EC_{50} (effective concentration producing a 50 % maximal response) and K_i values were calculated as described previously (section 2.2.2). The maximum possible stimulation was routinely given by determining the response to 3 μM fentanyl.

2.2.6 Tolerance studies in SH-SY5Y cells

The level of opioid agonist-stimulated [^{35}S]GTPγS binding was examined in control and chronically opioid-exposed cells. Cells were grown as described above (section 2.2.4) and on the fifth day after passaging (when the cells were confluent) the cells were fed and either morphine (10 μM) or DAMGO (10 μM) was added to the medium. After 2 days the cells were harvested and experiments conducted. In parallel, control cells were also fed on the fifth day and prior to harvesting either morphine (10 μM) or DAMGO (10 μM) was added to the medium for 5 min. This was designed to demonstrate that any effect observed in the tolerant cells was a chronic rather than an acute effect of the opioid treatment.

To determine the effect of enzyme inhibitors on the development of morphine-induced tolerance (10 μM, 24h) cells were incubated in the absence or presence of morphine together with suramin (8,8'-[carbonylbis[imino-3,1-phenylenecarbonyl-imino(4-methyl-3,1-phenylene)carbonyl-imino]]bis-1,3,5-naphthalenetrisulfonic acid) or H7 (1-(5-isoquinolinesulfonyl)-2-methyl-piperazine). Thus the level of tolerance was tested in control cells and in the presence of either suramin or H7.

Statistical comparisons were made where appropriate by Student's t-test, where $P < 0.05$ was considered significant.

CHAPTER 3
μ / δ INTERACTIONS IN ISOLATED TISSUES

The great tragedy of science - the slaying of a beautiful hypothesis by an ugly fact.

T.H. Huxley (1825 - 95)

3.1 Introduction

The ability of δ-opioid agonists to modulate the *in vivo* actions of μ-opioid agonists to promote or decrease their activity has been demonstrated. In addition, evidence from ligand binding assays and biochemical experiments suggests the existence of μ / δ interactions, as discussed in section 1.3.1. Importantly, interactions between co-administered μ- and δ-opioid agonists could be of major clinical value since it may be possible to overcome tolerance and the reduced pain-relieving ceiling of certain lower efficacy μ-opioid ligands. For example, it has been shown that while [D-Pen^2,D-Pen^5]-enkephalin (DPDPE) and [Leu^5]enkephalin increase the potency of morphine, if either compound is co-administered with morphine it is only the amount of morphine that determines the rate of development of tolerance [Jiang *et al.*, 1990b]. To investigate and understand this phenomenon it would be helpful if such interactions could be observed *in vitro.*

This study examines whether two isolated tissue preparations which respond to opioid agonists by inhibition of electrically-induced contractions, namely the mouse vas deferens and the guinea-pig myenteric plexus-longitudinal muscle, might be suitable models to address this phenomenon. The mouse vas deferens contains a predominantly δ-opioid receptor population, which recent studies suggest may be of the putative δ_2-opioid subtype [Wild *et al.*, 1993a], with μ- and κ-opioid receptors also present [Lord *et al.*, 1977]. In this tissue, evidence for an interaction between μ-and δ-opioid receptors has been obtained by examining the ability of various ligands to protect the δ-opioid site from alkylation by the affinity ligand β-chlornaltrexamine [Sheehan *et al.*, 1986]. The guinea-pig ileum myenteric plexus-longitudinal muscle contains functional μ- and κ-opioid receptors [Chavkin *et al.*, 1982]. Whilst δ-opioid receptors have been shown in ligand binding [Leslie *et al.*, 1980] and electrophysiological studies [Egan & North, 1981], ligand occupation of these sites does not lead to a blockade of electrically induced contractions [Lord *et al.*, 1977]. Therefore we have considered whether this δ-opioid receptor population might be able to modulate the actions of μ-opioid agonists.

Since modulation of μ-opioid agonists has been observed both in the presence of sub-effective [Jiang *et al.*, 1990a] and EC_{50} [Malmberg & Yaksh, 1992] doses of δ-opioid agonists each of these methodologies were tested. In

addition, evidence of a μ- / δ-opioid complex has been illustrated using β-endorphin, which may bind simultaneously to μ- and δ-opioid sites [Bals-Kubik *et al.*, 1990] thus this ligand was evaluated in the mouse vas deferens preparation.

3.2 Results

3.2.1 Mouse vas deferens preparation

The δ-opioid agonists DPDPE [Mosberg *et al.*, 1983], [D-Ala^2,Glu^4]deltorphin II (DELT II) [Erspamer *et al.*, 1989], [Met^5]- and [Leu^5]enkephalin [Lord *et al.*, 1977] were all full agonists in the field stimulated mouse vas deferens preparation with potencies (IC_{50} values) of 0.70 ± 0.06 , 0.28 ± 0.03 , 5.7 ± 1.2 and 6.6 ± 0.9 nM respectively (Fig. 3.1). Concentrations of δ-opioid agonists affording < 10 % inhibition were chosen as sub-effective concentrations, i.e., 0.1 nM for DPDPE and DELT II or 1.0 nM for [Met^5]- and [Leu^5]enkephalin, in subsequent experiments. Concentration-effect curves were obtained for the μ-opioids [D-Ala^2,MePhe^{4}Gly(ol)^5]enkephalin (DAMGO) [Handa *et al.*, 1981] and morphine and repeated in the presence of a co-administered δ-opioid ligand at the chosen sub-effective concentration. There was no significant difference in the potencies for the μ-opioid ligands under the two conditions (Table 3.1), nor in the slope of the curves (Fig. 3.2). Similarly no attenuation or enhancement of the effect was observed when the δ-opioid agonists were added 2, 4 or 15 min prior to the re-determination of the μ-opioid agonist response curve (Table 3.1).

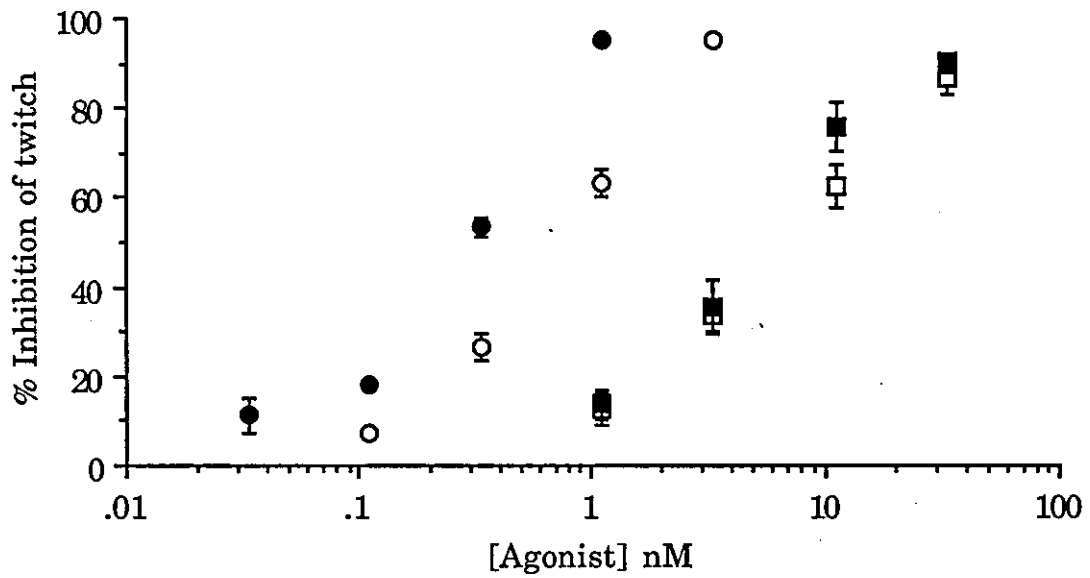


Figure 3.1 Dose-response curves in the mouse vas deferens for DELT II (●), DPDPE (O), [Leu^5]enkephalin (■) and [Met^5]-enkephalin (□). Values represent means ± s.e.mean where n ≥ 3.

Table 3.1 Potency (IC_{50}) of DAMGO and morphine in the mouse vas deferens preparation, in the absence and presence of sub-effective concentrations of δ-opioid agonists added at different times prior to the μ-opioid agonist.

μ-agonist	δ-agonist	Time (min)	IC_{50} (nM)
DAMGO			15.3 ± 1.7
	DPDPE	0 (coadministered)	12.1 ± 1.1
		2	12.1 ± 0.9
		4	11.4 ± 1.6
		15	18.3 ± 1.3
	DELT II	0 (coadministered)	16.0 ± 2.4
		2	10.5 ± 1.4
		4	10.5 ± 0.6
		15	13.2 ± 0.7
Morphine			180 ± 13
	DPDPE	0 (coadministered)	200 ± 16
		2	130 ± 21
		4	122 ± 17
		15	195 ± 30
	DELT II	0 (coadministered)	150 ± 6.5
		2	192 ± 44
		4	161 ± 24
		15	248 ± 60
	[Leu^5]enkephalin	0 (coadministered)	233 ± 26
	[Met^5]enkephalin	0 (coadministered)	182 ± 12

Sub-effective concentration of 0.1 nM for DPDPE and DELT II, and 1.0 nM for [Met^5]- and [Leu^5]enkephalin. Values represent means ± s.e.mean where $n \geq 3$. None of the results are significantly different from additive where $P < 0.05$.

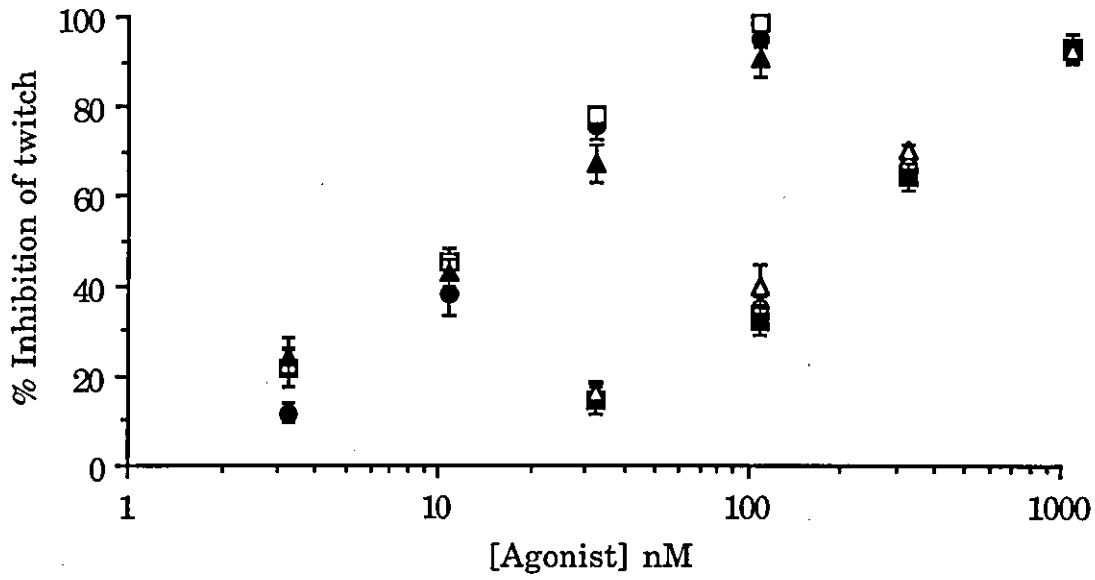


Figure 3.2 Dose-response curves for DAMGO and morphine coadministered with sub-effective concentrations of DPDPE (0.1 nM) or DELT II (0.1 nM) in the mouse vas deferens. DAMGO (●), DAMGO + DPDPE (□), DAMGO + DELT II (▲), morphine (O), morphine + DPDPE (■) and morphine + DELT II (Δ). Values represent means ± s.e.mean where n ≥ 3.

DPDPE added at a concentration causing 50 % inhibition of the electrically-induced twitch also failed to significantly alter the IC_{50} value for the μ-opioid agonists morphine (180 ± 13 nM in the absence of DPDPE compared to 212 ± 22 nM in the presence of DPDPE) and DAMGO (15.3 ± 1.7 nM in the absence of DPDPE compared to 14.2 ± 3.6 nM in the presence of DPDPE) when co-administered. The same lack of effect was observed using DELT II and also [Met^5]- and [Leu^5]enkephalin. The results are summarised in Table 3.2. The dose-response curves for DAMGO and morphine in the presence of an IC_{50} dose of DPDPE are shown in Fig. 3.3.

Table 3.2 Potency (IC_{50}) of DAMGO and morphine in the mouse vas deferens preparation, alone and in the presence of IC_{50} concentrations of DPDPE, DELT II, [Met^5]- and [Leu^5]enkephalin.

μ-agonist	δ-agonist	IC_{50} (nM)
DAMGO		15.3 ± 1.7
	DPDPE	14.2 ± 3.6
	DELT II	13.0 ± 6.0
Morphine		180 ± 13
	DPDPE	212 ± 22
	DELT II	132 ± 6.4
	[Met^5]enkephalin	232 ± 27
	[Leu^5]enkephalin	213 ± 53

IC_{50} concentrations of δ-opioid ligands used were (nM): DPDPE (0.7), DELT II (0.3), [Met^5]- and [Leu^5]enkephalin (6). Values represent means ± s.e.mean from 3 or more separate experiments. None of the results are significantly different from controls, where $P < 0.05$.

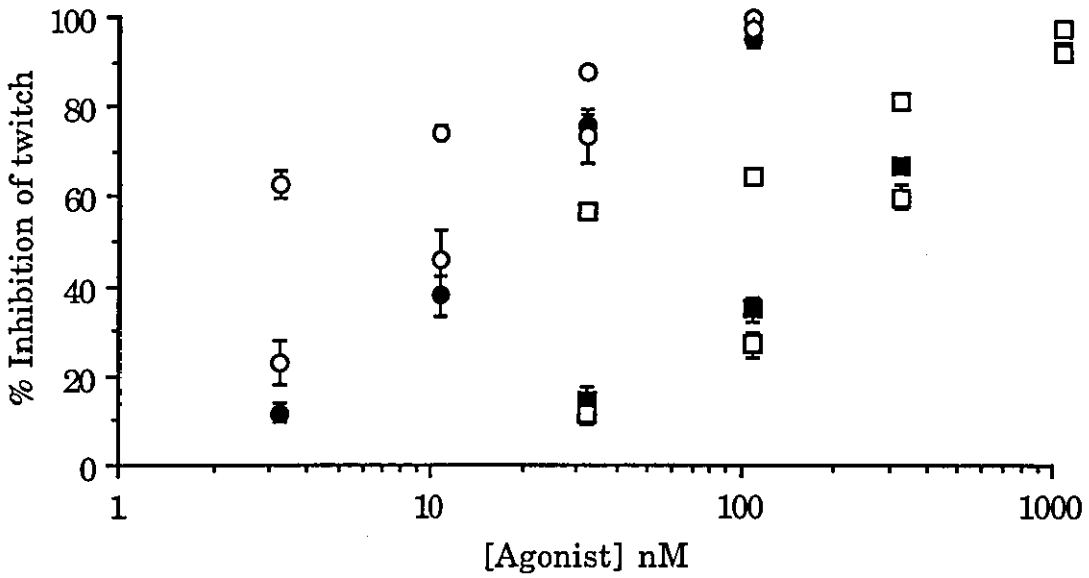


Figure 3.3 Dose-response curves for DAMGO and morphine coadministered with IC_{50} concentration of DPDPE (0.7 nM) in the mouse vas deferens. DAMGO (●), DAMGO + DPDPE (O), DAMGO curve adjusted for presence of IC_{50} concentration of DPDPE (O), morphine (■), morphine + DPDPE (□) and morphine curve adjusted for presence of IC_{50} concentration of DPDPE (□). Values represent means ± s.e.mean where n ≥ 3.

β-Endorphin has similar affinity for μ- and δ-opioid receptors [Kosterlitz *et al.*, 1986; Rothman & Westfall, 1982]. This compound was a full agonist in the mouse vas deferens affording an IC_{50} of 31.3 ± 2.7 nM. The dose-effect curve for β-endorphin was shifted to the right in the presence of the non-selective opioid antagonist naloxone (200 nM), affording an apparent K_e for naloxone of 26.0 ± 4.3 nM, indicative of an action at δ-opioid receptors (Fig. 3.5). This was confirmed by the effectiveness of the δ-opioid selective antagonist naltrindole [Rogers *et al.*, 1990b] in antagonising the β-endorphin response affording an apparent K_e of 0.86 ± 0.1 nM, and the lack of antagonism by the μ-opioid selective antagonist cyprodime [Schmidhammer *et al.*, 1989] affording an apparent K_e > 300 nM (Fig. 3.4).

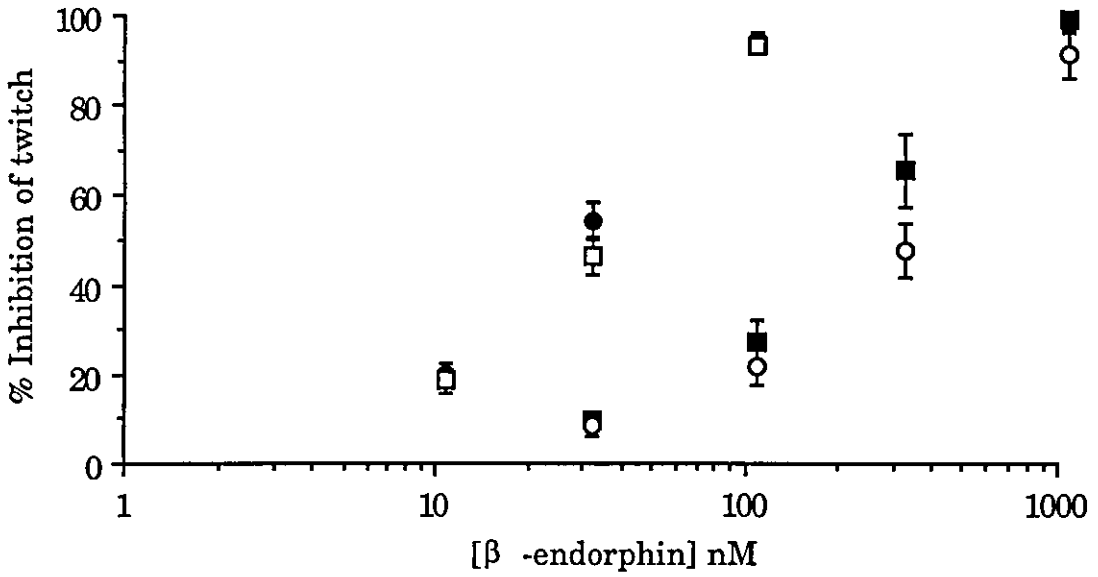


Figure 3.4 Dose-response curves for β-endorphin alone and in the presence of various antagonists in the mouse vas deferens. β-endorphin (●), β-endorphin + 300 nM cyprodime (□), β-endorphin + 200 nM naloxone (■) and β-endorphin + 6 nM naltrindole (O). Values represent means ± s.e.mean where n ≥ 3.

3.2.2 Guinea-pig ileum myenteric plexus-longitudinal muscle

The δ-opioid agonists DPDPE (IC_{50} = 4740 nM) and DELT II (IC_{50} = 2180 nM) were very weakly active in inhibiting the electrically induced twitch of this tissue compared to values of morphine (IC_{50} = 100 nM) and DAMGO (IC_{50} = 8.3 nM) (Fig. 3.5). Co-administration of a concentration of DPDPE of 0.3 μM (which was just sub-effective) with the μ-opioid ligands caused no alteration in the IC_{50} values of DAMGO (8.3 ± 2.0 nM in the absence of DPDPE and 7.3 ± 1.2 nM in the presence of DPDPE) or morphine (100 ± 34 nM in the absence of DPDPE and 92 ± 37 nM in the presence of DPDPE). A sub-effective concentration of DELT II was also unable to modulate the potency of DAMGO, which gave an IC_{50} of 24.1 ± 5.7 nM. Similary no change was observed when either DPDPE or DELT II were used at a concentration which inhibited 50 % of the twitch response (Table 3.3).

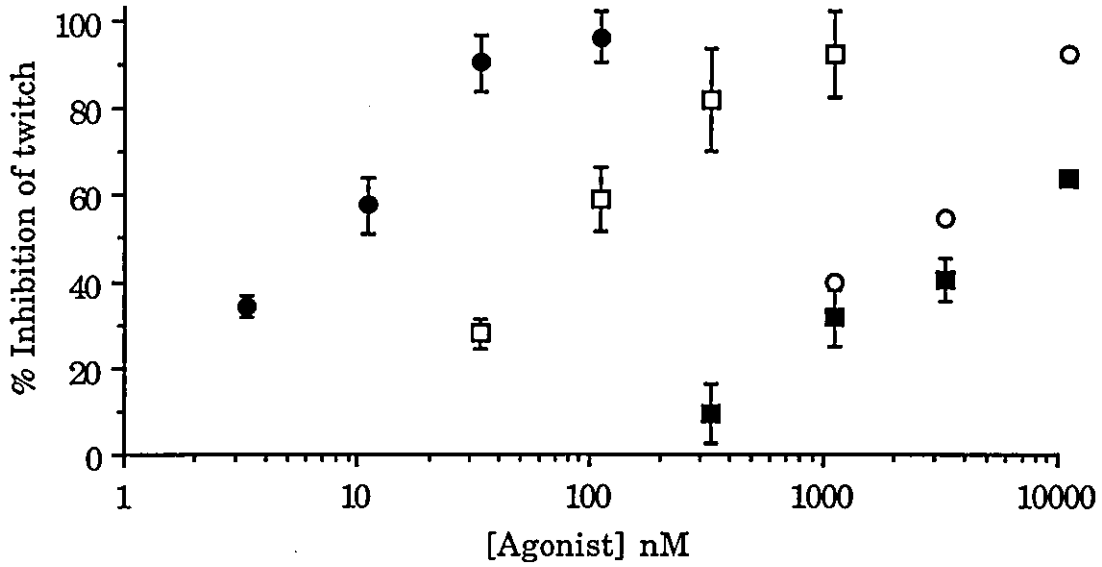


Figure 3.5 Dose-response curves for selective μ- or δ-opioid agonists in the guinea-pig ileum myenteric plexus-longitudinal muscle, DAMGO (●), morphine (□), DELT II (O) and DPDPE (■). Values represent means ± s.e.mean where n ≥ 3.

Table 3.3 Potency (IC_{50}) of DAMGO and morphine in the guinea-pig ileum myenteric plexus-longitudinal muscle preparation, alone and in the presence of IC_{50} concentrations of DPDPE and DELT II.

μ-agonist	δ-agonist	IC_{50} (nM)
DAMGO		8.3 ± 2.0
	DPDPE	13.3 ± 3.2
	DELT II	10.7 ± 5.5*
Morphine		100 ± 34
	DPDPE	120 ± 48
	DELT II	116 ± 37*

IC_{50} concentrations of δ-opioid ligands used were (μM): DPDPE (5) and DELT II (2). Values represent means ± s.e.mean where $n \geq 3$ or *where $n = 2$ the range. None of the results are significantly different from controls, where $P < 0.05$.

3.3 Discussion

The results show that in the mouse vas deferens preparation modulation of the action of μ-opioid agonists by δ-opioid agonists cannot be demonstrated. This applies to both peptide and non-peptide μ-opioid agonists using either sub-effective levels of δ-opioid agonists or concentrations of δ-opioid agonists which by themselves cause 50 % inhibition of the electrically-induced twitch. This is perhaps surprising since both μ- and δ-opioid receptors exist in this tissue and indeed have been shown to exist on the same neuron [Rogers & Henderson, 1990a]. The results thus support the suggestion of an anatomical but not functional coupling of the receptors [Sheehan *et al.*, 1986a]. Furthermore, in brain tissues μ- and δ-opioid receptors may be functionally coupled since interactions have been shown to occur in the inhibition of the release of neurotransmitters from brain slice preparations. Thus the selective δ-opioid agonist DPDPE blocks the morphine-mediated inhibition of μ-opioid D-1 dopamine receptor-stimulated cAMP efflux [Schoffelmeer *et al.*, 1987]. Furthermore, preincubation of the slices with the irreversible δ-opioid receptor selective antagonist fentanyl isothiocyanate (FIT) inhibits the direct effect of DPDPE, has no effect on DAMGO but does prevent the antagonism of DAMGO by naloxone [Schoffelmeer *et al.*, 1988].

The results may suggest that differences exist in opioid receptor systems between the brain and periphery. Indeed there is evidence that the δ-opioid receptor populations in the brain and periphery are different. The conformationally constrained peptide [D-Ala^2,(2R,3S)-V^{E}Phe^4,Leu^5]enkephalin (CP-OH) is very weakly active in the mouse vas deferens although it has high affinity for the δ-opioid receptor in rat brain and can modulate the antinociceptive effects of morphine [Shimohigashi *et al.*, 1987; 1988]. Additionally, whereas the tritiated ligand *p*-Cl-DPDPE has similar affinity for δ-opioid receptors in the mouse vas deferens and rat brain, the methyl ester of CP-OH (CP-OMe) has a 33-fold lower affinity for δ-opioid receptors in the mouse vas deferens than the brain [Vaughn *et al.*, 1990].

Recent *in vivo* evidence has suggested a division of the δ-opioid receptor population in the central nervous system into putative δ_1 and δ_2 subtypes; for review see section 1.4.2. There is evidence to suggest that the δ-opioid receptor involved in modulating centrally μ-opioid mediated effects is of the putative δ_2 subtype [Porreca *et al.*, 1992; Kamei *et al.*, 1993]. However in the mouse vas

deferens preparation, experiments with selective putative δ-opioid subtype antagonists point to a single homogeneous δ-opioid receptor population [Wild *et al.*, 1993a]. The present finding that no difference in modulatory effects is seen with either a putative δ_1-opioid agonist (i.e., DPDPE) or a putative δ_2-opioid agonist (i.e., DELT II) would support this. If indeed δ_2-opioid receptors are able to modulate μ-opioid receptor mediated effects then DELT II would be expected to potentiate μ-opioid agonists to a greater extent than DPDPE. Since no moduation was observed with either agonist it can be suggested that no δ_2-opioid receptors are complexed with μ-opioid receptors in the mouse vas deferens preparation.

The endogenous opioid peptide β-endorphin has similar affinity for μ- and δ-opioid receptors [Kosterlitz *et al.*, 1986; Rothman & Westfall, 1982]. The peptide has been demonstrated to bind to both μ- and δ-opioid sites simultaneously in rat brain membrane preparations [Schoffelmeer *et al.*, 1990] and can be inhibited by both μ- and δ-opioid antagonists, providing evidence for receptor interaction. In addition the β-endorphin mediated dose-dependent increase of dopamine in rat nucleus accumbens has been demonstrated to result from activation of both μ- and δ-opioid receptors [Spanagel *et al.*, 1990]. However this does not appear to be the case in the mouse vas deferens since the present studies with the selective antagonists naltrindole and cyprodime indicate that β-endorphin was exerting its actions solely through activation of δ-opioid receptors. (Naltrindole has a 20-fold higher affinity for δ- over μ-opioid receptors whereas cyprodime possesses over 100-fold selectivity for μ- as opposed to δ-opioid receptors in the mouse vas deferens [Rogers *et al.*, 1990b; Schmidhammer *et al.*, 1989]). These results are in direct disagreement with those of Shook *et al.*, [1988] where β-endorphin was blocked by both the μ-opioid selective antagonist CTP (D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-NH_2) and the δ-opioid selective antagonist ICI 174,864, in *in vivo* analgesia tests in the mouse and *in vitro* in the mouse isolated vas deferens preparation. The value of these results is unclear since the high dose of CTP used (1 μM) is known to have agonist effects in the mouse vas deferens preparation [Wire *et al.*, 1987].

Although the mouse vas deferens contains both functional δ- and μ-opioid receptors, the level of μ-opioid receptors is low compared to the level of δ-opioid receptors which have a large receptor reserve [Leslie, 1987]. Thus only low numbers of δ-opioid receptors need to be activated by high affinity δ-opioid ligands in order to give a response. However, in order to see interactions similar levels of receptor expression may be important.

In the myenteric plexus of the guinea-pig ileum the studies confirmed the lack of potency of δ-opioid ligands using both putative δ_1 preferring and putative δ_2 preferring opioid ligands. Consistent with previous studies the δ-opioid ligands did have agonist properties but only at high concentrations which suggests an action *via* μ-opioid receptors in this tissue [Schulz *et al.*, 1981]. The lack of antagonism of the μ-opioid agonists in the presence of δ-opioid ligands confirmed the very low affinity of δ-opioid ligands for μ-opioid receptors. Any δ-opioid receptors present in the tissue were also unable to modulate the actions of the μ-opioid agonists since with either DPDPE or DELT II, at doses well in excess of those expected to fully occupy δ-opioid receptors, no changes in the μ-opioid agonist dose-response curves were observed.

In conclusion the results indicate a lack of interaction between μ- and δ-opioid agonists at any level in the pathway from receptor to effector in either the mouse vas deferens or the guinea-pig myenteric plexus-longitudinal muscle. Although the range of ligands studied was not exhaustive these results lend support to the proposal [Shimohigashi *et al.*, 1987; Vaughn *et al.*, 1990; Portoghese *et al.*, 1992; Fang *et al.*, 1994] that differences exist between types of opioid receptors, or opioid systems in the periphery and those involved centrally in antinociception [Vaught *et al.*, 1979; Lee *et al.*, 1980; Jiang *et al.*, 1990a], inhibition of gut propulsion [Heyman, 1987], volume induced contractions of the bladder [Sheldon *et al.*, 1989] and antitussive activity [Kamei *et al.*, 1993; 1991].

CHAPTER 4
STUDIES OF δ-OPIOID RECEPTORS / SUBTYPES

I know nothing except the fact of my ignorance.

Socrates (469 - 399 BC)

4.1 Introduction

There is considerable evidence from both *in vivo* studies and ligand binding assays to support the hypothesis of the existence of δ-opioid receptor subtypes, see section 1.4.2 (for review see Traynor & Elliott, 1993).

However, to date, cloning experiments have only revealed one type of δ-opioid receptor across a range of species [Uhl *et al.*, 1994]. Some evidence exists to suggest that the clone currently isolated may be of the δ_2 subtype since putative δ_2 ligands, [D-Ser^2,Leu^5,Thr^6]enkephalin (DSLET), [D-Ala^2]deltorphin II (DELT II) and naltriben (NTB), have slightly higher affinity than putative δ_1 ligands, [D-Pen^2,Pen^5]enkephalin (DPDPE) and 7-benzylidenenaltrexone (BNTX), at this cloned δ-opioid receptor as assessed by ligand-binding assays [Raynor *et al.*, 1994]. For instance the K_i values of DPDPE and DSLET were found to be 14 and 4.8 nM respectively, whereas the widely accepted K_i values are namely, 2.7 and 1.8 nM [Leslie, 1987].

The aim of the present studies was to seek further evidence of the existence or otherwise of δ-opioid receptor subtypes.

Table 4.1 Classification of putative δ_1- and δ_2-opioid ligands.

δ_1		δ_2	
Agonists	Antagonists	Agonists	Antagonists
DPDPE	BNTX	DELT I & II	Naltriben
DADLE	DALCE	DSLET	5'NTII

4.2 Results

4.2.1 Evidence for δ-opioid receptor subtypes

Displacement of [^{3}H]DPDPE by Deltorphin I

As reported earlier (see section 1.4.2) the displacement of [^{3}H][D-Ala^2]deltorphin I (DELT I) in rat brain homogenates by DPDPE yields a biphasic curve providing evidence for two subtypes of δ-opioid receptors [Negri *et al.*, 1991]. DPDPE and DELT I receptor binding heterogeneity was further investigated by conducting the reverse experiment, the displacement of [^{3}H]DPDPE from mouse brain by DELT I.

Specifically bound [^{3}H]DPDPE (2 nM) was displaced from mouse brain homogenates by DELT I. However, although all known parameters were kept the same e.g., pH, temperature of incubation, preparation time and method, and the animals were of the same age, sex, from the same supplier and housed under identical conditions, the results were not consistent. On seven out of ten occasions DELT I was unable to displace approximately 20 % of the specifically bound [^{3}H]DPDPE affording an IC_{50} of 37.5 nM and a Hill slope of 0.68 but in the remainder of the experiments all the specifically bound [^{3}H]DPDPE was displaced (IC_{50} = 46.2 nM and slope = 1.21) (Figs 4.1 to 4.6). Thus in some experiments DELT I appparently recognised two δ-opioid binding sites or possibly two affinity states labelled by [^{3}H]DPDPE. When the results from all ten experiments were combined an IC_{50} of 29 nM and a slope of 0.75 were obtained from the Hill plot.

One possible explanation for the inconsistent results is that the binding site / state not recognised by DELT I is labile and is sometimes lost during the preparation of the membranes. Therefore, rather than use a Polytron to homogenise the tissue a milder treatment with a glass Potter Elvejham homogeniser was employed. However, similar inconsistent results were achieved.

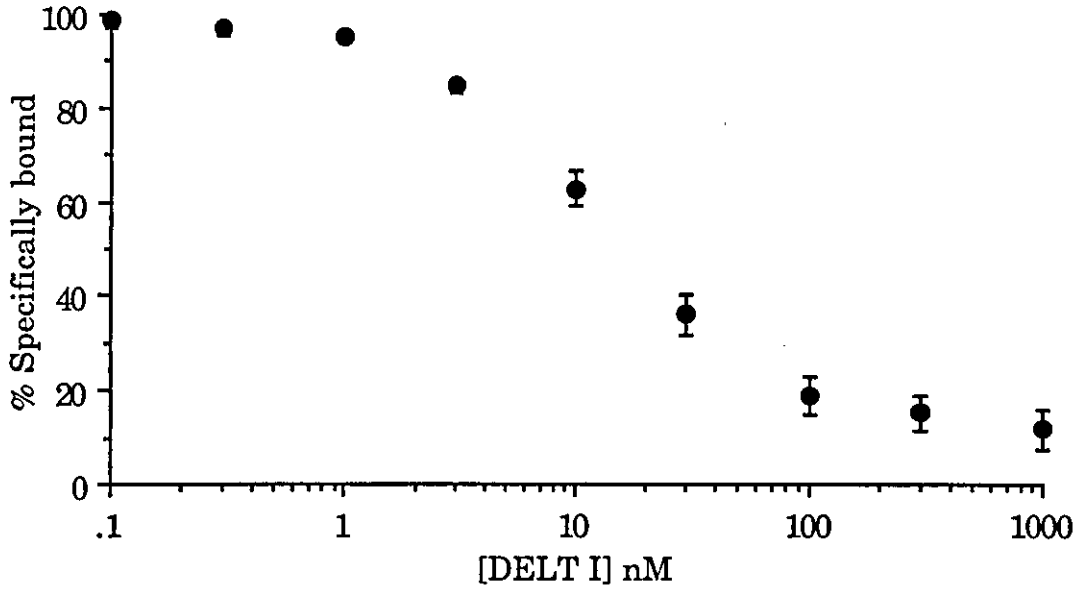


Figure 4.1 Displacement of [^{3}H]DPDPE (2 nM) specific binding by DELT I in homogenates of mouse brain (17 mg wet tissue / ml Tris-HCl buffer), incubated at 25°C for 40 min (n = 10). Values represent means ± s.e.mean.

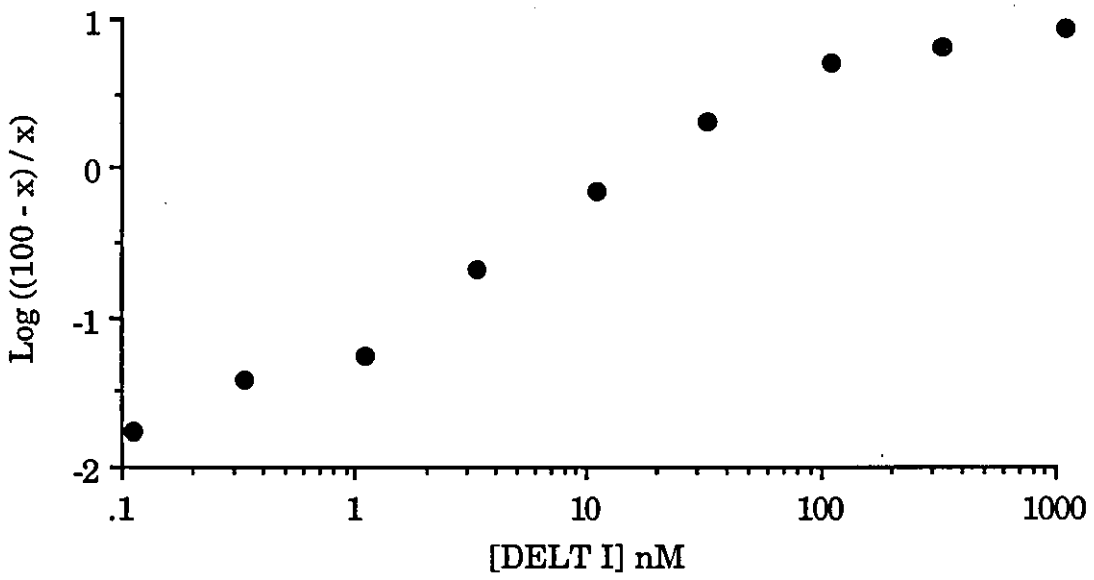


Figure 4.2 Hill plot of the displacement of [^{3}H]DPDPE (2 nM) specific binding by DELT I in homogenates of mouse brain (17 mg wet tissue / ml Tris-HCl buffer), incubated at 25°C for 40 min (n = 10).

