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An Investigation into the Regulatory Mechanisms Associated with the Recombinant Human 5-hydroxytryptamine$_{1A}$ (5-HT$_{1A}$) Receptor

By

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ABSTRACT

An Investigation into the Regulatory Mechanisms Associated with the Recombinant Human 5-hydroxytryptamine$_{1A}$ (5-HT$_{1A}$) Receptor

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Classically, acute agonist challenge results in a concomitant loss of G protein-coupled receptor signalling function and high affinity membrane ligand binding sites. Expression of the human 5-HT$_{1A}$ receptor in CHO-K1 cells allowed the study of a homogeneous receptor population expressed at high density. Exposure of CHO-K1 cells expressing the cloned 5-HT$_{1A}$ receptor to 1μM 8-hydroxy-2-(di-*n*-propylamino)-tetralin (8-hydroxy-DPAT) resulted in receptor internalisation (20% of receptors after 20 minutes) to an undefined intracellular compartment. Receptor internalisation was found to be sensitive to, hypertonic sucrose (0.7-0.8M) and Concanavalin A (0.5-1mg/ ml), both of which are inhibitors of clathrin-mediated endocytosis. Removal of 8-hydroxy-DPAT from the incubation medium allowed 5-HT$_{1A}$ receptors to recycle back to the plasma membrane, this process occurred in the presence of the protein synthesis inhibitor cycloheximide and in a time dependent fashion (40-50 minutes). Mutagenesis of the putative PKC phosphorylation motifs, KR**T**$_{149}$**A**PR and RK**T**$_{229}$**A**VK located in the second and third intracellular loops had an insignificant effect upon receptor binding characteristics however, complete abolition of receptor redistribution in response to acute agonist challenge was observed. In a similar manner conservative and radical mutation of the internalisation motif NPVI**Y** (Y$_{400}$F and Y$_{400}$A respectively) had no effect on receptor binding characteristics but they did abolish receptor internalisation in response to short term agonist incubation. Conversely, mutation of the putative PKC phosphorylation site, KK**S**$_{253}$**G**VN located in the third intracellular loop resulted in a significantly increased rate of receptor internalisation (80% of receptors after 20 minutes) with no observable effect on receptor ligand binding affinity. Following removal of 8-hydroxy-DPAT from the incubation medium the S$_{253}$G mutant 5-HT$_{1A}$ receptors were observed to recycle to the plasma membrane in the presence of cycloheximide in a time dependent fashion (40-50 minutes). Short-term incubation (40 minutes) of CHO-K1 cells expressing the unmutated human 5-HT$_{1A}$ receptor with 8-hydroxy-DPAT also resulted in translocation of the transcription factors NF-κB and CREB to a nuclear environment in an active form. In conclusion, these data show that the human 5-HT$_{1A}$ receptor is able to undergo clathrin-mediated endocytosis and recycle back to the plasma membrane independently of new protein synthesis, in response to acute agonist incubation. Putative PKC phosphorylation motifs KRT$_{149}$PR and RKT$_{229}$VK represent critical sites for the promotion of receptor internalisation, as does the putative NPVI**Y** internalisation motif located in the seventh transmembranous domain. Conversely, the KK**S**$_{253}$VN motif represents a novel attenuating signal that arrests receptor internalisation in response to incubation with 8-hydroxy-DPAT. The human 5-HT$_{1A}$ receptor is also linked to the activation of the transcription factors NF-κB and CREB, suggesting a mechanism by which activation of the human 5-HT$_{1A}$ receptor may modulate transcription of one or more genes including its own.

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I would like to dedicate this work to my Family and Lien, thanks Mum, Dad, Ben and Joe for being there, always willing to help. Thanks to Lien for love, support and patience without which I would not have come this far.

ABBREVIATIONS

5-HT	5-hydroxytryptamine
$5\text{-HT}_{1A}R$	5-hydroxytryptamine$_{1A}$ receptor
8-hydroxy/OH-DPAT	8-hydroxy-2-(di-*n*-propylamino)-tetralin
AC	Adenylate cyclase
APS	Ammonium persulphate
$AT_{1A}R$	Angiotensin$_{1A}$ receptor
B_{max}	The amount of drug (fmol/ mg) which can bind specifically to the receptors in a membrane preparation
bp	Base pair
cAMP	3',5'-cyclic adenosine monophosphate
CAT	Chloramphenicol-acetyl-transferase
cGAMP	3',5'-cyclic guanosine monophosphate
CBF1	CCAAT-Binding Factor 1
CHO-K1	Chinese hamster ovary cell
Con A	Concanavalin A
CRE	Cyclic AMP response element
CREB	Cyclic AMP response element binding protein
DAG	Diacylglycerol
DHFR	Dihydrofolate reductase
dpm	Disintegrations per minute
DTT	Dithio-L-threitol
EC_{50}	Concentration of a drug which causes 50% of the maximum possible inhibition of a response of a given drug
ECL	Enhanced chemiluminescent
EDTA	Ethylenediaminetetraacetic acid
Erk	Extracellular signal-regulated kinase
G protein	Guanine nucleotide regulatory binding protein
GDP	Guanosine diphosphate
G_i	G protein that inhibits adenylate cyclase
G_o	G protein that serves functions other than regulation of adenylate cyclase

GPCR	G protein-coupled receptor
G_q	G protein that activates phospholipase C
GRK	G protein-coupled receptor kinase
GRP	G protein-coupled receptor phophatase
G_s	G protein that stimulates adenylate cyclase
GTP	Guanosine triphosphate
GTPγS	Guanosine 5'-O-(3-thiotriphosphate)
HEPES	N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulphonic acid)
I.U.P.H.A.R.	International union of pharmacology committee on drug classification and receptor nomenclature
Ig	Immunoglobulin
IL	Interleukin
IP_3	Inositol 1,4,5-triphosphate
I-κB	Inhibitor of kappa B
K_d	The dissociation constant for a radiolablled drug determined by its saturation analysis. The concentration of drug which at equilibrium occupies 50% of the receptors (nM).
K_i	The inhibition constant for a drug; the concentration of competing ligand in a competition assay which would occupy 50% of the receptors if no radioligand were present
LDL	Low density lipoprotein
LPS	Lipopolysaccaride
MAPK	Mitogen activated protein kinase
MAPKAP	Mitogen activated protein kinase activating protein
MASP	Mitogen activated signal pathway
MPPI	4-(2'-methoxyphenyl)-1-[2'-[N-(2'-pyridinyl) -p-iodobenzamido] ethyl] piperazine
NAD(P)H	Nicotinamide adenine dinucleotide phosphate
NFAT	Nuclear factor of activated T cells

NF-κB	Nuclear factor- kappa B
NPY	Neuropeptide Y
PAR1	Protease-activated receptor 1
PC-PLC	Phosphatidylcholine-dependent phospholipase C
PIP_2	Phosphatidylinositol 4,5,-biphosphate
PI-PLC	Phosphatidylinositol-dependent phospholipase C
PKA	Cyclic AMP-dependent protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PMA	4β-phorbol-myristate-acetate
POPOP	(1,4-bis[5-phenyl-2-oxazolyl] benzene-2,2'-p-phenylene-bis [5-phenoxyloxazole])
PPO	2,5-Diphenyloxazole
RHD	Rel homolgy domain
RME	Receptor mediated endocytosis
ROI	Reactive oxygen intermediate
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
TEMED	N,N,N',N'-tetramethylethlenediamine
TNFα	Tumour necrosis factor alpha
TPA	12-O-tetradecanoylphorbol-13-acetate
WAY100635	N-[2-[4-(2-methoxyphenyl)-piperazinyl]ethyl-*n*-(2-pyridiynl) cyclohexanecarboxamide trihydrochloride
α_1AR	$Alpha_1$ adrenergic receptor
β_1AR	$Beta_1$ adrenergic receptor
β_2AR	$Beta_2$ adrenergic receptor

CHAPTER 1

1.1. 5-HYDROXYTRYPTAMINE (5-HT); SEROTONIN

Studies performed during the mid nineteenth century led to the discovery of a serum-borne factor which induced powerful contraction of smooth muscle. It wasn't until 1948, that Rapport *et al.*, identified and isolated serotonin, a vasoconstrictor released during blood clotting and platelet congregation (Rapport *et al.*, 1948). Once the chemical structure of serotonin was elucidated, it subsequently became known as 5-hydroxytryptamine (5-HT) although today both names are used interchangeably. 5-HT is mainly concentrated within the wall of the stomach and small intestine; with as much as 90% of total body 5-HT being found here. 5-HT is also located in the central nervous system where it has been shown to function as an important neurotransmitter. In the blood stream, platelets actively take up 5-HT and it is here where it reaches relatively high concentrations. In response to tissue damage and trauma, platelet cells aggregate to form a clot and upon doing so release 5-HT which acts as a vasoconstrictor, reducing local blood flow. As a neurotransmitter 5-HT is found highly concentrated in localised regions of the midbrain, these include the cortex, caudate-putamen, limbic system and hypothalamus (Imai *et al.*, 1986; Molliver, 1987). Recent studies have shown that 5-HT is often localised and released with other active peptide hormones and neurotransmitters. The co-release of neurotransmitters and peptide hormones is thought to be physiologically relevant in a process known as cross talk, a process by which co-released neurotransmitters modulate the neuronal responses to each other. Since 5-HT cannot cross the blood brain barrier due to its size and charged nature it must be synthesised *de novo*. Tryptophan is the precursor amino acid and is hydrolysed and decarboxylated in a two step, enzyme catalysed process to form 5-HT (**Figure 1**). 5-HT is rapidly deaminated to an aldehyde and this intermediate is oxidised to 5-hydroxyindoleacetic acid (**Figure 1**). 5-HT is known to play an essential role in many physiological functions and is implicated in a number of clinical conditions.

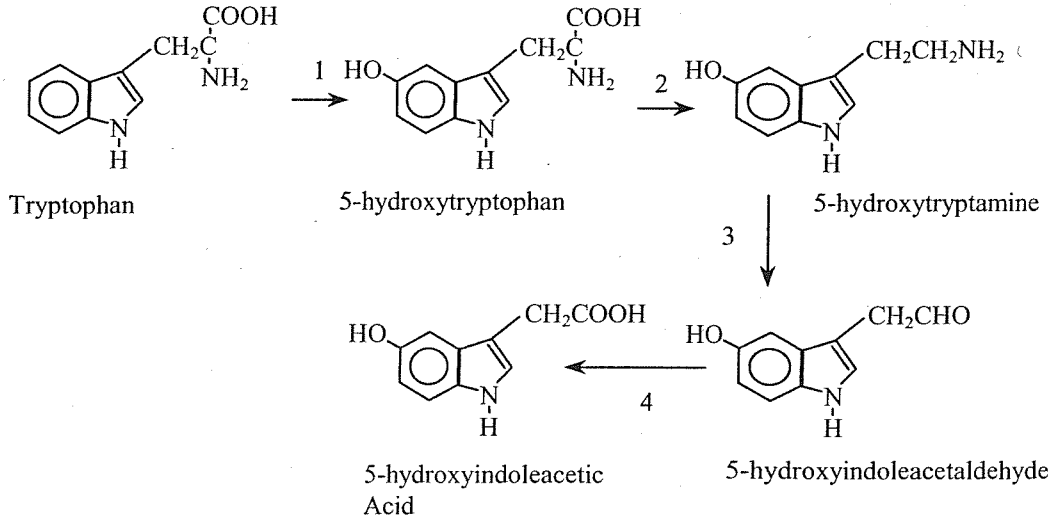


Figure 1: The biosynthesis and metabolism of 5-hydroxytryptamine (serotonin)

The enzymes involved in each step of 5-HT synthesis and breakdown are (1) Tryptophan hydroxylase, (2) L aromatic amino acid decarboxylase, (3) Monoamine oxidase, and (4) Aldehyde dehydrogenase.