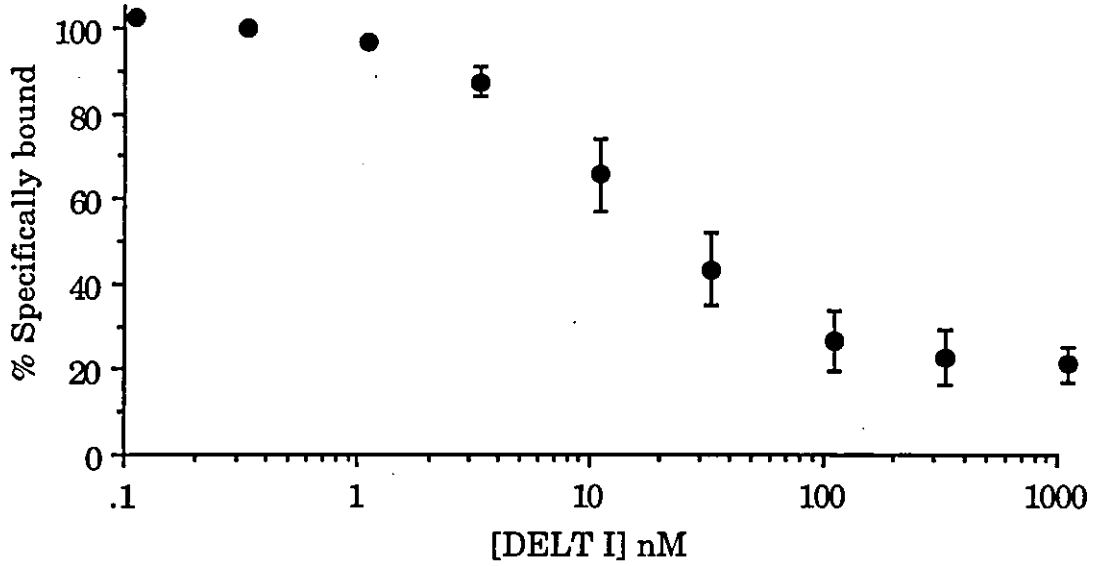


Figure 4.3 Displacement of [^{3}H]DPDPE (2 nM) specific binding by DELT I in homogenates of mouse brain (17 mg wet tissue / ml Tris-HCl buffer), incubated at 25°C for 40 min (n = 7 out of 10 occasions when not all the [^{3}H]DPDPE was displaced). Values represent means ± s.e.mean.

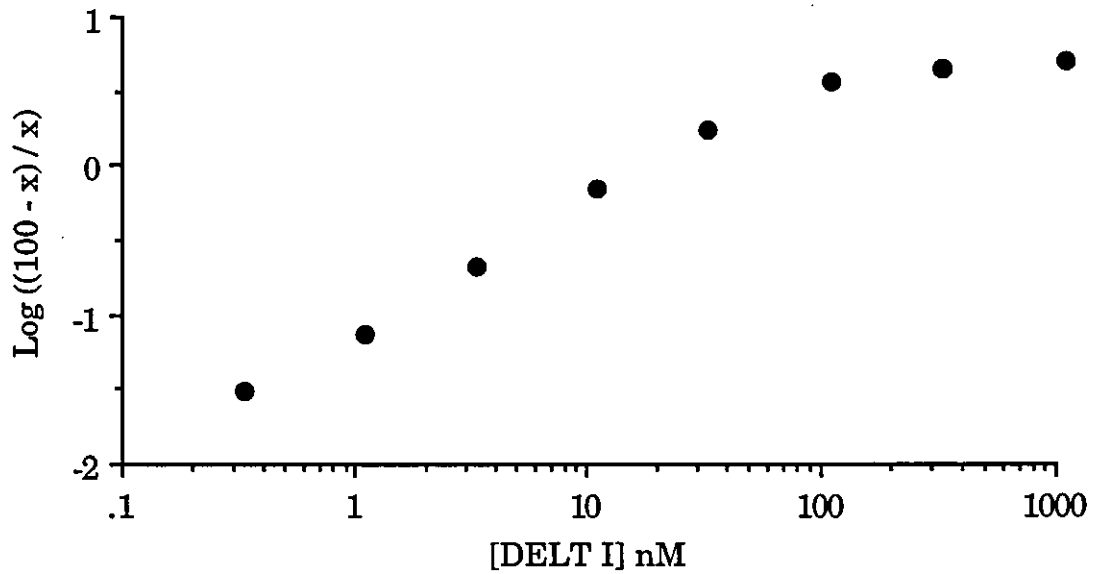


Figure 4.4 Hill plot of the displacement of [^{3}H]DPDPE (2 nM) specific binding by DELT I in homogenates of mouse brain (17 mg wet tissue / ml Tris-HCl buffer), incubated at 25°C for 40 min (n = 7 out of 10 occasions when not all the [^{3}H]DPDPE was displaced).

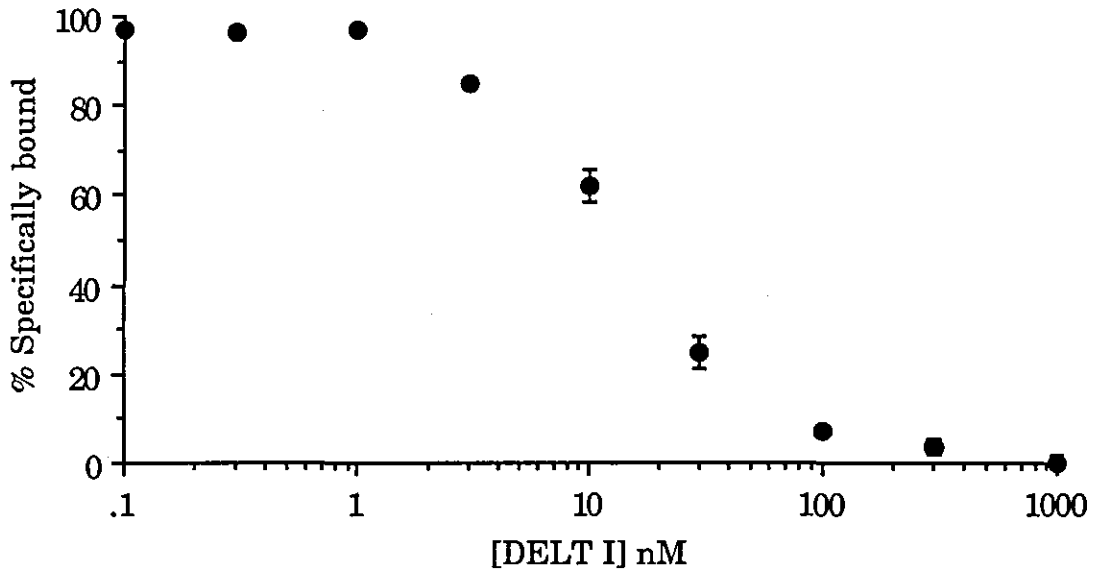


Figure 4.5 Displacement of [^{3}H]DPDPE (2 nM) specific binding by DELT I in homogenates of mouse brain (17 mg wet tissue / ml Tris-HCl buffer), incubated at 25°C for 40 min (n = 3 out of 10 when all the [^{3}H]DPDPE was displaced). Values represent means ± s.e.mean.

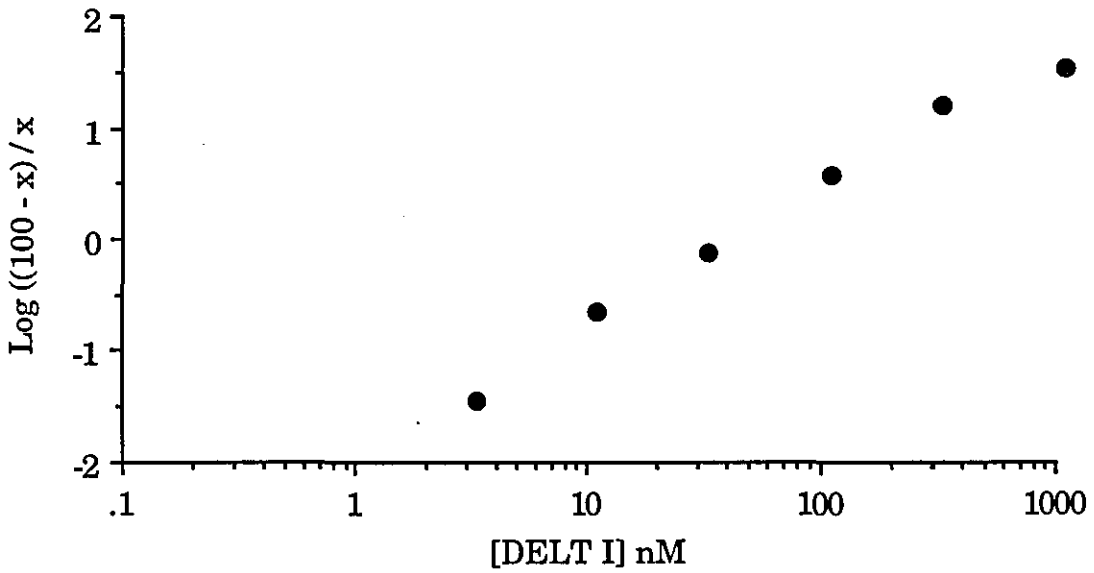


Figure 4.6 Hill plot of the displacement of [^{3}H]DPDPE (2 nM) specific binding by DELT I in homogenates of mouse brain (17 mg wet tissue / ml Tris-HCl buffer), incubated at 25°C for 40 min (n = 3 out of 10 when all the [^{3}H]DPDPE was displaced).

Evidence for labile δ-opioid sites

Previous work in our laboratory has shown that repeatedly washing mouse brain homogenates decreases δ-opioid receptor binding, as measured by [^{3}H]DPDPE, but that after several washings binding no longer decreases [Bardaji & Traynor, 1992]. One possible explanation for these results could be the existence of stable and labile binding sites. Importantly the loss of binding was not caused by the loss of significant amounts of protein as determined by the Lowry method [Lowry *et al.*, 1951].

Consistent with these previous findings, four washings of a mouse brain homogenate decreased the specific binding of [^{3}H]DPDPE (1.5 nM) by 33 % compared to control values. This loss of specific binding was not due to an equivalent loss of protein.

In order to determine whether this labile binding was being lost in the supernatant after each centrifugation, the supernatants were collected, combined and then spun at 100,000 g. No specific binding was obtained from the resulting pellet. Any very small amount of protein must be presumablely routinely discarded in the supernatants and could be vital for specific binding. However when the washed pellet and the very small pellet obtained from the combined supernatants were reconstituted no significant difference was found, i.e., the specific binding had decreased by 40 % compared to control values, while the protein content, within the limits of measurement, was unchanged.

In order to investigate the possible existence of stable and labile δ-opioid receptor binding sites further a more precise and consistent method of differentiating the labile sites was required. Thus the effects of temperature on the homogenate were examined. The homogenate was prepared as described previously (section 2.2.2) and subjected to a further incubation period at a raised temperature, recentrifuged and specific binding assayed with [^{3}H]DPDPE as before.

In an initial experiment heat treatment at 60°C for 2 min had virtually no effect, however, an incubation time of 5 or 10 min was too severe and specific binding was all but abolished compared to the control incubated at 4°C (Fig. 4.7). Experiments conducted at a temperature of 55°C for varying time periods gave a gradual degradation of specific binding with time (Fig. 4.8).

At this point the investigation of stable and labile δ-opioid binding sites was abandoned in favour of finding a more distinct method of characterising putative δ-opioid receptor subtypes.

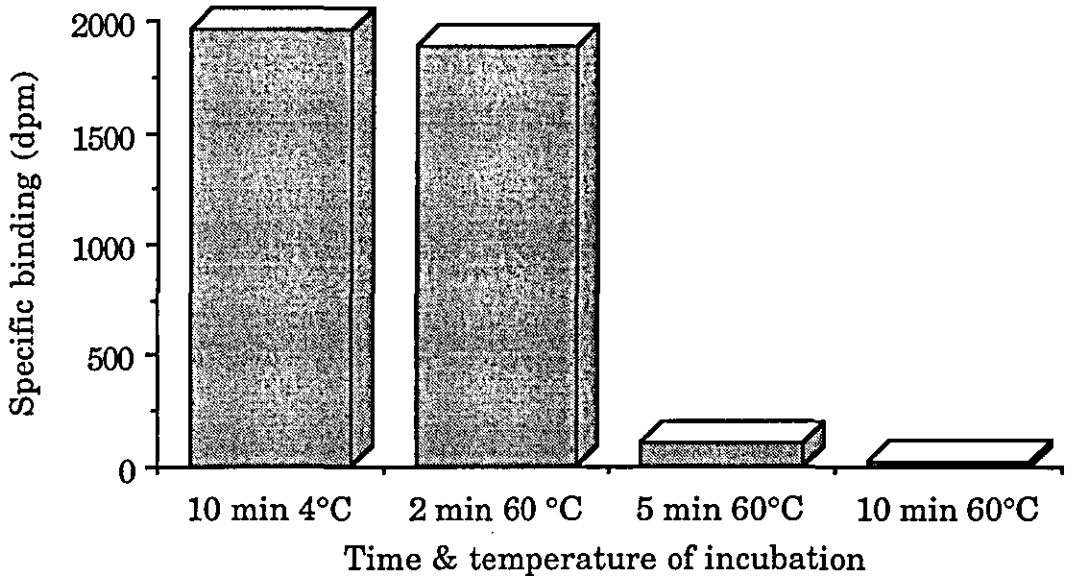


Figure 4.7 Effect of a 60°C incubation period on the specific binding of [^{3}H]DPDPE (2 nM) in homogenates of mouse brain (17 mg wet tissue / ml Tris-HCl buffer), n = 1.

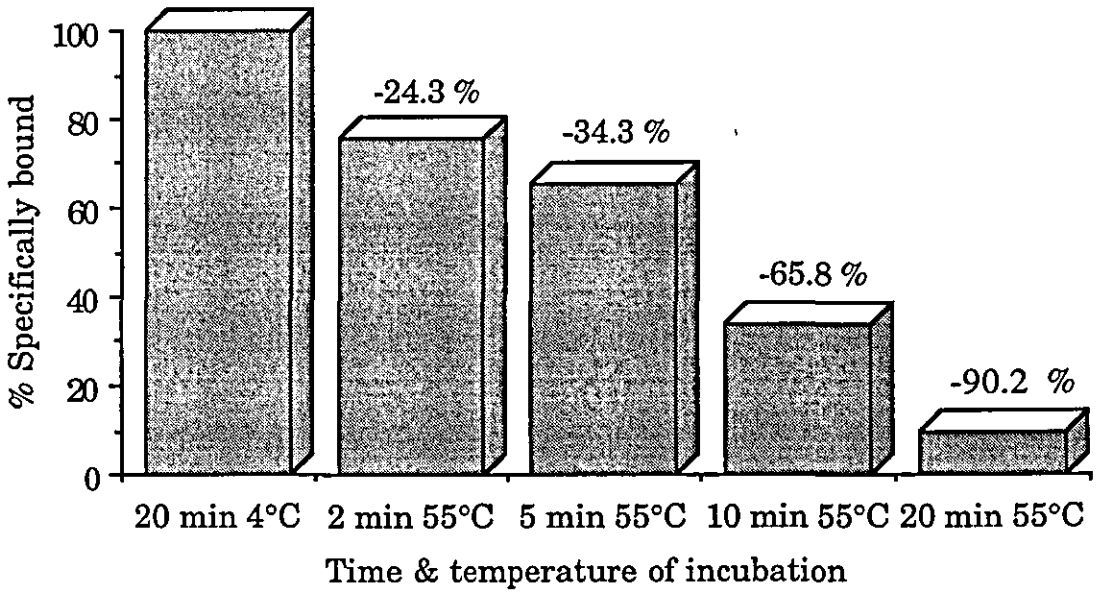


Figure 4.8 Effect of a 55°C incubation period on the specific binding of [^{3}H]DPDPE (2 nM) in homogenates of mouse brain (17 mg wet tissue / ml Tris-HCl buffer). Values represent means where n = 2 and the labels represent loss of specific binding.

4.2.2 Use of δ-opioid selective ligands: naltrindole analogues

In order to fully characterise any receptor and / or subtype it is essential to make use of highly selective ligands, especially antagonists. Naltrindole is a non-peptide δ-opioid antagonist with a reported selectivity in binding assays to guinea-pig brain membranes of more than 100 - and 10000 - fold over μ- and κ-opioid binding sites respectively. In isolated tissue preparations naltrindole displays a 200 - 400 greater antagonism at δ- compared to μ- and κ-opioid sites [Portoghese *et al.*, 1990]. In addition unlike other currently available δ-opioid antagonists it has the added possible potential of being useful in *in vivo* experiments since it is a non-peptide [Portoghese *et al.*, 1988]. Unfortunately at high doses naltrindole does possess some agonist activity [Jackson *et al.*, 1989b; Staplefield *et al.*, 1992] and other workers have not reported such a high degree of δ-opioid receptor selectivity [Rogers *et al.*, 1990b].

Examination of structure-activity relationships has shown that if an opiate has a cyclopropyl nitrogen substituent then antagonist actions are conferred, while a methyl group bonded to the nitrogen atom conveys agonist actions [Casy & Parfitt, 1985]. Furthermore, it has been reported that introduction of a 14-O-alkyl group into N-methylmorphinan-6-ones increases receptor affinity [Schmidhammer *et al.*, 1984]. In addition 14-methoxymetopon, which contains both a 5-Me group and a 14-O-Me group, has appproximately 1500 times higher potency than oxymorphone [Schmidhammer *et al.*, 1993]. At the commencement of this work selective non-peptide δ-opioid agonists were confined to the naltrindole analogue oxymorphindole, which displays only partial agonist activity [Portoghese *et al.*, 1990]. Therefore in the hope of finding novel non-peptide δ-opioid ligands with agonist activity and high selectivity over μ- and κ-opioid receptors Dr. H. Schmidhammer (Innsbruck University) synthesised twelve naltrindole analogues (the majority of which possessed N-Me groups) with various 14- and 5-substituents for evaluation.

The structures of naltrindole and the analogues (HS series) are given in Fig. 4.9 and Table 4.2.

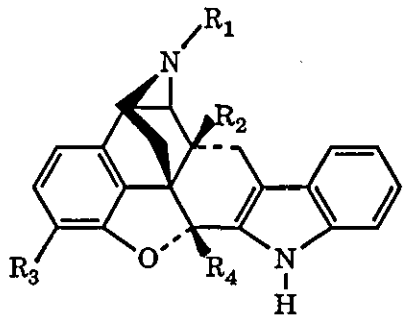


Figure 4.9 Structure of naltrindole (R_1 = CPM, R_2 = OH, R_3 = OH and R_4 = H) and the HS series of analogues.

Table 4.2 Structure of naltrindole and the HS series of analogues.

	R_1	R_2	R_3	R_4
Naltrindole	$CH_2C(CH_2)_2$	OH	OH	H
Naloxindole	$CH_2CH{=}CH_2$	OH	OH	H
HS 305	CH_3	H	OCH_3	H
HS 306	CH_3	OH	OCH_3	H
HS 326	CH_3	OH	OH	H
HS 327	CH_3	OCH_3	OCH_3	H
HS 328	CH_3	OC_2H_5	OCH_3	H
HS 329	CH_3	OCH_3	OH	H
HS 330	CH_3	OC_2H_5	OH	H
HS 331	CH_3	OCH_3	OCH_3	CH_3
HS 333	CH_3	OCH_3	OH	CH_3
HS 350	CH_3	OC_2H_5	OH	CH_3
HS 378	$CH_2C(CH_2)_2$	OC_2H_5	OCH_3	CH_3

a) Binding assays

In order to determine the opioid receptor selectivity of the HS compounds, competition ligand binding assays were performed in guinea-pig brain membranes using three selective radioligands, to label μ, δ and κ opioid sites, [^{3}H]DAMGO ([D-Ala^2,MePhe^4,Gly(ol)^5]-enkephalin), [^{3}H]DPDPE and [^{3}H]CI977 ((5R-(5α,7α,8β)-N-methyl-N[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]-4-benzofuranacetamide)) [Hunter *et al.*, 1990] were used respectively. The results of these experiments are summarised in Table 4.3.

Table 4.3 Inhibition constants, K_i values (nM), of the HS series of naltrindole analogues using receptor selective radioligands in guinea-pig brain.

	[^{3}H]DAMGO (1 nM)	[^{3}H]DPDPE (2 nM)	[^{3}H]CI977 (0.5 nM)
Naltrindole	21 ± 3	1.5 ± 0.31	14 ± 3
Naloxindole	103 ± 8	4.6 ± 1.5	170 ± 40
HS 305	5800 ± 1200	180 ± 20	5700 ± 50
HS 306	1100 ± 80	68 ± 5	5800 ± 900
HS 326	41 ± 8	8.8 ± 2.6	620 ± 270
HS 327	1700 ± 400	65 ± 11	1700 ± 300
HS 328	1800 ± 400	39 ± 9	2100 ± 40
HS 329	54 ± 4	2.6 ± 0.6	38 ± 6
HS 330	30 ± 2	3.3 ± 0.8	98 ± 10
HS 331	820 ± 250	250 ± 50	4100 ± 400
HS 333	76 ± 10	80 ± 10	1400 ± 100
HS 350*	664	4.9	367
HS 378*	38.5	0.73	60.5

Binding assays incubated at 25°C for 40 min, using 10 mg wet tissue / ml Tris-HCl buffer. *Conducted in rat brain (3 - 4 mg protein / ml Tris-HCl buffer) by S. Nevin & Dr. A. Borsodi, Institute of Biochemistry, Biological Research Centre of the Hungarian Academy of Sciences, Szeged, Hungary, δ-sites were labelled with [^{3}H]naltrindole and κ-sites with [^{3}H]U69593 ((5α,7α,8β)-(+)-N-methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]-dec-8-yl]-benzeneacetamide). The K_i values were calculated as described in section 2.2.2, using the following K_D values (nM): DAMGO (1.9) and DPDPE (2.7) [Leslie, 1987], U69593 (2.9) [Lahti, *et al.*, 1985] and CI977 (0.29) [K. Bell, 1994]. Values represent means ± s.e.mean where n ≥ 3.

From the K_i values quoted in Table 4.3 the selectivity of each of the compounds can be determined. These figures are given in Table 4.4 with the most selective compounds (μ / δ) listed first.

Table 4.4 Selectivity profiles of the HS series of naltrindole analogues.

	μ / δ	κ / δ
HS 350	140	75
HS 378	54	81
HS 328	46 (29 - 73)	54 (42 - 74)
HS 305	32 (23 - 44)	32 (28 - 36)
HS 327	26 (17 - 39)	26 (18 - 37)
Naloxindole	22 (16 - 36)	37 (21 - 68
HS 329	21 (16 - 29)	15 (10 - 22)
HS 306	16 (14 -19)	85 (67 - 106)
Naltrindole	14 (10 - 20)	9.3 (6 - 14)
HS 330	9.1 (6.8 - 13)	30 (21 - 43)
HS 326	4.7 (2.9 - 7.9)	70 (31 - 140)
HS 331	3.3 (1.9 - 5.4)	16 (12 - 23)
HS 333	1.0 (0.8 - 1.4)	18 (14 - 21)

$\mu / \delta = K_i\mu / K_i\delta$ and $\kappa / \delta = K_i\kappa / K_i\delta$, the figures in brackets indicate the range according to the error in the K_i values.

As can be seen from the above table the most selective compounds are HS 350, HS 378 and HS 328 (Fig 4.10). These compounds were tested in isolated tissue preparations to determine agonist or antagonist properties, and also whether the selectivity profile was consistent with the results obtained from ligand-binding studies.

In the guinea-pig myenteric plexus-longitudinal muscle preparation all three compounds showed only weak agonist activity, HS 328 had an IC_{50} of 9600 ± 2000 nM (Fig 4.11) and 10 μM HS 350 and HS 378 inhibited the twitch height by 21 ± 12 and 32 ± 7 %, respectively.

In the mouse vas deferens preparation HS 378 was once again only very weakly active, producing 23 ± 9 % inhibition of twitch height at a concentration of 10 μM. The antagonist selectivity profile of HS 378 was determined in the mouse vas deferens by obtaining affinity values (K_e values, by the single dose method) against selective μ-, δ- and κ-opioid agonists, i.e., DAMGO, DPDPE and CI977 respectively. The results are given in Table 4.5 and are compared with naltrindole [Rogers *et al.*, 1990b].

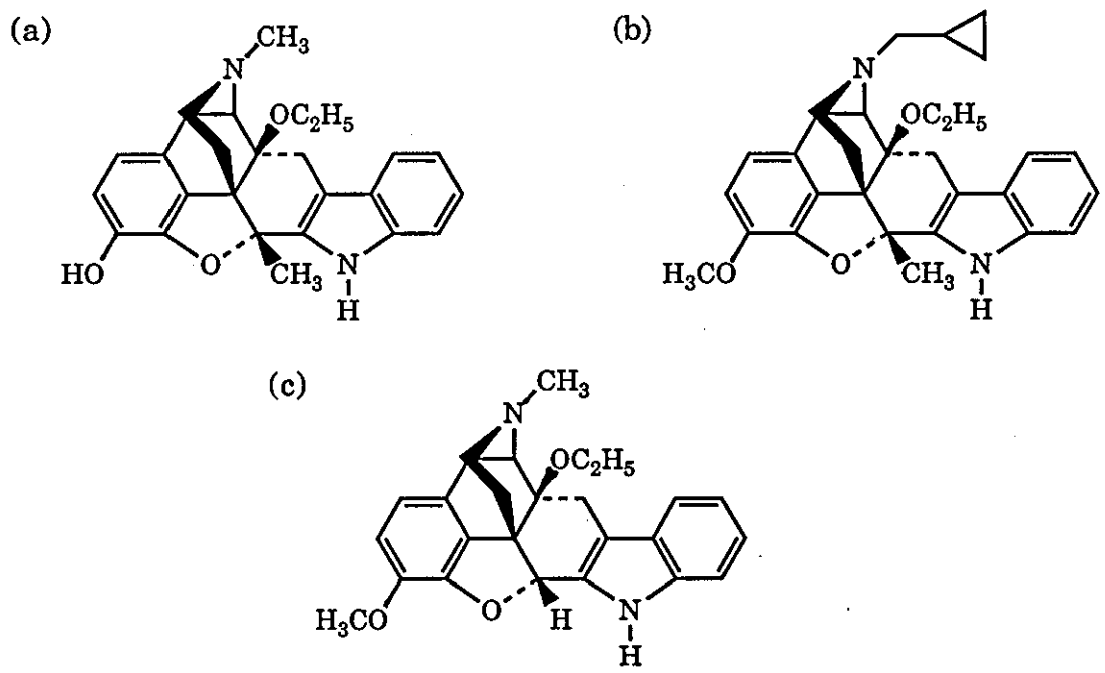


Figure 4.10 (a) HS 350, (b) HS 378 and (c) HS 328

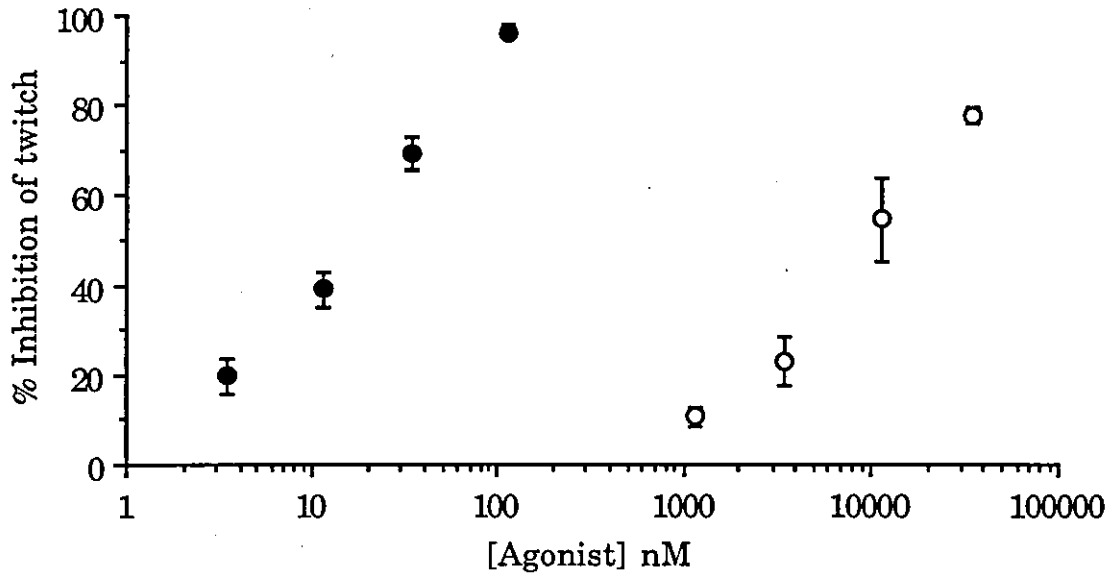


Figure 4.11 Dose-response curves for DAMGO (●) and HS 328 (O) in the guinea-pig myenteric plexus-longitudinal muscle preparation. Values are means ± s.e.mean where n ≥ 3.

Table 4.5 Affinity values (K_e) and selectivity profiles of HS 378 and naltrindole determined in the mouse vas deferens preparation using the selective μ-, δ- and κ-opioid agonists, DAMGO, DPDPE and CI977 respectively.

	K_e (nM)			Selectivity	
	μ	δ	κ	μ / δ	κ / δ
HS 378	133 ± 42	*1.3 ± 0.3	529 ± 92	102	455
Naltrindole	5.25 ± 0.68	0.182 ± 0.016	32.4 ± 1.13	28.8	178

Values represent means ± s.e.mean where n ≥ 3. $\mu / \delta = K_e\mu / K_e\delta$ and $\kappa / \delta = K_e\kappa / K_e\delta$. *Within experimental error the same apparent δ K_e value was obtained by using the putative δ_2 selective agonist DELT II, i.e., 1.6 ± 0.2 nM.

Hence although HS 378 had approximately 7 times less affinity for δ-opioid receptors, it also has much lower affinity for both μ- and κ-opioid receptors and therefore it was approximately 3 times more selective for δ-opioid sites than naltrindole. To determine whether HS 378 displayed any selectivity for the putative δ_1 or δ_2 sites, its ability to antagonise both the putative δ_1-opioid agonist DPDPE and the putative δ_2-opioid agonist DELT II was compared. There was no significant difference, i.e., K_e values of 1.3 ± 0.3 and 1.6 ± 0.2 nM against DPDPE and DELT II respectively, were obtained.

In contrast, the N-methyl substituted compounds HS 328 and HS 350 were full agonists in the mouse vas deferens preparation with IC_{50} values of 71 ± 9 and 104 ± 33 nM, respectively. Since HS 328 and HS 350 exhibited much more potent agonist activity in the mouse vas deferens than the guinea-pig myenteric plexus-longitudinal muscle preparation this suggested that they were acting *via* δ-opioid receptors in the mouse vas deferens. This proposal was further tested using antagonists for the more potent of the two compounds, i.e., HS 328.

ICI 174,864 (N,N-diallyl-Tyr-Aib-Aib-Phe-Leu, where Aib = α-aminoisobutyric acid) is a δ-opioid selective antagonist, with a K_e value of approximately 30 nM at δ-opioid sites but no antagonism at μ- or κ-opioid sites up to concentrations of 5 μM [Cotton *et al.*, 1984; Kreil *et al.*, 1989]. In the mouse vas deferens preparation ICI 174,864 afforded a K_e value of 37 ± 9 nM versus HS 328, indicative of an action mediated *via* δ-opioid receptors (Fig. 4.12). In contrast the naloxone K_e value yielded by HS 328 in the guinea-pig myenteric plexus-longitudinal muscle preparation was 10 ± 4 nM, suggesting HS 328 was not acting *via* μ-opioid receptors in this preparation. The relatively non-selective antagonist naloxone exhibits approximately 10-fold higher affinity for μ- (1-3 nM) as opposed to δ- (20-30 nM) opioid sites, and an intermediate affinity for κ-opioid sites [Leslie, 1987].

The 3-hydroxy analogue of HS 328, HS 330, was also examined in the same manner. The results were appreciably different. HS 330 was a full potent agonist in both the mouse vas deferens and guinea-pig myenteric plexus-longitudinal muscle preparations, yielding IC_{50} values of 23 ± 4 and 190 ± 30 nM respectively (Figs. 4.13 & 4.14). ICI 174,864 in the mouse vas deferens preparation antagonised HS 330 with an apparent K_e value of 120 ± 30 nM (Fig. 4.13) and against naloxone in the guinea-pig myenteric plexus-longitudinal muscle preparation an apparent K_e value of 2.9 ± 0.7 nM was obtained (Fig. 4.14). In combination these results suggest that HS 330 is probably acting *via* δ-opioid sites in the mouse vas deferens preparation and

μ-opioid sites in the guinea-pig myenteric plexus-longitudinal muscle preparation.

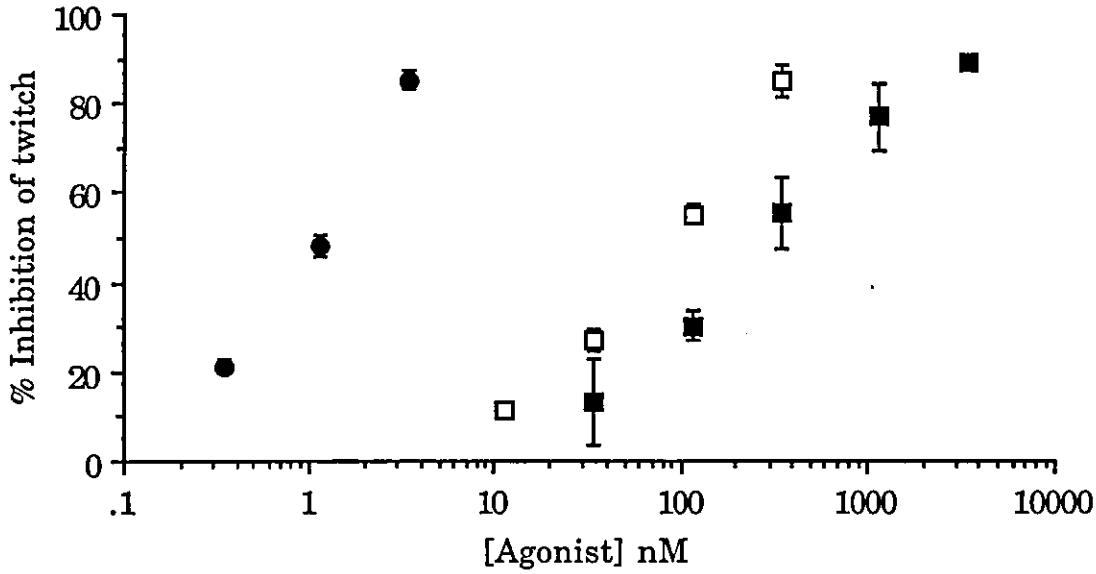


Figure 4.12 Dose-response curves for DPDPE (●), HS 328 (□) and HS 328 in the presence of 100 nM ICI 174,864 (■) in the mouse vas deferens preparation. Values represent means ± s.e.mean where n ≥ 3.

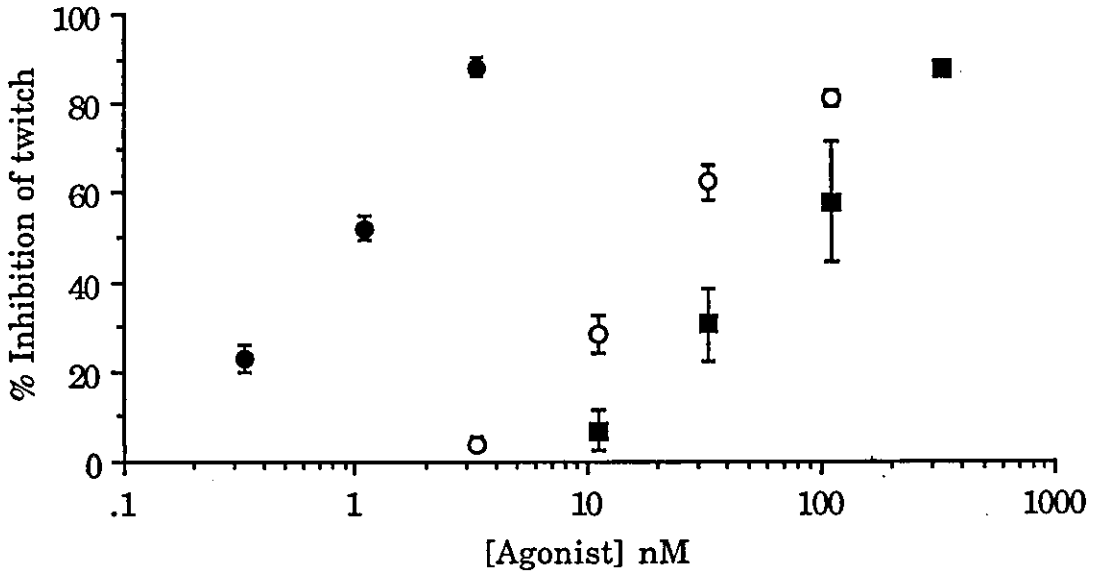


Figure 4.13 Dose-response curves for DPDPE (●), HS 330 (O) and HS 330 in the presence of 300 nM ICI 174,864 (■) in the mouse vas deferens preparation. Values represent means ± s.e.mean where n ≥ 3.

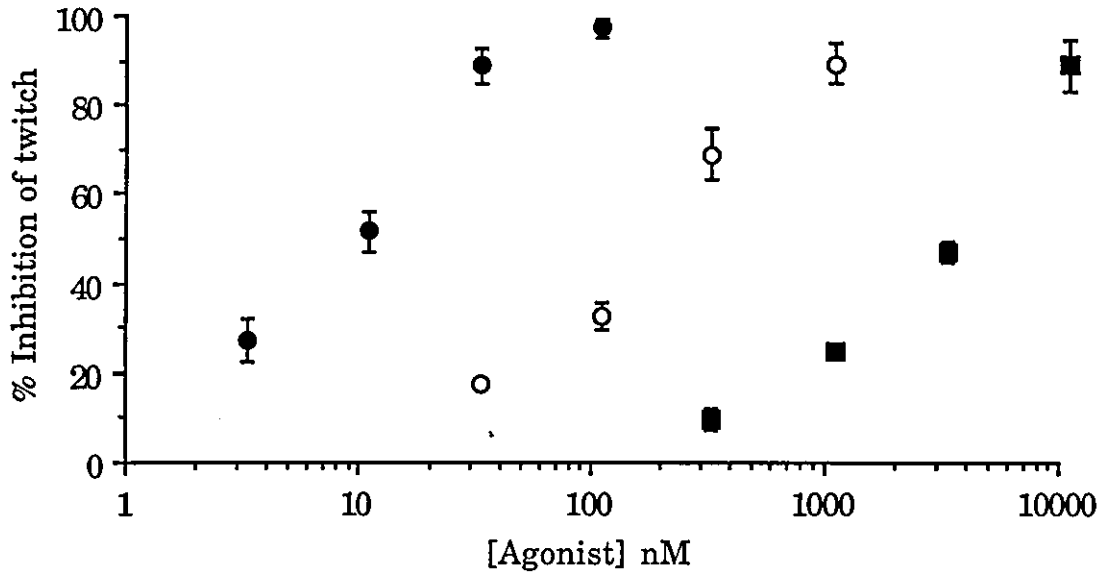


Figure 4.14 Dose-response curves for DAMGO (●), HS 330 (O) and HS 330 in the presence of 30 nM naloxone (■) in the guinea-pig myenteric plexus-longitudinal muscle preparation. Values represent means ± s.e.mean where n ≥ 3.

In addition, the effect on potency of altering the 14-O-alkyl group of HS 328 was evaluated in the mouse vas deferens preparation. There was no advantage in substituting OEt (HS 328, IC_{50} = 71 ± 9 nM) in favour of OMe (HS 327, IC_{50} = 292 ± 40 nM), OH (HS 306, IC_{50} = 217 ± 21 nM) or H (HS 305, IC_{50} = 98 ± 24 nM) although full agonist activity was maintained in each case.

4.2.3 Use of oripavines: studies of RX 8008M and etorphine in isolated tissues

The hamster vas deferens is reported to contain only opioid receptors of the δ type as only δ-opioid selective agonists, and not μ- or κ-opioid agonists, are able to inhibit electrical field stimulated contractions [McKnight *et al.*, 1984]. Interestingly the δ-opioid receptor in this tissue does not display an identical pharmacology to the δ-opioid receptor in mouse vas deferens. Two oripavines with bridged structures and 7-substituted tertiary alcohols, the highly potent agonist etorphine (Fig. 4.15) [Leslie, 1987] and the antagonist 16-methyl cyprenorphine (RX 8008M) (Fig. 4.15) appear to be able to discriminate between δ-opioid receptors in the mouse and those in the hamster vas deferens [Smith, 1987].

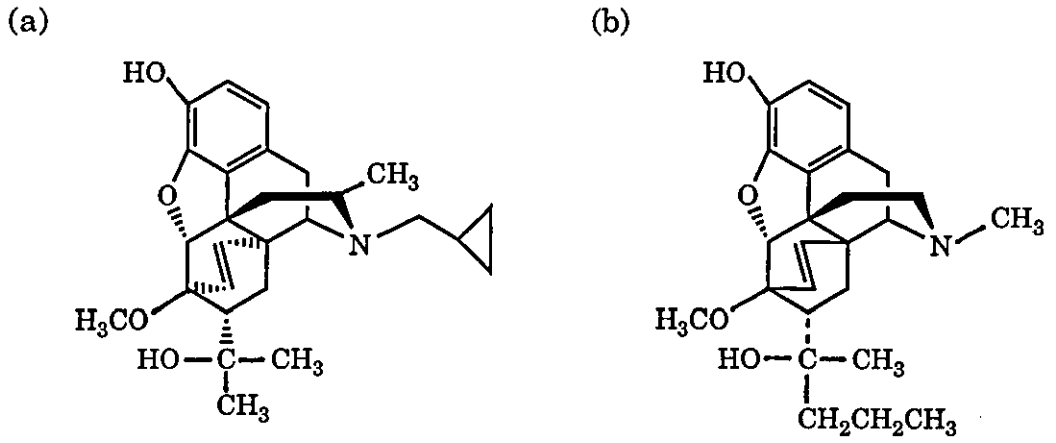


Figure 4.15 Structure of (a) RX 8008M (16-Me cyprenorphine) and (b) etorphine

In the mouse vas deferens RX 8008M has much higher affinity for δ- and μ- than κ-opioid receptors, its K_e values being 0.73, 1.77 and 59.6 nM respectively. Similar affinities are obtained when examining μ- and κ-opioid receptors in other isolated tissues, i.e., μ-opioid receptors in rat vas deferens and guinea-pig ileum, and κ-opioid receptors in the rabbit vas deferens, mouse ileum and guinea-pig ileum. However, although the K_e value of RX 8008M at δ-opioid receptors in the mouse ileum is the same as in the mouse vas deferens, in the hamster vas deferens preparation the K_e value of RX 8008M is 17.0 nM, i.e., it exhibits 23 times less affinity for the δ-opioid receptor in hamster vas deferens as opposed to mouse vas deferens [Smith, 1987].

Another compound with differing activity in the hamster vas deferens as opposed to the mouse vas deferens preparation is etorphine. Etorphine is a full and highly potent agonist in the mouse vas deferens [Leslie, 1987] but a partial agonist in the hamster vas deferens preparation, only causing a maximum inhibition of twitch height of 60 %. In the hamster vas deferens preparation naloxone and ICI 174,864 K_e values are three times higher against etorphine than the values obtained using DADLE and DSLET. In addition, the affinity of etorphine for δ-opioid receptors in the hamster vas deferens preparation is twenty times lower than the affinity of DADLE or DSLET [Sheehan *et al.*, 1986b]. Other workers found that a concentration of 4 μM etorphine sometimes inhibited as much as 50 % of the twitch, but that this action could not be reversed by either naloxone or ICI 174,864 [Miller & Shaw, 1985]. These results suggest a heterogeneity in δ-opioid receptors, since in other systems etorphine

does act *via* δ-opioid receptors. For example, in NG108-15 cells etorphine acts as a potent full agonist to inhibit cAMP accumulation [Law *et al.*, 1983]. Ligand binding assays in guinea-pig brain have suggested that etorphine is a potent displacer of the δ-opioid ligand [^{3}H]DADLE exhibiting a K_i of 0.56 nM, similar to displacement of μ- and κ-opioid ligands [Magnan *et al.*, 1982].

The reasons underlying the reported differential effects of these oripavines in the hamster vas deferens were therefore further examined. To investigate the possibilty of differences between δ-opioid receptors in the mouse vas deferens and the hamster vas deferens, the ability of RX 8008M to antagonise several δ-opioid agonists in the mouse vas deferens preparation was initially tested. The potencies (IC_{50}) and affinities (K_e values, determined from Schild plots) are listed in Table 4.6. All the δ-opioid agonists act at a receptor population recognised as a single type by RX 8008M. A graph of the dose-response curves illustrating the shift of the potent δ-opioid peptide DELT II, for example, by various concentrations of RX 8008M is given in Fig 4.16 and the corresponding Schild plot is also shown (Fig. 4.17).

Table 4.6 IC_{50} values for various δ-opioid agonists in the mouse vas deferens preparation and antagonism by RX 8008M (as K_e values).

Agonist	IC_{50} (nM)	K_e (nM)
DADLE	0.32 ± 0.01	1.07 ± 0.01
DELT II	0.35 ± 0.08	1.62 ± 0.25
DPDPE	1.96 ± 0.26	0.84 ± 0.08
DSLET	0.32 ± 0.14	0.90 ± 0.11

Values represent means ± s.e.mean where n ≥ 3.

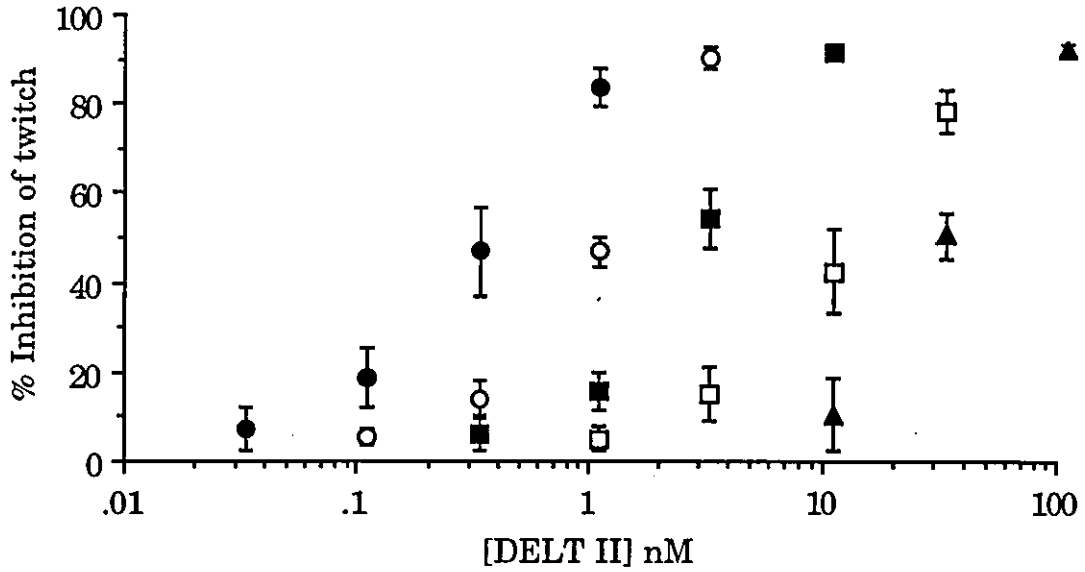


Figure 4.16 Dose-response curves for DELT II alone (●), or in the presence of 3 nM RX 8008M (O), 10 nM RX 8008M (■), 30 nM RX 8008M (□) and 100 nM RX 8008M (▲) in the mouse vas deferens preparation. Values represent means ± s.e.mean where n ≥ 3.

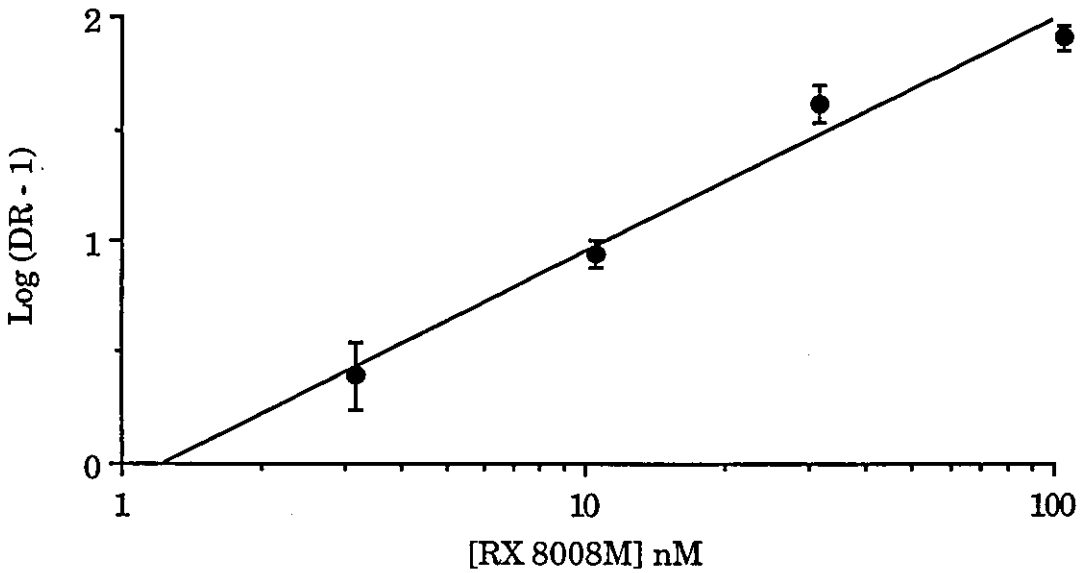


Figure 4.17 Mean Schild plot of the antagonism of DELT II by RX 8008M in the mouse vas deferens preparation. Slope = 1.04. Values represent means ± s.e.mean where n ≥ 3.

The K_e values of RX 8008M versus DADLE and DPDPE were determined in the hamster vas deferens preparation as 7.5 ± 1.3 and 11.6 ± 4.4 nM respectively. In some cases K_e values were determined from a single concentration of antagonist. In other experiments Schild plots were obtained but on each occasion the slope was steep (1.26 ± 0.04). An example is given in Figs. 4.18 and 4.19. Steep Schild slopes can result from inadequate incubation times of antagonist or agonist effects of the antagonist at high concentrations [Kenakin, 1984]. The period of incubation with the antagonist was increased from 10 min to 60 min but steep slopes were still obtained.

Etorphine was confirmed to be a full and potent agonist in the mouse vas deferens and guinea-pig myenteric plexus-longitudinal muscle preparation yielding IC_{50} values of 0.081 ± 0.006 (Fig 4.20) and 0.11 ± 0.04 nM respectively. It was also confirmed to be a partial agonist in the hamster vas deferens preparation. At 10 μM etorphine only 60 % of the opiate sensitive twitch was inhibited (Fig. 4.21).

Before determining through which receptor etorphine was mediating its effects in the hamster vas deferens preparation, receptor selectivity was examined in the mouse vas deferens preparation. The K_e values of naloxone (Fig. 4.20) and three receptor selective antagonists, versus etorphine were determined, namely cyprodime (Fig. 4.22), nor-binaltorphimine (nBNI) [Portoghese *et al.*, 1987] (Fig. 4.23) and naltrindole (Fig. 4.24). The results are summarised in Table 4.7. The results obtained were not totally consistent. The naloxone K_e value suggested an action *via* δ-opioid receptors while the K_e values of cyprodime, nBNI and naltrindole point towards the μ-opioid receptor being responsible for etorphine-mediated actions. To confirm that the antagonists were acting in the manner previously reported in the literature, affinities of the antagonists versus the selective agonists DAMGO, U69593 and DPDPE were also determined (Table 4.7).

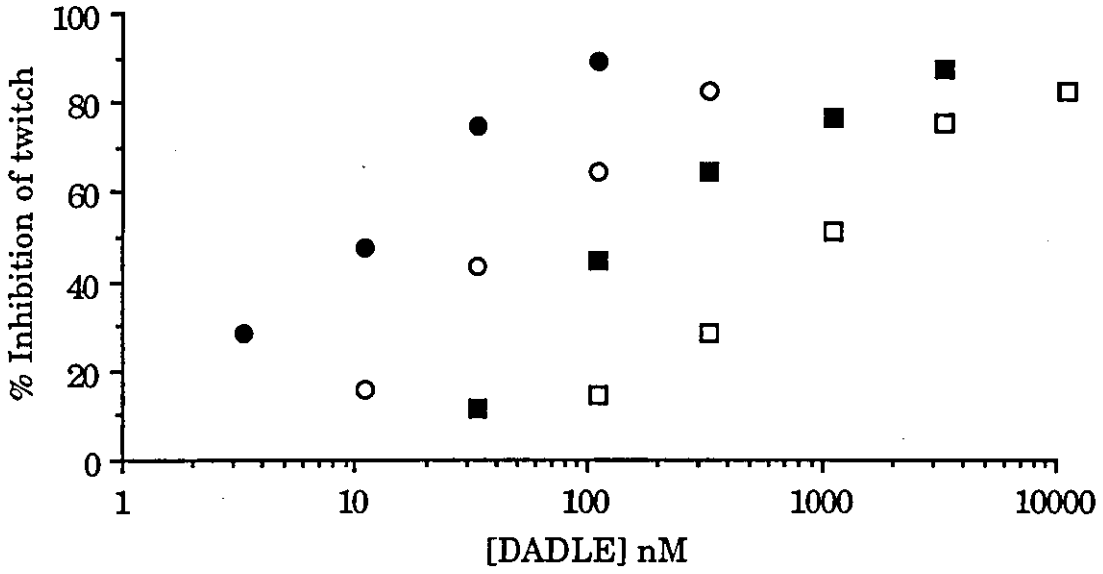


Figure 4.18 A representative experiment of dose-response curves for DADLE alone (●), or in the presence of 30 nM RX 8008M (O), 100 nM RX 8008M (■) and 300 nM RX 8008M (□) in the hamster vas deferens preparation.

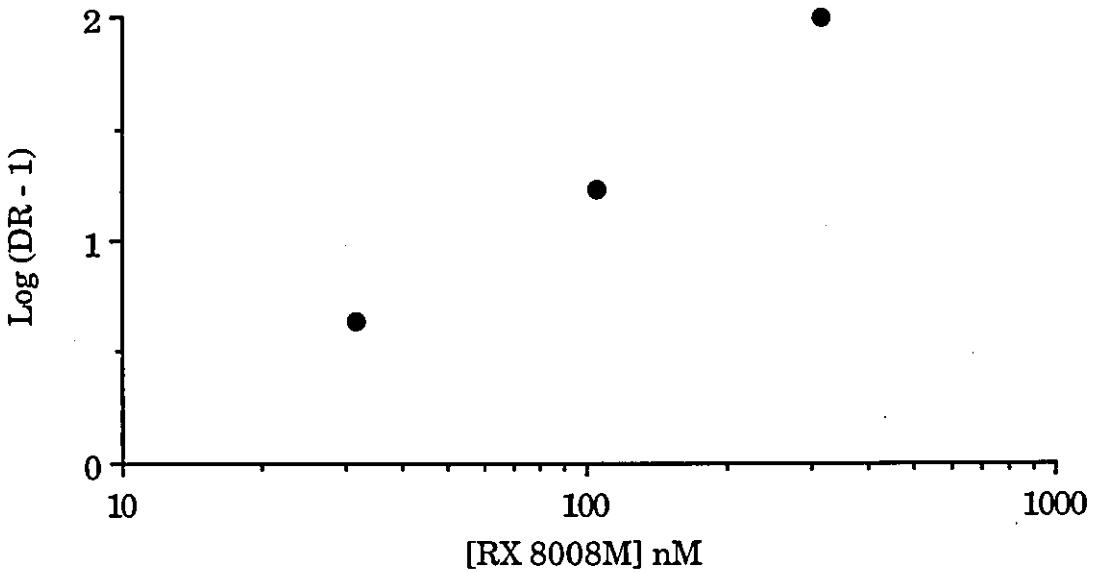


Figure 4.19 Schild plot of the antagonism of DADLE by RX 8008M in the hamster vas deferens preparation. Slope = 1.36.

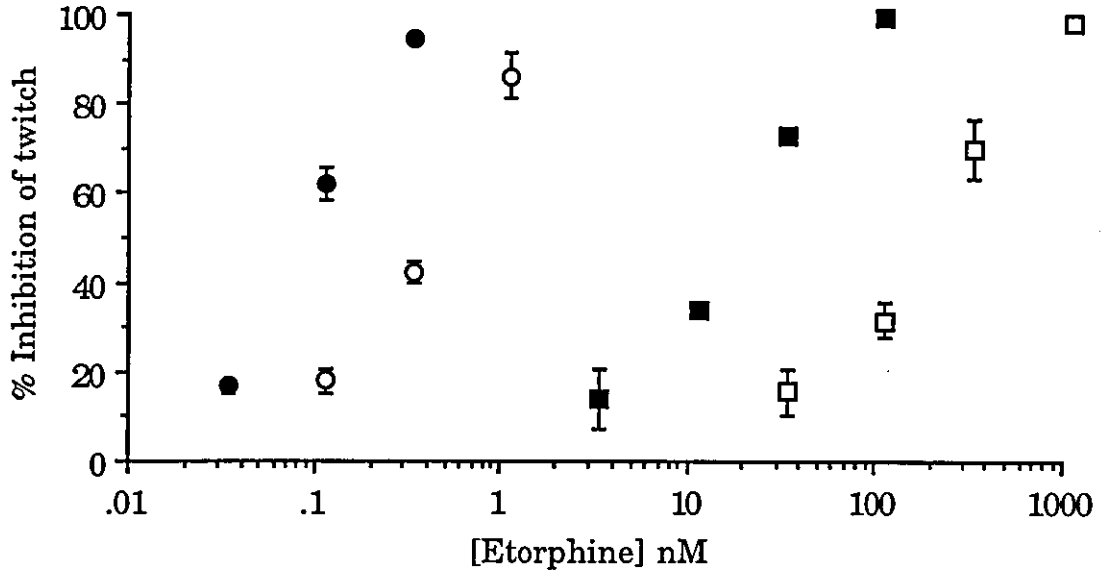


Figure 4.20 Dose-response curves for etorphine (●), etorphine + 100 nM naloxone (O), DAMGO* (■) and DAMGO + 30 nM naloxone* (□) in the mouse vas deferens preparation. Values represent means ± s.e.mean where n ≥ 3, or *where n = 2 ± the range.

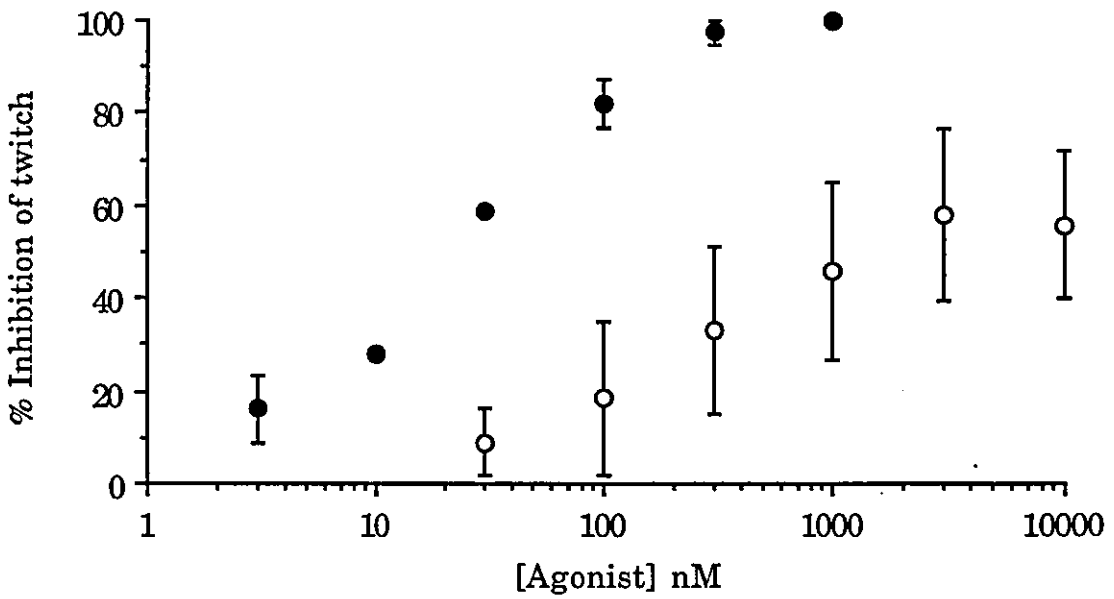


Figure 4.21 Dose-response curves for DADLE (●) and etorphine (O) in the hamster vas deferens preparation. Values represent means ± range where n = 2.

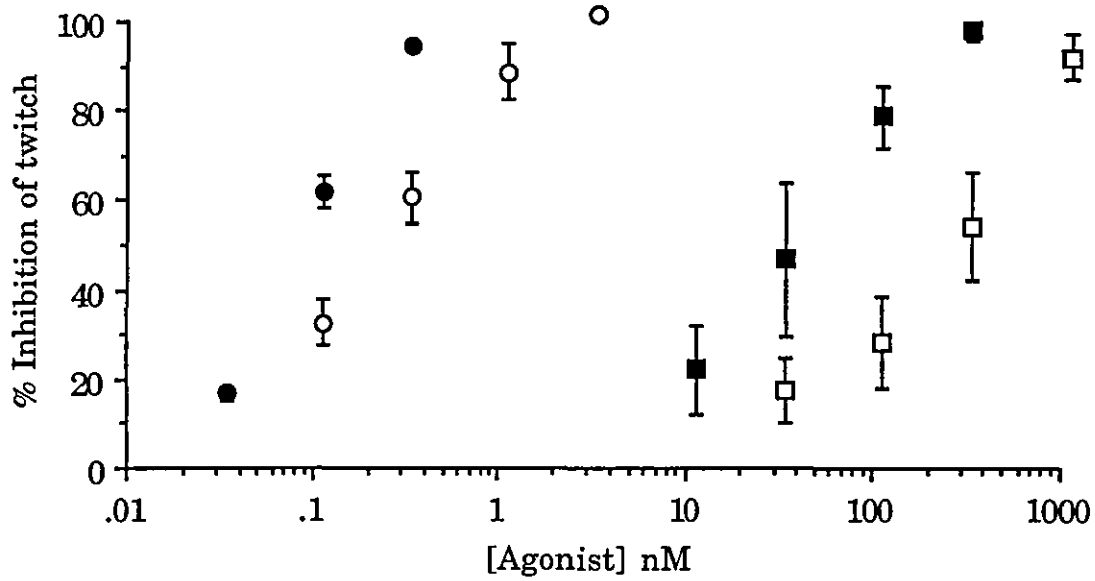


Figure 4.22 Dose-response curves for etorphine (●), etorphine + 300 nM cyprodime (O), DAMGO (■) and DAMGO + 300 nM cyprodime (□) in the mouse vas deferens preparation. Values represent means ± s.e.mean where n ≥ 3.

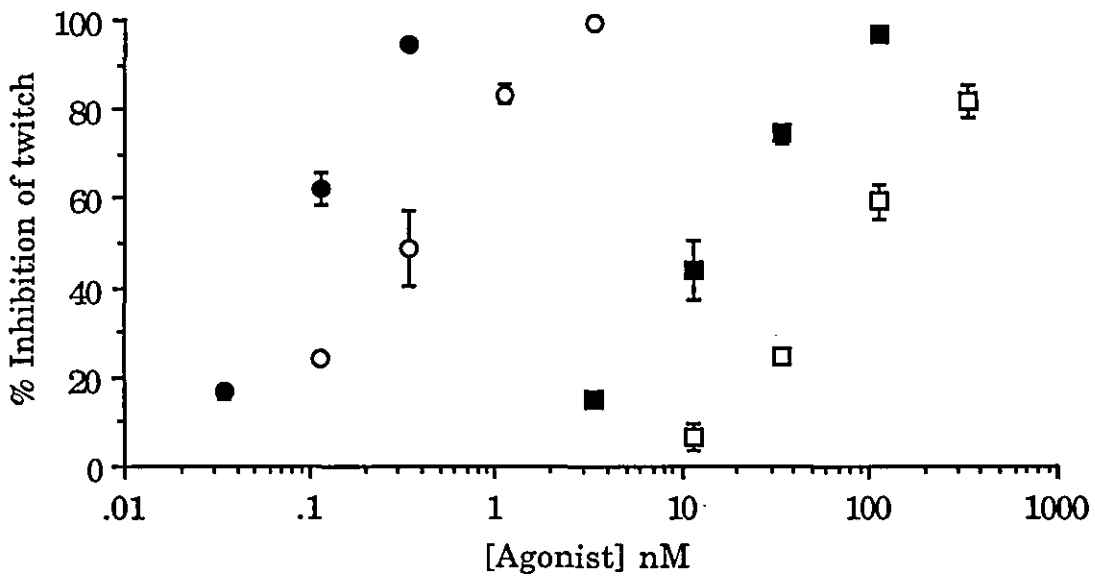


Figure 4.23 Dose-response curves for etorphine (●), etorphine + 100 nM nBNI* (O), U69593* (■) and U69593 + 1 nM nBNI* (□) in the mouse vas deferens preparation. Values represent means ± s.e.mean where n ≥ 3 or *where n = 2 ± the range.

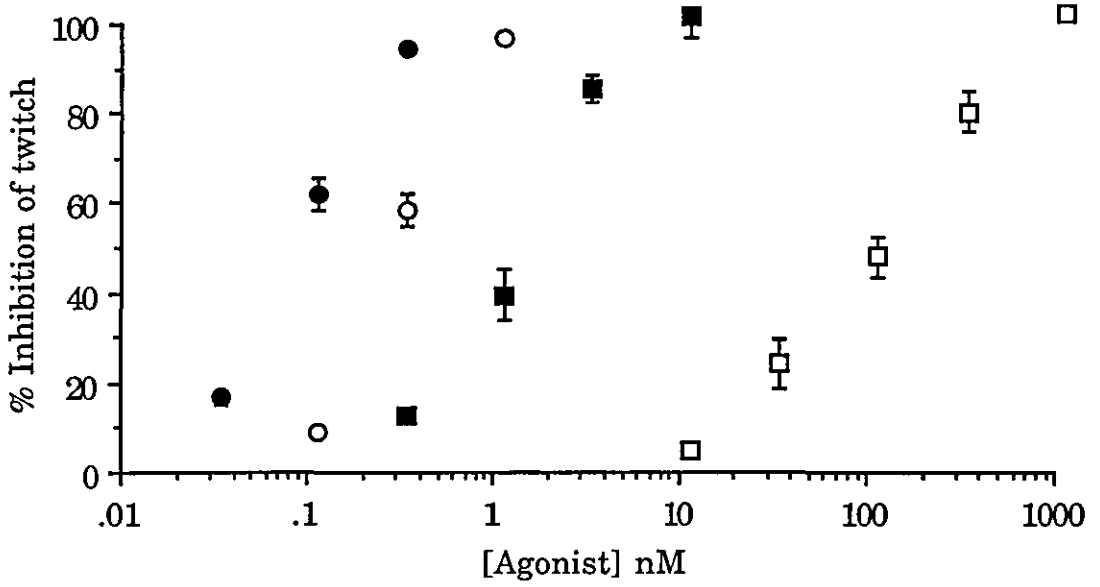


Figure 4.24 Dose-response curves for etorphine (●), etorphine + 10 nM naltrindole (O), DPDPE (■) and DPDPE + 10 nM naltrindole (□) in the mouse vas deferens preparation. Values represent means ± s.e.mean where n ≥ 3.

Table 4.7 Affinity (K_e values (nM)) of naloxone and receptor-selective antagonists using etorphine and receptor-selective agonists in the mouse vas deferens.

Antagonists / Agonists	Naloxone	Cyprodime (μ)	nBNI (κ)	Naltrindole (δ)
Etorphine	34.8 ± 3.4	153 ± 45	51.5 ± 7.5*	3.30 ± 0.07
DAMGO (μ)	3.15 ± 0.45*	59.6 ± 10.8	32.8 ± 1.1*	6.5 ± 1.7*
U69593 (κ)	14.9 ± 1.0	850 ± 106*	0.35 ± 0.03*	17.3 ± 0.7*
DPDPE (δ)	59.4 ± 20	2640 ± 615*	17.5 ± 1.5*	0.14 ± 0.03

Values represent means ± s.e.mean where n ≥ 3, or *where n = 2 ± the range.

4.2.4 Effects of strain differences and stress on δ sites in rat brain

A survey of the literature suggests that central δ-opioid receptor levels vary between species and even between strains of the same species [Cotton *et al.*, 1985; Yoburn *et al.*, 1991]. Such differences may be real or may represent alterations induced by housing conditions or handling since δ-opioid systems have been implicated in various behavioural responses. Stressful events give rise to opiate-like induced phenomena, e.g., analgesia [Wood *et al.*, 1981], catalepsy [Ling & Pasternak, 1982], changes in body temperature [Rosow *et al.*, 1980] and locomotor activity [Tepper & Woods, 1978]. In addition, electroconvulsive shock (ECS) is also known to produce both analgesia and catalepsy. These phenomena can be blocked by naloxone implying that endogenous opioid systems at least partially regulate such events [Nabeshima *et al.*, 1985]. The mechanism of action is as yet unclear but the role of the δ-opioid receptor has been implicated. For example, in rats, electric footshock decreases [^{3}H][D-Ala^2,Met^5]enkephalinamide (DAMEA) binding [Nabeshima *et al.*, 1985] and social isolation reduces levels of [^{3}H][Met^5]enkephalin binding in some brain regions, [Petkov *et al.*, 1985] especially in aggressive animals. *In vivo* experiments have shown that swim-stress-induced antinociception can be blocked by selective δ-opioid antagonists, i.e., ICI 174,864 and naltrindole [Jackson & Kitchen, 1989a; Jackson *et al.*, 1989b] and both ICI 154,129 (N,N-diallyl-Tyr-Gly-Gly-Ψ-(CH_2S)-Phe-Leu) and ICI 174,864 have been reported to attenuate stress-induced analgesia in mice [Hart *et al.*, 1983; 1985].

Strain differences

Levels of δ-opioid binding sites (B_{max}) assayed using [^{3}H]DPDPE showed differences across a variety of rat strains, though K_D values were similar (with the possible exception of those from Aberdeen), as shown in Table 4.8. These experiments were conducted in various laboratories but under the same conditions by the same operative (except those experiments conducted at St. John's [Yoburn *et al.*, 1991]). In order to investigate these apparent differences more thoroughly, binding in several strains was examined in house. All rats were male (200 - 250 g), fed on a standard laboratory diet and kept on a 12 h light / dark cycle at a temperature of 20°C. Under such conditions differences were not apparent, as shown in Table 4.9.

Table 4.8 Characteristics of [^{3}H]DPDPE binding to homogenates of brains from various rat strains (all male and 200 - 250 g). (W = Wistar, HL = Hooded Lister and SD = Sprague Dawley.)

Strain	Laboratory	B_{max} (fmol / mg brain)	K_D (nM)
W (B&K)	Bristol	2.31 ± 0.39	5.37 ± 0.48
W (Interfauna)	PDRU	12.4 ± 1.90	4.82 ± 0.72
HL	ICI*	2.47 ± 0.17	5.42 ± 0.69
HL	Aberdeen	6.30 ± 0.81	1.60 ± 0.16
SD	St. John's†	11.1 ± 0.88	6.72 ± 0.67

Values represent means ± s.e.mean where n ≥ 3. *Cotton *et al.*, 1985. †Yoburn *et al.*, 1991.

Table 4.9 Characteristics of [^{3}H]DPDPE binding to homogenates of brains from various rat strains, all studied at Loughborough University.

Species	Source	B_{max} (fmol/mg brain)	B_{max} (fmol/mg protein)	K_D (nM)
Hooded Lister	Nott.	4.51 ± 0.29	49.1 ± 3.8	5.28 ± 1.20
Hooded Lister*	B&K	4.26 ± 0.19	52.4 ± 3.5	3.35 ± 0.08
Sprague Dawley	B&K	3.95 ± 0.68	50.6 ± 4.5	3.14 ± 0.58
Wistar*	Nott.	4.48 ± 0.58	46.9 ± 5.1	2.43 ± 0.44
Wistar*	B&K	5.29 ± 0.28	55.5 ± 4.5	2.47 ± 0.10

Values represent means ± s.e.mean where n ≥ 3 or *where n = 2 ± the range. None of the results were significantly different where $p < 0.05$.

Swim-stress and electroconvulsive shock (ECS) treatment

Scatchard analyses using [^{3}H]DPDPE were conducted on brains from control rats affording a B_{max} of 2.31 ± 0.39 fmols / mg brain and an affinity (K_D) of 5.37 ± 0.48 nM, Fig. 4.25. The level of binding in the swim-stressed rats was determined using a single concentration of [^{3}H]DPDPE (1.7 nM). This level was chosen as it gave the highest levels of specific binding, resulting in 0.95 ± 0.14 fmols / mg brain binding in control animals and should also give an indication of a change in the level or affinity of binding. No significant changes were observed in [^{3}H]DPDPE binding during a 24 hour period following swim-stress, while peak swim-stress-analgesia is noted after 10 min. Similarly no changes were observed in the 60 min following ECS treatment, (Table 4.10).

Table 4.10 Specific binding (fmol / mg brain) of 1.7 nM [^{3}H]DPDPE, in homogenates of Wistar rat brain (150 - 200 g), following swim-stress and ECS treatment.

Time post-swim	Binding	Time post-ECS	Binding
control	0.95 ± 0.14	control	0.95 ± 0.14
10 min	0.78 ± 0.07	10 min	0.75 ± 0.04
20 min	0.91 ± 0.21	60 min	0.77 ± 0.02
60 min	0.98 ± 0.17		
24 hrs	0.97 ± 0.07		

Values are means ± s.e.mean where $n \geq 3$. None of the results were significantly different where $p < 0.05$.

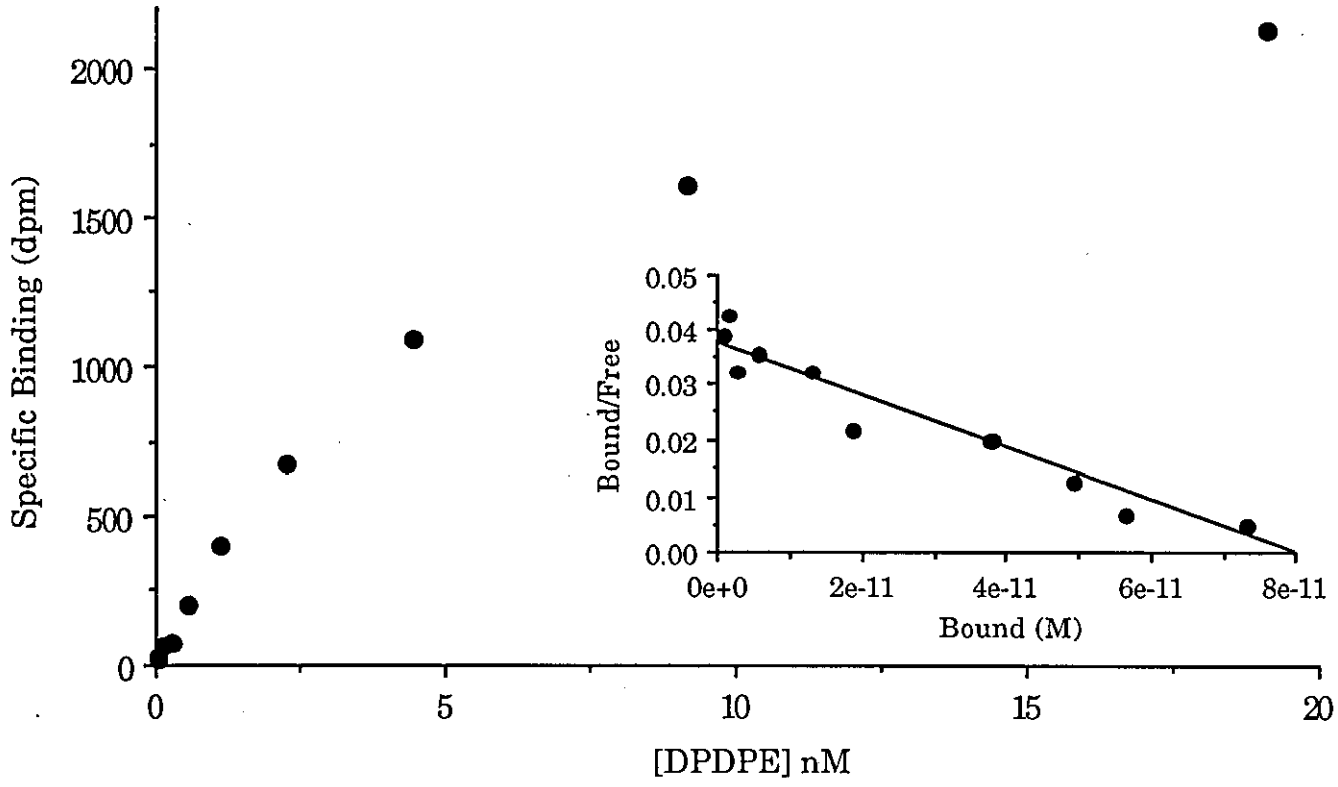


Figure 4.25 A representative graph showing saturation [^{3}H]DPDPE binding to rat brain homogenate (1 : 70 w / w Tris-HCl buffer) from an unstressed animal. Inset is the corresponding Scatchard plot.