5-HT increases gut motility (by exciting smooth muscle), induces contraction of smooth muscle (aortic smooth muscle, bronchi), platelet aggregation, vasoconstriction (directly by activating arterial-sphincters) stimulation of peripheral nociceptors and excitation/inhibition of central nervous system neurones. Serotonin in conjunction with other neurotransmitters is thought to play a role in sleep (Koella, 1988), feeding (Curzon, 1990), biological rhythms (Wesemann & Weiner, 1990), thermoregulation (Myers, 1981) and endocrine secretion (Tuomisto & Mannisto, 1985). Disturbances in 5-HT function manifest in clinical symptoms such as migraine, carcinoid syndrome, mood disorders and anxiety (Rang *et al.*, 1995).

Recent advances in gene cloning techniques and selective ligand design have revealed the existence of multiple 5-HT receptor subtypes. Seven subtypes have been described to date and the are further subdivisions within these subtypes (**Table 1**). The reason for this receptor diversity is unclear, but it has been suggested that multiplicity of the receptors allows 5-HT to have its diverse effect in the nervous system and that different subtypes may be important at different times of foetal development (Saudou & Hen, 1994).

1.2. 5-HT RECEPTOR BIOLOGY

Gaddum & Picarelli (1957) were the first to report the existence of two separate 5-HT receptors on smooth muscle preparations. The receptors were named after the selective antagonists which blocked musculotropic (**D**ibenzyline) and neurotropic (**M**orphine) responses of the smooth muscle. This "**D** and **M**" classification remained until 1979 when Peroutka & Snyder, described the selective binding of various tritiated ligands. They demonstrated that one receptor subtype (5-HT_1) had a high affinity for [^{3}H]-5-HT whereas the other receptor (5- HT_2) had a comparatively low [^{3}H]-5-HT affinity. The first unifying scheme for naming and classifying the rapidly growing number of 5-HT receptors was proposed by Bradley *et al.*, (1986). Bradley was the first to look at and classify the receptors according to their second messenger coupling system (for example 3',5'-cyclic adenosine monophosphate (cAMP) and phosphoinositol turnover and ion channel modulation).

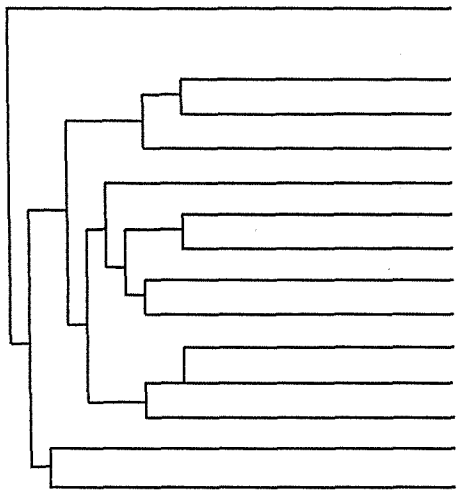
The most recent 5-HT receptor classification scheme has been proposed by the International Union of Pharmacology Committee on Drug Classification and Receptor Nomenclature (I.U.P.H.A.R.). I.U.P.H.A.R. divided the 5-HT receptors into seven subclasses (5-HT_{1-7}) based upon; operational, (actions of agonists and antagonists), transductional (coupling to second messenger systems), structural (amino acid sequence) and gene structure (introns and exons) characteristics (Martin & Eglen 1998). When these criteria were applied to the 5-HT receptors some rearrangement and relocation of receptor subtypes resulted. In the case of the 5-HT_{1C} receptor it was reclassified as the 5-HT_{2C} receptor on the basis of its' transductional and structural identity with the 5-HT_2 receptor family.

1.3. EVOLUTION OF THE 5-HT RECEPTOR FAMILY

The rate of protein evolution can be determined by correlating the percentage amino acid homology between species and the dates of evolutionary divergence of each species. The established G protein-coupled receptor (GPCR) rate of evolution is approximately 1% for every 10 million years, making the primordial G protein-coupled 5-HT receptor roughly 750 million years old (Peroutka & Howell, 1994).

Yeast and moulds that contain GPCRs (Guan, 1994) are thought to have appeared 1 thousand million years ago. 5-HT receptors have been identified in worms which are thought to have evolved from yeast about 700 million years ago. The early 5-HT receptor is thought to have evolved sometime around the differentiation of yeast and worms (Wilson *et al.*, 1973). The primitive 5-HT receptor then appears to have differentiated into three major subtypes of 5-HT receptor, 5-HT_1 (including 5-HT_5 and 5-HT_7 receptors), 5-HT_2 receptors and 5-HT_6 receptors (**Table 1**). The 5-HT_5 and 5-HT_7 receptors both appeared to have differentiated before the evolution of vertebrates from invertebrates, which was around 650-700 million years ago (Erlander *et al.*, 1993; Peroutka & Howell, 1994). The 5-HT_1 receptor subtype was the first to begin divergence. The 5-HT_{1A} receptor is thought to have branched at around 600 million years ago and evolved into 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E} and 5-HT_{1F} receptors.

Table 1: 5-HT receptor phylogeny and pharmacology



Sub-Type	Chromosomal Location	Structure	Effector	Agonists	Antagonists
5-HT_3	11	478aa, α-sub-unit homopentamer	cation channel	SR7227, *m*-chlorophenylbiguanide	granisetron, ondansetron, tropisetron
5-HT_{2A}	13q14-21	471aa, 7TM	$G_{q/11}$	α-Me-5-HT	ketanserin, MDL100907
5-HT_{2C}	Xq24	458aa, 7TM	$G_{q/11}$	α-Me-5-HT	mesulergine, SB242084, RS102221
5-HT_{2B}	2q36.3-2q37.1	481aa, 7TM	$G_{q/11}$	BW723C86, α-Me-5-HT	SB200646, SB204741
5-HT_{1A}	5q11.2-q13	422aa, 7TM	$G_{i/o}$	8-hydroxy-DPAT	WAY100635
5-HT_{1B}	6q13	390aa, 7TM	$G_{i/o}$	sumatriptan, L694247	GR55562, SB216641
5-HT_{1D}	1p34.3-36.3	377aa, 7TM	$G_{i/o}$	sumatriptan, L694247	BRL15572
5-HT_{1E}	6q14-15	365aa, 7TM	$G_{i/o}$	-	-
5-HT_{1F}	-	366aa, 7TM	$G_{i/o}$	LY334370	-
5-ht_{5A}	7q36	357aa, 7TM	unknown	-	-
5-ht_{5B}★	2q11-q13	370aa, 7TM	unknown	-	-
5-HT_7	10q23.3-24.3	445aa, 7TM	G_s	-	SB258719
5-HT_4	5q31-33	387aa, 7TM	G_s	BIMU8, RS67506, ML10302	GR113808, SB204070, RS100235
5-ht_6	1p35-36	440aa, 7TM	G_s	-	Ro046790[5]

★Rat receptor: potentially a pseudogene- no human equivalent has been identified. Lower case nomenclature is used for receptors for which no endogenous expression has been demonstrated (Martin & Eglen, 1998).

The 5-HT_{1D} receptor is believed to have evolved from the 5-HT_{1B} receptor 400 million years ago and the 5-HT_{1F} receptor may have evolved from the 5-HT_{1E} receptor, approximately 450 million years ago (Peroutka & Howell, 1994; **Table 1**).

1.4. 5-HYDROXYTRYPTAMINE $_{1A}$ RECEPTOR (5-HT_{1A}R)

1.4.1. History of the 5-HT_{1A}R

The 5-HT_{1A} receptor was first described by Pedigo *et al.*, (1981), but the real break through did not occur until 1987, when the gene was cloned. Kobilka and colleagues who had originally set out to clone the human β_1 adrenergic receptor (β_1AR) stumbled across a mysterious, intronless section of cDNA which they termed G-21 (Kobilka *et al.*, 1987). Sequence analysis indicated that the G-21 gene encoded a membrane bound protein, which shared a high degree of sequence homology with the β_2 adrenergic receptor (β_2AR) and other GPCRs.

G-21 remained a mystery until Fargin *et al.*, (1988) provided experimental evidence showing that it encoded the human 5-HT_{1A}R. Fargin and colleagues had expressed the G-21 gene in COS-7 cells and then screened the cell membranes for binding of various 5-HT receptor ligands. One such radioligand that bound was the selective β adrenergic receptor antagonist [^{125}I] Iodocyanopindolol; this was found to bind to the protein with an affinity that was more representative of a 5-HT_1-type receptor (K_d approximately10nM). The binding of the 5-HT_{1A} selective agonist, [^{3}H] 8-hydroxy-2-(di-*n*-propylamino)-tetralin (8-hydroxy/OH-DPAT) provided evidence that the G-21 gene encoded the human 5-HT_{1A}R.

1.4.2. Localisation of the 5-HT_{1A}R

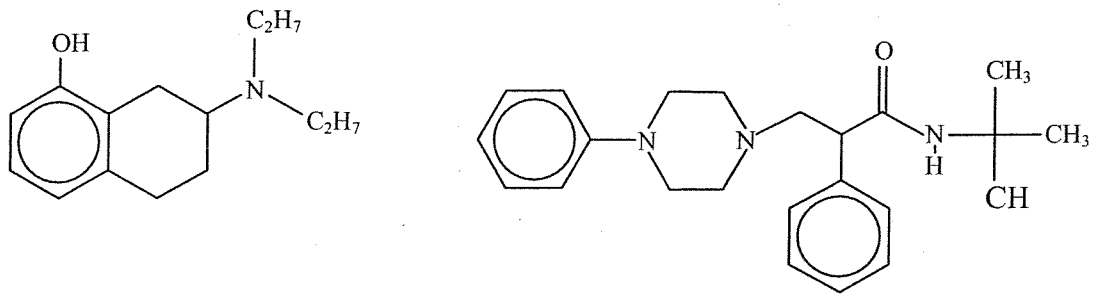
Combinations of autoradiographic, *in situ* hybridisation, immunological techniques and radioligand binding studies have been used to define the anatomical distribution of the 5-HT_{1A}R. Consistent in both animal and human models was the preferential distribution of radioligands to the limbic areas (i.e. hippocampus, lateral septum and frontal cortex) and the nucleus raphe dorsalis of the central nervous system. Messenger RNA (mRNA) transcripts for the 5-HT_{1A}R have also been isolated in the

amygdala, hypothalamus, and medulla. The correlation of 5-HT_{1A} R radioligand binding and mRNA distribution suggests a mainly postsynaptic location for the receptor. In a postsynaptic location (within for example, the hippocampus) the 5-HT_{1A}R acts to hyperpolarise pyramidal cells by opening K^+ channels, via heterotrimeric GTP binding proteins, resulting in the formation of an excitatory post synaptic potential. Distribution of the 5-HT_{1A}R to the limbic areas of the brain is consistent with its hypothesised role in moods and anxiety.