4.3 Discussion

Labile δ-opioid sites and displacement assays with DELT I

The results of this study confirmed that [^{3}H]DPDPE bound to homogenates of mouse brain but that a portion of this binding was unstable to washing. In addition, no binding was observed in the washings and also binding did not return to control levels when the ultracentrifuged washings from the supernatants and the washed pellet were recombined.

Washed and unwashed homogenates show equal affinities for δ- and κ-opioid ligands and μ-opioid antagonists but interestingly can be distinguished by the μ-opioid agonists DAMGO and morphine [Bardaji *et al.*, 1992]. The loss of binding following the procedure is accompanied by a decrease in the number of binding sites as measured by [^{3}H]DPDPE; this could be due to a real loss of δ-opioid sites or alternatively a conversion of high affinity binding sites to low affinity ones.

The results could be explained by the existence of δ-opioid subtypes (see section 1.4.2). The results imply that there are two different forms of the δ-opioid receptor which can be differentiated by μ-opioid agonists, i.e., DAMGO displaces [^{3}H]DPDPE binding to the labile δ-binding site with high affinity but displaces [^{3}H]DPDPE binding to the stable δ–binding site with much lower affinity [Bardaji *et al.*, 1992]. One classification of δ-opioid receptors proposes the existence of mu-complexed delta sites (δ_{cx}) and non-complexed delta sites (δ_{ncx}) [for review see Traynor & Elliott, 1993]. These findings fit with such a classification, i.e., perhaps the labile δ-binding site is equivalent to δ_{cx}, while the stable δ–binding site corresponds to δ_{ncx}. The presence of δ-opioid subtypes is also suggested by the competition assays between [^{3}H]DPDPE and DELT I. In the majority of experiments even 1 μM DELT I could not displace all of the [^{3}H]DPDPE binding to mouse brain homogenates. This is consistent with previous ligand binding studies showing biphasic inhibition of [^{3}H]DELT I specific binding by DPDPE [Negri *et al.*, 1991]. Alternatively since cloning studies have as yet only isolated a single form of the δ-opioid receptor [Uhl *et al.*, 1994] perhaps subtypes arise from differences between systems down-stream of the receptor, e.g., differential coupling of receptor to one or more G proteins, or variable interactions between G proteins and effectors. Thus the results could possibly be indicative of the accessibility of the agonist to the binding domain of the receptor in the membrane or indeed to some post-translational modification.

Novel natrindole analogues

In order to further investigate δ-opioid subtypes better tools are required. Naltrindole was synthesised using the message-address concept [Portoghese *et al.*, 1990]. An indole system was fused to naltrexone in order that the benzene moiety of indole mimicked the Phe^4 group of enkephalin and acted as an address component of the δ-opioid receptor, while the pyrrole system acted as a spacer to the naltrexone component which was viewed as the message portion of the compound. Unfortunately other workers [Rogers *et al.*, 1990] have been unable to reproduce the high selectivity initially reported by Portoghese and futhermore at high doses naltrindole does possess some agonist activity [Jackson *et al.*, 1989b; Staplefield *et al.*, 1992]. Selective and potent non-peptide δ-opioid agonists and antagonists are essential to gain further insights of the δ-opioid receptor and its subtypes. In the hope of discovering suitable ligands, twelve naltrindole analogues were evaluated. A large amount of data was produced from the binding assays and by carefully comparing the results of pairs of compounds, where only one substituent varied, a number of trends in binding affinity were observed.

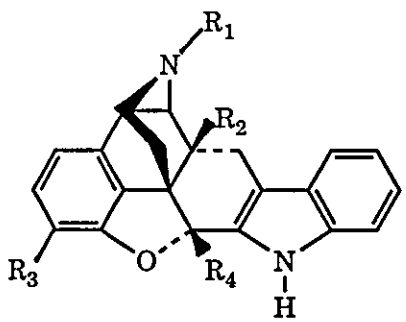


Figure 4.26 Structure of the HS series of naltindole analogues.

With reference to the structure given above:

i) On changing the R_4 substituent from H to Me there was a consistent loss in affinity for the κ-opioid site. Depending on other substituents sometimes there was no change, or a loss, in affinity at μ- and δ-opioid sites.

ii) When considering the R_3 substituent, OH was preferable to OMe at all three opioid sites. On average there was a 32 - fold loss in affinity

at the μ-opioid site, 20 - fold at the κ-opioid site and 12 - fold at the δ-opioid site on replacing OH by OMe.

iii) The substituent at R_2 had the least affect on affinity. At the μ- and κ-opioid sites there was no difference whether the substituent was OEt, OMe, OH or H. At the δ-opioid site OEt was prefered over OMe and OH.

iv) The best κ / δ selectivity was achieved when the R_2 substituent was either OH or OEt but not OMe or H (see Fig. 4.27) When the R_2 group was OEt high μ / δ selectivity was possible but unlike κ / δ selectivity an OH group at R_2 was not favourable. Thus δ-opioid selectivity was favoured by the bulkiest substituent at R_2, and possibly an even larger substituent would have conferred an even greater degree of selectivity, while H or OMe gave non-selective ligands. In general better μ / δ and κ / δ selectivity was achieved when the R_3 substituent was OMe as opposed to OH. There were no apparent trends in the effects R_4 on selectivity.

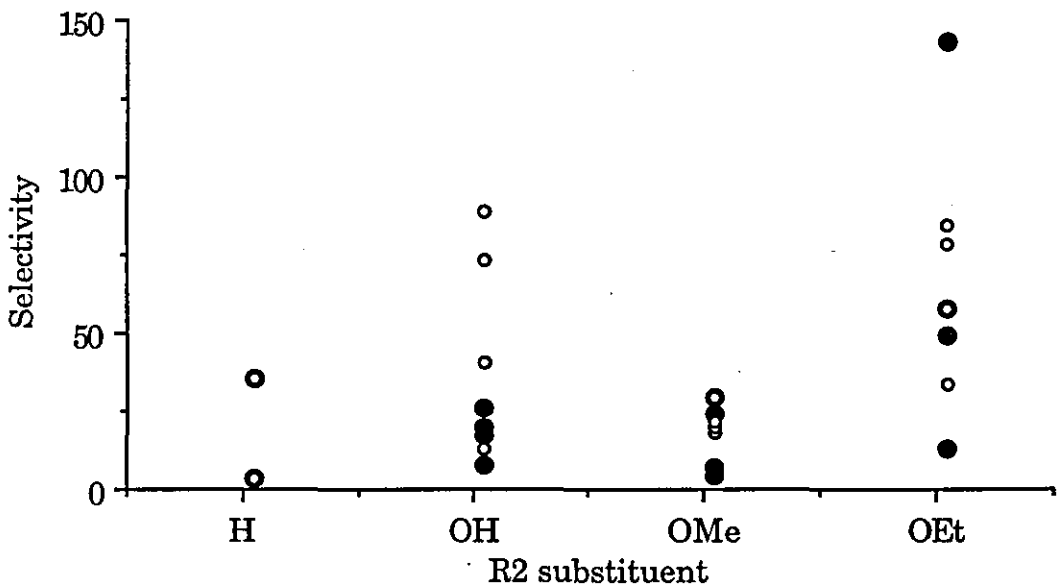


Figure 4.27 Effect of the R_2 substituent in the HS series of naltrindole analogues on μ /δ (●) and κ / δ selectivity (O), from ligand binding assays conducted in guinea-pig brain (see Table 4.3).

The most promising compounds from binding experiments were HS 328, HS 350 and HS 378 and so these were selected for evaluation in isolated tissue preparations. In agreement with highly selective δ-opioid binding and the N-methyl substituent both HS 328 and HS 350 were full δ-opioid agonists in the mouse vas deferens preparation, but with no appreciable activity in the guinea-pig myenteric plexus-longitudinal muscle preparation which contains only functional μ- and κ-opioid receptors [Chavkin *et al.*, 1982]. In comparison to the partial agonist oxymorphindole (a naltrindole analogue) [Portoghese *et al.*, 1990] both HS 328 and HS 350 appear to be selective δ-opioid full agonists. Unfortunately in common with oxymorphindole they possess rather low δ-opioid receptor affinity.

If a stable compound was available, able to pass through the blood brain barrier, it would be extremely useful in understanding the function of the δ-opioid receptor *in vivo* and possible therapeutic usefulness of ligands selective for this site.

Since this study was undertaken, another non-pepide δ-opioid agonist has been developed; BW373U86 ((±)-4-(α-R)-α-((2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-hydroxy-benzyl-N,N-diethylbenzamide) (see Fig. 4.28) [Chang *et al.*, 1993]. In the same manner as HS 328 and HS 350, BW373U86 is a full and potent δ-opioid agonist in the mouse vas deferens prepartion (ED_{50} = 0.20 nM). However, it also acts as a full μ-opioid agonist in the guinea-pig ileum (ED_{50} = 143 nM). Therefore from isolated tissue studies BW373U86 is approximately 700 times more selective for δ- as opposed to μ-opioid receptors. This high selectivity is not maintained in ligand binding assays to rat brain where its μ / δ ratio = 10, and κ / δ ratio is 20. In addition, BW373U86 does not possess antinociceptive activity when administered s.c. to rats. Thus overall BW373U86 appears to be of limited use. Indeed further *in vivo* studies suggest that BW363U86 is a partial agonist with δ-opioid mediated actions at spinal sites but μ-opioid mediated actions at supraspinal sites [Wild *et al.*, 1993b].

A cyclopropyl group on the nitrogen atom of opiates confers antagonist activity [Casy & Parfitt, 1985]. In agreement with this theory a cyclopropyl N-substituent in HS 328 yielded the antagonist HS 378 (14-ethoxy-5-methylnaltrindole). HS 378 showed similar selectivity to the parent compound naltrindole in binding assays. However, in bioassays the affinity of HS 378 was approximately 7 - fold weaker, but it was more selective, especially over μ- / δ-opioid sites.

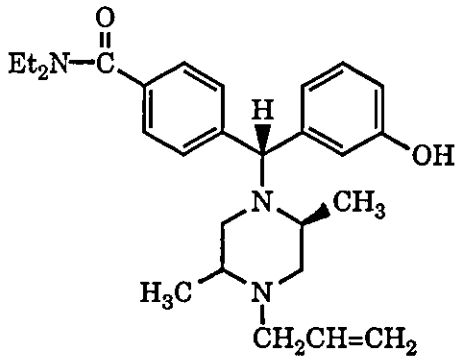


Figure 4.27 Structure of BW373U86

Studies of RX 8008M and etorphine in isolated tissues

Both putative δ_1- (DPDPE and DADLE) and δ_2- (DELT II and DSLET) opioid agonists mediate their actions *via* a receptor population recognised as a single site by the antagonist RX 8008M. Thus RX 8008M is unable to distinguish δ-opioid subtypes in the mouse vas deferens. This finding is perhaps not unexpected since another study using antagonists selective for the putative δ-opioid subtypes suggests that the δ-opioid population in the mouse vas deferens consists of a homogeneous subtype [Wild *et al.*, 1993a]. However, investigations in the hamster vas deferens revealed that RX 8008M had 7-fold lower affinity for the δ-opioid receptor in the hamster vas deferens as opposed to the mouse vas deferens. This differential affinity activity of RX 8008M was not as large as previously reported [Smith, 1987]. Unfortunately Smith did not quote the concentration of RX 8008M used for antagonist studies. For some unknown reason in this study steep Schild plots were consistently obtained. If the antagonist possesses agonist activity at high concentrations then the dose-response curve of the agonist will be shifted to a lesser degree than predicted, thus the dose-ratio in the presence and absence of antagonist will be smaller than expected and a steep Schild plot is obtained [Kenakin, 1984]. However, no agonist activity even by high concentrations of RX 8008M was observed.

Another compound very similar in structure to RX 8008M but with a different N-substituent group is etorphine (Fig. 4.15). Consistent with previous studies etorphine was found to be a potent and full agonist in the mouse vas deferens and guinea-pig myenteric plexus-longitudinal muscle preparation but only a partial agonist in the hamster vas deferens preparation.

The opioid receptor population in the hamster vas deferens is reported to be exclusively of the δ type [McKnight *et al.*, 1984]. Since etorphine is regarded as a potent but non-selective opiate there should be no obvious reason why it is only weakly active at the δ-opioid receptor of the hamster vas deferens. However, the results suggested that etorphine is not only a low efficacy agonist at the δ-opioid receptor in the hamster vas deferens preparation (10 μM only inhibited 60 % of the opiate sensitive twitch) but also prefers the μ- to the δ-opioid receptor in the mouse vas deferens preparation. The K_e value of naloxone determined against etorphine was 34.8 ± 3.4 nM (consistent with previous findings [Ward *et al.*, 1982a]) and is indicative of activity mediated through δ- or κ- but not μ-opioid receptors (naloxone exhibits approximately 10-fold higher affinity for μ- (1-3 nM) as opposed to δ- (20-30 nM) opioid sites, and an intermediate affinity at κ-opioid sites [Leslie, 1987]). However, in direct contrast, the K_e values of cyprodime, nBNI and naltrindole, versus etorphine, all suggest etorphine exerted its effects *via* the μ-opioid receptor in the mouse vas deferens preparation. A μ-opioid receptor mediated action of etorphine in this tissue has been previously suggested since etorphine is antagonised by the irreversible alkylating agent β-funaltrexamine (β-FNA) (at a dose of β-FNA which antagonised the μ-opioid agonist methadone but not the δ-opioid agonists [Met^5]enkephalin and DADLE) [Ward *et al.*, 1982b]. Since etorphine can act *via* δ-opioid receptors in binding assays conducted in guinea-pig brain [Magnan *et al.*, 1982], stimulate δ-opioid receptors in the spinal cord of mice following i.c.v. injection [Xu & Tseng, 1992] and can inhibit cAMP accumulation in NG108-15 cells [Law *et al.*, 1983] these findings may further highlight the differences between δ-opioid receptors in the periphery and those in the CNS and cell lines [Shimohigashi *et al.*, 1987; 1988; Vaughn *et al.*, 1990]. Particularly since the mouse vas deferens has a larger receptor reserve for δ- than μ- or κ-opioid receptors and δ-opioid ligands are very potent in this tissue. These differences may not be due to actual δ-opioid subtypes but may illustrate pharmacokinetic differences, as etorphine is a very lipophilic compound which may have difficulty gaining access to δ-opioid receptors in the lipid membrane of certain tissues and cells. Alternatively, if etorphine does mediate its actions *via* μ-opioid receptors in the mouse vas deferens preparation the high K_e value of naloxone may be a reflection of its poor selectivity and thus the need for highly selective antagonists is highlighted.

Effects of strain differences and stress on δ sites

In this study δ-opioid receptor levels in brain homogenates from various rat strains housed in the same or different laboratories was examined. The effects of swim-stress and electroconvulsive shock (ECS) on the level of [^{3}H]DPDPE binding were also investigated [Elliott *et al.*, 1992].

Differences were apparent in binding experiments using [^{3}H]DPDPE in rat brain species, in which the maximum level of binding appears to vary froma low of 2.31 to a high of 12.4 fmol / mg brain. However, these discrepancies may be related to the housing conditions of the animals, rather than strain variation since no differences were seen in rat strains from ligand binding assays of animals housed in the same place.

Since these deviations may involve stress responses to housing conditions the effects of swim-stress analgesia on δ-opioid binding were investigated. In order to conserve materials and animals the specific binding of [^{3}H]DPDPE was only tested at one concentration, but a significant increase in B_{max} or K_D would result in a higher level of binding, and if apparent this could be investigated further using a range of concentrations of [^{3}H]DPDPE. Although δ-opioid receptor systems are involved in swim-stress analgesia, since the effect can be blocked with the δ-selective opioid antagonists naltrindole and ICI 174,864 [Jackson & Kitchen, 1989a; Jackson *et al.*, 1989b], no significant change in the level of δ-opioid specific binding was noted, which implies no alteration in receptor numbers or K_D values occurred.

More recent studies utilising putative δ_1-opioid and δ_2-opioid agonists and antagonists in the mouse have inferred that antinociceptive responses to cold water swim-stress (CWSS (5°C)) are mediated *via* the activation of the δ_2-opioid receptor [Vanderah *et al.*, 1992]. This finding is based on the fact that cold water-induced antinociception is selectively antagonised by the δ_2-opioid antagonist naltrindole-5'isothiocyanate (5'NTII) but not the δ_1-opioid antagonist [D-Ala^2,Leu^5,Cys^6]enkephalin (DALCE), and also there is two-way antinociceptive cross-tolerance between the δ_2-opioid agonist DELT II and CWSS. Furthermore, the increase in morphine potency following CWSS is also mediated *via* the δ_2-opioid receptor as shown by the selective antagonism by 5'NTII but not DALCE, and therefore this subtype of δ-opioid receptor may be functionally coupled to μ-opioid receptors [Vanderah *et al.*, 1993].

ECS treatment also failed to alter δ-opioid binding. This latter result is perhaps not unexpected. Jackson & Nutt [1991] have shown that ECS-induced antinociception and catalepsy are blocked by naloxone and RX 8008M but not by naltrindole. They therefore proposed that these responses are mediated *via* the

release of an endogenous opioid active at μ-opioid sites, e.g., β-endorphin. This is consistent with the increased levels of this peptide found in plasma after ECS treatment.

In conclusion, the washing and displacement studies do constitute some evidence for the existence of δ-opioid receptor subtypes. However, neither the naltrindole analogues developed nor studies of receptors conducted allow for expansion of these ideas.

CHAPTER 5
OPIOID BINDING IN SH-SY5Y CELLS

Every form of addiction is bad, no matter whether the narcotic be alcohol, morphine or idealism.

C.G. Jung (1875 - 1961)

5.1 Introduction

Human neuroblastoma SH-SY5Y cells are a subclone of the SK-N-SH cell line. The parent cell line was isolated from a tumour in the bone marrow of a 4 year old girl [Biedler *et al.*, 1973]. SH-SY5Y cells are known to possess various receptor types, i.e., α_2-adrenergic [Kazmi & Mishra, 1989], M_1-, M_2- [Serra *et al.*, 1988] and M_3-muscarinc [Lambert *et al.*, 1989] and also μ- and δ-opioid receptors [Kazmi & Mishra 1986; 1987].

The ratio of μ- / δ-opioid binding sites is between 2 : 1 [Kazmi & Mishra, 1987] and 5 : 1 [Yu & Sadee, 1988], and, importantly, electrophysiological and fluorescence microscopy studies have confirmed that cells express both receptor types [Seward *et al.*, 1989; Agarwal & Glasel, 1993]. Activation of μ- and δ-opioid pertussis toxin-sensitive G protein coupled receptors leads to inhibition of adenylate cyclase [Kazmi & Mishra, 1987], activation of phospholipase C, increased levels of the second messenger inositol(1,4,5)-triphosphate [Smart *et al.*, 1994] and inhibition of calcium channels [Seward *et al.*, 1989]. Using photoaffinity labelling of the receptor activated G proteins, with the guanosine triphosphate analogue [α-^{32}P]GTP azidoanilide, it has been found that both μ- and δ-opioid receptors in the cells interact with various different G proteins, namely G_{i1}, G_{i2}, G_{i3}, G_{o1} and G_{o2} [Laugwitz *et al.*, 1993]. Although these different receptor types have not been shown to activate different effector systems they do preferentially activate different G proteins as confirmed by immunoprecipitation of the photolabelled G protein α-subunits with subtype-specific antisera, i.e., μ-opioid receptors are more effectively coupled to G_{i3}, whereas δ-opioid receptors preferentially couple to G_{i1}. Another study utilising Western-blot analysis also revealed the existence of $G_{s\alpha}$, $G_{z\alpha}$ and Gβ G protein subunits in these cells [Ammer & Schulz, 1993]. Incubation of cells with various anti-G protein antibodies followed by adenylate cyclase assays has also endorsed the idea of opioid receptors being capable of activating various subtypes of G proteins [Carter & Medzihradsky, 1993a].

Thus SH-SY5Y human neuroblastoma cells provide a very useful tool for the further characterisation of both μ- and δ-opioid receptors. For example, using ligand binding studies and inhibition of PGE_1-stimulated cAMP accumulation as a form of response, a range of μ-opioids has been evaluated, illustrating that SH-SY5Y cells offer a good system in which to evaluate functional responses of μ-opioid agonists [Costa *et al.*, 1991]. While the small magnitude of the biochemical response of cAMP assays in these cells limits its

use for drug screening purposes this method does provide a useful technique of evaluating signal transduction pathways. In this investigation the level of μ- and δ-opioid sites was determined using ligand binding assays and the possible existence of μ-opioid subtypes (see sections 1.4.3) was examined using the irreversible antagonists naloxonazine and β-funaltrexamine.

5.2 Results

5.2.1 Saturation binding assays in SH-SY5Y cells

Initial experiments were designed to illustrate the opioid receptor population on the SH-SY5Y cells was the same as that commonly reported in the literature. Saturation binding utilising various radioligands was conducted and both the levels (B_{max} values) and affinities (K_D values) obtained were consistent with previous findings [Kazmi & Mishra, 1987; Yu *et al.*, 1986]. Studies employed the following radioligands: [^{3}H]diprenorphine, a non-selective antagonist [Leslie, 1987], [^{3}H]DAMGO ([D-Ala^2,MePhe^{4}Gly(ol)^5]-enkephalin), a μ-selective peptide agonist [Handa *et al.*, 1981], [^{3}H]DPDPE ([D-Pen^2,D-Pen^5]enkephalin), an agonist peptide [Akiyama *et al.*, 1985] and [^{3}H]natrindole [Yamamura *et al.*, 1992], a non-peptide antagonist. The latter two being selective for the δ-opioid receptor. The results are summarised in Table 5.1.

Table 5.1 Radioligand saturation binding in SH-SY5Y membranes

Opioid	B_{max} (fmol / mg protein)	K_D (nM)
Diprenorphine	206 ± 36	0.24 ± 0.07
DAMGO	140 ± 11	1.45 ± 0.31
DPDPE	56 ± 11	0.97 ± 0.07
Naltrindole	70 ± 16	0.072 ± 0.012
Diprenorphine*	60 ± 10	0.22 ± 0.02

Assays conducted at 25°C for 40 min, Tris-HCl buffer pH 7.4, 100 μg protein / ml, *performed in whole cells in Krebs / Hepes buffer. At the 95 % confidence limit: the B_{max} of naltrindole compared to DPDPE is not significantly different. Values represent means ± s.e.mean where $n \geq 3$.

5.2.2 *Characterisation of μ-opioid receptors on SH-SY5Y cells using naloxonazine and β-funaltrexamine*

The μ-opioid receptor from rat brain has been cloned [Chen *et al.*, 1993] but no indication of distinct subtypes is yet apparent. However biochemical and pharmacological experiments do suggest μ-opioid receptor heterogeneity may exist (see section 1.4.3).

The human neuroblastoma SH-SY5Y cell line is known to express both μ- and δ-opioid receptors, with μ-opioid receptors predominating, see section 5.2.1 [Yu & Sadee, 1988; Kazmi & Mishra, 1987]. The possibility of different μ-opioid receptor subtypes being expressed by SH-SY5Y cells has yet to be determined, though based on studies with the selective μ-opioid receptor antagonist [D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2] (CTOP) [Pelton *et al.*, 1985] it has been suggested that a single μ-opioid receptor site in a single affinity state exists [Toll, 1990]. The irreversible μ_1-opioid antagonist naloxonazine and the μ-opioid alkylating agent β-funaltrexamine should enable distinction of multiple μ-opioid receptors on whole undifferentiated SH-SY5Y human neuroblastoma cells.

Binding of naloxonazine to rat cortical membranes and SH-SY5Y cells

Naloxonazine was able to displace specifically bound [^{3}H]diprenorphine from whole undifferentiated SH-SY5Y cells, affording a K_i of 3.4 ± 0.7 nM (Fig. 5.1). Pretreatment of the cells with naloxonazine over the concentration range 3 - 300 nM inhibited the binding of [^{3}H]DAMGO (3 - 3.5 nM) in a concentration-dependent manner, but this was reversed by repeated washing, with specific binding returning to control values within four washes (Table 5.2). In contrast, washing experiments showed that naloxonazine (300 nM) inhibited the binding of [^{3}H]DAMGO (3 - 3.5 nM) to rat cortical membranes but a portion of this binding was irreversible and allowed only a 71 % recovery of specific ligand binding even after six washes (Table 5.3).

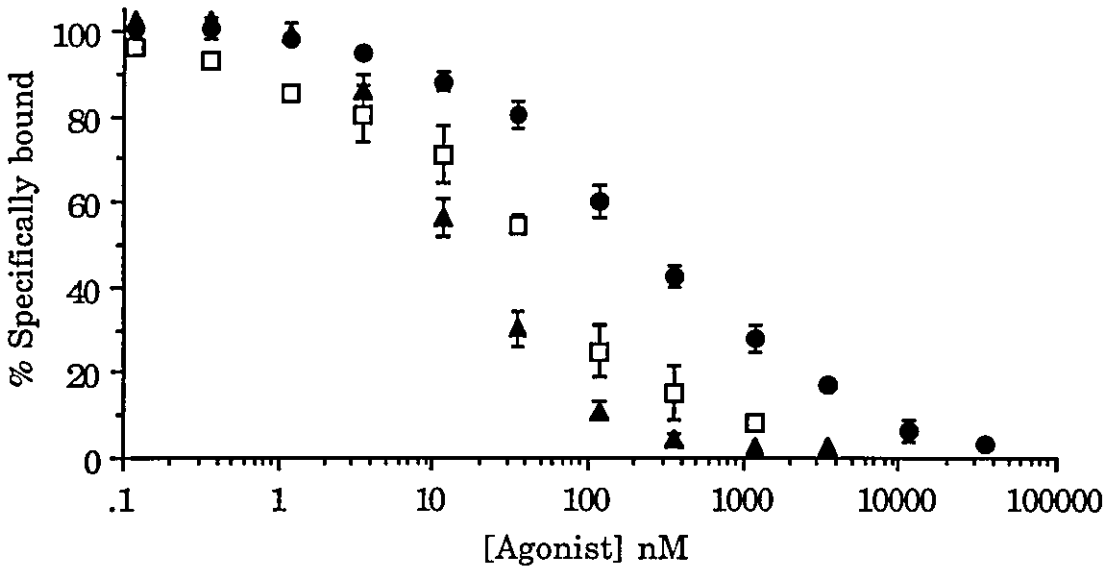


Figure 5.1 Competitive inhibition of [^{3}H]diprenorphine (0.4 - 0.7 nM) specific binding to SH-SY5Y whole cell suspensions by naloxonazine (▲), cyprodime (●) and naltrindole (□). Values represent means ± s.e.mean where n ≥ 3.

Table 5.2 Inhibition of specific [^{3}H]DAMGO (3 - 3.5 nM) binding to rat cortical membranes and SH-SY5Y whole cells by naloxonazine.

	[Naloxonazine] (nM)			
	SH-SY5Y cells			Rat brain*
Wash No.	3	30	300	300
0	74.3 ± 2.2†	54.3 ± 6.5†	13.7 ± 3.2†	0 ± 0†
2	92.8 ± 2.9	68.4 ± 9.9†	31.8 ± 5.2†	4.8 ± 0.6†
4	100 ± 0	98.9 ± 2.5	90.7 ± 1.3	23.8 ± 1.1†
6	101.4 ± 1.4	101.4 ± 1.4	98.5 ± 5.2	71.4 ± 2.3†

All experiments using naloxonazine were conducted by Dr. D. Smart, Dept. of Anaesthesia, Leicester University, Leics., LE1 5WW. Values represent % of control values and are means ± s.e.mean where n ≥ 3. *Rat cortical membranes. †$P < 0.05$ compared to controls.

Treatment of SH-SY5Y cells with β-funaltrexamine

Pretreatment of cells with β-funaltrexamine caused a dose-related reduction in the specific binding of [^{3}H]diprenorphine (Fig. 5.2) which was not reversed by extensive washing. However, the loss of ligand binding sites was not total and even following treatment with 1 μM β-funaltrexamine 39 ± 6 % specific [^{3}H]diprenorphine binding still remained.

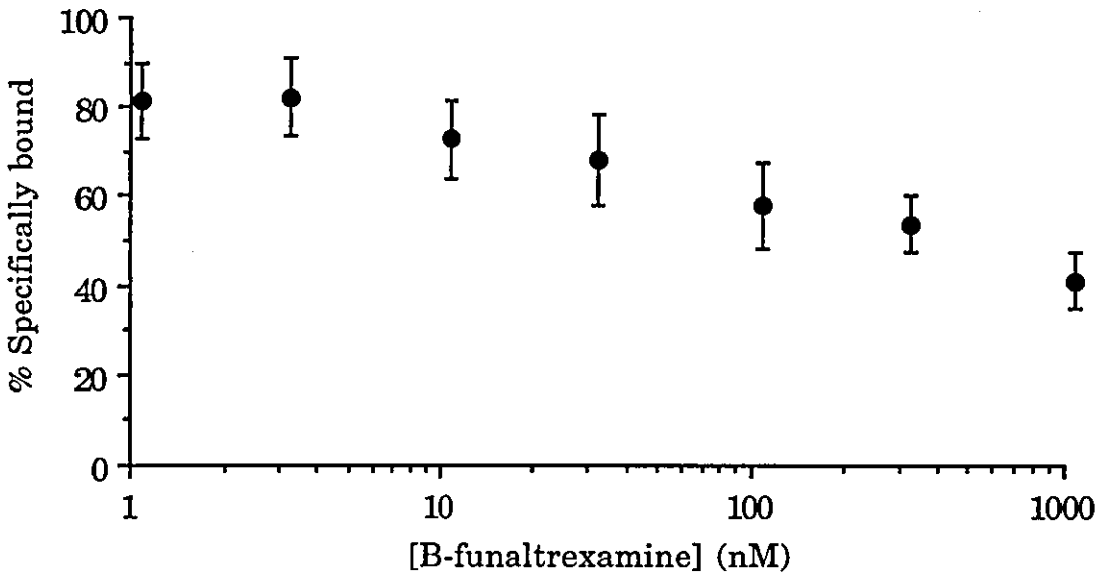


Figure 5.2 Dose-dependent inhibition of [^{3}H]diprenorphine (0.8 nM) specific binding to SH-SY5Y whole cell suspensions by β-funaltrexamine. Values represent means ± s.e.mean where n ≥ 3. At the 95 % confidence level the value at 1000 nM is not significantly different from that at 100 nM.

Since [^{3}H]diprenorphine binds to both μ- and δ-opioid receptors it is possible that the β-funaltrexamine resistant sites may be δ-opioid receptor binding sites. However, competition assays with the μ-opioid receptor antagonist cyprodime and the μ-opioid receptor antagonist naltrindole afforded K_i values typical of interactions with μ-opioid receptor sites, i.e., 51.8 ± 1.8 nM and 11.7 ± 3.6 nM respectively (Fig. 5.1), confirming that the [^{3}H]diprenorphine

was binding largely to μ-opioid receptor sites on the cells at the radioligand concentrations used. To further establish that the β-funaltrexamine resistant sites were μ-opioid receptor sites, binding of [^{3}H]DAMGO (2.1 - 2.3 nM) was performed in membranes following treatment of whole cells with 1 μM β-funaltrexamine for 1 h. Specific [^{3}H]DAMGO binding was reduced to only 40 ± 13 %, thus the same level of sites remained insensitive to β-funaltrexamine whether sites were labelled with [^{3}H]diprenorphine or [^{3}H]DAMGO (Fig. 5.3).

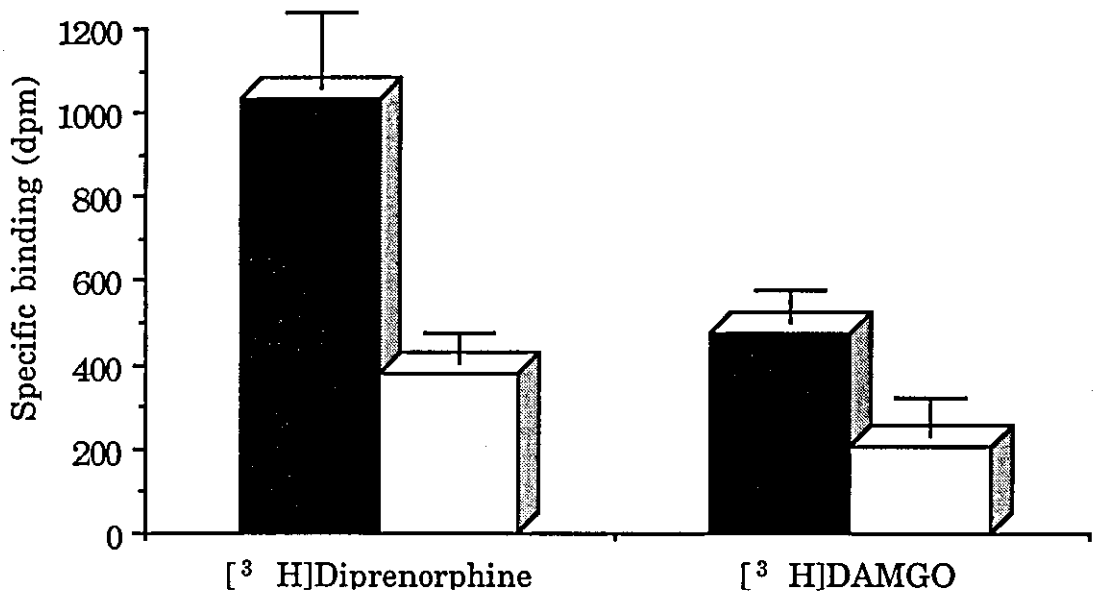


Figure 5.3 Specific binding of [^{3}H]diprenorphine (0.87 nM) and [^{3}H]DAMGO (2.22 nM) in control (black) and β-funaltrexamine- (1 μM, 1 h) treated SH-SY5Y cells (white). Values are means ± s.e.mean where n ≥ 3.

[^{3}H]DAMGO (2.3 - 2.4 nM) binding to the β-funaltrexamine insensitive site was displaced by DAMGO affording an IC_{50} of 2.7 ± 0.4 nM (n = 2, the error equals the range), identical to the IC_{50} attained in untreated membranes (Fig. 5.4).

Treatment of cells with pertussis toxin (100 ng / ml for 40 h) resulted in a reduction of 56 ± 3 % in the specific binding of [^{3}H]DAMGO to membranes. β-funaltrexamine treatment of the cells, following pertussis toxin treatment, again maximally afforded a 60 % reduction in the μ-opioid receptor binding sites, as measured by [^{3}H]DAMGO specific binding (Fig. 5.5).

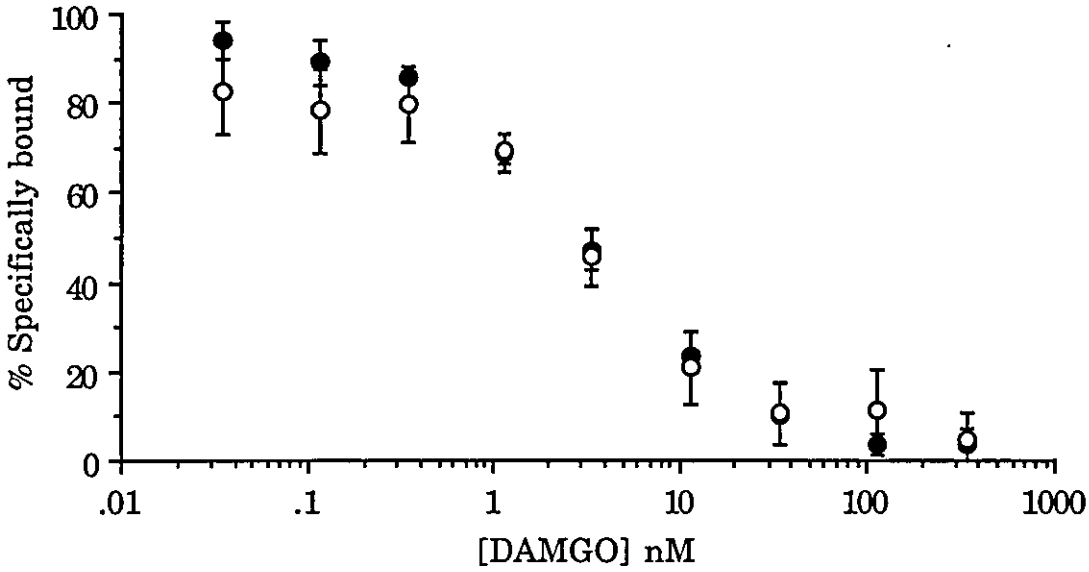


Figure 5.4 Displacement of [^{3}H]DAMGO (2.3 nM) specific binding by DAMGO in control (●) and β-funaltrexamine- (1 μM, 1 h at 37°C) treated SH-SY5Y cells (O). Each concentration point was conducted in duplicate and values are means ± the range where n = 2.