5-HT_{1A}Rs are also located peripherally, on white blood cells (Hellstrand & Hermodsson, 1987), in the spinal cord (Daval *et al.*, 1987) and the cardiovascular system (McCall & Clement, 1994). As a result of its' location on T lymphocytes (Aune *et al.*, 1993), the 5-HT_{1A}R is suspected to play an important regulatory role in the immune system. Although much work has been carried out on 5-HT_{1A}R localisation *in vivo*, the function of the more peripherally located receptors is still relatively unexplored.

1.4.3. 5-HT_{1A}R pharmacology

Radioligands allow a receptor to be labelled in a specific manner and from this, much information can be gained. In 1983, it was discovered that the tritiated tetralin derivative [^{3}H] 8-hydroxy-DPAT bound with high affinity to the 5-HT_{1A}R; this led to a revolution in receptor study. 8-hydroxy-DPAT was the first, and to date remains the only specific 5-HT_{1A}R agonist (**Figure 2**), although other ligands are known to bind with a reasonably high affinity but with less specificity. Included within this list are other radiolabelled tetralin derivatives such as [^{3}H] 5-MeO-DPAC and [^{125}I] BH-8-MeO-N-PAT, the latter has also proven useful as a specific 5-HT_{1A}R agonist. Other non-tetralin derivatives that bind the receptor include 5-HT, ipsapirone, WB4101, buspirone and spiroxatrine. Non-tetralin derivatives can also be radiolabelled, but 5-HT will bind to every 5-HT receptor type, whereas ipsapirone and WB4101 will bind the α_1 -adrenergic receptor (α_1AR) and spiroxatrine binds D_2-dopamine receptors and μ-opioid receptors, thus rendering these ligands useless as 5-HT_{1A}R specific radioligands in mixed receptor populations. Another commonly used tritiated 5-HT_{1A}R ligand is spiperone.



8-hydroxy-DPAT

WAY100635

Figure 2: The structures of the 5-HT_{1A}R specific agonist, 8-hydroxy-DPAT and antagonist WAY100635

Spiperone displays antagonistic properties at the 5-HT_{1A}R although it also binds antagonistically to the dopaminergic D_2 and D_3 receptor (Gardner *et al.*, 1997; Sartania & Strange, 1999).

Until quite recently (1990), no specific 5-HT_{1A}R antagonists were available. Compounds that were once thought to possess antagonistic properties were also demonstrated to act as partial agonists in separate systems (for example, BMY 378 and propranolol on somatodendric autoreceptor function; Fornal *et al.*, 1994). The discovery of the first truly selective 5-HT_{1A}R antagonist, (s)-n-tert-butyl-3-(4-(2-methoxyphenyl)-piperazin-1-yl)-2 phenylpropanamide (WAY100635; **Figure 2**) was made by Fletcher *et al.*, (1993). This phenylpiperazine derivative has two enantiomers. The 5-HT_{1A}R was found to be stereoselective for the (+) enantiomer, and is more functional in behavioural studies. Recent studies have also demonstrated that derivatives of WAY100635 such as 1,3-dimethyl-9-[3-(4-phenyl-1-piperazinyl)propyl]-2,4,8-trioxo-1,3,9-trihydropyrimidino[2,1-f]purine 8, as have specific 5-HT_{1A} postsynaptic receptor antagonist characteristics (Chojnackawojcik *et al.*, 1998).

Results presented by Sundaram *et al.*, (1993) demonstrated the ability of spiperone to distinguish between G protein-coupled and G protein uncoupled forms of the receptor. 5-HT_{1A}Rs expressed in Ltk^- fibroblasts were observed to have a lower affinity for the agonist 8-hydroxy-DPAT in the presence of guanine nucleotides (Sundaram *et al.*, 1993). Unexpectedly the affinity of the receptor for the antagonist spiperone was found to increase in the presence of guanine nucleotides. Guanine nucleotides (for example, guanosine 5'-O-(3-thiotriphosphate) GTPγS) are able to destabilise receptor/ G protein interactions, mimicking receptor desensitisation, which is often a result of phosphorylation of the receptor in response to agonist challenge (covered in depth in a later section). These findings present pharmacologists with a very useful device for differentiating receptors that are desensitised from those that are not.

1.4.4. Clinical correlates of 5-HT_{1A}R activity

The serotonergic system has been implicated in a number of clinical disorders including, depression, pain sensitivity and anxiety. Benzodiazepine drugs which are

used in the regulation of anxiety and insomnia act directly on the GABA/ Cl^- channel and this action is known to be modulated by 5-HT. One the other hand benzodiazepines are also known to decrease the activity of central 5-HT neurones thus forming a negative feed back loop (Chopin & Briley, 1987). Non benzodiazepine anxiolytics (such as buspirone) also act directly on serotonergic neurones to modulate firing rate (Dourish *et al.*, 1987). Compounds mentioned here are also known to modulate other receptor types and a neuronal pathway (for example, dopaminergic) so it seems that serotonin may play an important co-ordinate role in physiological disorders.

The serotonergic system is also implicated in the regulation of both ascending and descending nociceptive mechanisms. Serotonergic projections from nucleus raphe magnus to the superficial layer of the dorsal horn are those thought to be involved in the descending control of nociception (Alhaider & Wilcox 1993). Autoradiographic studies in animals lacking nociception have demonstrated the presence of 5-HT_{1A}R on primary afferent fibres in the dorsal horn of the spinal cord (Daval *et al.*, 1987). This evidence along with that demonstrating 8-hydroxy-DPAT and buspirone as having an analgesic effect (Eide *et al.*, 1988) would suggest the 5-HT_{1A}R has an important role in the perception and response to pain.

1.4.5. Signalling systems

Surface bound receptors are vital in relaying extracellular messages into intracellular responses. The function of the surface bound receptor is respond to and amplify the initial signal into a large intracellular response, each step along the pathway involving proteins that exist in multiple copies. Intracellular responses can for example manifest as changes in metabolite concentration with concomitant widespread effects. To date numerous categories of signal transducer have been identified, these include; GPCRs, receptor tyrosine kinases, cytokine receptor activated kinases, ligand gated ion channels and receptor serine kinases. All of these signal transducers have complex mechanisms of action and intricate cellular consequences, which have yet to be fully elucidated. Matters are made even more complex by the ability of each

receptor to interact with others of the same or different signalling type in a process known as "cross talk" (Hill, 1998; Selbie & Hill, 1998 for review).

The human 5-HT_{1A}R is a member of the GPCR family and by definition, the receptor relays its intracellular signalling via a G protein. Such proteins are able to bind guanine triphosphate (GTP) molecules. One such class of G proteins exists as hetrotrimers in a membrane-associated cytosolic environment. Of the three subunits, the α subunit is the largest at approximately 40kDa on SDS PAGE and binds GTP. The β subunit is approximately 35kDa and the γ subunit is approximately 8kDa, β and γ subunits coexist to form the $\beta\gamma$ complex (Alberts *et al.*, 1994). The second type of G protein are 20kDa monomeric proteins which also bind GTP and are thought to regulate processes central to normal gene expression, one example being Ras (Nathanson & Harden, 1989).

G protein coupling can lead directly or indirectly to activation or inhibition of an effector enzyme, thereby directly affecting concentrations of second messenger molecules. Included in this list of second messenger molecules are for example cAMP, 3',5'-cyclic guanine monophosphate (cGMP), inositol phosphates and arachidonate. These second messengers then usually interact with a protein kinase, which results in phosphorylation of downstream proteins. Phosphorylation of cellular proteins plays an important role in the control of cell growth and differentiation. Numerous serine/threonine kinases have been identified that may be responsible for these phosphorylation reactions (Robinson & Cobb, 1997).

Primary effector activities of heteromeric G proteins are ascribable to the GTP-bound α subunit. In addition the various isoforms of the α, β and γ subunits have diverse effector profiles. There are at least 5 β subunit isoforms and 11 γ subunit isoforms but the α subunit is the most diverse with 23 different isoforms identified to date (Sugden & Clerk, 1997). When inactive, G proteins form membrane bound $\alpha\beta\gamma$ hetrotrimers, with GDP tightly bound to the α subunit. Upon activation by extracellular signals (for example 5-HT), the receptor catalyses the exchange of bound guanine diphosphate (GDP) for cytosolic GTP. The GTP-bound form of the heterotrimer exist in a less

stable conformation and heterolytically dissociates to form active GTP-α and $\beta\gamma$ complexes. Acting either co-ordinately or independently these two species bind and modulate the activity of downstream effector molecules. G proteins are released from the effector protein upon the hydrolysis of GTP that results from the slow intrinsic GTPase activity of the α subunit. The inactive α subunit then has a far higher affinity for the $\beta\gamma$ complex and the two recombine. The reformed heterotrimer can then re-associate with its receptor and undergo a new cycle of signal transduction (Coleman & Sprang, 1996; **Figure 3**).

The multiplicity of α β and γ subunits allows the formation of many heterotrimeric species that may confer increased specificity or flexibility of signalling to different GPCRs. For example G_s stimulates adenylate cyclase (AC; which converts ATP to cAMP, an important second messenger) and increased calcium channel activity (regulating intracellular Ca^{2+}). Conversely, G_i acts as an inhibitor of AC. G_q stimulates phospholipase C_β (PLC) leading to inositol phospholipid turnover, which in turn activates a downstream (Protein kinase C) PKC. GPCRs can also alter gene transcription via interaction with transcription factors such as the cAMP response element binding protein (CREB). CREB is activated by phosphorylation which can be mediated by either cAMP-dependant protein kinases, Protein kinase A (PKA) or Ca^{2+} /calmodulin dependant protein kinases. Multiple forms of AC, PLC, PKA and PKC have also been identified and it is possible that more than one isoenzyme is involved in the signal transduction of one receptor type (Coleman & Sprang, 1996). There are two signal transduction pathways, which have been shown to associate with the 5-HT_{1A}R family: a multistep enzyme mediated pathway and a direct regulation of ion channels.

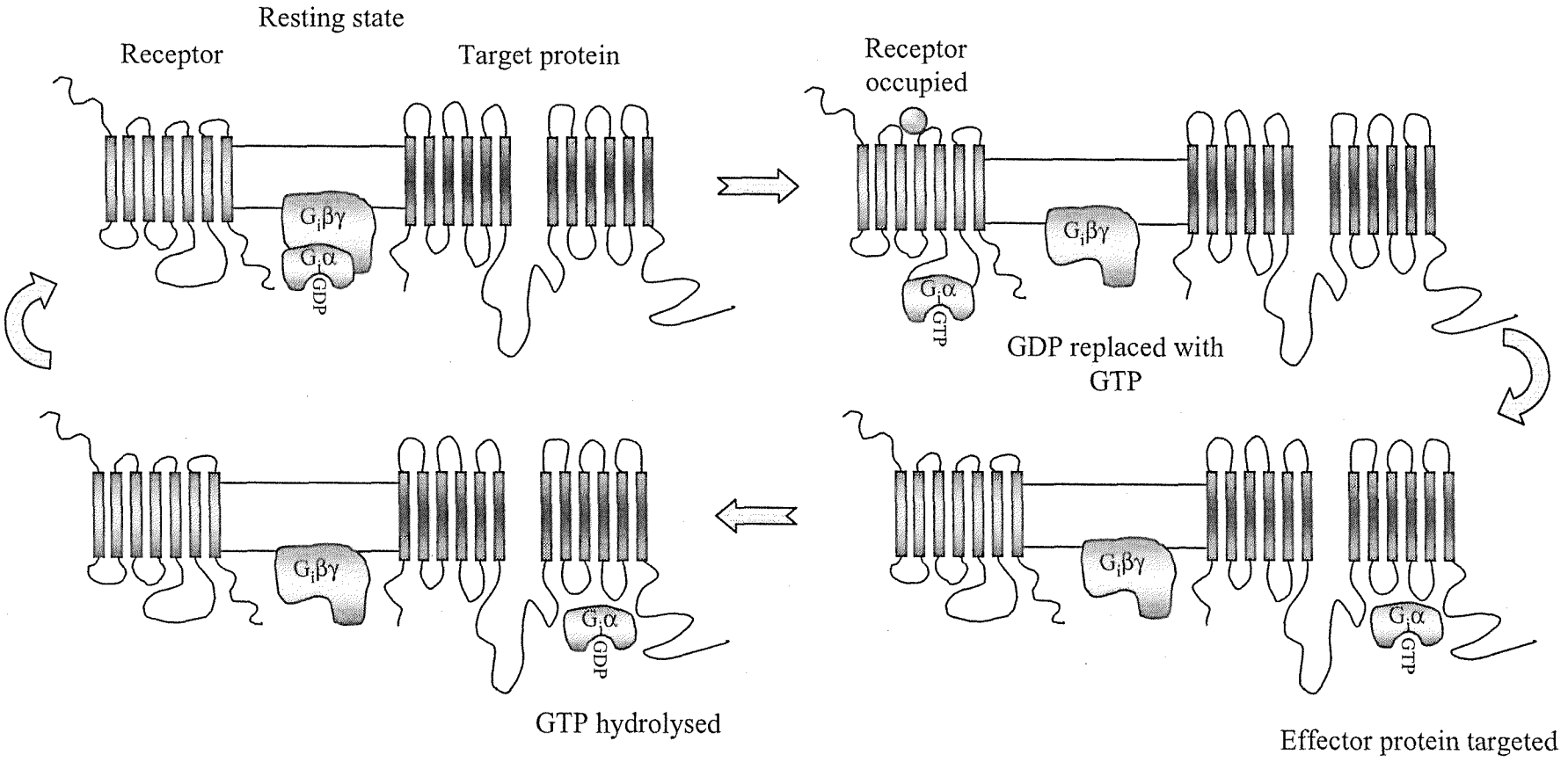


Figure 3: A current model of target protein activation by a GTP binding protein

As long as the ligand remains bound to the receptor the G protein can continue to activate target enzymes such as AC. Once ligand dissociation occurs the GTP binding protein can continue to act upon its target proteins for a number of seconds. This being an important amplification process allowing a small signal to elicit a large response (adapted from Alberts *et al.*, 1994).

The 5-HT_{1A}R was shown to couple to a number of different effector systems, resulting in the inhibition (De Vivo & Mayani, 1986) or stimulation of AC (De Vivo & Mayani, 1990), the opening of K^+ channels (Andrade & Nicol, 1987), stimulation (Raymond, 1991) or inhibition of PLC (Claustre *et al.*, 1988) and decreased Ca^{2+} influx by closing voltage-gated Ca^{2+} channels (Colino & Halliwell, 1987). The most consistently observed pathway across all cell systems and species is the inhibition of forskolin stimulated AC activity (Newman-Tancredi *et al.*, 1992).

In addition to AC coupling, the 5-HT_{1A}R expressed in rat atrial myocytes was also shown to be directly linked to an endogenous atrial rectifier K^+ current with no intervening second messenger signal, suggesting a role for the G protein, G_o (Karschin *et al.*, 1991). The dual coupling to AC and K^+ channels is recognised as the hallmark of G_i-linked receptors and also occurs with other members of the 5-HT_1 receptor family (Sanders-Bush & Canton, 1995; Martin & Eglen 1998). In addition, expression of the 5-HT_{1A}R in Ltk^- fibroblasts, NIH-3T3 cells, Cos-7 cells, GH_4C_1 pituitary cells and *Xenopus* oocytes all demonstrated a functional coupling of the 5-HT_{1A}R to PLC (Liu & Albert, 1991; Varrault *et al.*, 1992; Fargin *et al.*, 1989; Ni *et al.*, 1997). Activation of PLC subsequently results in an increased hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) leading to an increase in cellular inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) concentrations. The increased IP_3 concentration results in the release of Ca^{2+} from intracellular calcium stores and DAG activates PKC (**Figure 4**; Fargin *et al.*, 1989; Claustre *et al.*, 1991). However, based on the high concentrations of agonist required to stimulate PLC activity, it is unlikely that this pathway has a significant role with regard to the physiological consequences of 5-HT_{1A} receptor activity (Harrington *et al.*, 1994).

The ability of the 5-HT_{1A}R to regulate several active transport processes has also been documented. Raymond *et al.*, (1989; 1990) demonstrated the ability of the 5-HT_{1A}R expresses in HeLa cells to regulate Na^+/ phosphate symport via a PKC mediated pathway. While Middleton *et al.*, (1990) reported the regulation of an Na^+/ K^+ ATPase by the 5-HT_{1A}R through a Ca^{2+} mediated cascade also in HeLa cells. Both of these pathways probably depend upon activation of PLC by $G_{\beta\gamma}$ stimulation.

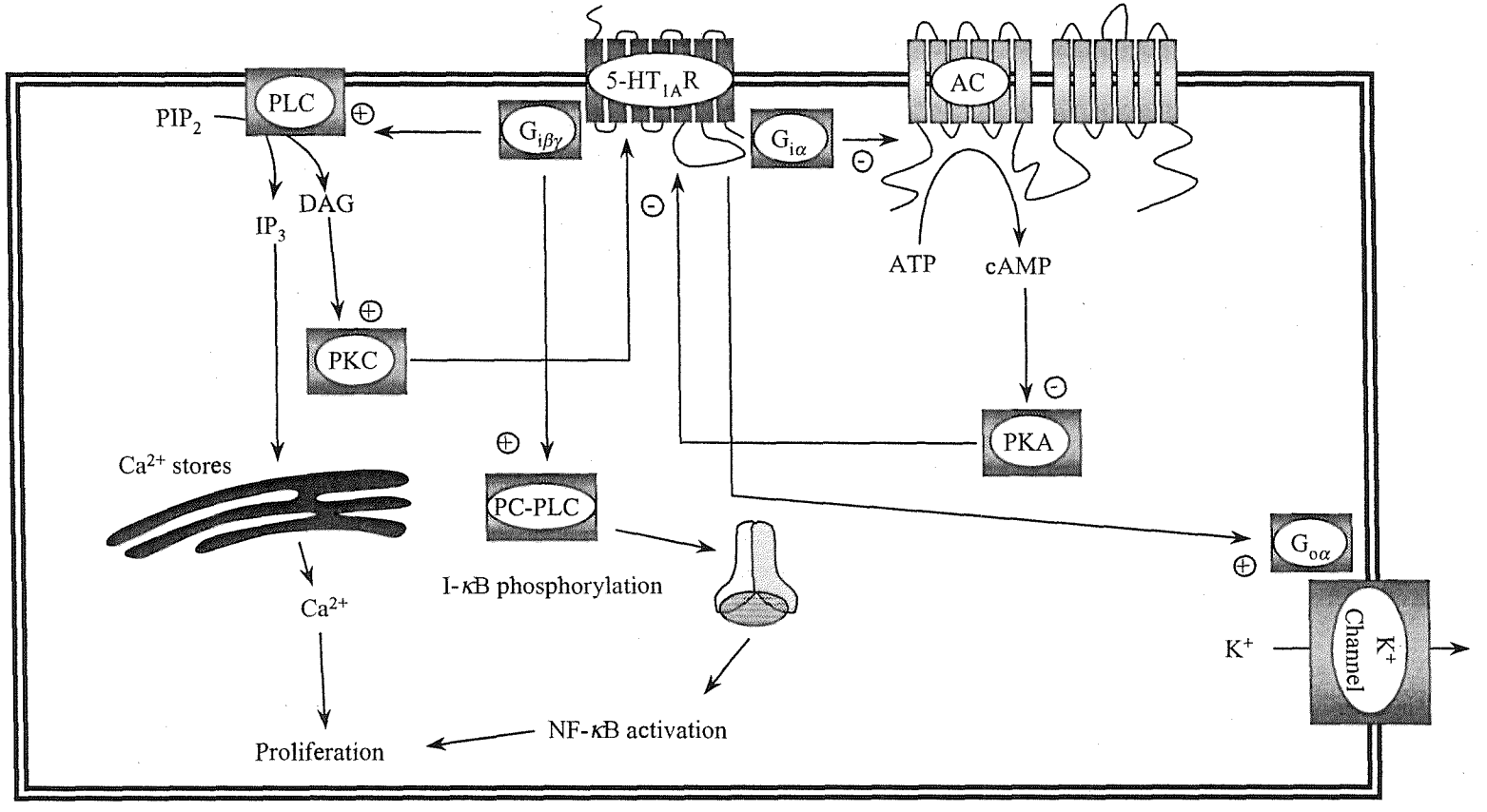


Figure 4: The second messenger effector system linked to the human 5-HT_{1A}R in various cell lines

Agonist activation causes $G_{i\alpha}$ mediated inhibition of adenylate cyclase causing a decrease in the production of cAMP, which in turn inhibits the activity of PKA. $G_{o\alpha}$ mediates the opening of K^+ channels allowing the efflux of K^+. $G_{i\alpha}$ mediated stimulation of PLC acts to increase the turnover of PIP_2 to DAG and IP_3. DAG in turn activates PKC which has direct stimulatory affects on the MAP kinase cascade. IP_3 is responsible for the release of Ca^{2+} from intracellular calcium stores resulting in the activation of Ca^{2+}/ calmodulin dependent kinases. PC-PLC activation by $G_{i\beta\gamma}$ also results in I-κB phosphorylation and NF-κB activation

In Chinese hamster ovary (CHO) cells the 5-HT_{1A}R has also been demonstrated to regulate an N^+/ H^+ ion exchange channel through a pathway involving $G_{i\alpha}$, Src tyrosine kinase and PI3-K (Garnovskaya *et al.*, 1997; 1998). These various processes would implicate the 5-HT_{1A}R in energy production as the exchange of Na^+ and H^+ along their respective concentration gradients results in the production of ATP (Raymond *et al.*, 1993). There are number of conflicting reports as to the intracellular consequences of 5-HT_{1A}R activation. An important question to ask is whether these different signal transduction pathways, which are mediated by G_i-like protein reflect multiple signalling mechanisms for a single receptor or multiple receptor subtypes that cannot be differentiated with available pharmacological techniques. One should also consider whether these results have been obtained from intact neurones or from transfected surrogate cell lines, where the receptor is unusually expressed at supraphysiological levels and may, therefore, promiscuously couple to many G proteins.