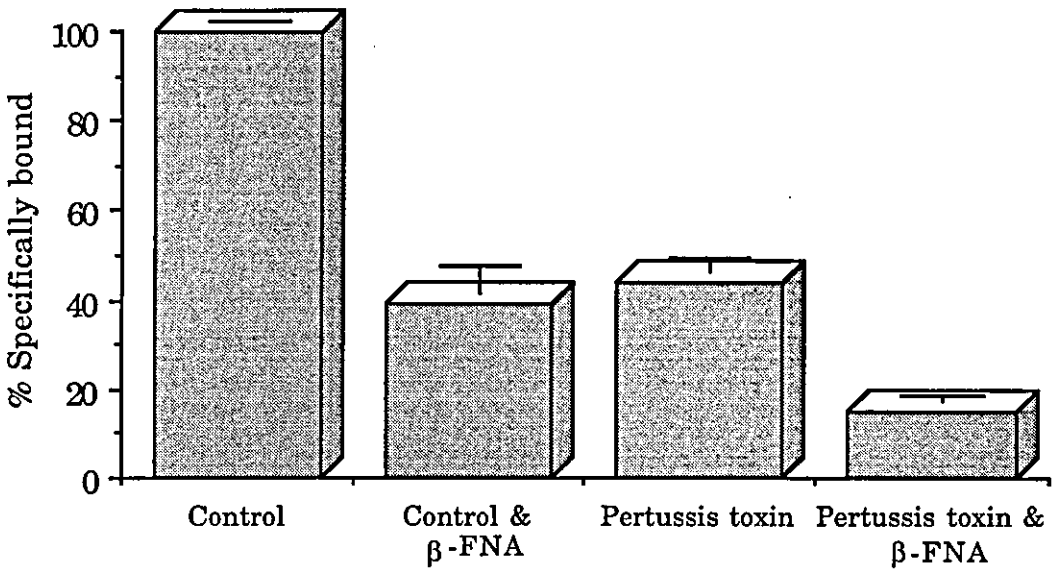


Figure 5.5 Specific binding of [^{3}H]DAMGO (3.4 nM) in control and pertussis toxin-treated SH-SY5Y cells (100 ng / ml, 40 h) with and without β-funaltrexamine treatment (1 μM, 1 h, 37°C). Values are means ± s.e.mean where n ≥ 3.

5.2.3 Characterisation of δ-opioid receptors on SH-SY5Y cells

At the commencement of these studies the δ-opioid receptor on SH-SY5Y cells had not been characterised in terms of the putative δ_1 and δ_2 classification (see section 1.4.2). Thus the displacement of the δ-selective antagonist [^{3}H]naltrindole by various ligands was examined (Table 5.3).

Table 5.3 Affinity (K_i values) of various opioids for the δ-opioid receptor (as labelled by [^{3}H]naltrindole) in SH-SY5Y membranes.

Opioid	K_i (nM)
Naltrindole	0.12 ± 0.04 (3)
DPDPE	0.99 ± 0.7 (2)
DELT II	3.7 ± 2.2 (2)
DAMGO	611 (1)
Cyprodime	1690 (1)

Assays conducted at 25°C for 40 min, Tris-HCl buffer pH 7.4, 100 μg protein / ml. The number of determinations is given in brackets. Errors represent s.e.mean where n = 3 or the range where n = 2.

The specific binding achieved using [^{3}H]naltrindole (or [^{3}H]DPDPE) was never greater than 40 %, and unfortunately a second batch of [^{3}H]naltrindole only gave 10 - 15 % specific binding. Thus accurate and reproducible results were unattainable.

5.3 Discussion

Ligand binding assays in SH-SY5Y cells

Both μ- and δ-opioid binding sites were observed in the SH-SY5Y cells with μ- opioid binding sites 2 - 3 times more predominant. The antagonists examined had approximately 10 - fold higher affinity for the opioid receptors than the peptidic agonists tested.

The non-selective antagonist [^{3}H]diprenorphine labels more sites (206 ± 36 fmol / mg protein) than any of the other ligands. One reason for this could be that it recognises both μ- and δ-opioid receptors. Alternatively this could be a relection of its antagonist activity as it would be able to label both high and low affinity opioid binding sites. In addition, the level of [^{3}H]diprenorphine binding sites is considerably higher in membranes than whole SH-SY5Y cells. This difference suggests the majority of [^{3}H]diprenorphine binding sites are intracellular. The affinity of [^{3}H]diprenorphine for cell surface receptors and intracellular receptors appears to be the same.

Characterisation of μ-opioid receptors on SH-SY5Y cells using naloxonazine and β-funaltrexamine

The irreversible nature of the binding of the μ_1-opioid receptor affinity label naloxonazine to a population of μ-opioid receptor sites was demonstrated in rat cortical membranes. The level of μ_1-opioid receptor sites observed is similar to that reported by Pasternak [Johnson & Pasternak, 1984]. On SH-SY5Y whole undifferentiated cells naloxonazine was able to displace [^{3}H]diprenorphine affording a K_i of 3.4 ± 0.7 nM, consistent with previous findings [Hahn *et al.*, 1982]. However, unlike rat cortical membranes, the binding of naloxonazine to SH-SY5Y cells was found to be fully reversible, suggesting that naloxonazine-sensitive μ_1-opioid receptors are not present on these cells. Thus the μ-opioid receptor populations on whole undifferentiated SH-SY5Y cells and rat cortical membranes differ and the SH-SY5Y cells appear to express only the μ_2-opioid receptor subtype of opioid receptor as defined by Pasternak & Wood (1986).

On the other hand the μ-opioid receptor binding sites on SH-SY5Y cells can be alkylated by β-funaltrexamine, but as in other systems approximately 40 % [Rothman *et al.*, 1983; Tam & Liu-Chen, 1986; Franklin & Traynor, 1991] of these sites appear to be insensitive to alkylation by this irreversible

antagonist. It is possible that these insensitive sites represent δ-opioid receptors since β-funaltrexamine is less able to alkylate such receptors [Ward *et al.*, 1982b] and [^{3}H]diprenorphine does bind to both μ- and δ-opioid receptors [Leslie, 1987]. However, application of the occupancy theory using the known affinity of diprenorphine for μ-and δ-opioid receptor sites (i.e., 0.31 and 1.48 nM respectively [Traynor *et al.*, 1987]) and a ratio of μ- and δ-opioid receptor sites of 2.5 : 1 (see section 5.2.1) indicates that ,at the most, only 10 - 20 % of the specifically bound [^{3}H]diprenorphine would be associated with δ-opioid receptor binding sites. Furthermore, competition assays with cyprodime, which has greater than 100-fold selectivity for μ-opioid receptor sites (K_i = 55.4 nM) as opposed to δ-opioid receptor sites (K_i = 6108 nM) [Schmidhammer *et al.*, 1989], and naltrindole which is 66 times more selective for δ-opioid receptor sites (K_i = 0.257 nM) than μ-opioid receptor sites (K_i = 17.0 nM) [Rogers *et al.*, 1990] afforded K_i values in the expected range for binding to μ-opioid receptors. In addition, following treatment of SH-SY5Y cells with 1 μM β-funaltrexamine, considerable specific binding of the highly μ-opioid receptor selective radioligand [^{3}H]DAMGO was still observed, accounting for 40 % of control levels. Finally, the same levels of β-funaltrexamine insensitive sites were observed whether binding sites were labelled with [^{3}H]diprenorphine or with [^{3}H]DAMGO. It was interesting to note that pertussis toxin treatment of the cells afforded a reduced μ-opioid receptor population, which still exhibited both β-funaltrexamine-sensitive and β-funaltrexamine-insensitive opioid binding sites.

In conclusion, these data suggest that whole undifferentiated SH-SY5Y cells do not express μ_1-opioid receptors and may represent a μ_2-opioid receptor system. The insensitivity of a percentage of the μ-opioid receptor population, labelled by [^{3}H]diprenorphine or [^{3}H]DAMGO, to alkylation by β-funaltrexamine does suggest that differences do exist in this μ_2-opioid receptor population. Similarly, 70 % of high affinity μ-opioid binding sites in guinea-pig brain tissue are β-funaltrexamine sensitive, whilst high affinity μ-opioid binding sites in myenteric plexus are insensitive to alkylation. These differences could be attributed to different coupling systems, variable binding sites, alternative G protein subtypes or variable interactions between G protein and receptor [Franklin & Traynor, 1991]. However while this may indicate a further subdivision, as has been proposed for rat brain μ-opioid receptors [Rothman *et al.*, 1983; 1987], the finding could equally be explained by a lack of access to the nucleophilic alkylation site of a certain proportion of the

homogeneous μ-opioid receptor population as has been suggested by other studies. In 7315c cells 30 % of solubilised, [^{3}H]etorphine prelabelled μ-opioid receptors remain sensitive to guanosine triphosphate (GTP) after pertussis toxin treatment, suggesting that perhaps solubilisation releases some G_i protein that was previously protected by the intact membrane [Frey *et al.*, 1989].

Characterisation of δ-opioid receptors on SH-SY5Y cells

The level of δ-opioid binding was the same whether assayed using [^{3}H]DPDPE or [^{3}H]naltrindole but since antagonists label all affinity states and [^{3}H]naltrindole labelled the δ-opioid site with higher affinity this ligand was used to investigate the possiblity of δ-opioid subtypes. Since the δ-opioid selective ligands were able to displace [^{3}H]naltrindole with ≥ 150 times higher affinity than the μ-selective ligands DAMGO and cyprodime [Schmidhammer *et al.*, 1989] the initial experiments suggest that [^{3}H]naltrindole is indeed selective for δ- as opposed to μ-opioid sites. However, there is no evidence that [^{3}H]naltrindole predominantly labels either the putative δ_1 or δ_2 sites in these cells since the δ_1 preferring agonist DPDPE and the δ_2 preferring agonist DELT II are able in these cells to displace [^{3}H]naltrindole with the same level of high affinity. Thus these studies do not provide evidence supporting the existence of δ-opioid subtypes on SH-SY5Y cells.

Recent investigations using the antagonists 7-benzylidenenaltrexone (BNTX) and naltriben (NTB) (see section 1.4.2) suggest that both the putative δ_1 and δ_2 subtypes are present on these cells but that DPDPE and DELT II exhibit cross-reactivity, i.e., NTB and BNTX can inhibit both agonists but NTB is more potent at inhibiting DELT II than DPDPE, while BNTX shows the reverse potency order [Toll & Polgar, 1994].

In conclusion, this series of experiments has demonstrated the existence of μ- and δ-opioid binding sites on SH-SY5Y cells and provided some evidence of μ-opioid subtypes, while the situation regarding δ-opioid subtypes is still unresolved. However, this system appears to be ideal one in which to investigate μ / δ interactions.

CHAPTER 6
APPLICATIONS OF [^{35}S]GTPγS BINDING

In science the credit goes to the man who convinces the world, not to the man to whom the idea first occurs.

Sir Francis Darwin (1848 - 1925)

6.1 Introduction

In order to develop better clinically potent antinociceptive opiates an understanding of receptor-ligand interactions and the function of these receptors is vital. Thus both *in vivo* and *in vitro* models have been widely tested. Opioid receptor selective animal tissues are routinely utilised to screen drugs and also to further characterise receptor types / subtypes. In terms of animals this process is very costly and in recent years the effectiveness of using cell lines has been evaluated. The future of cell lines in this regard depends upon the development of reliable, reproducible and meaningful assays especially in terms of correlating results from animal systems. At present the most commonly used functional assay for opioids is inhibition of stimulated cAMP accumulation. This assay does not lend itself to large scale drug screening due to the small magnitude of response [Costa *et al.*, 1991] and has the disadvantage that cell differentiation (a time-consuming process) may be necessary to see any viable response [Pålman *et al.*, 1984; Yu & Sadee, 1988; Seward *et al.*, 1989]. In addition GTPase assays have been examined [Burns *et al.*, 1983; Carter & Medzihradsky, 1992] but these have the similar drawbacks.

As discussed previously (see section 1.3) both μ- and δ-opioid receptors are coupled to G proteins. Agonist binding to receptor causes activation of G proteins which in turn mediates effector systems. If GTP is replaced by its non-hydrolysable analogue GTPγS then the G protein remains activated [Higashijima *et al.*, 1987] (Fig. 6.1). Use of [^{35}S]GTPγS (Fig. 2.1) allows the level of activation of G protein, following agonist occupation, to be determined since association of [^{35}S]GTPγS is an essentially irreversible process and the level of [^{35}S]GTPγS - G protein complex can be measured as [^{35}S]GTPγS specifically bound. This assay has been used in several receptor systems to examine functional responses, such as, muscarinic receptors in cardiac membranes and CHO cells [Hilf *et al.*, 1989; Lazareno *et al.*, 1993], chemotactic peptide receptors in HL-60 granulocytes [Giershik *et al.*, 1991], adenosine receptors in bovine brain [Lorenzen *et al.*, 1993] and μ-opioid receptors in SH-SY5Y cells [Traynor & Nahorski, 1994].

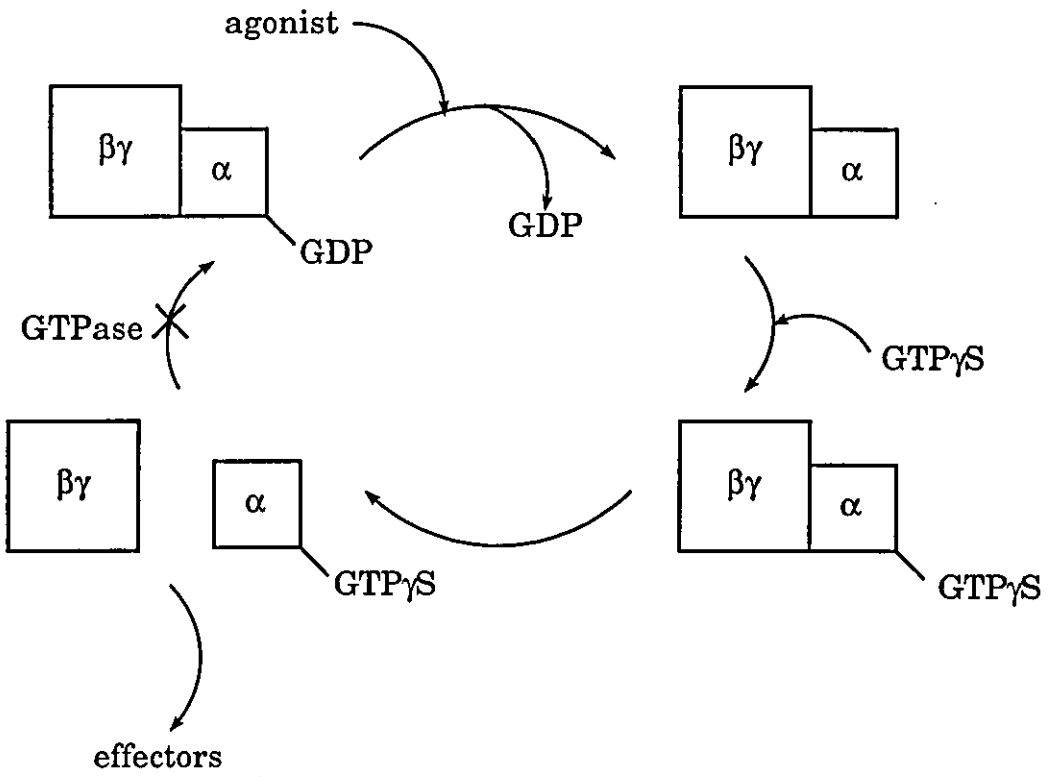


Figure 6.1 A diagramatic representation of agonist-induced G protein turnover in the presence of GTPγS.

μ-Opioid agonists have been shown to modulate [^{35}S]GTPγS binding in SH-SY5Y human neuroblastoma cells in a naloxone-reversible manner [Traynor & Nahorski, 1994]. Using this assay the efficacy and potency of μ-opioids can be quickly determined and thus this assay can potentially be used to screen large numbers of opiates. In order for stimulation to be observed the addition of the nucleotide guanosine diphosphate (GDP) is required. The optimal concentration of GDP is 3 μM and maximal stimulation is observed when the assay is conducted at 30°C for 1 h.

6a.1 Introduction

[^{35}S]GTPγS binding in neuroblastoma x glioma NG108-15 hybrid cells

In this study the aim was to see whether [^{35}S]GTPγS binding could be stimulated by opioids in the mouse neuroblastoma x rat glioma hybrid NG108-15 cells. These cells have been shown to possess opioid receptors which appear to be exclusively of the δ type [Chang & Cuatrecasas, 1979] and which have so far been shown to be coupled to the G proteins $G_{i2\alpha}$, $G_{i3\alpha}$ and $G_{o\alpha}$ [McKenzie & Milligan, 1990; Roerig *et al.*, 1992]. Optimisation of δ-opioid stimulated [^{35}S]GTPγS binding in NG108-15 cells could potentially provide a useful method of determining the δ-opioid effects of compounds.

6a.2 Results

6a.2.1 Characterisation of [^{35}S]GTPγS binding in neuroblastoma x glioma NG108-15 hybrid cells

Ligand binding assays

Ligand binding experiments on the NG108-15 cells were conducted in order to ascertain whether results consistent with literature values could be achieved and to confirm that these cells had essentially the same δ-opioid receptor profile as those used in other laboratories.

TIPP (Tyr-Tic-Phe-Phe, Tic = tetrahydroisoquinoline-3-carboxylic acid) (Fig. 2.1) is a δ-opioid highly selective peptide antagonist with a reported affinity (K_i) of 1.2 nM in rat brain [Schiller *et al.*, 1992]. The presence of δ-opioid receptors on the NG108-15 cells was confirmed by the displacement by TIPP of the non-selective antagonist [^{3}H]diprenorphine (0.28 nM), affording a K_i of 3.6 nM and a slope for the displacement of 1.0 (Fig. 6.2).

Having demonstrated a single δ-opioid binding site by the displacement of [^{3}H]diprenorphine with TIPP, the level of opioid binding sites on the NG108-15 cells was determined by saturation binding of [^{3}H]diprenorphine (Fig. 6.3). The receptor number (B_{max}) was 364 ± 33 fmols / mg protein and the affinity (K_D) of [^{3}H]diprenorphine was 0.31 ± 0.09 nM.

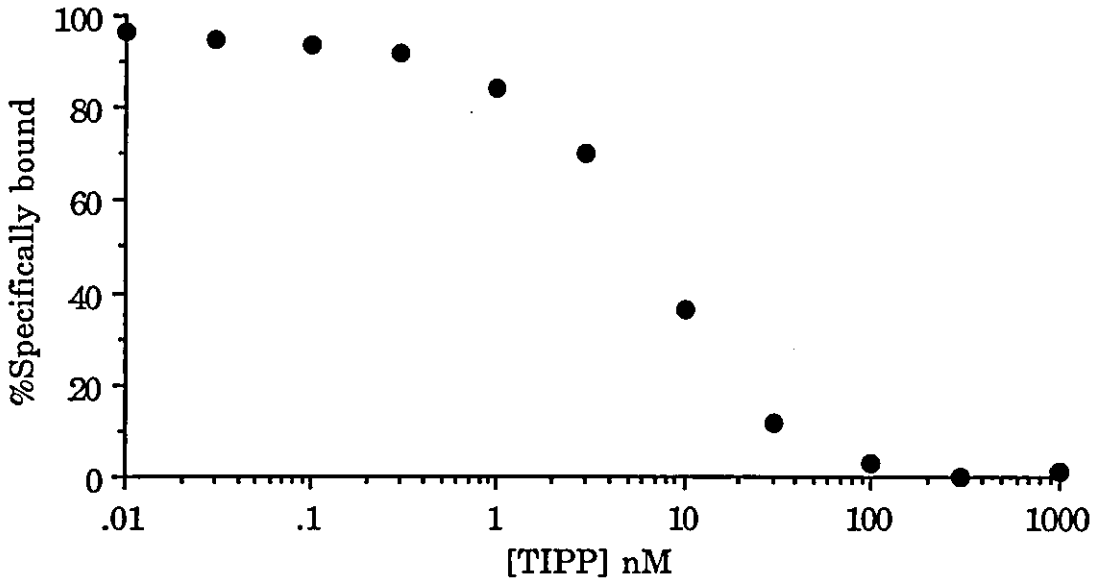


Figure 6.2 Displacement of [^{3}H]diprenorphine (0.28 nM) specific binding by TIPP in NG108-15 cell membranes (Tris-HCl buffer, pH 7.4), incubated at 25°C for 40 min, n = 1. Specific binding represents 70 % of total binding.

In order to confirm that all the receptors labelled by [^{3}H]diprenorphine even at high radioligand concentrations were of the δ-opioid type the level of [^{3}H]TIPP binding was assayed. Previously [^{3}H]TIPP binding has been characterised in rat brain yielding > 70 % specific binding [Nevin *et al.*, 1993]. Equally high levels of specific [^{3}H]TIPP binding were achieved in NG108-15 membranes, yielding a B_{max} of 395 ± 54 fmols / mg protein and a K_D of 0.29 ± 0.01 nM (Fig. 6.4). Thus all the evidence points to the opioid receptors in the NG108-15 cells being of the δ type.

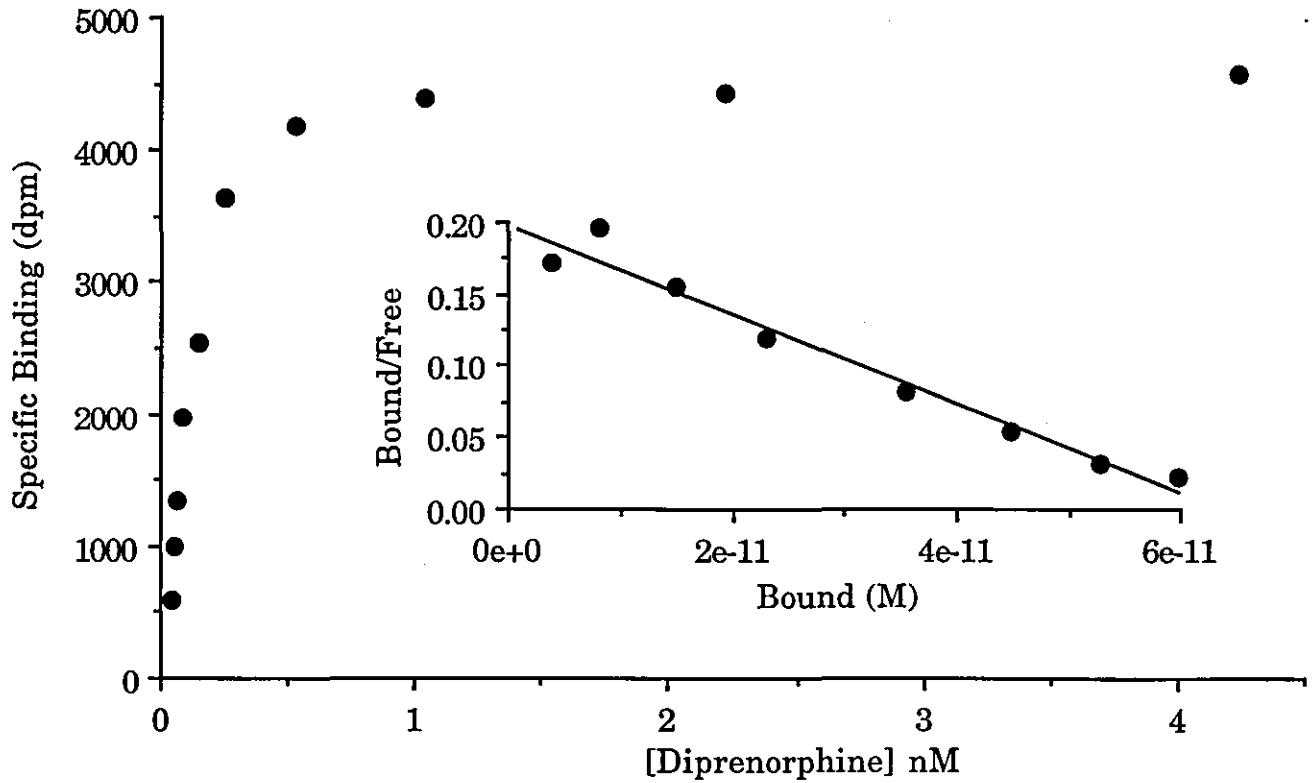


Figure 6.3 A representative graph showing saturation [^{3}H]diprenorphine binding to NG108-15 membranes (150 µg protein / ml, Tris-HCl buffer, pH 7.4). Inset is the corresponding Scatchard plot.

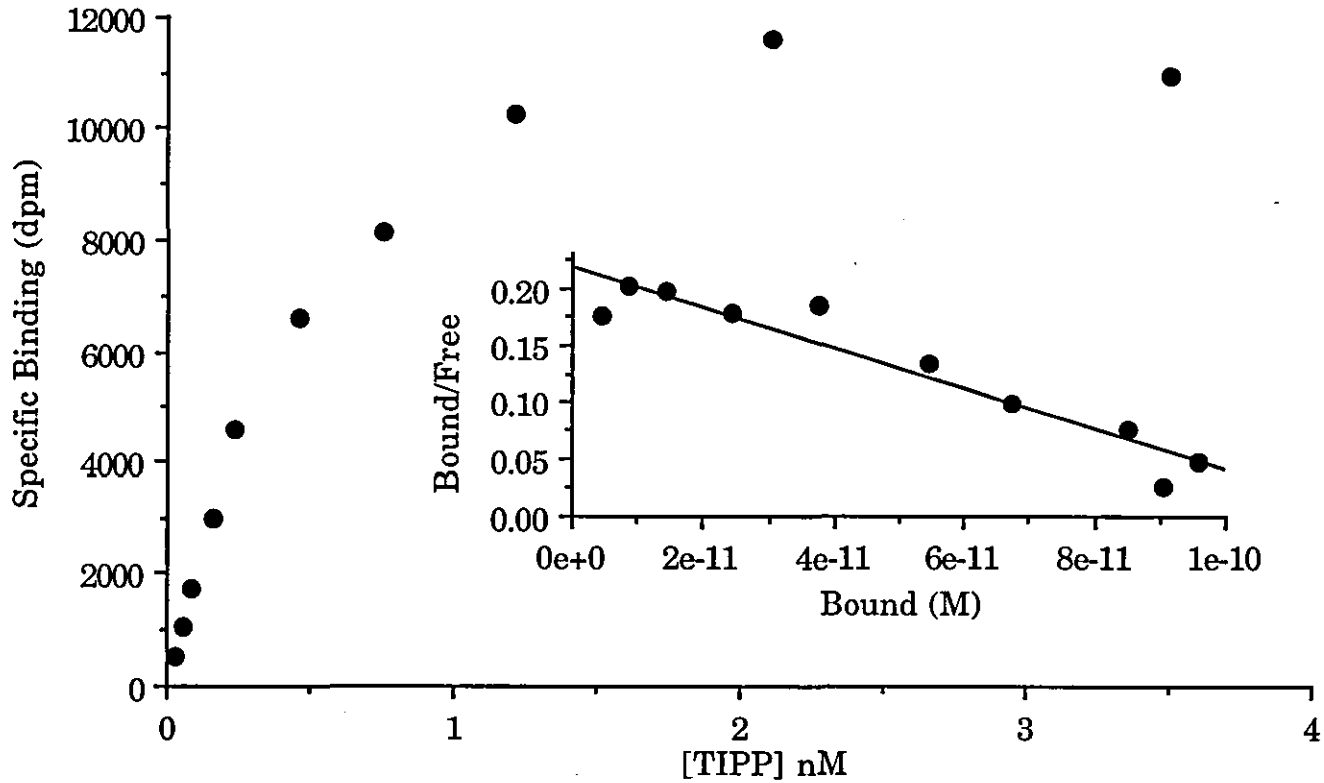


Figure 6.4 A representative graph showing saturation [^{3}H]TIPP binding to NG108-15 membranes (150 µg protein / ml, Tris-HCl buffer, pH 7.4). Inset is the corresponding Scatchard plot.

[^{35}S]GTPγS binding

When performing [^{35}S]GTPγS binding assays it is important that the concentration of GDP employed is correct in order to gain maximal agonist-stimulated [^{35}S]GTPγS binding. In NG108-15 cells the optimal signal to noise ratio for δ-opioid stimulated [^{35}S]GTPγS binding was determined. The highly selective δ-opioid agonist [D-Pen^{2},D-Pen^{5}]enkephalin (DPDPE) (1 μM) was added to the NG108-15 membranes in the presence of varying concentrations of GDP in order to stimulate [^{35}S]GTPγS binding. The level of binding was compared to membranes incubated in the absence of DPDPE (Figs. 6.5 & 6.6). Agonist-stimulated [^{35}S]GTPγS binding was observed at concentrations of 10 μM GDP or more. The higher the concentration of GDP the greater the extent of stimulation. Thus maximal stimulation was observed at the highest concentration of GDP evaluated, i.e., 100 μM.

Having illustrated that [^{35}S]GTPγS binding could be stimulated by the δ-opioid agonist DPDPE the next criteria to be established was that this effect is dose-dependent and naloxone reversible. DPDPE was found to stimulate [^{35}S]GTPγS binding in the presence of 100 μM GDP, incubated at 30°C for 1 h, with an EC_{50} of 7.75 ± 0.45 nM (± range), and afforded a K_e of 12 nM versus naloxone (Fig. 6.7).

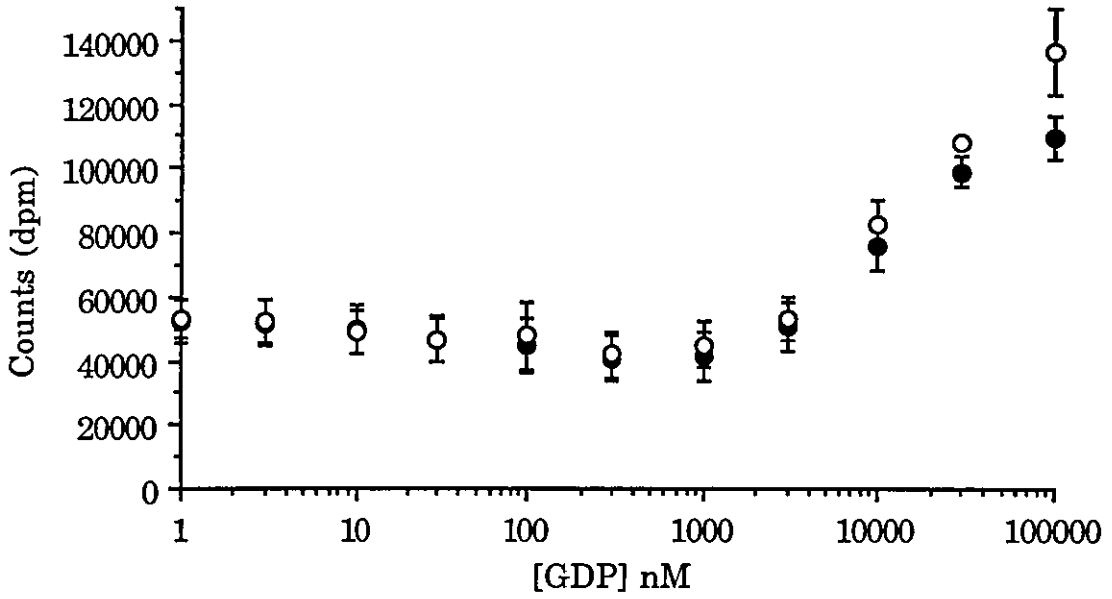


Figure 6.5 Effect of varying the concentration of GDP in the absence (●) and the presence (O) of DPDPE (1 μM) on [^{35}S]GTPγS binding in NG108-15 membranes (200 μg protein / ml), incubated at 30°C for 1 h. Values represent means ± s.e.mean where n ≥ 3.

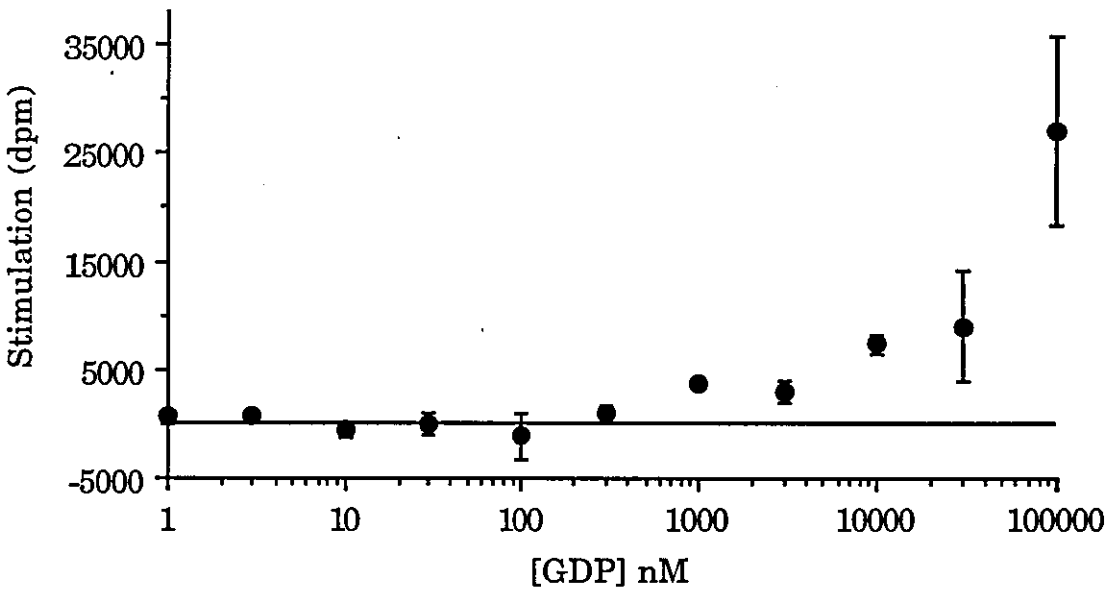


Figure 6.6 Stimulation of [^{35}S]GTPγS binding in NG108-15 membranes (200 μg protein / ml), incubated at 30°C for 1 h, in the presence of DPDPE (1 μM) and varying concentrations of GDP. Values represent means ± s.e.mean where n ≥ 3.

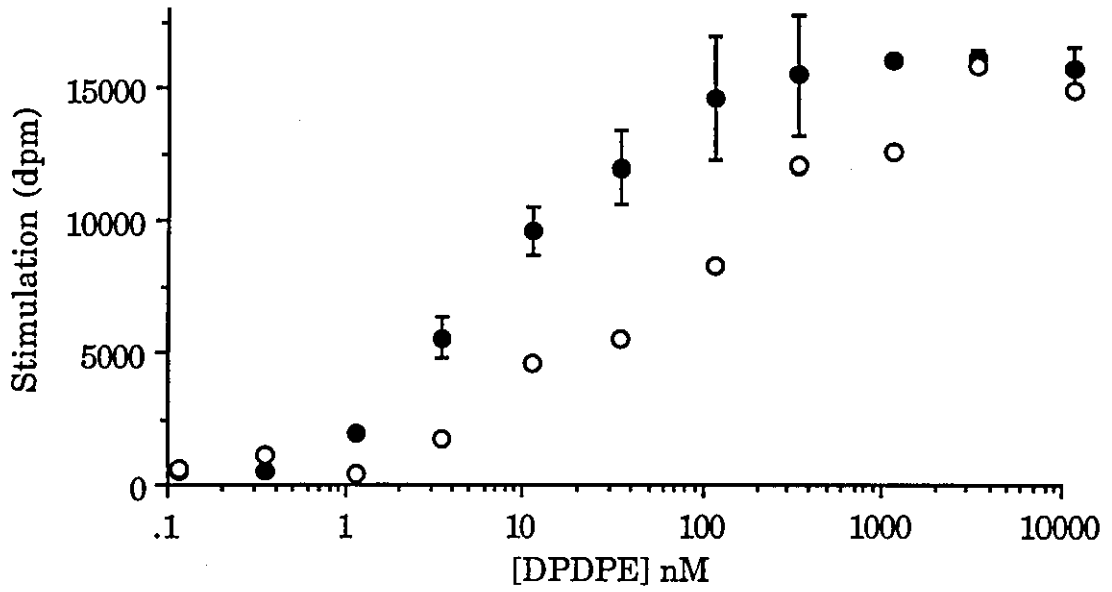


Figure 6.7 Dose-response curve of DPDPE in the absence (●) and the presence* (O) of 100 nM naloxone on the stimulation of [^{35}S]GTPγS binding in NG108-15 membranes (200 μg protein / ml), incubated at 30°C for 1 h, in the presence of GDP (100 μM). Values represent means ± range where n = 2, *n = 1.

As a starting point the above experiments were conducted under the same conditions (200 μg protein / ml, incubated at 30°C for 1 h) as for μ-opioid stimulated [^{35}S]GTPγS binding in SH-SY5Y cells [Traynor & Nahorski, 1994]. In order to optimise the conditions for δ-opioid stimulated [^{35}S]GTPγS binding in NG108-15 cells, the concentration of protein was varied in the presence of 100 μM GDP. Optimal stimulation was achieved at 80 μg protein / ml although satisfactory levels of stimulation were observed in the range 35 - 120 μg protein / ml (Fig. 6.8). Finally the best conditions of temperature and time were investigated (Fig. 6.9). The optimal signal to noise ratio was achieved at 25 and 30°C over 90 min where binding was increased by 70 - 85 % above controls (approximately 20000 counts higher than basal levels, which were approximately 100000 dpm, see Fig. 6.5).

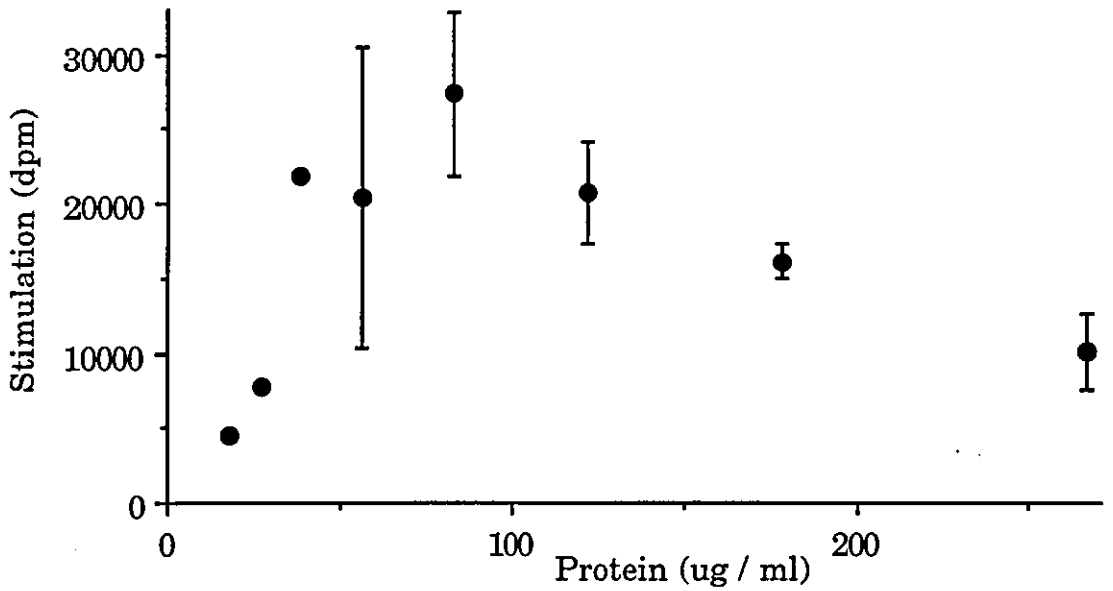


Figure 6.8 Effect of protein concentration on the stimulation of [^{35}S]GTPγS binding in NG108-15 membranes, incubated at 30°C for 1 h, in the presence of DPDPE (1 μM) and GDP (100 μM). Values represent means ± s.e.mean where n ≥ 3.

6a.2.2 μ / δ interactions as measured by [^{35}S]GTPγS binding in SH-SY5Y cells

The concept of μ- / δ-opioid receptor interactions has been extensively discussed in section 1.4.1 and evaluated in isolated tissue preparations as described in chapter 3 (also see [Elliott & Traynor, 1994]). In this part of the study the possibility of μ- / δ-opioid receptor interactions was examined in SH-SY5Y using a [^{35}S]GTPγS binding assay. SH-SY5Y cells are known to possess both μ- and δ-opioid receptors (see section 5.2) and indeed electrophysiological experiments have suggested they can exist on the same cell [Seward *et al.*, 1989].

Initial experiments were conducted to demonstrate that similar results could be achieved to those originally reported in SH-SY5Y cells [Traynor & Nahorski, 1994]. Thus the action of the μ-opioid agonists morphine, [D-Ala^2,MePhe^4,Gly(ol)^5]enkephalin (DAMGO), and fentanyl were investigated and resulted in the following EC_{50} values 63.9 ± 7.9, 30.4 ± 12, and 15.7 nM respectively (Figs. 6.10 & 6.11). In a single experiment the dose-response curve to fentanyl was shifted to the right in the presence of naloxone affording a K_e of 2.73 nM, consistent with μ-opioid mediated actions.

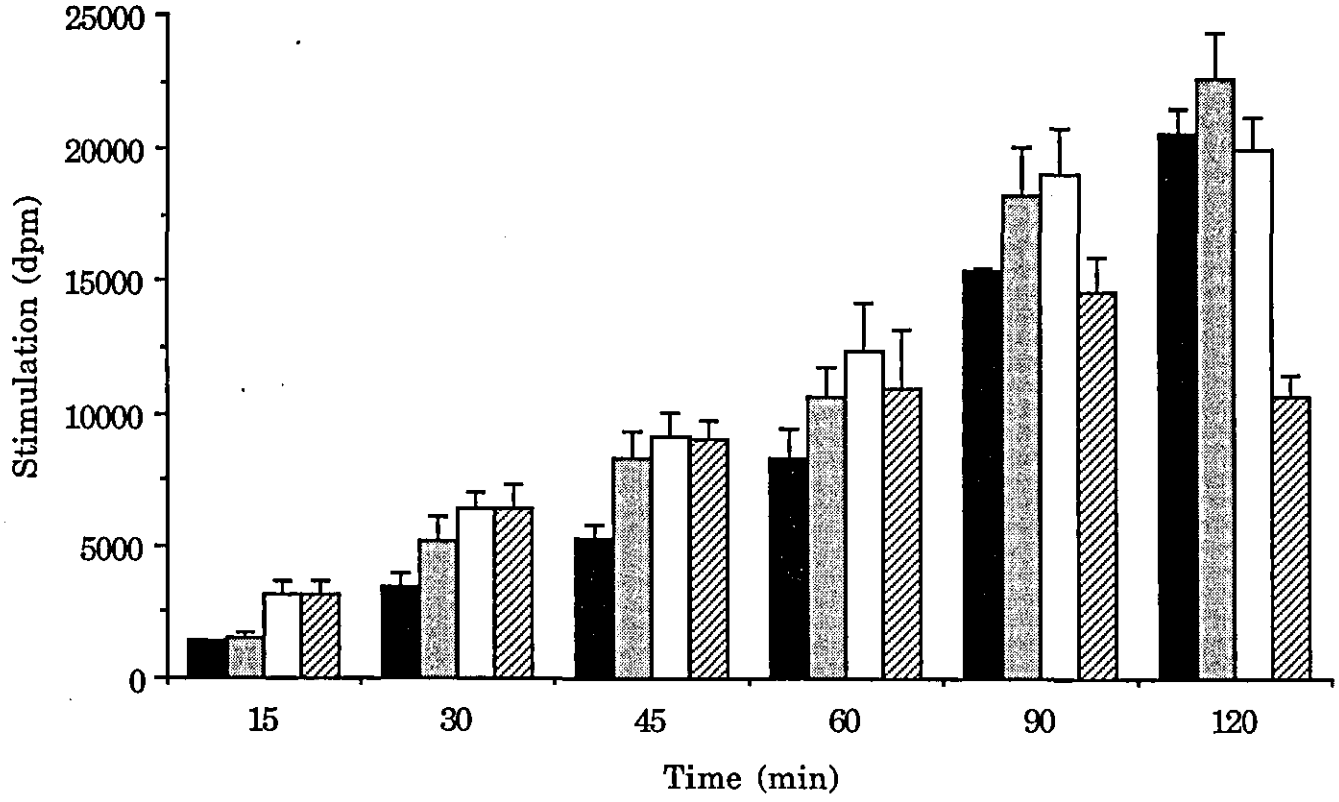


Figure 6.9 Effect of the temperature 20°C (black), 25°C (grey), 30°C (white) and 37°C (hatched) and time of incubation on the stimulation of [^{35}S]GTPγS binding in NG108-15 membranes, in the presence of DPDPE (1 μM) and GDP (100 μM). Values represent means ± s.e.mean where n ≥ 3.

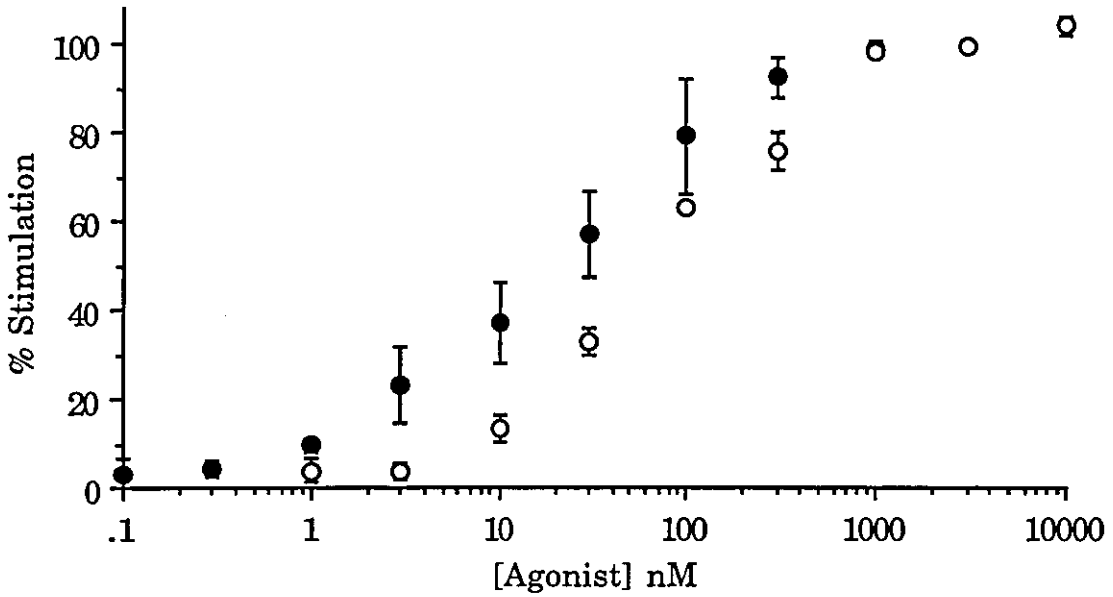


Figure 6.10 Stimulation of [^{35}S]GTPγS binding to SH-SY5Y membranes, incubated at 30°C for 1 h, by DAMGO (●) and morphine (O). Values represent means ± s.e.mean where n ≥ 3.

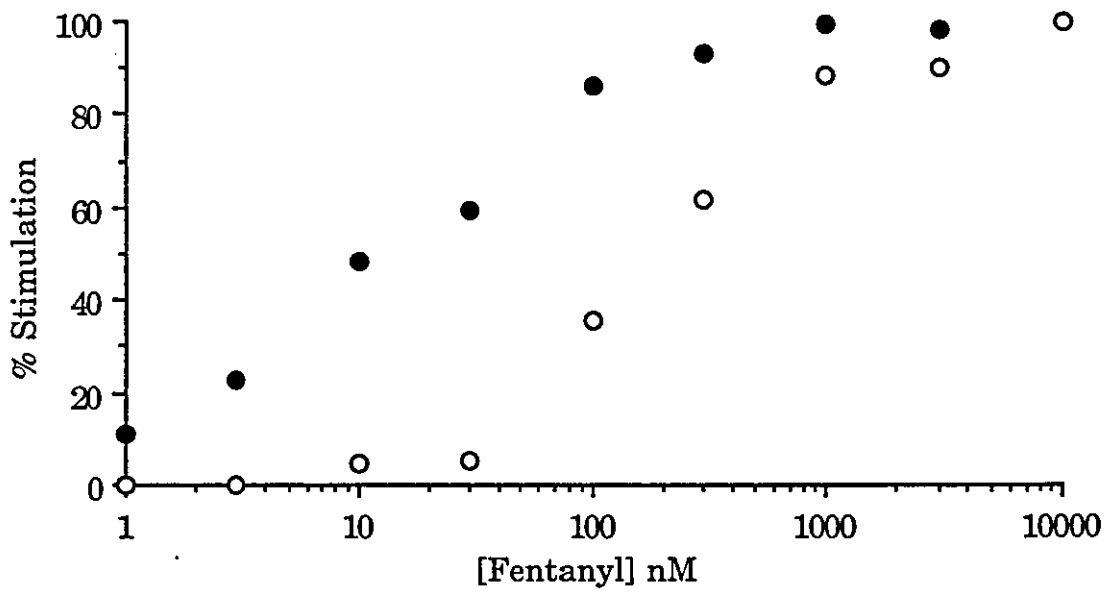


Figure 6.11 Stimulation of [^{35}S]GTPγS binding in SH-SY5Y membranes, incubated at 30°C for 1 h, by fentanyl in the absence (●) and the presence of 30 nM naloxone (O), n = 1.

In a similar manner dose-response curves for the putative δ_1-opioid agonist DPDPE and the putative δ_2-opioid agonist [D-Ala^2, Glu^4]deltorphin II (DELT II) were observed in SH-SY5Y cells. The results were very variable, but neither peptide was very potent nor was able to effect the same maximal binding as fentanyl (3 μM). A detailed dose-response curve to DPDPE was carried out in the hope of clarifying the situation (Fig. 6.12); in a single experiment a similar curve was obtained using DELT II. Both δ-opioid agonists were partial agonists with shallow slopes and low potencies. From the shape of the curve it appears plausible that DPDPE is binding to two receptor sites, possibly δ-opioid receptors at low concentrations (≤ 300 nM) and μ-opioid receptors at high concentrations (≥ 1 μM).

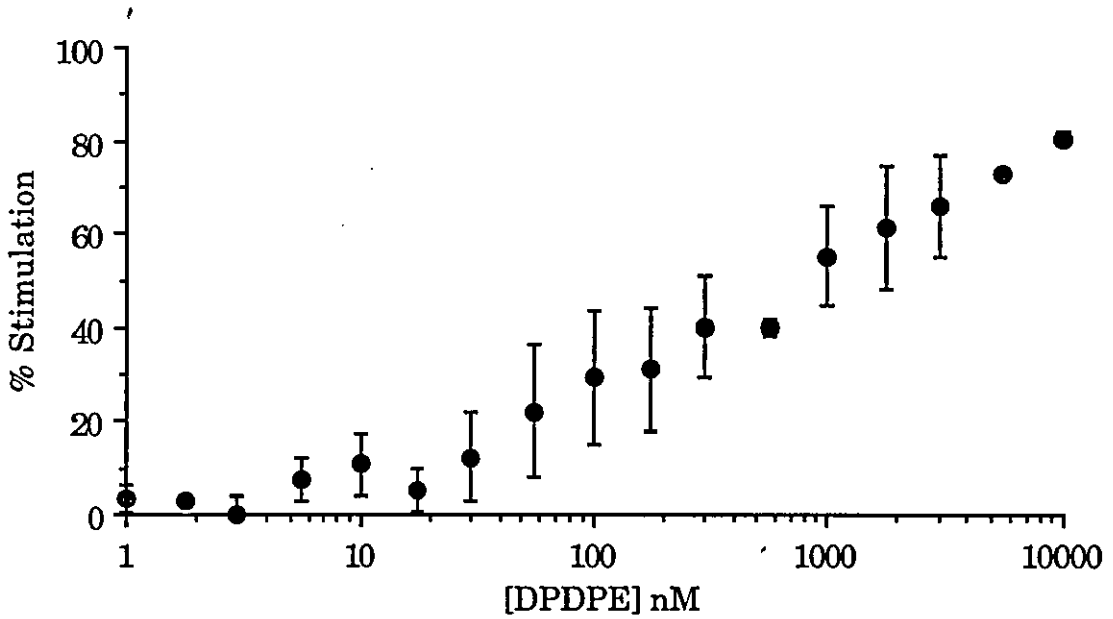


Figure 6.12 Stimulation of [^{35}S]GTPγS binding in SH-SY5Y membranes by DPDPE, expressed as a % of the stimulation achieved by 3 μM fentanyl, incubated at 30°C for 1 h. Values represent means ± s.e.mean where n ≥ 3.

Thus δ-opioid stimulation of [^{35}S]GTPγS binding was dramatically different in SH-SY5Y cells compared to NG108-15 cells where DPDPE was a highly potent agonist, EC_{50} = 7.75 ± 0.45 nM, see section 6.2.1. One possible reason might be the the level of GDP present in the assay. Therefore the effect of varying the concentration of GDP on DPDPE-induced [^{35}S]GTPγS stimulation in SH-SY5Y cells was examined (Figs. 6.13 & 6.14).

As can be seen from the graph the highest level of stimulation occurred at 3 μM GDP, the same as for μ-opioid agonists although the stimulation was not to such a great extent.

Having established that DPDPE produced optimal stimulation of [^{35}S]GTPγS binding in the presence of 3 μM GDP, the same as μ-agonists, the effect of combinational doses of μ- and δ-opioid agonists was examined in a similar way as for the isolated tissue preparations. In order to conserve materials rather than construct complete concentration-response curves selected concentrations of μ- and δ-agonists were combined. Two concentrations of each of the μ-opioid agonists morphine and DAMGO were chosen, one which gave a 30 - 50 % response, and another which gave a maximal response. These concentrations were combined with either DPDPE or DELT II (putative δ_1 and δ_2 selective agonists) at concentrations of 1, 100 and 10000 nM, i.e., a sub-effective concentration, a concentration yielding an approximately 30 % response and a concentration of maximal effect (approximately 80 %). The results are summarised in Figs. 6.15 & 6.16.

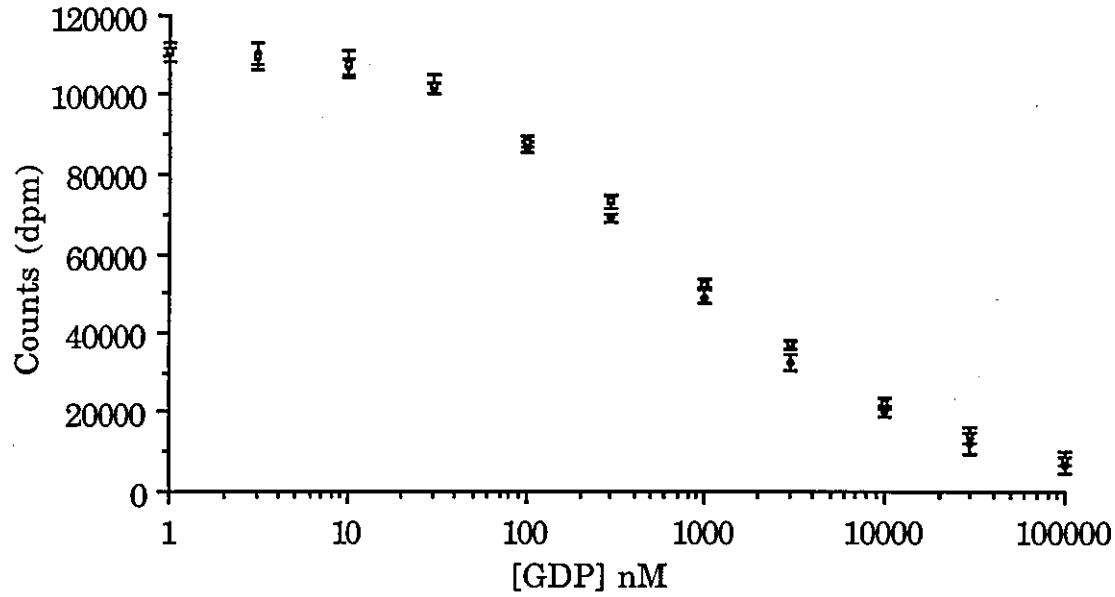


Figure 6.13 Effect of varying the concentration of GDP in the absence (●) and the presence (O) of DPDPE (1 μM) on [^{35}S]GTPγS binding in SH-SY5Y membranes, incubated at 30°C for 1 h. Values represent means ± s.e.mean where n ≥ 3.

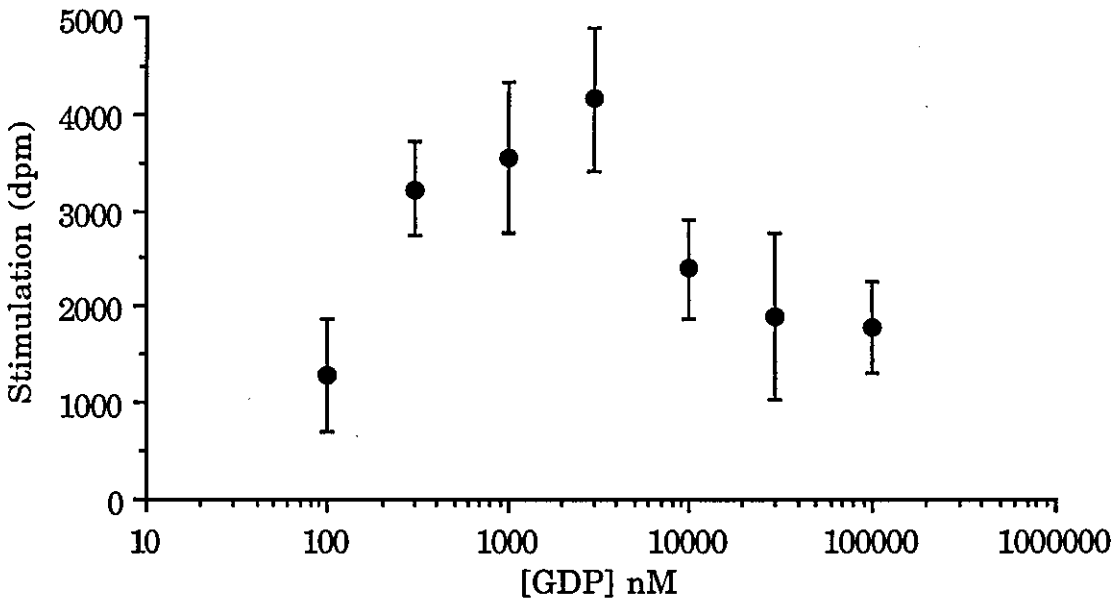


Figure 6.14 Stimulation of [^{35}S]GTPγS binding in SH-SY5Y membranes in the presence of DPDPE (1 μM) and varying concentrations of GDP, incubated at 30°C for 1 h. Values represent means ± s.e.mean where n ≥ 3.

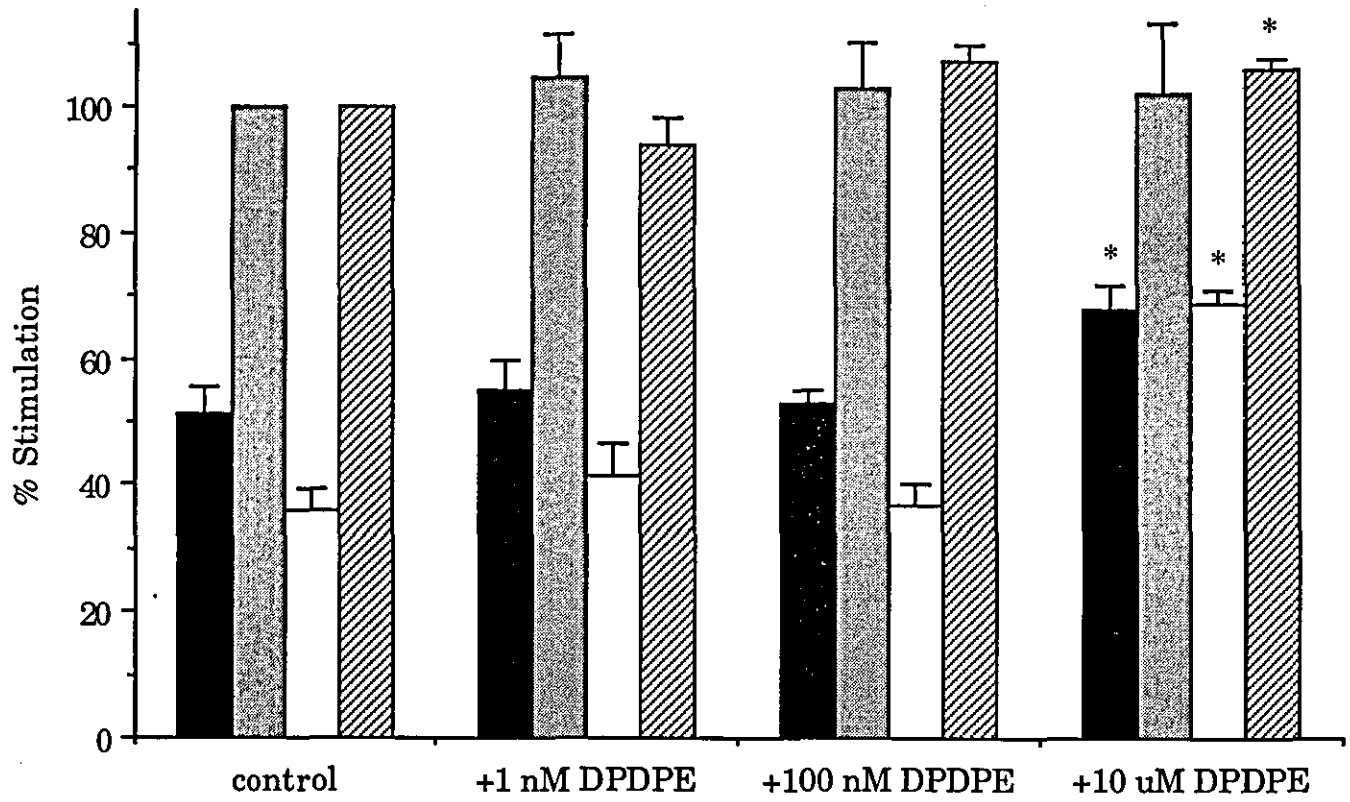


Figure 6.15 Effect of combinational doses of DPDPE on 64 nM morphine (black), 10 μM morphine (grey), 10 nM DAMGO (white) and 10 μM DAMGO (hatched) stimulated [^{35}S]GTPγS binding in SH-SY5Y membranes. *At the 95 % confidence limit: significant increase compared to controls (i.e., μ-agonist alone). Values represent means ± s.e.mean where n ≥ 3.

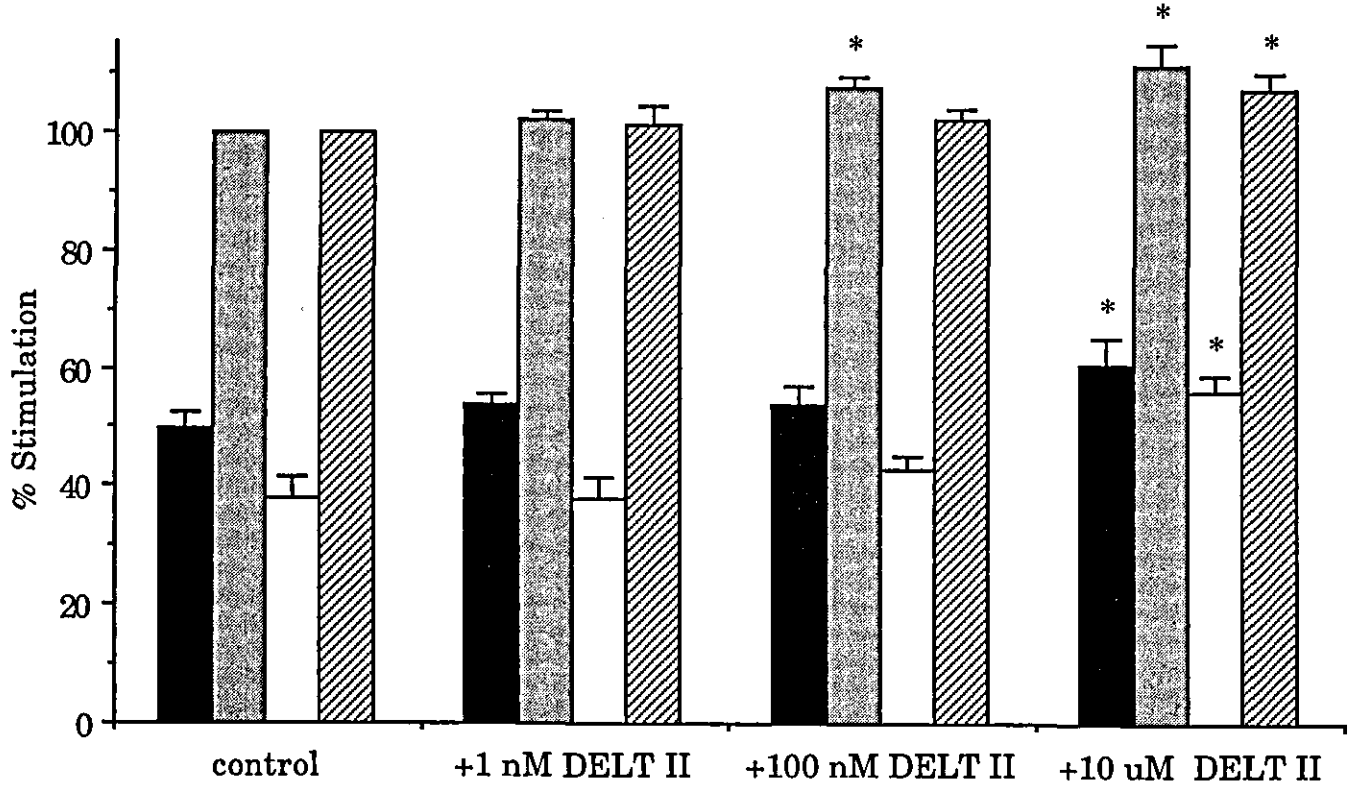


Figure 6.16 Effect of combinational doses of DELT II on 64 nM morphine (black), 10 μM morphine (grey), 10 nM DAMGO (white) and 10 μM DAMGO (hatched) stimulated [^{35}S]GTPγS binding in SH-SY5Y membranes. *At the 95 % confidence limit: significant increase compared to controls (i.e., μ-agonist alone). Values represent means ± s.e.mean where n ≥ 3.

Significant increases in μ-opioid stimulated [^{35}S]GTPγS binding were only observed in combination with the highest dose (10 μM) of DPDPE or DELT II (10 μM morphine + 100 nM DELT II being the exception). At such high concentrations the effects of DPDPE and DELT II are probably no longer selective for the δ-opioid receptor [Mosberg *et al.*, 1983; Erspamer *et al.*, 1989] as implied by Fig. 6.12. In a single experiment this was confirmed to be the case since antagonism of the increased effect observed with 10 μM DPDPE could not be reduced back to control levels either in the presence of TIPP (100 nM) or the putative δ_1 antagonist BNTX (7-benzylidenenaltrexone (5 nM) [Portoghese *et al.*, 1992] (Table 6.1).

Table 6.1 Effect of a combinational concentration of 10 μM DPDPE, in the absence and presence of the δ-opioid antagonists TIPP (100 nM) or BNTX (5 nM), on the level of stimulated [^{35}S]GTPγS binding in SH-SY5Y membranes.

	% Stimulation			
	A	D	T	B
64 nM morphine	50	63	83	62
10 μM morphine	100	111	105	107
10 nM DAMGO	38	56	68	58
10 μM DAMGO	100	107	117	107
10 μM DPDPE	43			

Where A = μ agonist alone, D = in the presence of 10 μM DPDPE, T = in the presence of 10 μM DPDPE + 100 nM TIPP and B = in the presence of 10 μM DPDPE + 5 nM BNTX. Stimulated [^{35}S]GTPγS binding was assayed at 30°C for 1 h in the presence of 3 μM GDP, n = 1.

6a.3 Discussion

Characterisation of [^{35}S]GTPγS binding in neuroblastoma x glioma NG108-15 hybrid cells

δ-Opioid stimulated [^{35}S]GTPγS binding has been successfully demonstrated in NG108-15 cells. The cells displayed a saturable population of δ-opioid receptors. The receptor number and the affinity of the site for [^{3}H]diprenorphine were consistent with literature values [Law *et al.*, 1983; Childers *et al.*, 1993; Polastron *et al.*, 1992; Werling *et al.*, 1988].

Various levels of GDP are required in different systems in order for agonist stimulation of [^{35}S]GTPγS binding to be observed. For example the optimum concentration of GDP required in various systems is: 3 μM by μ-opioid agonists in SH-SY5Y cells [Traynor & Nahorski, 1994], 0.1 μM for m1 and m3 and 1 μM for m2 and m4 muscarinic agonists in CHO cells [Lazareno & Birdsall, 1993] and 10 μM by adenosine A_1 agonists in bovine brain membranes [Lorenzen *et al.*, 1993]. In NG108-15 cells 10 μM GDP increased the level of [^{35}S]GTPγS binding but the highest level of δ-opioid stimulation of [^{35}S]GTPγS binding occurred in the presence of the highest level of GDP tested i.e., 100 μM. The reason why GDP needs to be added is as yet unknown. One obvious possibility is to reduce basal binding of GTPγS so that the stimulated binding is a significant proportion and can be readily seen. Clearly the coupling of various types of receptor or even subtype, as in the case of muscarinic receptors expressed in CHO cells, to their G protein(s) must differ considerably as variable amounts of GDP are necessary in this assay [Lazareno & Birdsall, 1993].

The conformationally constrained δ-opioid selective ligand DPDPE is a potent and naloxone reversible agonist in this assay. The affinity of DPDPE for the δ-opioid receptor in a single experiment, as measured by the non-selective antagonist naloxone (K_e = 12 nM), is perhaps a little higher than expected [Leslie, 1987] but until the experiment has been repeated the significance of this result cannot be evaluated.

Finally the optimal conditions for stimulation of [^{35}S]GTPγS binding were determined. The best results were obtained using 80 μg protein / ml, but the level of protein is not crucial within the range of 35 - 120 μg protein / ml.

The level of stimulation increased over the time course of 2 h at 20, 25 and 30°C. However, the degree of stimulation was decreasing after 2 h at 37°C when the rate of dissociation mediated by GTPase must be greater than the rate of GTPγS to α-subunit association. Although the stimulation in counts (dpm) was highest after 2 h the best signal to noise ratio, i.e., binding increased by 70 - 85 %, was achieved after 90 min at either 25 or 30°C.

Thus δ-opioid stimulated [^{35}S]GTPγS stimulated binding has been demonstrated in NG108-15 cells and the optimal conditions were over 90 min, at 25 - 30°C, in the presence of 100 μM GDP using 80 μg protein / ml. The period of incubation needed to be longer for δ-opioid mediated stimulation in NG108-15 cells compared to μ-mediated [^{35}S]GTPγS stimulated binding in SH-SY5Y cells.

μ / δ interactions as measured by [^{35}S]GTPγS binding in SH-SY5Y cells

Despite the fact that SH-SY5Y cells possess both μ- and δ-opioid receptors the response due to activation of these individual receptor types is markedly different in [^{35}S]GTPγS binding assays. μ-Opioid agonists give a large stimulation of binding and are highly potent but δ-opioid agonists only appear to be partial agonists of relatively low potency. This may be simply due to low δ-opioid receptor numbers, i.e., 70 ± 16 fmol / mg protein in SH-SY5Y cells compared to 395 ± 54 fmol / mg protein in NG108-15 cells. Alternatively the poor response of δ-opioid agonists could be the level of GDP in the assay, since earlier experiments conducted in NG108-15 cells had shown that optimal δ-opioid stimulation was observed in the presence of 100 μM GDP, (see section 6.2.1). However, varying the level of GDP did not increase the level of δ-opioid mediated [^{35}S]GTPγS stimulation and indeed best stimulation was found to occur in the presence of 3 μM GDP; the same concentration used for optimal stimulation of [^{35}S]GTPγS binding by μ-opioid agonists. Alternatively, the low maximal response observed with δ-opioid agonists may be a reflection of receptor numbers as the population of μ-opioid receptors is 2.5 - times greater than that of δ-opioid receptors (see section 5.2.1). Another reason may be that δ-opioid receptors in the two cell lines are different or that they are differentially coupled to G proteins, although evidence to date does suggest that both receptors work through similar G proteins [Laugwitz *et al.*, 1993; McKenzie & Milligan, 1990; Roerig *et al.*, 1992].

The results demonstrate that μ / δ interactions cannot be observed in SH-SY5Y cells as measured by [^{35}S]GTPγS binding assays. Although the level of stimulation produced by the μ-opioid agonists morphine and DAMGO was increased by the presence of the δ-opioid agonists DPDPE and DELT II, this

was not due to the δ-opioid mediated effects of these agonists. At δ-opioid selective doses DPDPE and DELT II had no effect on μ-opioid mediated responses, only at non-selective doses of 10 μM, at which DPDPE and DELT II have μ-opioid effects [Mosberg *et al.*, 1983; Erspamer *et al.*, 1989] was any significant increase in [^{35}S]GTPγS stimulation recorded. Furthermore, the highest dose of δ-opioid agonist produced approximately 80 % stimulation on its own, but in combination with μ-agonists this level of stimulation was decreased suggesting some form of antagonism.

Although μ / δ interactions were not implicated by the results of this study, they have been demonstrated in the parent cell line to SH-SY5Y cells. The SK-N-SH cell line possesses μ- and δ-opioid receptors in the ratio of 1.5 : 1 [Baumhaker *et al.*, 1993]. Ligands selective for either μ- (DAMGO) or δ- (DPDPE) opioid receptors down-regulated their respective receptors selectively as measured by ligand binding assays. Morphine, which is able to bind to both μ- and δ-opioid receptors, down-regulates both receptor types but is unable to down-regulate δ-opioid receptors in NG108-15 cells. Alkylation of μ-opioid receptors using the irreversible antagonist β-funaltrexamine inhibited the down-regulation of μ-opioid receptors by DAMGO, while not altering the down-regulation of δ-opioid receptors caused by DPDPE. However, down-regulation of δ-opioid receptors by morphine was inhibited, thus suggesting a functional μ / δ complex in the same cell. This may suggest differences in receptor populations or G protein coupling in SK-N-SH cells compared to its subclone SH-SY5Y, but since different methods were employed further investigation is required.

6b.1 Introduction

Tolerance

When a drug is administered continuously or repeatedly there is often a decrease in its effectiveness. If this phenomenon occurs within a short time period (minutes to a few hours) then desensitisation is said to have developed. Alternatively if the potency of the drug diminishes over a longer time period of perhaps many hours or days then the term tolerance is used. The distinction between these two terms is not clearly defined. There appears to be two stages in the development of a tolerant state in G protein-linked receptors. In the initial stage of desensitisation the receptor becomes uncoupled from the GTP-binding protein with an accompanying reduction of affinity for agonist binding. This stage is then followed by the process of down-regulation during which receptors are removed from the cell surface causing a decrease in B_{max}. The internalised receptors are either degraded or later recycled to the surface [Brown *et al.*, 1982].

Tolerance to morphine in humans usually occurs after 2 - 3 weeks at normal therapeutic doses [Burgen & Mitchell, 1985]. Larger doses of morphine are required to produce the same degree of analgesia. The respiratory depressant effects of morphine are also subject to tolerance whereas the effects of morphine on the pupil and on the intestine are unaltered. If it were possible to modulate tolerance in a suitable model then this could be of great clinical value if extended to man for the management of chronic pain. The chronic pain syndrome most commonly treated by opiates is cancer pain, in which long-term treatment is required to improve the quality of life of the patient [Wells & Woolf, 1991].

The mechanisms involved in opioid tolerance have been extensively studied but as yet have not been fully ascertained, partly because of the lack of suitable experimental models. Since antinociception cannot be measured in a cell line or an *in vitro* experiment, these types of models do have limitations regarding the complete understanding of opiate actions in whole animals. However, compared to a whole animal model, experimental models based on cells or isolated tissues or even membrane preparations do not have the complications of, for example, route of drug administration, drug distribution and clearance, drug metabolism and time consuming assessments of effector response. Therefore cellular processes can be more readily analysed.

Opioid tolerance has been shown to occur at the second messenger level in SH-SY5Y cells since inhibition of cAMP accumulation is decreased following chronic opioid treatment [Yu & Sadee, 1988] and receptors are down-regulated [Carter & Medzihradsky, 1993b; Zadina *et al.*, 1993]. This study investigated whether opioid tolerance could also be observed at a site higher in the cascade, i.e., at the G protein level as measured by changes in the stimulation of agonist binding of [^{35}S]GTPγS to G proteins of SH-SY5Y cells.

6b.2 Results

6b.2.1. [^{35}S]GTPγS binding as a measure of opioid tolerance in SH-SY5Y cells

SH-SY5Y cells were treated with morphine (10 μM, 48 h) as described in section 2.2.6. The cells were then harvested and [^{35}S]GTPγS binding assays were conducted as outlined in section 2.2.5. On each occasion a parallel experiment on untreated cells was conducted to provide a direct comparison with a naive state. Morphine was found to be an agonist in naive untreated cells with a potency (EC_{50} value) of 76 ± 10 nM, affording an increase in binding over basal levels of 15 000 dpm, equivalent to 30 fmols / mg protein [^{35}S]GTPγS bound. In comparison an EC_{50} of 174 ± 39 nM was obtained in morphine-treated cells. Thus the potency of morphine was significantly reduced 2.4 - fold. In addition morphine pretreatment of cells also decreased the maximum [^{35}S]GTPγS stimulation by 33 ± 10 % (Fig. 6.17).