Neurotransmitters are often released in conjunction with other neurotransmitters and can act as neuromodulators, amplifying the response to a co-released neurotransmitter (Burnstock, 1976; Asano & Hidaka, 1980). There are many examples of co-localisation and co-release of chemical messengers resulting in an amplified response on target cells suggesting that such synergy may have an important regulatory function in normal physiological functions. These interactions may serve to modulate or fine-tune multiple receptor-signalling pathways. Neurotransmitter release serves to activate GPCRs, but activation does not always lead to a direct effect on a particular signalling pathway but rather to amplification of the response produced by a separate simultaneous signal within the same cell or tissue. The interactions of signalling pathways, which originate from different sources, may result in the augmentation of a cellular response or an increase in the maximal response (Stupecky *et al.*, 1986; Flavahan & Vanhoutte, 1988). Synergistic cross talk interactions between GPCRs specific for direct neurotransmitters can result in the augmentation of physiological responses such as smooth muscle contraction (Hill & Kendal, 1989; Dickenson & Hill, 1994). For example, blood vessels can be sensitised to noradrenaline by pre-treatment with neuropeptide Y (NPY), or by co-stimulation with concentrations of NPY which on their own do not elicit potent contractile effects (Edvinsson *et al.*,

1984). The noradrenaline response is mediated through G_q-coupled receptors and the effect can be significantly augmented at low noradrenaline concentrations by activation of G_i-coupled receptors by NPY. 5-HT is also known to act as an amplifier (Van Nueten *et al.*, 1985). The amplifying effects of G_i coupled receptors on G_q stimulated PLC activity (which eventually leads to the release of Ca^{2+} from intracellular calcium stores) are often accompanied by a small direct stimulation of PLC by the G_i coupled receptor. Both direct and indirect effects of these receptors are sensitive to pertussis toxin treatment, confirming a role for $G_{i/o}$ proteins (Gilman, 1987; Selbie *et al.*, 1997; Felder *et al.*, 1991; Selbie *et al.*, 1995; Dickenson & Hill, 1997). Following GPCR stimulation enhanced PLC activity leads to an increase in (Burnstock 1976; Lunberg & Hokfelt, 1993; Hokfelt, 1991) IP_3 production and resultant increases in the release of Ca^{2+} from intracellular stores. Stimulation of NPY G_i-coupled receptors results in the augmentation of noradrenaline induced increase in IP_3 (Gilman, 1987; Megson *et al.*, 1995; Selbie *et al.*, 1995; Dickenson & Hill, 1997). Contractions of smooth muscle cells are regulated by the concentration of cytosolic Ca^{2+}, which activate myosine light chain kinases (Somlyo & Somlyo 1994; Kitazwa *et al.*, 1991). Generally the larger the intracellular calcium concentration the greater the contractile force.

GPCRs also interact with other receptor classes such as tyrosine kinase receptors (e.g. insulin receptors). The actions of receptor tyrosine kinase signalling pathways can result in effects on cellular proliferation and cytoskeletal rearrangement such as contraction. For example NPY and 5-HT receptors have conserved tyrosine residues which, if phosphorylated, are putative binding sites for a number of proteins involved in the transmission of signals from receptor tyrosine kinases to the nucleus: these include Src, Shc, Grb2 and tyrosine kinase growth factor receptor sites (Baltensperger *et al.*, 1996). There is evidence that intrinsic tyrosine kinase growth factor receptors can phosphorylate GPCRs at these putative binding sites both *in vivo* and *in vitro* (Karoor *et al.*, 1995; Baltensperger *et al.*, 1996). Stimulation of GPCRs may also result in cross talk regulation of receptor tyrosine kinase (RTK)-mediated signal transduction at the level of the receptor and at downstream sites. Stimulation of endothelin 1 and thrombin receptors (both GPCRs) results in tyrosine phosphorylation

of the epidermal growth factor receptor and subsequent transactivation of the mitogen-activated protein kinase (MAPK) pathway (Van Biesen *et al.*, 1996; Daub *et al.*, 1996).

Receptors linked to G_i, G_q and G_s, G proteins have all been shown to stimulate MAPK activity (or extracellular signal regulated kinases [ERKs]) as well as other kinases leading to changes in gene expression (Van Biesen *et al.*, 1996; Crespo *et al.*, 1994; Hawes *et al.*, 1995; Faure *et al.*, 1994; Cadwallader *et al.*, 1997). The 5-HT_{1A}R has been shown to regulate MAPK activity (Garnovskaya *et al.*, 1996) in cell culture but it remains unclear whether this occurs in a physiological (*in vivo*) context. G_q-mediated effects on MAPK are distinct and are sensitive to inhibition of PKC activity, but unlike G_i mediated MAPK activation, are not inhibited by the expression of the $G_{\beta\gamma}$ subunit scavenger β-adrenergic receptor kinase (Hawes *et al.*, 1995; Faure *et al.*, 1994).

The potent ability of GPCR to cross talk with the tyrosine kinase pathway also results in synergistic effects on receptor tyrosine kinase function. Co-expression of $G_{\beta\gamma}$ subunits results in significant synergy of MAPK activity, suggesting that the $G_{\beta\gamma}$ dependant G_i stimulated MAPK activation may augment MAPK activity stimulated by other GPCR and receptor tyrosine kinases (Hawes *et al.*, 1996). Importantly, the downstream proliferation effects of receptor tyrosine kinase activation are modulated by GPCR activation. It appears that relatively small changes in the production of second messengers can be amplified by coincident receptor activation. Such signals can converge at sites further downstream with signalling pathways stimulated by other receptor classes. The expanding numbers of GPCRs that are being identified by gene cloning and the availability of more discriminatory pharmacological agents are being matched by an increasing awareness of the modulatory role of these GPCRs.

1.4.6. Protein structure of the human 5-HT_{1A}R

The 5-HT_{1A}R belongs to a superfamily of protein receptors that share structural, functional and sequence characteristics with the β_2AR, rhodopsin, and the chloride channel involved in cystic fibrosis. The 5-HT_{1A}R was originally estimated to be 421

amino acids in length based on primary sequence analysis (Kobilka *et al.*, 1987). Full re-sequencing of the G-21 clone in our laboratory and by other authors (Page *et al.*, 1996; Chanda *et al.*, 1993) has confirmed the receptor is actually 422 amino acids in length.

The amino acid sequence encoding the 5-HT_{1A}R has seven distinct regions each composed of approximately 25 hydrophobic amino acids. These are believed to be the membrane spanning segments of the protein, which are arranged into α helices. Separating the membrane spanning segments are eight hydrophilic regions, which are believed to form the intracellular and extracellular domains involved in receptor function. The long amino terminus, which is thought to be extracellular, has a number of potential sites for asparagine-linked glycosylation at positions N_{10}, N_{11} and N_{24}. On the other hand the carboxyl terminal is relatively short (19 amino acids) and is orientated intracellularly. The 5-HT_{1A}R has a proposed molecular weight of 46kDa when unmodified and possesses a single disulphide bond between positions C_{109} and C_{186} (El Mestikawy *et al.*, 1991 and citations therein; **Figure 5**). Evidence derived from β_2AR deletion mutants, β_1/β_2AR chimeric receptors, and rhodopsin suggest the 5-HT binding site is likely to be located within a deep pocket comprised of the transmembrane domain (Strange, 1996). New evidence also indicates that the first and third extracellular loops may have a role in the discriminatory binding of selective receptor ligands, by controlling their entry into the transmembrane binding site (Dietrich *et al.*, 1998).

The 5-HT_{1A}R has a long third intracellular loop (128 amino acids) that is a common feature of receptors coupled to the inhibition of AC. Regions bordering the plasma membrane in the second and third intracellular loops are extremely well conserved amongst GPCR. This is thought to be because these areas interact with G proteins, and G proteins are known to bind with some promiscuity to various receptors, thus a common signal is needed. The first intracellular loop has a non-conserved sequence of amino acids, and it probably plays an important structural role. Contained within second and third intracellular loops are putative substrate amino acid sequences for interaction with PKC and PKA (**Figure 5**). Within the third intracellular loop are also found multiple G protein-coupled receptor kinase (GRKs) targets (Zhang *et al.*, 1997).

The consensus sequences are based around serine or threonine (S/T) residues which facilitate the binding of PKC, PKA and GRKs which may be subject to phosphorylation (KRTPR, position 147-151, RKTVK, position 227-231, KKS, position 251-253 and RKTVK, position 341-345; **Figure 5**). Raymond has shown PKC activation via agonist binding to the 5-HT_{1A}R and receptor phosphorylation at two sites (Raymond, 1991). Receptor phosphorylation by PKC, PKA and GRKs is though to play an important role in receptor regulation by uncoupling the receptor from its G protein and facilitating receptor desensitisation and/ or internalisation.

1.4.7. Structure of the 5-HT_{1A}R gene

The structure of the rat, human and mouse 5-HT_{1A}R genes have been described. In addition, those transcription factors important in the regulation of 5-HT_{1A}R gene expression have been studied in rat, human and mouse-derived cell lines.. The use of both receptor positive and negative cells has also allowed for study of specific and non-specific transcription regulatory elements.

The initial work into 5-HT_{1A}R gene structure by Parks & Shenk (1996) was performed on the mouse and human 5-HT_{1A}R gene. They demonstrated using three separate techniques (RNA 5' end mapping, DNA protein interaction and transient expression studies) that the 5'-flanking DNA sequence lacked a typical TATA box element and was rich in guanine and cytosine. This guanine-cytosine rich DNA motif in the 5-HT_{1A}R gene was related to the 5'-GGGG(C/A)GGGG-3' (Parks & Shenk 1996). Crude HeLa cell nuclear extracts were used to try to identify proteins that may interact with this 5' sequence. A cDNA was subsequently cloned and identified as that encoding a protein known as "Maz". DNase I footprinting of the 5' flanking region of the 5-HT_{1A}R gene demonstrated the existence of four Maz sites of which three were found to bind the SP-1 transcription factor (**Figure 6**). When transiently expressed both SP-1 and Maz were found to increase the expression of the 5-HT_{1A}R gene directed by the human 5' flanking sequence (Parks & Shenk 1996). Conflicting findings came from more detailed reports on the rat 5-HT_{1A}R gene structure.

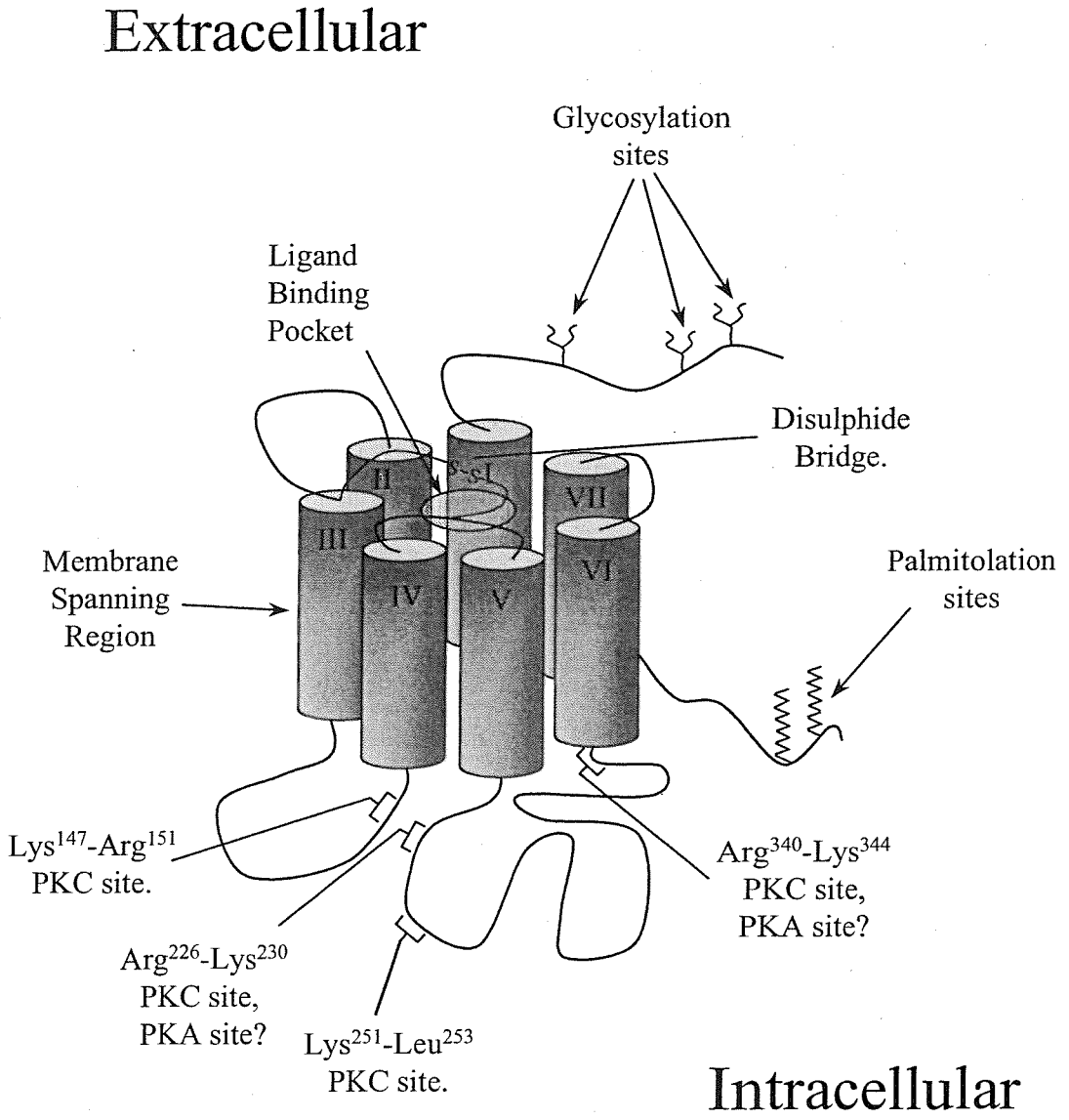


Figure 5: Three Dimensional representation of the human 5-HT_{1A} R

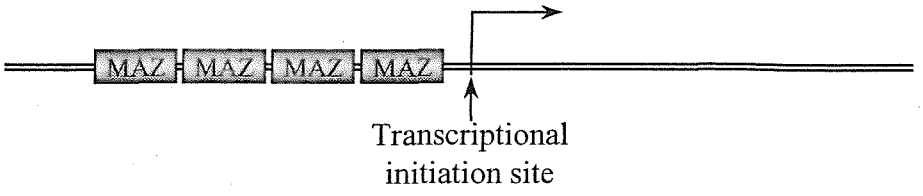
Figure includes putative phosphorylation sites, disulphide bridges, ligand binding pocket and palmitolation sites.

Storring *et al.*, (1999) using 5-HT_{1A}R positive (rat raphe RN46A and septal SN-48) and negative (pituitary GH_4C_1 L6 myoblast and C6 glioma) cell lines dissected out those base pairs (bp) involved in 5-HT_{1A}R gene regulation. Nine hundred and sixty seven bp upstream of the 5-HT_{1A}R-gene start codon was found a site of transcriptional initiation which appears to be brain-specific.

The site of transcription initiation was 58bp downstream from a consensus TATA box, suggesting TATA driven transcription of the rat 5-HT_{1A}R (Storring *et al.*, 1999). However, in almost every case of genes containing a TATA box it is located between 25-30bp from the transcription start site a distance set by the locality of various proteins involved in transcription (Alberts *et al.*, 1994). This might suggest that the authors have misinterpreted the data or have misplaced the transcriptional initiation site. The possible existence of a TATA box in the upstream region of the rat 5-HT_{1A}R would be in stark contrast to both the mouse and human 5-HT_{1A}R upstream regions which as already mentioned lacked a consensus TATA element (Parks & Shenk 1996). Located within the upstream 426-117bp fragment of the rat 5-HT_{1A}R is an enhancer region, which was only active in 5-HT_{1A}R positive cell lines (rat RN46A and SN-48; Storring *et al.*, 1999). Also located in the upstream region (1519-426bp) of the rat 5-HT_{1A}R gene are non-specific enhancer/ promoter elements, containing consensus TATA, CCAAT, SP-1 and AP-1 elements which are active in receptor negative cell lines (**Figure 6**; Storring *et al.*, 1996).

A repressor element has also been located in the upstream 1590-1519bp fragment of the rat 5-HT_{1A}R gene (**Figure 6**; Storring *et al.*, 1999; Ou *et al.*, 2000). Deletion of a 71bp segment between -1590 and 1-519bp resulted in a 10-fold enhancement of transcriptional activity in rat 5-HT_{1A}R positive and negative cell lines. Of the same71bp fragment of the rat 5-HT_{1A}R gene 31bp were protected from DNase I digestion by rat RN46A and L6 crude nuclear extracts (Ou *et al.*, 2000). Further analysis of the 31bp fragment demonstrated a single protein complex that bound a novel 14bp DNA element in rat 5-HT_{1A}R positive cells. Also contained within the same 31bp fragment adjacent to the 14bp fragment was a 12bp fragment that conferred non-specific 5-HT_{1A}R gene repression in receptor negative cell lines (Ou *et al.*, 2000).

(**A**) Upstream region of mouse and human 5-HT_{1A}R gene



(**B**) Upstream region of rat 5-HT_{1A}R gene

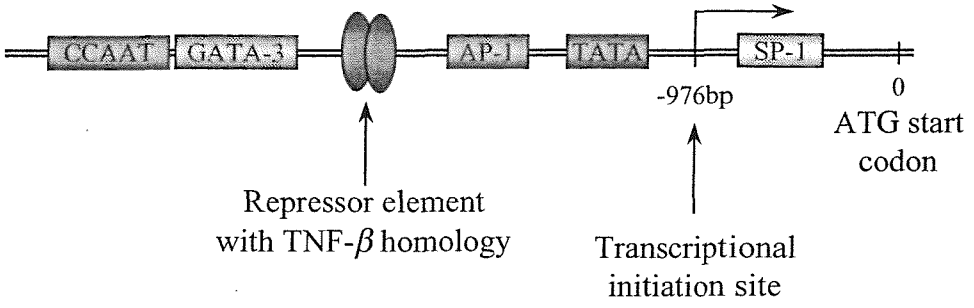


Figure 6: Topology of the upstream regions of the human, mouse (A) and rat (B) 5-HT_{1A}R gene

Indicated are the approximate positions of putative binding sites for the DNA binding proteins, MAZ, AP-,1 SP-1 and the regulatory sequences TATA, CCAAT and GATA-3

In summary, results demonstrate the human and mouse 5-HT_{1A}R gene lack a TATA element but do contain four novel guanine-cytosine rich domains that bind MAZ, of these four domains, three also bind SP-1 (Parks & Shenk 1996). Both MAZ and SP-1 enhanced transcriptional activity of the human and mouse 5-HT_{1A}R gene but MAZ was substantially more effective (**Figure 6**; Parks & Shenk 1996). However, the rat 5-HT_{1A}R gene does contain a TATA box, 1025bp from the start codon suggesting TATA driven gene expression (Storring *et al.*, 1999). Other specific enhancer elements for the rat 5-HT_{1A}R gene are located between nucleotides 426 and 117 (still to be identified). Non-selective enhancer elements (including CCAAT SP-1 and AP-1) in the rat 5-HT_{1A}R gene are located upstream of the start codon between nucleotides 1519 and 426 (**Figure 6**; Storring *et al.*, 1999). Also located between 1590-1519bp upstream from the rat 5-HT_{1A}R gene are two repressor elements, the first 14bp unit confreres specific regulation to the gene whereas the adjacent 12bp unit confers non-specific gene regulation (Ou *et al.*, 2000).

1.4.8. Surrogate expression systems

The 5-HT_{1A}R has been studied in a wide range of cell systems; some of those endogenously express the receptor, whilst the majority of cells used, are receptor negative. Cell expression systems are used for a wide range of applications including pharmacological profiling, G protein coupling to effector systems, and regulation of receptor gene expression. Once isolated and cloned, the G-21 clone was expressed in Cos-7 cells thus becoming the first artificial expression system for the 5-HT_{1A}R (Fargin *et al.*, 1988). Cells that are known to naturally express the 5-HT_{1A}R include rat raphe RN46A cells, septal SN-48 cells and Jurkat T lymphocytes, (Storring *et al.*, 1999; Aune *et al.*, 1993).

The cell lines that have been used to study the recombinant 5-HT_{1A}R include, pituitary GH_4C_1 cells, L6 myoblast cells, C6 glioma cells, Ltk^- fibroblasts, *Xenopus* oocytes, rat atrial myocytes, CHO cells, HeLa cells and NIH-3T3 cells (Storring *et al.*, 1999; Liu & Albert, 1991; Ni *et al.*, 1997; Karshin *et al.*, 1991; Newman-Tancredi *et al.*, 1992; Harrington *et al.*, 1994; Varrault *et al.*, 1992).

The main reason for using cell lines and artificial expression systems is convenience, cells are relatively easy to grow and maintain. In addition, they provide for optimal quality control, growth can be quickly scaled up, they reduce the requirement for animal use for some types of experiment and offer a reductionist approach to the study of receptors. In the case of receptor negative lines, they offer the chance to introduce a homogenous population of receptors (if expressed at high levels) that can easily be isolated. However, there are drawbacks of artificially expressing a receptor in a cell line, which is normally receptor negative. The cost of setting up and maintaining cell-culture facilities can be large and the day-to-day maintenance of cell lines that can be time consuming, expensive and wasteful. In addition, because the receptor is expressed in a foreign environment usually at supraphysiological levels it is not necessarily going to respond in a manner that is comparable to the *in vivo* situation. Careful characterisation of receptors in surrogate lines is essential and the interpretation of the data should always be considered carefully. The use of cells that are receptor positive helps to overcome some of these problems but the expression of the receptor tends to be much lower i.e. at more physiologically relevant levels. In these cell lines, responses may often be below the level of sensitivity of many analytical techniques (including radioligand analysis).

1.5. RECEPTOR REGULATION AT THE PROTEIN LEVEL

Regulation of receptor function is a vital cellular role as it results in the termination of a set of sequential reactions set in motion by an extracellular stimulus. Receptor function can be regulated at several levels from the transcription of new receptor mRNA to translation of receptor mRNA to new protein (long-term), through to direct modification of the existing receptor protein (short-term). The long-term regulation of receptor function is normally achieved via the induction or inhibition of various transcription factors. Such transcription factors that are influenced by the human 5-$HT_{1A}R$ include CREB and nuclear factor kappa B (NF-κB) (Nishi & Azmitia, 1999; Cowen *et al.*, 1997). CREB and NF-κB have also been demonstrated as having a role in directly regulating the expression of the 5-$HT_{1A}R$ thus forming a postulated autoregulatory feedback loop (Nishi & Azmitia, 1999; Cowen *et al.*, 1997). Regulation of receptor function that occurs within seconds to minutes after ligand

challenge is known as short-term receptor regulation. Short-term receptor regulation is transient and enables the cell to effectively switch off the response to the extracellular stimulus. The mechanism involved may include phosphorylation of the receptor thus disabling its' ability to interact with G proteins. The agonist promoted phosphorylation of GPCRs is termed desensitisation and will be discussed in greater depth in later sections.