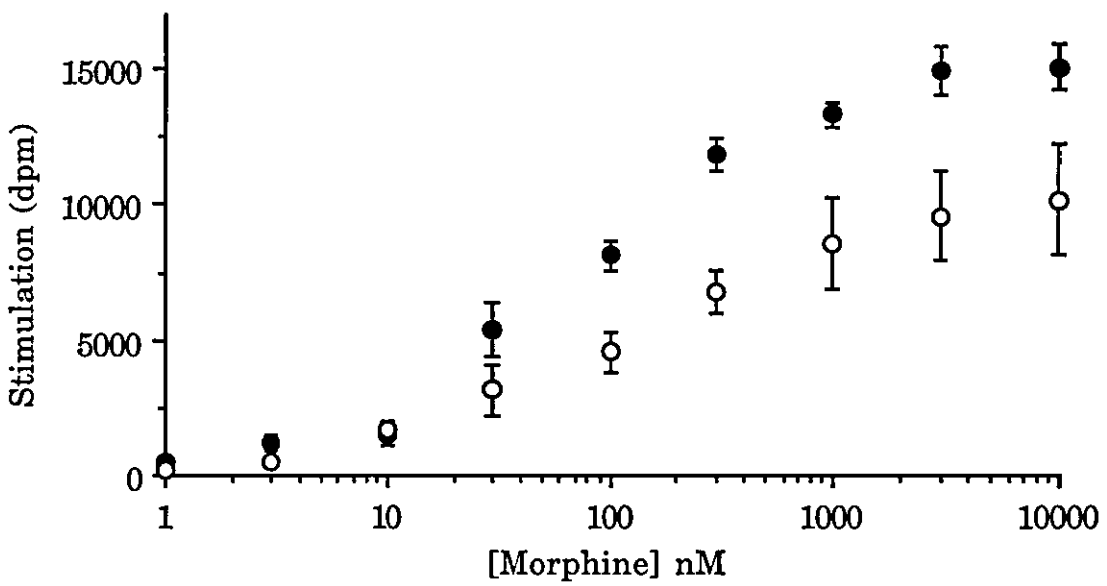


Figure 6.17 Stimulation of [^{35}S]GTPγS binding in SH-SY5Y membranes, incubated at 30°C for 1 h, by morphine in naive (●) and morphine-treated (10 μM, 48 h) (O) cells. Values represent means ± s.e.mean where n ≥ 3.

Morphine is a μ-preferring agonist but does possess some affinity for both δ- and κ-opioid receptors [Magnan *et al.*, 1982]. Since SH-SY5Y cells also express δ-opioid receptors (see section 5.2) it is possible that at high concentrations morphine mediates its effect *via* δ-opioid sites. To confirm the role of μ-opioid receptors the dose-response curve of the μ-selective agonist DAMGO was also examined in naive and morphine-treated cells. The EC_{50} for DAMGO was not significantly altered from naive values (98 ± 33 nM) in morphine-treated cells (112 ± 31 nM). The maximum effect produced by 10 μM DAMGO was reduced by 29 ± 5 % following morphine treatment (Fig. 6.18).

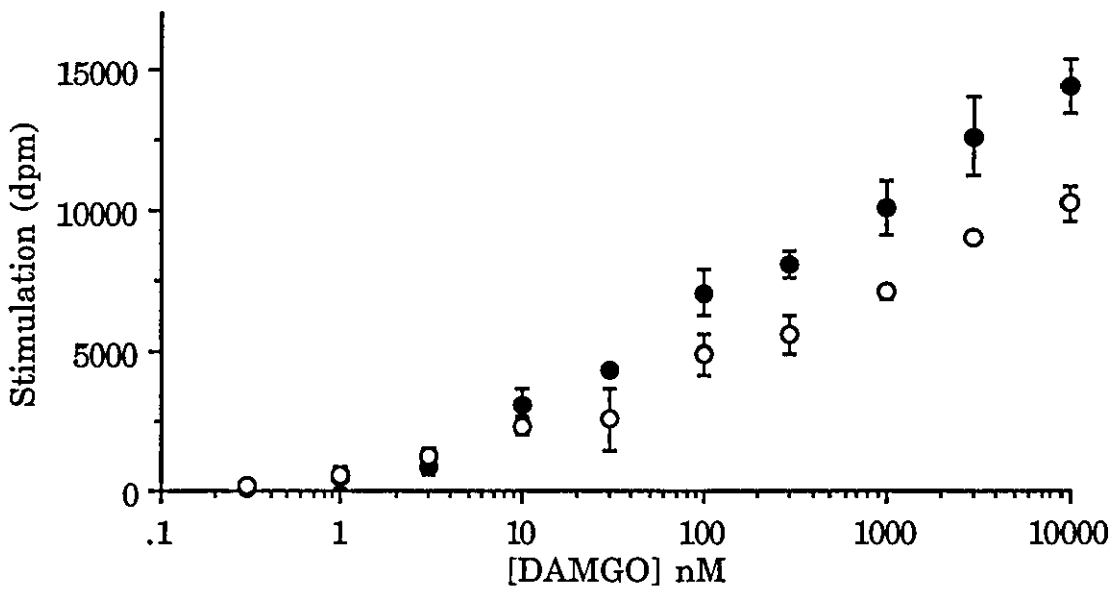


Figure 6.18 Stimulation of [^{35}S]GTPγS binding in SH-SY5Y membranes, incubated at 30°C for 1 h, by DAMGO in naive (●) and morphine-treated (10 μM, 48 h) (O) cells. Values represent means ± s.e.mean where n ≥ 3.

In the same manner dose-response curves before and after morphine (10 μM, 48 h) pretreatment were also conducted with the partial agonist pentazocine [Casy & Parfitt, 1985]. This led to a marked loss in potency in morphine-treated cells compared to naive cells (EC_{50} = 129 ± 51 nM) (Fig. 6.19).

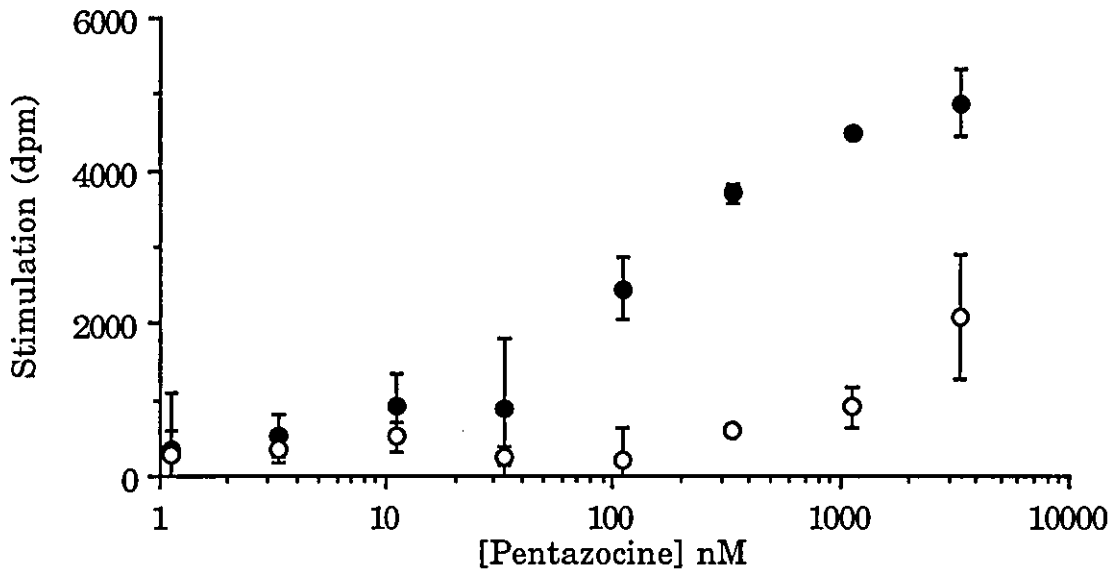


Figure 6.19 Stimulation of [^{35}S]GTPγS binding in SH-SY5Y membranes, incubated at 30°C for 1 h, by pentazocine in naive (●) and morphine-treated (10 μM, 48 h) (O) cells. Values represent means ± s.e.mean where n ≥ 3.

To confirm that this apparent morphine-tolerant state was indeed opioid-induced the effect of adding 10 μM naloxone to the cell media (in the presence of 10 μM morphine) was examined. In these cells the potency of morphine was 99 ± 3 nM, i.e., not significantly different to the value of 89 ± 7 nM obtained in naive cells (Fig. 6.20), in addition there was no reduction in the maximal stimulation of binding.

Since the reduction in potency of morphine was only 2.4 - fold and the maximal effect was decreased by 33 % in morphine-treated cells, the cells were also pre-incubated with a higher efficacy drug than morphine to determine whether a greater degree of tolerance could be induced. DAMGO (10 μM, 48 h) which has higher efficacy than morphine [Handa *et al.*, 1981] was used (Figs. 6.21 - 6.23).

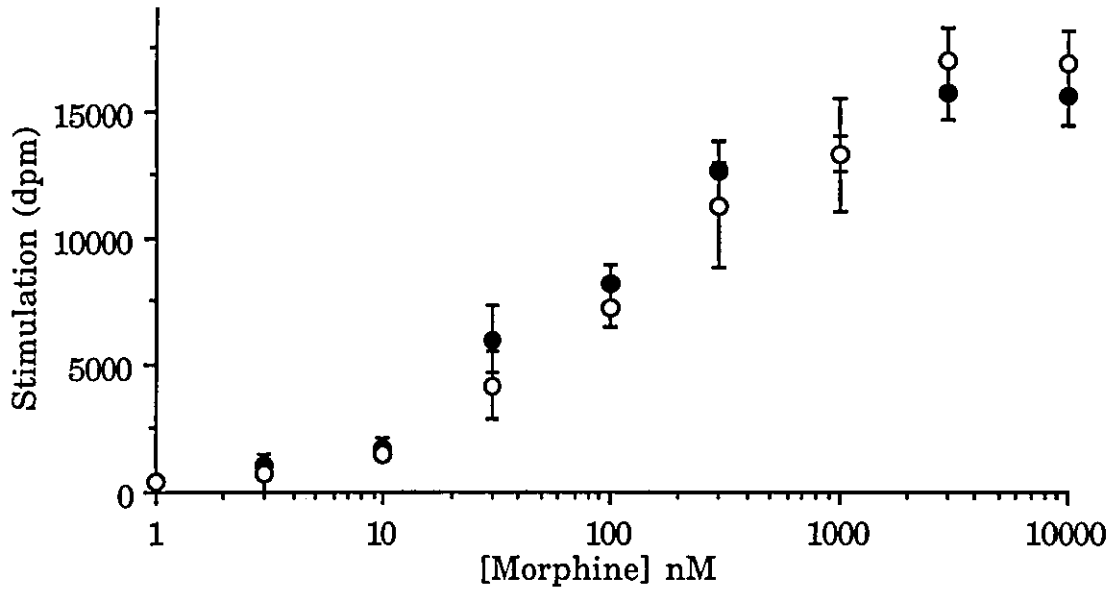


Figure 6.20 Stimulation of [^{35}S]GTPγS binding in SH-SY5Y membranes, incubated at 30°C for 1 h, by morphine in naive (●) and morphine- (10 μM, 48 h) + naloxone- (10 μM, 48 h) treated (O) cells Values represent means ± s.e.mean where n ≥ 3.

The EC_{50} of morphine was not significantly altered, i.e., 116 ± 61 nM in the naive state and 286 ± 62 nM in the DAMGO-tolerant state. However, the maximum effect was significantly reduced by 46 ± 7 % (Fig. 6.21). The effect was the same when considering the DAMGO dose-response curves. The maximal response was significantly decreased by 35 ± 8 % but there was no significant change in EC_{50} values, i.e., 49 ± 25 nM in the naive state and 128 ± 63 nM in the DAMGO tolerant state (Fig. 6.22). Once again the greatest degree of loss of effect was observed with pentazocine, where the dose-response curve was virtually non-existent in the DAMGO-induced tolerant state (Fig. 6.23).

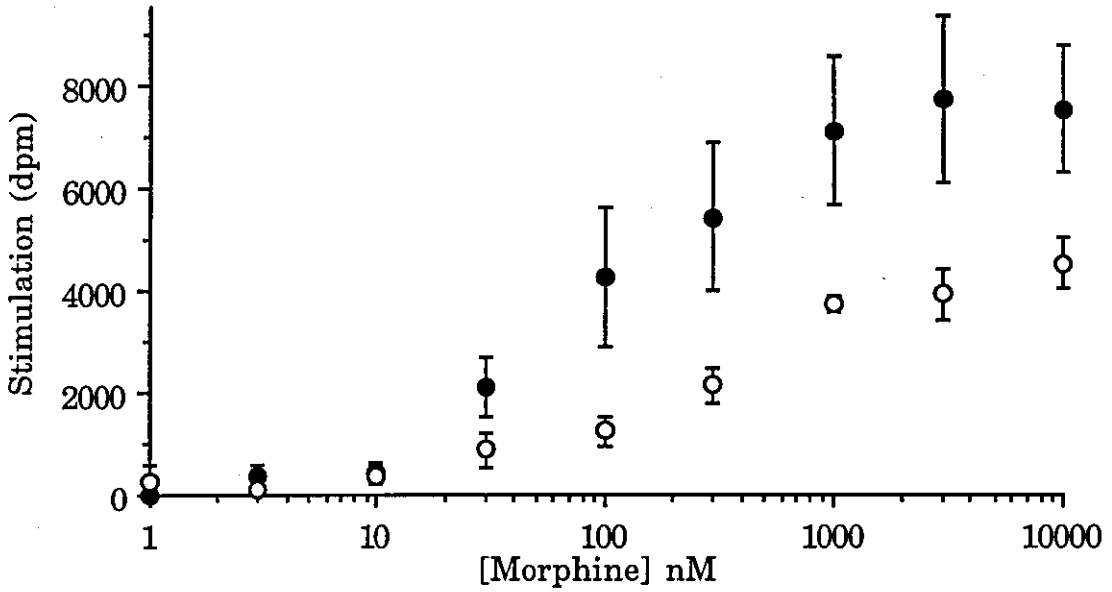


Figure 6.21 Stimulation of [^{35}S]GTPγS binding in SH-SY5Y membranes, incubated at 30°C for 1 h, by morphine in naive (●) and DAMGO-treated (10 μM, 48 h) (O) cells. Values represent means ± s.e.mean where n ≥ 3.

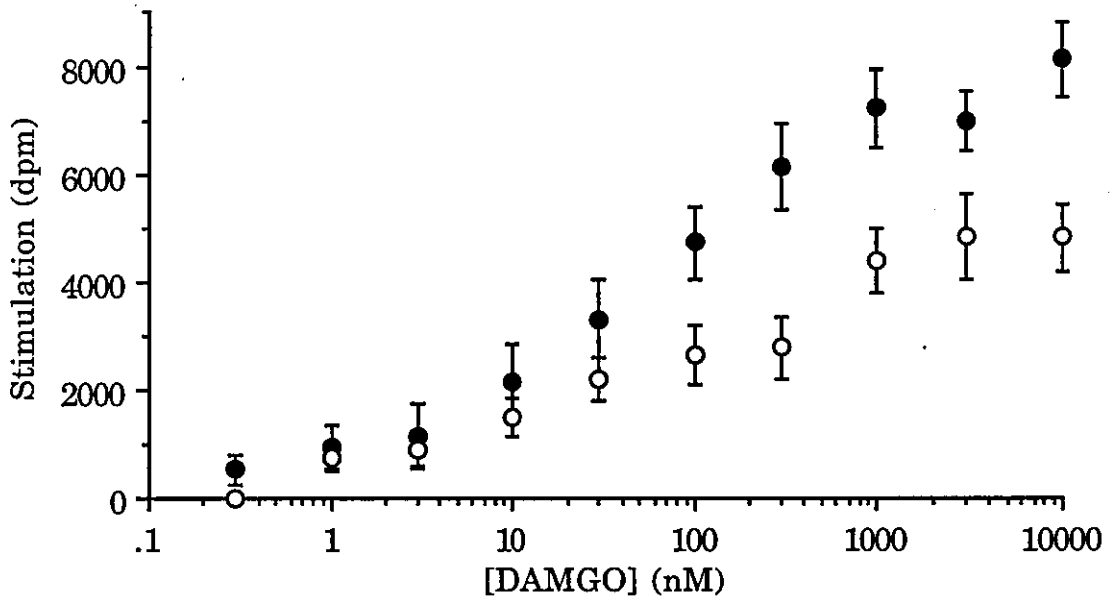


Figure 6.22 Stimulation of [^{35}S]GTPγS binding in SH-SY5Y membranes, incubated at 30°C for 1 h, by DAMGO in naive (●) and DAMGO-treated (10 μM, 48 h) (O) cells. Values represent means ± s.e.mean where n ≥ 3.

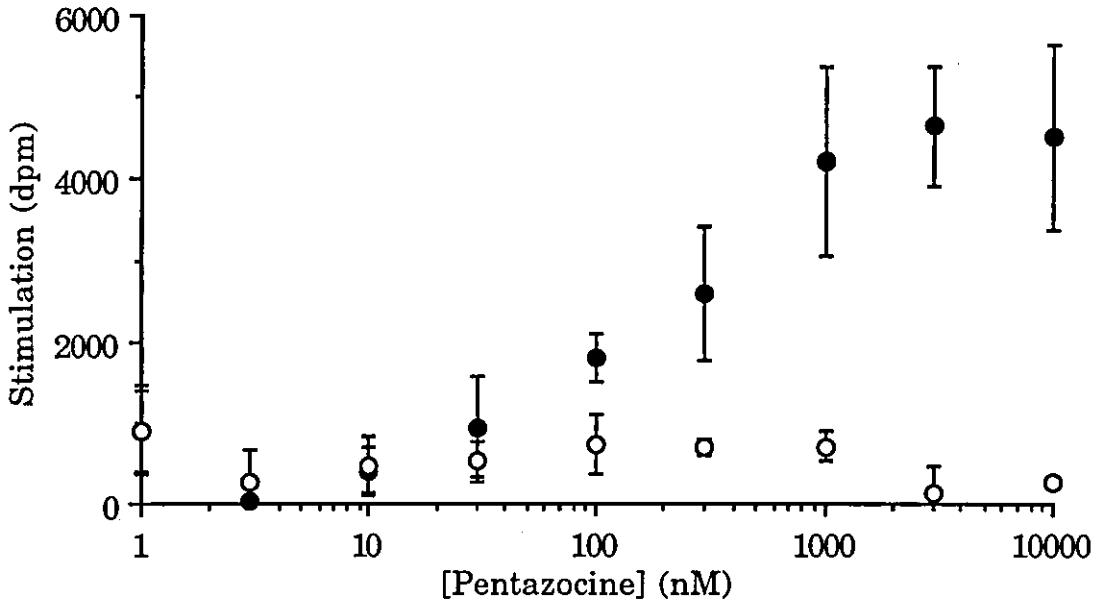


Figure 6.23 Stimulation of [^{35}S]GTPγS binding in SH-SY5Y membranes, incubated at 30°C for 1 h, by pentazocine in naive (●) and DAMGO-treated (10 μM, 48 h) (O) cells. Values represent means ± s.e.mean where n ≥ 3.

Time course of the development of tolerance

The development of morphine tolerance was examined in the [^{35}S]GTPγS binding assay model by adding morphine (10 μM) to the cell media at various time intervals (0 - 72 h). The cells were all harvested at the same time and the level of [^{35}S]GTPγS binding determined at five concentrations of morphine (1 - 10000 nM). The decrease in [^{35}S]GTPγS stimulated binding was time-dependent, with a half-maximum decrease time of approximately 6 h. The binding decrease was maximal at 24 h with no further significant change observed up to 72 h (Fig. 6.24). It can also be seen from the figure that the potency of morphine does not significantly vary over the time scale used, EC_{50} values of morphine after various time periods are given in Table 6.2.

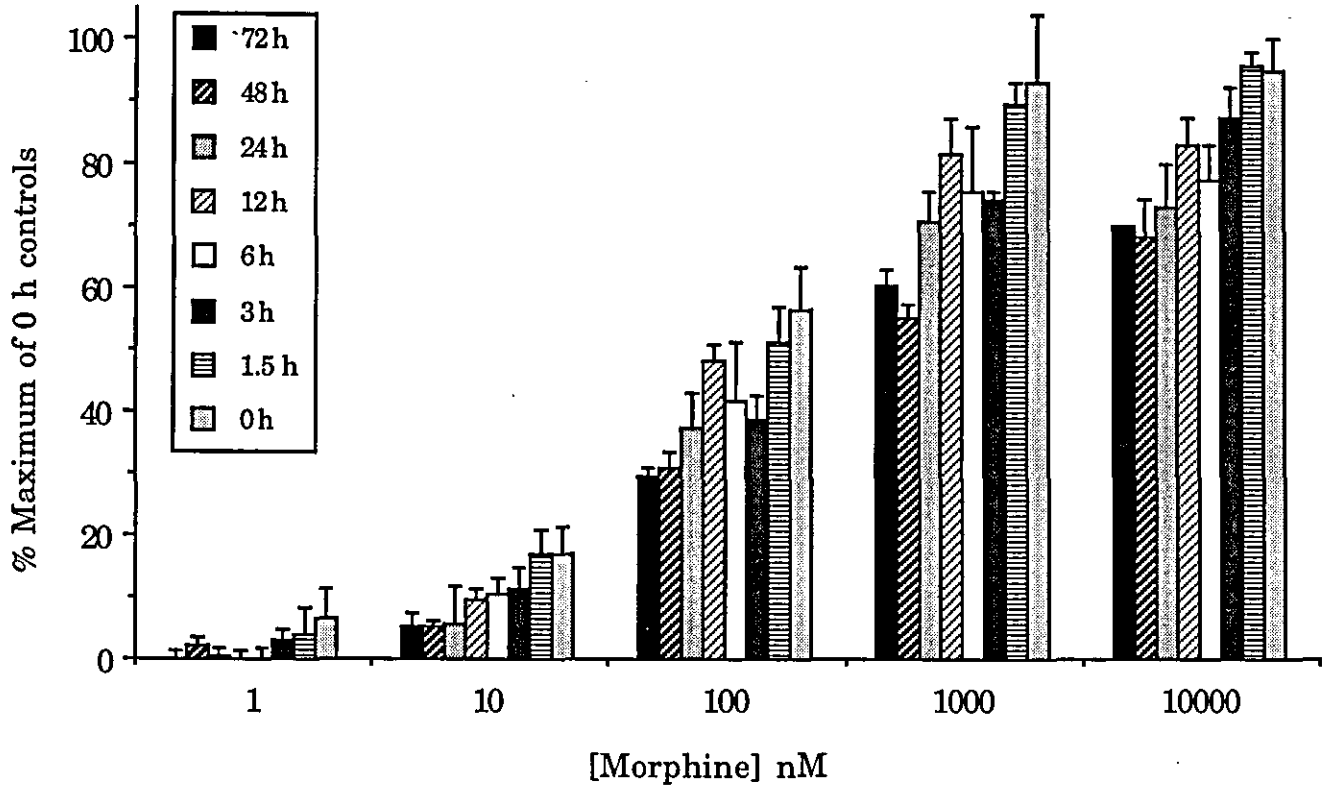


Figure 6.24 A time-course of the level of morphine-stimulated [^{35}S]GTPγS binding in SH-SY5Y cells incubated with 10 μM morphine for various time intervals. Values represent means ± s.e.mean where n ≥ 3.

Table 6.2 Potency (EC_{50} values) of morphine in SH-SY5Y cells pre-treated with morphine (10 μM) for varying time periods.

Length of morphine pretreatment (h)	Potency (nM)
0	76 ± 27
1.5	134 ± 41
3	131 ± 30
6	140 ± 35
12	101 ± 26
24	129 ± 19
48	225 ± 87
72	157 ± 14

Stimulated [^{35}S]GTPγS binding was assayed at 30°C for 1 h. Values represent means ± s.e.mean where $n \geq 3$. None of the results were significantly different where $P < 0.05$.

Illustration of an opiate dependent state

Clinically in an opiate-dependent state the effect of naloxone administration is to precipitate an opiate withdrawal syndrome. In this study the level of [^{35}S]GTPγS binding was measured in the presence of three concentrations of naloxone (100, 1000 and 10000 nM) both in naive and morphine-treated (10 μM, 24 h) cells (Fig 6.25). Once again there was reduced stimulation achieved by morphine in the morphine-treated as compared to naive cells, confirming that a tolerant state existed. Interestingly, naloxone caused a slight stimulation of [^{35}S]GTPγS binding in naive cells whereas in morphine-tolerant cells the reverse was true, i.e., small but significant inhibition of [^{35}S]GTPγS binding had occurred. The effects of naloxone are not any greater at 10000 nM as opposed to 100 nM. However, the effects of naloxone are very small (approximately 1000 dpm) compared to the agonist effects of morphine (approximately 15000 dpm) both in the tolerant and non-tolerant state.

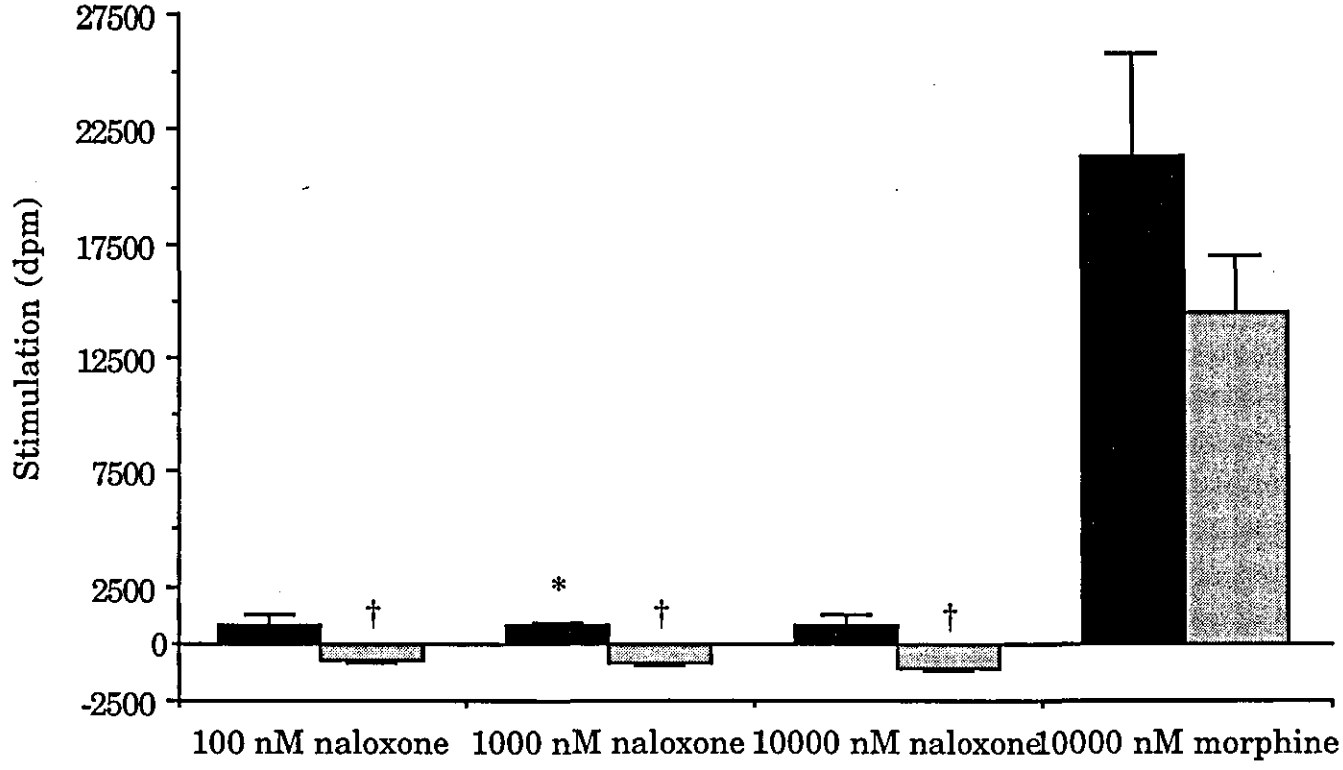


Figure 6.25 Level of naloxone- (or 10000 nM morphine- as control) stimulated [^{35}S]GTPγS binding in control (black) and morphine-treated (grey) (10 μM, 24 h) SH-SY5Y cells, incubated at 30°C for 1 h. Values represent means ± s.e.mean, where n ≥ 3. At the 95 % confidence limit compared to basal levels: *significant stimulation of [^{35}S]GTPγS binding and †significant inhibition of [^{35}S]GTPγS binding.

Mechanisms of opioid tolerance in this model

In order to examine μ receptor down-regulation [^{3}H]DAMGO was used to determine the receptor number (B_{max}) and the affinity (K_D) of the μ receptor both in naive and DAMGO-treated (10 μM, 48 h) SH-SY5Y cells (Fig. 6.26). In naive cells [^{3}H]DAMGO labelled 140 ± 11 fmols / mg protein, and only 71 ± 7 fmols / mg protein in DAMGO-treated cells (experiments were conducted in parallel and n = 3). Thus approximately 50 % of [^{3}H]DAMGO binding sites were lost following long-term exposure to DAMGO. The affinity of the μ-opioid receptor in the naive and tolerant state was not significantly different, i.e., the respective K_D values for naive and DAMGO treated cells were 1.45 ± 0.31 nM and 0.88 ± 0.18 nM.

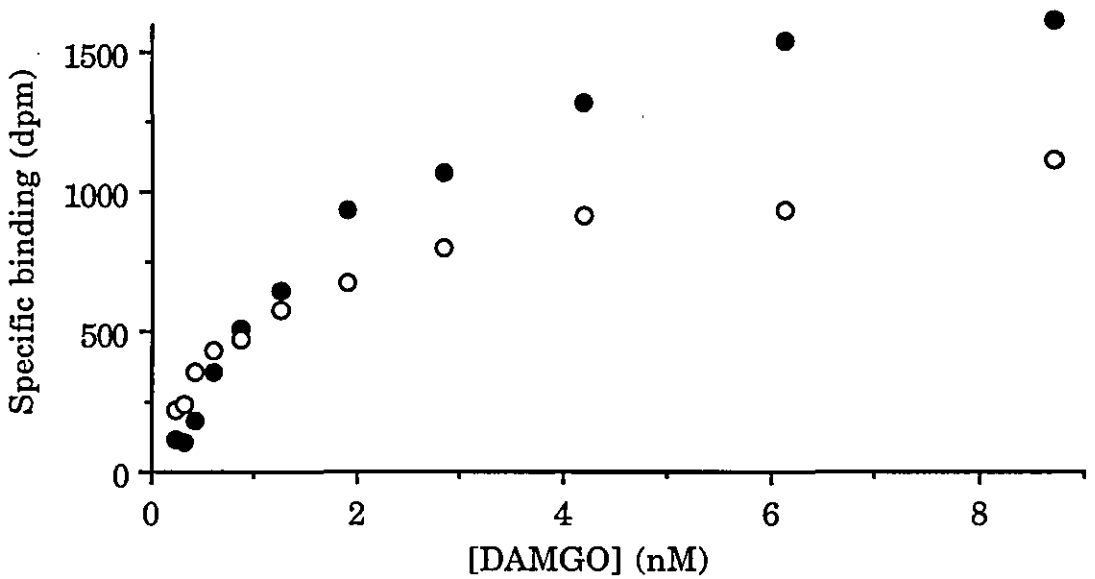


Figure 6.26 A representative graph showing saturation [^{3}H]DAMGO binding to naive (●) and DAMGO-treated (10 μM, 48 h) (O) SH-SY5Y cells, incubated at 25°C for 40 min (Tris-HCl buffer, pH 7.4).

Agonists such as [^{3}H]DAMGO at levels up to 10 nM will only label high affinity μ-opioid binding sites since their affinity at low affinity sites is approximately 1 μM. Thus the observed loss of specific [^{3}H]DAMGO binding sites could represent either a loss of μ-opioid receptors or a change in conformation of the receptors from high to low agonist affinity states. In order to determine whether down-regulation has occurred binding at the μ-opioid receptor was assessed utilising the antagonist [^{3}H]diprenorphine which should label both high and low affinity states.

[^{3}H]Diprenorphine has affinities for μ- and δ-opioid sites of 0.31 and 1.48 nM, respectively [Traynor *et al.*, 1987]. Therefore prior to saturation analysis of [^{3}H]diprenorphine binding it is necessary to determine the level of [^{3}H]diprenorphine binding to δ-opioid receptors in SH-SY5Y cells. The displacement of [^{3}H]diprenorphine by the highly selective δ-antagonist TIPP was examined. This compound has affinities (K_i values) of 1.22 and 1720 nM for δ- and μ-opioid receptors respectively [Schiller *et al.*, 1992]. TIPP (up to a concentration of 100 nM) was unable to displace any significant amount of [^{3}H]diprenorphine specific binding (Fig. 6.27).

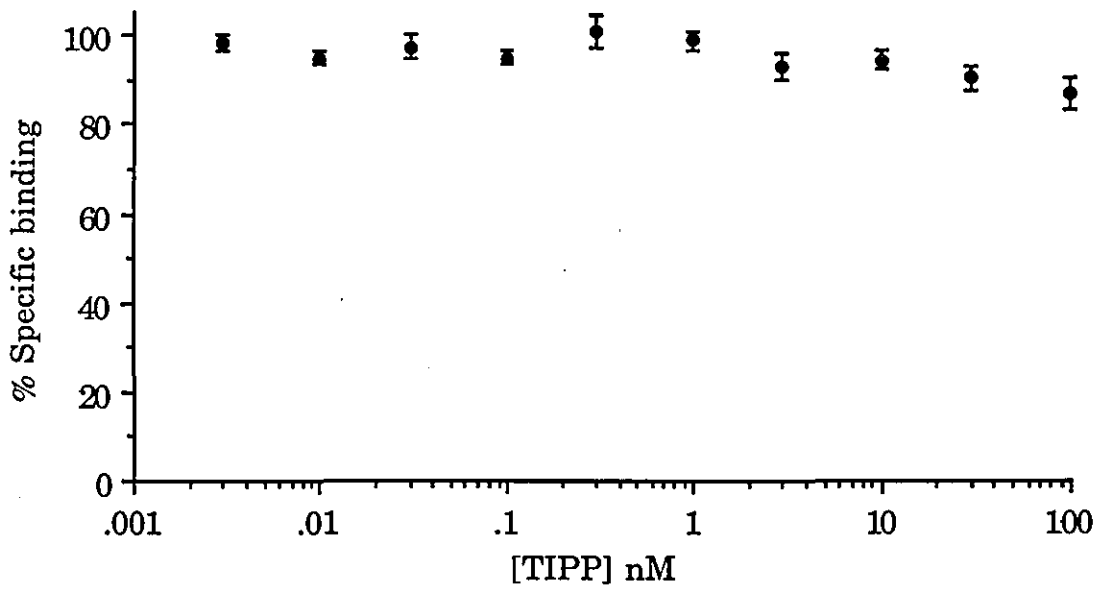


Figure 6.27 Displacement of [^{3}H]diprenorphine (0.90 ± 0.13 nM) by TIPP in SH-SY5Y membranes (Tris-HCl buffer, pH 7.4), incubated at 25°C for 40 min. Values represent means ± s.e.mean where n ≥ 3.

Having confirmed that [^{3}H]diprenorphine essentially only labels μ-opioid receptors on these SH-SY5Y cells (as suggested earlier based on the occupany theory, chapter 5), the receptor number and the affinity of the μ-opioid receptors in the naive and DAMGO-induced tolerant states were assessed.

Consistent with previous studies it was found that [^{3}H]diprenorphine labels significantly more opioid sites in SH-SY5Y membranes than [^{3}H]DAMGO [Kazmi & Mishra, 1987]. Furthermore in naive cells the B_{max} was 206 ± 36 fmols / mg protein and in tolerant cells the B_{max} was reduced yielding a B_{max} of 65 ± 14 fmols / mg protein (Figs. 6.28 & 6.29). Therefore chronic exposure of SH-SY5Y cells to DAMGO decreases the number of receptors labelled by [^{3}H]diprenorphine by approximately 68 %. As with [^{3}H]DAMGO the affinity of [^{3}H]diprenorphine was consistent in the two populations of cells, i.e., 0.24 ± 0.07 nM in naive and 0.33 ± 0.17 nM in tolerant cells. These results suggest that there is a real loss of receptor number in an opioid-induced tolerant state.

To confirm that there was no change in affinity due to tolerance, the displacement of [^{3}H]diprenorphine by DAMGO under the exact conditions of [^{35}S]GTPγS binding was examined, that is, the experiment was conducted in [^{35}S]GTPγS buffer in the presence of 3 μM GDP and was incubated at 30°C for 1 h. The IC_{50} values were not siginificantly different in naive as compared to tolerant cells, i.e., 211 ± 95 and 277 ± 41 nM respectively (Fig. 6.30). Thus these results confirm that the tolerance observed in [^{35}S]GTPγS binding assays is not a function of a change in affinity of μ-opioid receptors.