The regulation of the signalling event also requires a fine balance between mechanisms contributing to the initiation and switching off, of the signalling cascade. Whereas the mechanisms by which the initiation of signal transduction through GPCRs have been fairly well defined, the relative physiological importance of the mechanisms involved in the turning off and re-establishment of signalling has not been fully resolved.

1.5.1. GPCR desensitisation

G protein signalling is regulated by a receptor/ G protein-uncoupling event that follows receptor phosphorylation upon ligand binding. The process of receptor phosphorylation, is mediated by either second messenger-dependent protein kinases such as PKA or G protein receptor kinases (GRKs) and occurs within seconds to minutes of agonist stimulation (Pronin & Benovic, 1997). GRKs are intracellular enzymes targeted to their substrate residues by heterotrimeric G proteins. They have an intrinsic ability to phosphorylate GPCRs (Inglese *et al.*, 1993; Pitcher *et al.*, 1995a).

Receptor phosphorylation mediated by GRKs is dependent upon agonist occupation and serves to regulate activated receptors. Phosphorylation mediated by second messenger dependant kinases is not necessarily dependent upon receptor occupancy. Phosphorylation of the receptor by GRKs, increases the affinity of the receptor protein for cytosolic "arrestin" proteins, which discriminate between receptors that have been activated and those which have not (Premont *et al.*, 1995). Arrestin proteins have an inhibitory role in regulating receptor function, effectively preventing the receptor from interacting with G proteins and facilitating further interactions with the internalisation

machinery (Alberts *et al.*, 1994). Of the six potential members of the arrestin family, only four full length clones have been identified, these include visual arrestin, cone arrestin, β-arrestin 1 and β-arrestin 2. Visual and cone arrestin are limited to cells involved with vision, whereas β-arrestin 1 and β-arrestin 2 demonstrate a more generalised pattern of expression (Ferguson *et al.*, 1996b).

Families of six individual GRKs (GRK 1-6) have been isolated to date including β-adrenergic receptor kinase 1 and 2 (now known as GRK2, 3; Premont *et al.*, 1995). These kinases (localised to the cytosol) share 53-93% sequence homology and are known to phosphorylate multiple serine/ threonine residues found on the third intracellular loop and carboxyl-terminus of activated GPCR, one such example being the $EES_{296}SSS_{299}$ motif located within the third intracellular loop of the α_{2A}adrenergic receptor (Eason *et al.*, 1995). The catalytic domains of these two GRKs share strong structural and sequence homology, whereas the flanking amino and carboxyl domains share little homology and are thought to play a role in substrate targeting and specificity (Premont *et al.*, 1995). The list of G_i-coupled receptors that become or are suspected to be phosphorylated by GRKs in response to agonist challenge includes, α_{2A}-adrenergic, α_{2C}-adrenergic receptor the 5-HT_{1A}R and adenosine$_{1A}$ receptor (Garcia-Sevilla *et al.*, 1999; Lembo *et al.*, 1999; Nie *et al.*, 1997; Nebigil *et al.*, 1995). Non-ligand induced receptor desensitisation can also be achieved with compounds that activate intracellular proteins responsible for receptor phosphorylation. One such class of compounds that have been reported to cause receptor desensitisation are phorbol esters. The phorbol ester 4β-phorbol-myristate-acetate (PMA) has been demonstrated to cause desensitisation of the α_2-adrenergic receptor in rat pancreatic islets by direct activation of PKC (El-Mansoury & Morgan 1998). The desensitisation of the α_2-adrenergic receptor resulted in a significant reduction in the inhibition of glucose-induced insulin secretion (El-Mansoury & Morgan 1998).

The desensitisation of the human 5-HT_{1A}R is a well-documented event and to date three proteins are thought to mediate receptor desensitisation, these include; PKC, PKA and GRK. Four putative PKC phosphorylation sites and two putative PKA sites are contained within the human 5-HT_{1A}R sequence and all of them are located on the

intracellular loops of the receptor (**Figure 5**). The phosphorylation of the receptor is associated with uncoupling of several signals in several cell types. In CHO cells transfected with the human 5-HT_{1A}R, activation of PKC by phorbol esters resulted in rapid receptor phosphorylation and uncoupling of AC inhibition (Raymond, 1991). In rat P11 pituitary cells, activation of PKA also resulted in the uncoupling of the 5-HT_{1A}R from AC inhibition (Hensler *et al.*, 1996). In contrast, pre-treatment of Ltk^- fibroblasts and GH_4C_1 cells (transfected with the 5-HT_{1A}R) with phorbol esters resulted in no uncoupling of the receptor from the inhibition of AC (Lembo & Albert, 1995; 1997; Liu & Albert, 1991). The stimulation of Ltk^- fibroblast with phorbol esters did however result in the uncoupling of the receptor from the activation of PLC and the resulting increase in intracellular Ca^{2+} concentrations (Lembo *et al.*, 1995). This study also showed that mutations to the third intracellular loop resulted in no uncoupling of the 5-HT_{1A}R from PLC (Lembo *et al.*, 1995), suggesting that in HeLa cells transfected with the 5-HT_{1A}R phorbol ester pre-treatment also induced rapid G protein uncoupling which was blocked by PKC inhibitors (Harrington *et al.*, 1994).

Liu & Albert (1991) were the first to document the ability of PKA to enhance the desensitisation of the 5-HT_{1A}R induced by phorbol ester treatment in Ltk^- fibroblasts. Harrington *et al.*, (1994) also demonstrated that the activation of PKA resulted in rapid AC uncoupling and a substantial loss of [^{3}H] 8-hydroxy-DPAT high affinity binding sites in HeLa cells expressing the 5-HT_{1A}R. The pre-treatment of HeLa cells with 8-hydroxy-DPAT also resulted in the rapid uncoupling of the 5-HT_{1A}R from AC inhibition (Harrington *et al.*, 1994). The stoichiometry of the phosphorylation of the receptor mediated by PKA is believed to be one mole of phosphate to one mole of receptor (Raymond *et al.*, 1999).

Pre-treatment of Sf9 insect cells expressing the 5-HT_{1A}R with 5-HT lead to rapid receptor phosphorylation at serine and threonine residues and an uncoupling of AC inhibition (Nebigil *et al.*, 1995). The phosphorylation of the 5-HT_{1A}R expressed in Sf9 cells was attenuated by heparin but not blocked by inhibitors of PKC and thus was attributed to GRKs (Nebigil *et al.*, 1995). Closer inspection of the human 5-HT_{1A}R amino acid sequence reveals 17 potential substrate residues for the GRK (Raymond *et*

al., 1999). Therefore, desensitisation in neurones is likely to involve more than one kinase.

1.5.2. Internalisation

Mammalian cells have evolved a variety of mechanisms to internalise small molecules, macromolecules, and other membrane bound proteins and target them to specific intracellular compartments. This whole process is termed endocytosis; under its broad definition, endocytosis includes various methods of uptake of extracellular material by cells, including phagocytosis, pinocytosis, clathrin-dependent receptor-mediated endocytosis, and clathrin-independent endocytosis. Phagocytosis and clathrin dependent endocytosis are the best-characterised internalisation mechanisms. Receptor-mediated endocytosis takes place in all nucleated vertebrate cells and plays an important role in many physiological processes. Phagocytosis, which involves the uptake of large particles, takes place in many cell types but is most important in specialised cells such as macrophages, where it plays an important role in cellular immunity.

Endocytosis is involved in uptake of extracellular nutrients, regulation of cell-surface receptor function, cellular cholesterol homeostasis, maintenance of cell polarity, antigen presentation, and many other physiological processes. Irregularities in endocytic processes play a role in several diseases including atherosclerosis and diabetes, and it follows therefore, that abberations in 5-HT receptor regulation may have implications for the development of disease states of the central nervous system.

Common fates awaiting internalised receptors include degradation (Protease-activated receptor-1 (PAR1); Trejo *et al.*, 2000) recycling back to the cell surface (β_2AR; Szekeres *et al.*, 1998), trafficking to organelles (for example, Golgi apparatus) or the cytosol (D_1 dopamine receptor; Dumartin *et al.*, 2000). Similar processes occur in other (non-mammalian) cell types, and specific aspects of those systems, especially in yeast (Schekman, 1992), have been utilised to understand the molecular mechanisms of membrane traffic.

Receptor mediated endocytosis (RME) via clathrin coated pits is an internalisation pathway for numerous varieties of receptor-ligand complexes. The coated pits and

vesicles in mosquito oocytes were first described in 1964 by Roth and Porter (Roth & Porter, 1964), who postulated that these structures help in yolk formation by taking up adsorbed proteins from the extracellular fluid. Kanaseki and Kadota (1969) were the first to describe the basket type structure now known as the clathrin coat. Structures such as the low-density lipoprotein (LDL) receptor are actively concentrated in coated pits (Anderson *et al.*, 1977; Anderson *et al.*, 1982), whereas some demonstrate random incorporation (various phospholipids and glycosphingolipids; Pagano, 1990, Dawidowicz, 1993), or even active exclusion from the coated pits (the influenza virus heamagglutinin protein, Roth *et al.*, 1986). Concentration of 60 - 70 %, of such proteins in these coated pits leads to an efficient clearance from the cell surface (Anderson *et al.*, 1977). Each coated pit is approximately 150nm in depth from the plasma membrane (Willingham *et al.*, 1981) and in the case of human fibroblasts and rat hepatocytes, they occupy about 2% of the plasma membrane surface (Anderson *et al.*, 1977; Carpentier *et al.*, 1985). Although these values can range from 0.4% (Goldberg *et al.*, 1987) to 3.8% (Nilsson *et al.*, 1983) depending on the cell type being studied.

Of the receptor proteins that are actively concentrated in coated pits, some are done so constitutively, for example the LDL receptor (Anderson *et al.*, 1982; Anderson *et al.*, 1977), and some become concentrated upon ligand binding, for example the epidermal growth factor receptor (Dunn & Hubbard, 1984). The targeting of receptors to coated pits, is thought to be due to interactions between so called "internalisation motifs" in the receptor and intracellular clathrin coat assembly proteins (Schmid, 1992; Kirchhausen, 1993). Internalisation motifs are also thought to be multi-functional. In addition to promoting internalisation, the motifs are thought to act as targeting sequences to various intracellular compartments (Voorhees *et al.*, 1995). Initial experiments on the regulation of the human 5-HT_{1A}R expressed in HeLa cells demonstrated a rapid PKC induced reduction of high affinity binding sites upon 8-hydroxy-DPAT incubation (Harrington *et al.*, 1994). These data indicate that the large loss (approximately 80%) of high affinity [^{3}H] 8-hydroxy-DPAT binding sites might be due to internalisation, thus warranting further investigation (See Aims and Objectives, section 1.7. and 1.8.).