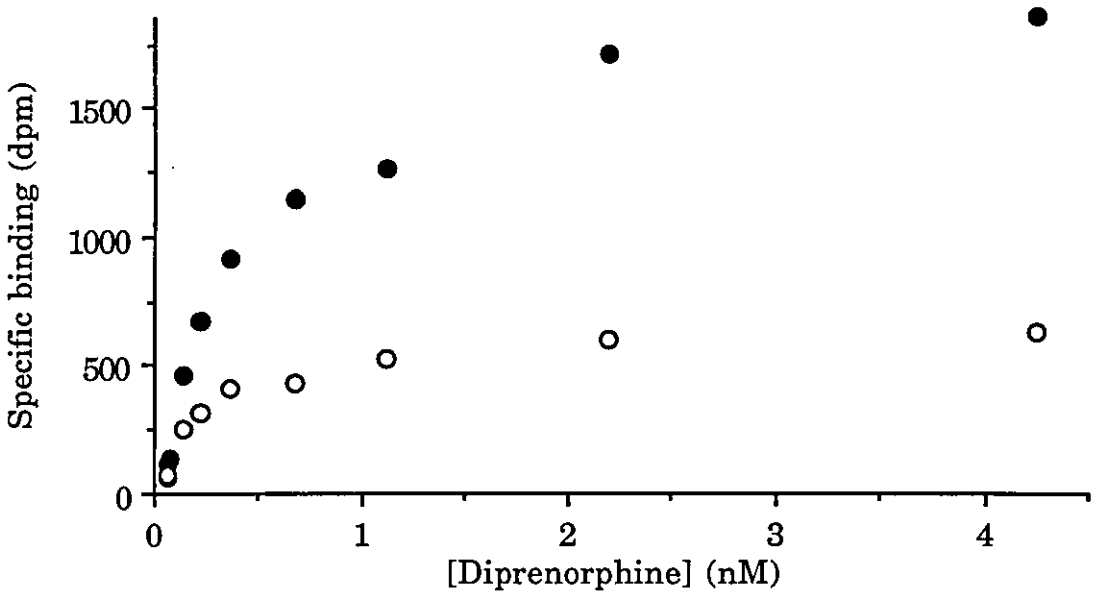


Figure 6.28 A representative graph showing saturation [^{3}H]diprenorphine binding to naive (●) and DAMGO-treated (10 μM, 48 h) (O) SH-SY5Y cells, incubated at 25°C for 40 min (Tris-HCl buffer, pH 7.4).

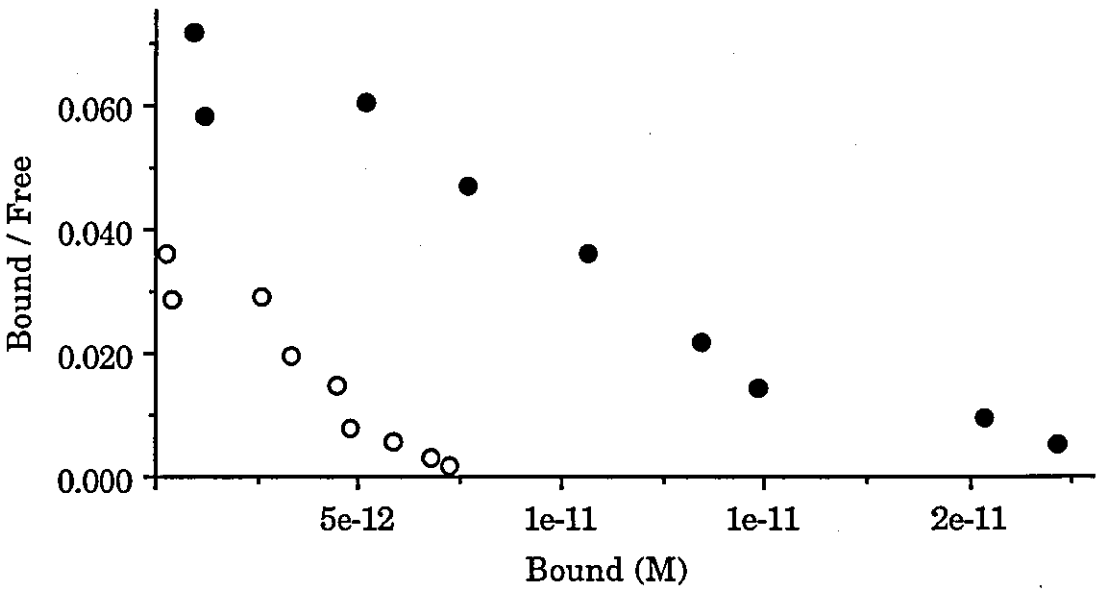


Figure 6.29 A Scatchard plot of the data shown in Fig. 6.12. Naive (●) and DAMGO-treated (10 μM, 48 h) (O) SH-SY5Y cells, incubated at 25°C for 40 min (Tris-HCl buffer, pH 7.4).

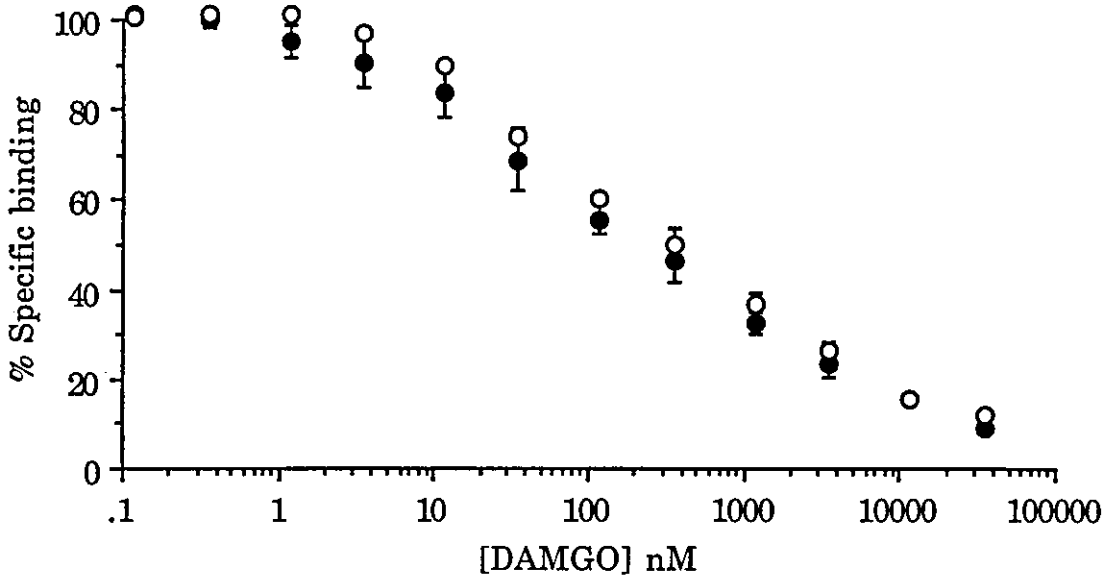


Figure 6.30 Displacement by DAMGO of [^{3}H]diprenorphine (0.49 ± 0.04 nM) in naive (●) and morphine-treated (10 μM, 24h) (O) SH-SY5Y membranes, conducted in [^{35}S]GTPγS buffer in the presence of 3 μM GDP, incubated at 30°C for 1 h. Values represent means ± s.e.mean where n ≥ 3.

Examination of partial tolerance

In the present model of opioid tolerance employing studies of [^{35}S]GTPγS binding the agonist-induced stimulation of [^{35}S]GTPγS binding is reduced but not abolished in the tolerant state. Thus partial tolerance has apparently occurred and some receptors remain functional.

Earlier studies (see section 5.2.2) [Elliott *et al.*, 1994] have demonstrated that the μ-opioid receptor binding sites on SH-SY5Y cells can be irreversibly inhibited by β-funaltrexamine, but as in other systems approximately 40 % [Rothman *et al.*, 1987; Tam & Liu-Chen, 1986; Franklin & Traynor, 1991] of these sites appear to be insensitive to this irreversible antagonist. Since similar receptor numbers appear insensitive to tolerance and to β-funaltrexamine pre-treatment, it may be that such "inaccessable" receptors may also be responsible for the situation of tolerance resistant sites.

In order to determine whether these β-funaltrexamine and tolerance resistant binding sites are the same, tolerant cells and naive cells were treated with β-funaltrexamine (1 μM for 1 h at 37°C) as previously described (see section 2.2.4). The specific binding of [^{3}H]diprenorphine (4 nM) was determined (a high enough concentration to label all receptor sites).

The findings of these experiments (Fig 6.31) are :

i) β-funaltrexamine significantly reduced the number of binding sites in naive cells; 56 ± 6 % of binding sites were found to be insensitive to β-funaltrexamine (1 μM).

ii) Morphine pre-treatment (10 μM, 48 h) significantly decreased receptor numbers; 68 ± 12 % were tolerance-insensitive.

iii) The level of specific binding sites remaining after β-funaltrexamine treatment of morphine-exposed SH-SY5Y cells was 50 ± 9 % of naive levels.

iv) The specific binding of [^{3}H]diprenorphine to tolerant cells was not significantly reduced by β-funaltrexamine treatment.

v) The level of β-funaltrexamine insensitive binding sites is not significantly different in naive as opposed to tolerant cells.

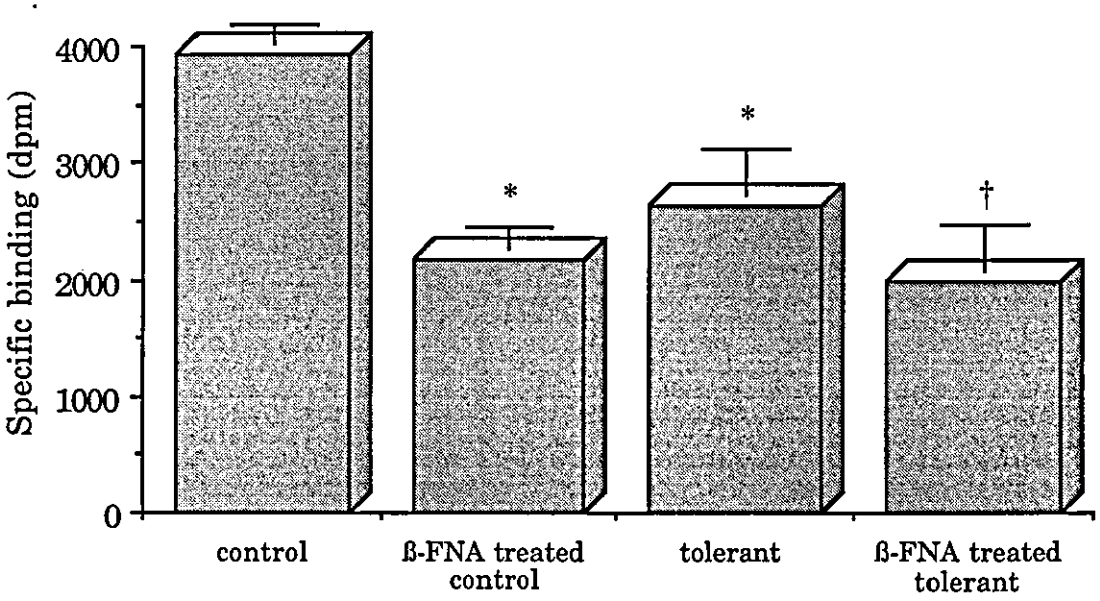


Figure 6.31 Specific binding of [^{3}H]diprenorphine (4.0 ± 0.3 nM) in SH-SY5Y membranes (Tris-HCl buffer, pH 7.4) , incubated at 25°C for 40 min. Values represent means ± s.e.mean where n ≥ 3. At the 95 % confidence limit: *significantly different to naive cells and †not significantly different to tolerant cells.

Modulation of opioid-induced tolerance

The mechanism(s) by which tolerance occurs is unknown. The process could involve specific enzymes, phosphorylation, protein synthesis or any combination of these. To determine whether a phosphorylation step *via* β-adrenergic receptor kinase (β-ARK) or protein kinase is involved, suramin (8,8'-[carbonylbis[imino-3,1-phenylenecarbonyl-imino(4-methyl-3,1-phenylene)-carbonyl-imino]]bis-1,3,5-naphthalenetri-sulfonic acid) and H7 (1-(5-isoquinolinesulfonyl)-2-methylpiperazine) (Fig. 6.32) were tested. H7 inhibits several protein kinases, e.g., PKC, as well as protein kinase dependent cAMP and cGMP [Hidaka *et al.*, 1984]. Suramin is reported to inhibit β-ARK (a cAMP-independent kinase) [Miller *et al.*, 1993], which phosphorylates both threonine and serine residues on the intracellular surface of receptors. This action is catalysed by agonist binding [Hausdorff *et al.*, 1990], thus coupling receptor activation and desensitisation.

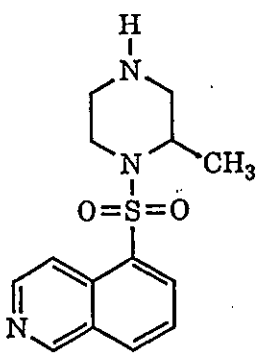


Figure 6.32 H7

In an initial experiment morphine-induced tolerance (10 μM, 24 h) was unaffected by 0.1 μM suramin when it was added to the cell media during morphine pretreatment. Therefore in subsequent experiments 10 μM suramin was used and in parallel experiments 50 μM H7 was utilised, as described in section 2.2.6. Single doses of 10 μM DAMGO, morphine and pentazocine were assayed. The level of tolerance in naive cells was 28.3 ± 0.3 % (as determined from the loss in maximum stimulation of [^{35}S]GTPγS compared to 3 μM fentanyl), was slightly but not significantly lower in suramin-treated cells

(20 ± 5%); however it was significantly reduced in H7-treated cells (7.2 ± 6.2 %) (Fig. 6.33). The development of morphine-induced cross-tolerance to the partial agonist pentazocine was virtually abolished by H7; only 3.9 ± 7.9 % tolerance in H7-treated cells compared to 33 ± 3 % tolerance in naive cells.

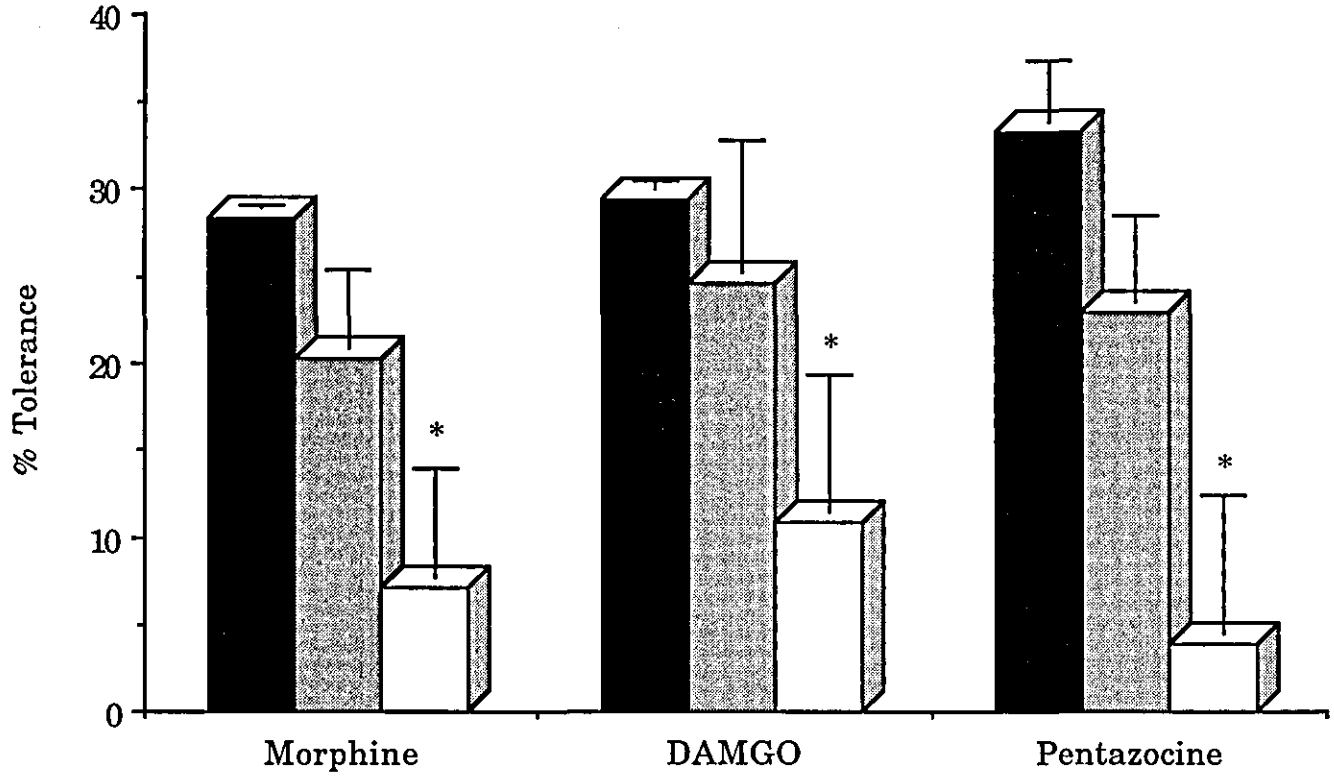


Figure 6.33 Percentage tolerance in morphine-induced tolerant cells (10 μM, 24 h) in naive (black), suramin- (10 μM) (grey) and H7- (50 μM) (white) treated SH-SY5Y cells, [^{35}S]GTPγS binding assays were conducted at 30°C for 1 h. Values represent means ± s.e.mean where n ≥ 3.

6b.3 Discussion

[^{35}S]GTPγS binding assays as a measure of opioid tolerance in SH-SY5Y cells

There are many experimental models of opiate tolerance, including those in whole animals [Duggan *et al.*, 1977; Tang & Collins, 1978], isolated tissues [Ward & Takemori, 1976; Schulz *et al.*, 1980] and neurons [Aghajanian, 1978]. In order to gain an understanding of tolerance at the cellular level single cell models are required [Christie *et al.*, 1987; Puttfarcken *et al.*, 1988; Law *et al.*, 1983]. More specifically there are models based on SH-SY5Y cells, i.e., inhibition of stimulated cAMP accumulation [Olasmaa & Terenius, 1988; Yu & Sadee, 1988] and intracellular electrophysiological recordings of calcium currents [Kennedy & Henderson, 1991]. By examining [^{35}S]GTPγS binding to SH-SY5Y membranes this study provides a further measure of the phenomenon of tolerance at the level of the receptor G protein. A major advantage of the model is that undifferentiated cells can be used. In the majority of previous studies SH-SY5Y cells have been differentiated using retinoic acid treatment which up-regulates the μ-opioid receptor, induces neuronal characteristics, increases levels of inhibitory G proteins and inhibits the growth rate [Pålman *et al.*, 1984; Yu & Sadee, 1988; Seward *et al.*, 1989; Ammer & Schulz, 1994]. This operation needs to carried out with care and is a time-consuming task.

The results demonstrated that agonist-induced stimulation of [^{35}S]GTPγS binding was reduced in SH-SY5Y cells chronically exposed to opioids. This tolerant state was shown to be μ-opioid induced and naloxone-reversible. The dose-response curve to morphine was shifted 2.4 - fold and the maximum effect was diminished by 33 ± 10 % in morphine-treated as opposed to naive cells. No significant shift of the DAMGO dose-response curve was observed although the maximum stimulation was once again reduced by approximately 30 %. The greatest effect was observed with pentazocine; this is likely to be a reflection of its lower intrinsic activity than either morphine or DAMGO [Casy & Parfitt, 1985]. Agonists with high intrinsic activity only need to occupy a relatively small proportion of the total receptor population in order to mediate their effects. In contrast, agonists with low intrinsic activity need to occupy a much greater percentage of the receptor population for maximal effects to be observed. This finding correlates with the results obtained from intracellular

recordings of potassium currents from rat locus coeruleus *in vitro*. The degree of tolerance induced by morphine is determined by the intrinsic efficacy of the opioid. In this system a greater level of tolerance to normorphine was exhibited than either to [Met^5]enkephalin or to DAMGO [Christie *et al.*, 1987]. Thus high efficacy agonists have been changed to partial agonists in tolerant cells and the partial agonist has even less efficacy than before.

Similar results were obtained when the tolerant state was DAMGO- rather than morphine-induced. In the morphine- and the DAMGO- induced tolerant state there was only approximately a 30 - 40 % reduction in the maximal effect afforded by either morphine or DAMGO. However, the stimulation of [^{35}S]GTPγS binding by the partial agonist pentazocine was inhibited to a greater extent in the DAMGO- as opposed to morphine-induced tolerant state. This is probably a relection of the higher intrinsic activity of DAMGO compared to morphine [Casy & Parfitt, 1985] affording a greater the level of tolerance.

Interestingly, chronic morphine treatment raises the levels of all G protein subunits identified in these cells, whereas DAMGO is without effect [Ammer & Schulz, 1993]. Morphiceptin, another compound with low intrinsic activity produed similar results to morphine. Thus low intrinsic activity opioids may be able to induce a compensatory effect, increasing the quantities of G proteins. This could offer an explanation as to why a lower degree of tolerance was induced by morphine compared to DAMGO in [^{35}S]GTPγS binding assays.

Apart from the problems of tolerance in human subjects there is the added drawback of the development of physical dependence when morphine is administered regularly for recreational use. Dependence is defined as a state in which regular doses of the drug are required to prevent withdrawal symptoms, these are characterised by restlessness, cramp, nausea, vomiting, diarrhoea, "goose flesh" and mydriasis. In addicts these symptoms start to occur within 15 - 20 h of withdrawal of the drug or within 30 min of the administration of an antagonist such as nalorphine [Burgen & Mitchell, 1985].

Opiate dependence can be characterised by an increase in responsiveness to opiate antagonists [Collier, 1980]. A physical "dependent-like" state can be observed in single cells since the addition of naloxone can result in a rebound effect. Opioids inhibit PGE_1- or forskolin-stimulated cAMP accumulation in SH-SY5Y cells. The level of inhibition is reduced in the tolerant state. When

naloxone is added to morphine pretreated SH-SY5Y cells, a cAMP overshoot is observed, i.e., the levels of cAMP are even higher than in naive cells [Yu *et al.*, 1990].

In this study the effect of naloxone was to slightly stimulate the level of [^{35}S]GTPγS binding in naive cells but to slightly and significantly inhibit binding in tolerant cells. The latter result may support the existence of constitutively active receptors. It has been proposed that chronic opioid exposure causes a gradual constitutive μ-opioid receptor activation, i.e., the receptor is converted to a form which no longer requires agonist for signal transduction. Thus tolerance occurs because fewer μ-opioid receptors remain able to be activated by agonists [Wang *et al.*, 1994b]. Therefore in the tolerant state [^{35}S]GTPγS binding would be already partially stimulated and this binding could be inhibited by naloxone. In this type of system an agent, such as naloxone, which lowers the basal activity of the receptor is called a negative antagonist [Lefkowitz *et al.*, 1993]. The importance of this result however is unclear since naloxone only inhibits 2 fmols of [^{35}S]GTPγS binding in the tolerant state, whereas morphine causes fiftteen times as much stimulation of [^{35}S]GTPγS binding, suggesting at best low levels of constitutive activity.

Previous studies of morphine tolerance in SH-SY5Y cells have demonstrated that morphine tolerance is observable after as little as 3 h and is usually complete after 24 h [Yu & Sadee, 1988; Carter & Medzihradsky, 1993b; Zadina *et al.*, 1993]. Experiments using this model show that the development of tolerance is a time-dependent process with a gradual decrease in maximal [^{35}S]GTPγS stimulated binding observable from 1.5 h onwards to 24 h when the decline was virtually complete. From this study the stages of desensitisation and down-regulation were indistinguishable. If desensitisation occurs within a matter of minutes rather than hours, then it is unlikely that this model could separate them since all naive cells were exposed to 10 μM morphine (or DAMGO) for 5 min prior to cell harvest to ensure that any effect was a result of chronic rather than acute exposure to opioid. In addition the [^{35}S]GTPγS binding assay itself was conducted over 60 min.

As previously mentioned the development of opioid tolerance is thought to progress in two stages. Firstly there is an uncoupling of the G protein from the receptor and then a subsequent down-regulation of receptors. High affinity binding occurs to the G protein-coupled receptor and is reduced in numerous systems following chronic exposure to opioids. For example, μ-opioid receptor

numbers are decreased, while there is no change in the affinity of the μ-opioid receptor in guinea-pig brain following long term morphine treatment [Werling *et al.*, 1989], there is also μ-opioid receptor loss in 7315c rat tumour cells following morphine pretreatment [Puttfarcken *et al*, 1988] and chronic etorphine exposure in rat brain reduces the levels of both μ- and δ-opioid receptors [Tao *et al.*, 1987]. In cellular systems expressed κ-opioid receptors are more easily desensitised than either μ- or δ-opioid receptors [Uhl *et al.*, 1994]. Interestingly, although tolerance was observed when examining the inhibition of cAMP accumulation in differentiated SH-SY5Y cells this was not accompanied by a significant change in μ-opioid receptor number or affinity, as measured by [^{3}H]DAMGO binding [Yu *et al.*, 1990]. This is in direct disagreement with another study of opioid tolerance in differentiated SH-SY5Y cells where both receptor-effector desensitisation and receptor down-regulation occurred, as measured by binding of the antagonist [^{3}H]naltrexone [Carter & Medzihradsky, 1993b]. The results of this latter study correlate well with the investigations by Zadina *et al.* (1993) and Baumhaker *et al.*, (1993) where μ-opioid-induced down-regulation was apparent but there was no change in the affinity of μ-sites labelled with [^{3}H]DAMGO. These findings agree with the present study in which the reduction in stimulation of [^{35}S]GTPγS binding, as measured by saturation antagonist binding, is accompanied with an approximately 68 % loss in μ-opioid binding sites, with no change in affinity and not just a conformational change in the receptor from a high to a low affinity state.

The affinity of agonists and antagonists for opioid receptors is differentially affected by the addition of sodium to the assay buffer [Pert & Snyder, 1974]. The binding of antagonists is unaffected whereas the binding of agonists is significantly decreased. This is because agonists bind to high rather than low affinity states and sodium ions stabilise the low affinity form of the receptor, thus reducing the levels of high affinity sites. Antagonists are unable to differentiate between high and low affinity states. For this reason [^{3}H]DAMGO binding was conducted in Tris-HCl buffer rather than a buffer containing a more physiological concentration of sodium ions. In order to maintain consistency the [^{3}H]diprenorphine binding experiments were also conducted in Tris-HCl buffer. However, the [^{35}S]GTPγS binding assays were carried out in a buffer containing high levels of sodium (100 mM).

To confirm that there is no change in the affinity of μ-opioid receptors in the tolerant state, the displacement of [^{3}H]diprenorphine by DAMGO, under the exact conditions used for [^{35}S]GTPγS binding was examined. The IC_{50} value in naive cells was not found to be significantly different to the IC_{50} value obtained in morphine-induced tolerant cells.

In untreated SH-SY5Y cells [^{3}H]DAMGO labels 140 ± 11 fmols / mg protein, whereas [^{3}H]diprenorphine affords a B_{max} of 206 ± 36 fmols / mg protein. However, of note is the fact that identical numbers of receptors remain in the tolerant state whether they are labelled with agonist or antagonist, even though there is a greater percentage loss of sites when they are labelled with [^{3}H]diprenorphine. One possible explanation for this result is that in addition to labelling the high affinity states recognised by [^{3}H]DAMGO, [^{3}H]diprenorphine also labels low affinity μ-opioid receptors which are lost in the tolerant-induced state. Thus approximately 70 fmols / mg protein of μ-opioid receptors are resistant to tolerance.

Chronic morphine exposure (100 μM for 24 h) in 7315c pituitary tumour cells, containing a solely μ-opioid receptor population, produces complete tolerance as measured by a total absence of opioid inhibition of adenylate cyclase [Puttfarcken *et al.*, 1988]. However, in the SH-SY5Y cells only partial tolerance develops and a considerable proportion of μ-opioid receptors are retained. Although this study gives a relatively small shift of 2.4 - fold in the dose-response of morphine following long-term morphine exposure, this result is not without precedent. Only a 2 - fold shift of DAMGO was observed (the EC_{50} increased from 91 to 201 nM) in potassium current recordings obtained from neurons of the rat locus coeruleus [Christie *et al.*, 1987]. In comparison a 4 - fold shift of the morphine dose-response curve was obtained in tolerant SH-SY5Y cells as measured by inhibition of stimulated cAMP accumulation (the IC_{50} of morphine was shifted from 78 to 316 nM) [Yu & Sadee, 1988] and a 7 - fold shift in the ability of DAMGO to inhibit calcium currents in morphine-tolerant SH-SY5Y cells was observed [Kennedy & Henderson, 1991].

The mechanism by which down-regulation occurs is still uncertain. It has been suggested that the ligand-receptor complex is internalised into sequestered regions of the membrane that are inaccessible to hydrophilic ligands [Brown *et al.*, 1982; Roth & Coscia, 1984]. Compared to opioid receptors β-adrenergic receptors have been extensively examined. The major mechanism of homologous desensitisation of the β-adrenergic receptor is

sequestration of the receptors away from their physiological effectors [Strasser *et al.*, 1985]. Whatever the process it must require certain parameters to be met and / or have limiting factors since some receptors are resistant to down-regulation. Previous work presented in this thesis (see section 5.2.2) has shown that the μ-opioid receptor population of SH-SY5Y cells is not fully homogeneous, i.e., approximately 40 % are β-funaltrexamine insensitive. One explanation is that a lack of access to the nucleophilic alkylation binding site of a certain proportion of the homogeneous μ-opioid receptor population was responsible for the β-funaltrexamine insensitive binding sites. The findings of this study suggest that tolerant resistant and β-funaltrexamine resistant sites may be one and the same since the receptor number of tolerant cells was not significantly reduced by β-funaltrexamine treatment and the level of β-funaltrexamine insensitive binding sites is not significantly different in naive compared to tolerant cells. Thus in some manner a proportion of μ-opioid receptors must be protected, possibly within the membrane so that they are not alkylated or down-regulated. However, these μ-opioid receptors are apparently functional and able to be bound by the ligands [^{3}H]diprenorphine and [^{3}H]DAMGO, although perhaps they would not be able to bind a membrane impermeable ligand. A recent study also provides evidence supporting different mechanisms by which morphine produces its agonist effects and those that cause tolerance to these effects [Bidlack *et al.*, 1994]. When β-funaltrexamine was coadministered with morphine (i.c.v. into mice) tolerance was prevented for up to 7 h, however, no antagonism of morphine analgesia was observed until 8 h or more after administration.

The most potentially valuable use of an experimental model of opioid tolerance is to devise a method by which the development of tolerance can be abolished or greatly dimished. Once such a system has been thoroughly characterised it could be extended for testing in whole animals and then eventually for use in a clinical situation.

The effects of suramin and H7 were selected for investigation since there is evidence to suggest that phosphorylation can be involved in the process of receptor desensitisation. The β-adrenoceptor has been extensively studied and the process of both heterologous and homologous forms of desensitisation have been shown to involve receptor phosphorylation [Sibley *et al.*, 1985]. Both *in vivo* and *in vitro* [Childers, 1991] studies have shown that μ- and δ-opioid receptors are desensitised following chronic morphine exposure. Importantly

previous work has implicated β-ARK in the mechanism of desensitisation of several agonist-occupied forms of G protein-linked receptors, such as κ-opioid [Reisine & Bell, 1993], β- and α_2-adrenergic, muscarinic and Substance P receptors [Benovic *et al.*, 1989; Hosey, 1992; Kwatra *et al.*, 1993]. Other workers have shown that in NG108-15 cells forskolin causes desensitisation of the adenylate cyclase response to adenosine A_2 agonists. When cells were pretreated in the presence of forskolin and 50 μM H7 the level of desensitisation was significantly reduced [Krane & Keen, 1991].

Our results also implicate phosphorylation in the process of opioid tolerance. Although suramin consistently reduced the level of morphine-induced tolerance this effect was not significant and considerably greater levels (one hundred times) were needed than were reportedly effective at inhibiting β-ARK in permeablised A_{431} cells [Miller *et al.*, 1993]. However, co-incubation of cells with H7 reduced the degree of tolerance markedly. This was especially apparent for pentazocine but was also significant for morphine and DAMGO. H7 is unselective for PKC, or cAMP- and cGMP-dependent protein kinases and so further work is necessary to determine exactly which protein kinase is responsible for causing tolerance and also the site of action.

The role of phosphorylation and its inhibition by H7 has previously been studied in a constitutively active model of tolerance both in SH-SY5Y cells and by using *in vitro* experiments [Wang *et al.*, 1994b]. Opioid inhibition of prostaglandin E_1 (PGE_1) stimulated cAMP accummulation in SH-SY5Y cells was examined in naive and morphine tolerant cells. Pretreatment of cells with morphine (1 μM for 12 h) in the presence of 100 μM H7 (12 h) increases the subsequent inhibition by 10 μM morphine compared to cells exposed to morphine alone, i.e., the morphine tolerance is capable of being reversed by H7. This study also tested the effect of H7 in an acute mouse model. H7 is able to reverse tolerance to subcutaneously administered morphine.

In conclusion, opioid agonist stimulation of [^{35}S]GTPγS binding in SH-SY5Y cells provides a useful measure of opioid tolerance and may be used to probe the mechansims of the development of tolerance.

CHAPTER 7
OVERVIEW

In research the horizon recedes as we advance, and is no nearer at 60 than it was at 20 ... and research is always incomplete.

Mark Pattison (1813 - 1884)

OVERVIEW

The recent cloning of the μ-, δ- and κ-opioid receptors will undoubtedly help to unravel the mysteries of their form and function. Of interest, is the fact that as yet there is no molecular biological evidence of opioid receptor subtypes. This is perhaps surprising as the existence of subtypes has often been proposed in order to explain pharmacological experiments

Indeed the results of ligand binding studies of the δ-opioid ligand [^{3}H][D-Pen^2,D-Pen^5]enkephalin (DPDPE) in mouse brain, presented in this thesis, are most easily reconciled in terms of δ-opioid subtypes. It was found that both stable and wash-sensitive [^{3}H]DPDPE sites exist and furthermore the highly selective δ-agonist [D-Ala^2]deltorphin I (DELT I) was unable to displace approximately 20 % of [^{3}H]DPDPE specific binding. In order to characterise opioid receptors highly selective ligands are required. During the course of the work presented in this thesis some newer ligands proposed to be selective for the putative δ-opioid subtypes became commercially available, such as, for δ_1-opioid receptors 7-benzylidenenaltrexone (BNTX) and for δ_2-opioid receptors naltrindole-5'-isothiocyanate (5'NTII). However, although a range of naltrindole analogues were investigated in this study and potent agonists found, no extra evidence for δ-opioid subtypes was observed. Nevertheless the development of non-peptide highly δ-selective agonists is important since this class of compounds is only in its infancy and may afford novel clinical compounds.

Another classification of δ-opioid subtypes, δ-complexed and δ-noncomplexed (δ_{cx} and $\delta_{ncx,}$) has arisen from observations of interactions between μ- and δ-agonists in whole animal studies. Initially, in the work covered in this thesis, potential interactions were studied in the mouse isolated vas deferens preparation. This tissue is known to contain all three types of opioid receptor, namely, μ, δ and κ. Neither sub-effective concentrations or IC_{50} concentrations of δ-agonists were able to modulate the action of μ-agonists. Although the range of μ- and δ-ligands was not exhaustive the results do not lend support to the idea of μ / δ interactions and therefore the existence of complexed and non-complexed δ-opioid receptors. The guinea-pig ileum myenteric plexus-longitudinal muscle preparation was similarly tested. This tissue contains both functional μ- and κ-opioid receptors. Despite the fact that δ-opioid receptors have been identified in this tissue by ligand-binding assays

no functional role for these receptors has been recognised. Therefore their possible modulatory role was investigated. Once again no interactions between μ- and δ-opioid agonists were detected. Previously only experiments utilising the central nervous system (CNS) have yielded results best explained by μ / δ interactions. Since no modulation was observed in this study, conducted in peripheral tissues, perhaps differences do exist between the peripheral and central branches of the nervous system with respect to opioid interactions, as has been proposed by other workers.

A recent development in the field of pharmacology is the use of agonist-stimulated binding of the GTP analogue [^{35}S]GTPγS to membranes as a functional assay. This assay was examined in the human neuroblastoma cell line SH-SY5Y, (using opioid receptor selective ligands these cells were found to express both μ- and δ-opioid receptors in the ratio of 2.5 : 1). The ability of μ-agonists to stimulate [^{35}S]GTPγS binding was unaffected by δ-selective concentrations of δ-agonists. Thus the results provided no evidence of μ / δ interactions in this CNS cell line. In addition, the mouse neuroblastoma x rat glioma NG108-15 cell line was found to express only δ-opioid receptors, as determined using a δ-opioid selective radioligand, in agreement with previous studies. δ-Opioid agonist-stimulated [^{35}S]GTPγS binding in this cell line was demonstrated and optimal conditions were established.

There are also many studies both *in vivo* and using ligand binding suggesting the existence μ-opioid subtypes. This possibility was studied in SH-SY5Y cells. In collaboration with Dr. D. Smart at Leicester University it was found that the μ-opioid population on these cells appeared to be of the μ_2-opioid subtype since it was insensitive to the reputed μ_1-opioid irreversible antagonist naloxonazine. However, this putative μ_2-opioid population displayed receptor heterogeneity since 40 % of these sites were not susceptible to alkylation by the irreversible μ-opioid antagonist β-funaltrexamine. This may be due to receptor differences or differences in coupling mechanisms.

It was established that μ-agonist-stimulated [^{35}S]GTPγS binding could be used as a suitable model of opioid tolerance. The potency of morphine was reduced 2.4 - fold and the maximal response was decreased by 33 % following chronic exposure of the cells to morphine (10 μM for 48 h). The potency of morphine was similarly effected by DAMGO-induced tolerance, although this higher efficacy agonist was able to afford a greater degree of tolerance than morphine as measured by the reduction in potency of the partial agonist

pentazocine. These tolerance effects were found to be accompanied by a 68 % loss of μ-opioid binding sites. The affinity of the remaining sites was unaltered as determined by tritiated antagonist ligand-binding studies.

Preliminary experiments were conducted in order to determine the mechanism of this phenomenon. Many previous investigations have implicated a role for phosphorylation. Two phosphorylation inhibitors suramin (a β-ARK inhibitor) and H7 (a non-selective protein kinase inhibitor) were studied. Initial results suggest that suramin did not but H7 did inhibit the development of tolerance in these cells. This latter result is in agreement with the effects of H7 on morphine tolerance as measured by opioid inhibition of PGE_1 stimulated cAMP accumulation in SH-SY5Y cells and suggests a role for phosphorylation in this process.

Thus, in conclusion while not providing any evidence to support the proposal of μ / δ interactions this study does support the hypothesis that both μ- and δ-subtypes exist. In addition, some applications of agonist stimulated [^{35}S]GTPγS binding have been highlighted and should provide a method by which to study the mechanism of δ-opioid agonists in NG108-15 cells and μ-opioid agonists in SH-SY5Y cells. The proof of the existence or otherwise of opioid receptor subtypes and the mechanism of the development of tolerance remain important goals for future opioid research.

CHAPTER 8
REFERENCES

If you steal from one author it's plagerism; if you steal from many, it's research.

Wilson Mizner (1876 - 1933)

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