1.5.2.i. Internalisation sequences

1.5.2.i.a. Peptide internalisation motifs

Entry to the cell via RME is a very rapid process (10-50% of total receptors per minute) suggesting that receptor concentration in the clathrin-coated pit is an efficient process. Evidence suggesting that so-called "internalisation" motifs play an important role, has been obtained from studies employing the site directed mutagenesis approach to study receptors. The first analytical work on residues important in internalisation was performed on a natural mutation of the human LDL receptor (cysteine replaced by a tyrosine; Dawidowicz, 1993) and this demonstrated a possible role for receptor residues located in the cytoplasmic domain as internalisation motifs (Goldstein *et al.*, 1985). Since then, systematic single site mutagenesis has been performed on a range of GPCRs and other receptors, indicating that a tyrosine or another aromatic amino acid is required at the membrane/ cytosol interface of the seventh/ final transmembranous domain for concentration into coated pits (Bohm *et al.*, 1997; Chuang *et al.*, 1997; Laporte *et al.*, 1996; Barak *et al.*, 1995; Hsu *et al.*, 1994). Although this residue is vital for the sequence to remain functional, there is also a requisite for a second important bulky hydrophobic group at a more membrane proximal location (Lazarovits & Roth, 1988; Chen *et al.*, 1990). Comparison of a number of GPCRs (including the human 5-HT_{1A}R) from various species indicates that the conserved sequence is a common motif and is necessary although not vital for internalisation (Wang *et al.*, 1997). Work on truncated mutants demonstrated that receptors lacking the cytoplasmic tail internalised at 23-50% of the wild-type receptor rate (Hukovic *et al.*, 1998; Huang *et al.*, 1995). Studies performed on the transferrin receptor which also contains an NPX_nY internalisation motif in the N-terminal 61 amino acid cytoplasmic portion, demonstrated the $Y_{31}XRF_{34}$ tetrapeptide of the transferrin receptor, was able to form a functional signal at several positions in the cytoplasmic domain. The internalisation motif was however only functional if it was separated from the membrane by at least seven amino acid residues (Collawn *et al.*, 1990; Collawn *et al.*, 1993).

Studies by Barak *et al.*, 1995 provided evidence that the common motif NPX_nY maybe a vital component for maintaining normal GPCR conformation, rather than acting as a general sequestration motif. Development of NPX_nY receptor mutants has

made it possible for the molecular mechanisms of GPCR internalisation to be elucidated and has given an insight into the role of GRKs and β-arrrestins in GPCR trafficking. The $Y_{326}A$ mutation in the β_2AR tail not only impaired sequestration but also greatly reduced GRK mediated phosphorylation of the receptor implying, that the proposed internalisation motif may have a more complex role than at first thought. This reduction in receptor phosphorylation and sequestration was reversed by the over-expression of GRK2 in HEK 293 cells, returning the receptor to unmutated characteristics (Ferguson *et al.*, 1995). Mutations to GRK2 ($K_{220}M$) had a similar affect as direct receptor mutation, in that it impaired both phosphorylation and internalisation of the unmutated β_2AR (Ferguson *et al.*, 1995). These findings clearly demonstrated that in the case of the β_2AR, receptor phosphorylation by GRK2 is vital for effective receptor internalisation. GRK mediated receptor phosphorylation and subsequent internalisation has been reported for a number of other GPCRs including the M_2 muscarinic receptor and the D_2 dopamine receptor (Tsuga *et al.*, 1994; Itokawa *et al.*, 1996). GRK mediated phosphorylation is not limited to a specific type or class of GPCRs as receptors coupled to $G_{i\alpha}$ (M_2 muscarinic receptor D_2 dopamine receptor) and $G_{s\alpha}$ (β_2AR) effector systems are both phosphorylated. This would suggest that phosphorylation might play a more general role in agonist promoted GPCR sequestration.

Contained within the 5-HT_{1A}R sequence are several consensus amino acid motifs that are thought to play a role in receptor internalisation. In the seventh transmembrane domain and the cytoplasmic carboxyl tail of the receptor is the NPVIY motif which, based on work performed on tyrosine kinase receptors, may play a direct role in 5-HT_{1A}R internalisation. Consensus PKA and PKC phosphorylation motifs located in the 2nd and 3rd intracellular loops of the receptor (see **Figure 5**) may also have a role in receptor endocytosis. Phosphorylation of the 5-HT_{1A}R at the consensus phosphorylation motifs is believed to result in uncoupling of the receptor from its' G protein and facilitate interactions with internalisation machinery.

Early indications were that β_2AR lacking an ability to undergo phosphorylation by GRK, could internalise in response to agonist binding: this might suggest that receptor phosphorylation is not a total requirement for internalisation (Hausdorff *et al.*, 1989).

β-arrestin is an intracellular protein that interacts with phosphorylated proteins and is thought to play an important role in receptor sequestration. The importance of β-arrestin in receptor endocytosis was illustrated by Ferguson *et al.*, (1996a) who demonstrated that the β_2AR-Y_{326}A mutant and other carboxyl terminus truncation mutants of the same receptor could internalise, if β-arrestin was over-expressed. It is thought that the role of receptor phosphorylation is to allow β-arrestin to interact with the receptor. In support of this evidence, unmutated β_2AR internalisation was shown to be inhibited by the over-expression of a β-arrestin mutant (β-arrestin 1-V_{53}D) which is a non-functional form of β-arrestin. This loss of internalisation could not be recovered by the over-expression of GRK2, which was able to recover the β_2AR-Y_{326}A mutants ability to sequester. Over expression of the β-arrestin 1-V_{53}D mutant and GRK resulted in the recovery of β_2AR-Y_{326}A mutant phosphorylation, without recovering its internalisation defect (Ferguson *et al.*, 1996a). These findings being consistent with studies that demonstrated GRK phosphorylation is not compulsory for β_2AR internalisation (Hausdorff *et al.*, 1989; Lohse *et al.*, 1990). In summary, it would seem that GRKs and β-arrestins have intercessory roles in both G protein uncoupling and ligand induced sequestration, with β-arrestins acting as adapter proteins mediating interactions with endocytic machinery.

Apart from the tyrosine-based internalisation signals, various leucine-based (Gabilondo *et al.*, 1997) and lysine-based (Itin *et al.*, 1995) cytoplasmic sequences also function as signals for coated pit concentration and lysosomal targeting. Proteins that lack the tyrosine motif but that still take part in RME (including CD4 receptor and human β_2AR) are thought to use leucine-based internalisation motifs (Miettinen *et al.*, 1989; Gabilondo *et al.*, 1997). The manner in which the leucine-based motif interacts with the internalisation machinery remains unclear.

1.5.2.i.b Ubiquitination

Ubiquitination of a protein involves the linkage of the 76 residue, protein ubiquitin, to the amino groups of lysyl residues contained within the carboxy-terminal of the target protein. Once ubiquitinated the protein is specifically targeted for degradation by a 26S protease termed a proteasome (Ciechanover, 1994). Recent studies also suggest

ubiquitination of cell surface proteins as a possible prerequisite for internalisation and degradation via lysosomal targeting (Hicke & Riezman, 1996).

When yeast α-factor binds to the GPCR Ste2p, it activates the signalling pathway associated with the receptor and, induces endocytosis of the receptor-ligand complex. Ligand binding also instigates the ubiquitination of the cytoplasmic portion of the Ste2p receptor. Mutants lacking the ubiquitination machinery or the lysine residues found within the cytoplasmic tail demonstrate a much lower rate of internalisation. Ubiquitination of other receptors upon ligand binding (Mori *et al.*, 1992; Yee *et al.*, 1994) and work done on growth hormone receptors transfected into CHO cell lines presented by Strous *et al.*, (1996) would lead to speculation that ligand-induced ubiquitination might be an important event leading to receptor internalisation and lysosomal degradation. Interestingly in the case of the β_2AR, Penela *et al.*, (1998) demonstrated that after GRK2 mediated phosphorylation GRK2 ubiquitination was markedly increased leading to GRK2 degradation via the proteasome pathway. This inactivation of GRK2 lead to an alteration in β_2AR signalling and internalisation indicating another important mechanism for modulating the cellular response to agonist challenge acting through GPCRs (Penela *et al.*, 1998).

1.5.2.i.c. Lipid domains and lipid-linked proteins

The endocytic process involves internalisation of proteins bound to the cell surface membrane and therefore, by definition, may involve the uptake of components intrinsic to the membrane. In many cases, this lipid uptake occurs in a non-selective, passive manner. It is however apparent that in several cases, lipid and lipid-linked proteins do not randomly congregate in clathrin coated pits or other endocytic structures, they may be selectively included or excluded. The toxin produced by *Shigella dysenteriae* demonstrates Receptor mediated endocytosis (RME) via a glycosphingolipid receptor on the plasma membrane. When the toxin binds to the glycosphingolipid receptor it induces aggregation of the receptor in the clathrin coated pits which can then undergo RME (Sandvig *et al.*, 1989). The possible explanations for this concentration within the coated pits may be that the glycosphingolipid is interacting with a membrane bound protein that contains an internalisation motif.

Alternatively, the glycosphingolipids may form aggregates upon ligand binding that have a preference for a coated pit localisation. However at this current time there is no evidence in the literature that GPCRs utilise this route of internalisation.

1.5.2.ii. Cellular pathways of GPCR internalisation

Dynamin is a large (100 kDa) GTPase first isolated as a microtubule binding protein and is now recognised as a major component in the clathrin-mediated RME pathway (reviewed by De Camilli *et al.*, 1995). The functional role of dynamin is to catalyse the pinching of membrane invaginations in a GTP dependent reaction. Consequently mutant dynamin proteins, lacking GTP binding abilities (dynamin 1-$K_{44}A$), block clathrin mediated RME (Van der Bliek *et al.*, 1993). Development of the β-arrestin mutant and dynamin 1 mutant ($K_{44}A$), both of which selectively block sequestration, has allowed the study of different endocytic mechanisms of different GPCRs in different cell type.

To look at the preferential internalisation pathways utilised by GPCRs upon agonist activation the role of dynamin and β-arrestin in the internalisation of two typical GPCRs (β_2AR and the angiotensin$_{1A}$ receptor [$AT_{1A}R$]) in both HEK 293 and COS-7 cells was examined (Zhang *et al.*, 1996). Sequestration of β_2AR in HEK 293 cells was effectively inhibited by the over-expression of the dynamin 1-$K_{44}A$ mutant, but had no effect on $AT_{1A}R$ internalisation, suggesting the two receptors internalise via two distinct pathways. Internalisation of the β_2AR was also found to be inhibited by expression of the β-arrestin 1-$V_{53}D$ whereas $AT_{1A}R$ sequestration remained unchanged, this was found to be the case in both cell types (Zhang *et al.*, 1996). It is therefore apparent that the β_2AR receptor internalised via a dynamin dependant pathway (clathrin mediated RME) and $AT_{1A}R$ internalised via an altogether separate pathway.

The exact sequestration pathway utilised by the $AT_{1A}R$ remains unclear, but there are several other possibilities, including the caveolae-mediated pathway, a non clathrin-coated vesicle pathway or perhaps a pathway, which is yet to be discovered. It is however apparent that GPCR receptor internalisation can occur through two separate pathways.

1.5.2.ii.a. Endocytosis via nonclathrin-coated pathways

Cells treated with inhibitors of clathrin mediated endocytosis (mentioned below) still demonstrate bulk membrane internalisation and the ability to uptake fluid phase markers (such as the enzyme horseradish peroxidase). This lead to the conclusion that there must be some other form of internalisation that does not involve clathrin coated pits. In studies, using human fibroblast cells it was calculated that approximately 50% of fluid was internalised via a non-clathrin mediated route (McKinley & Wiley, 1988). Methods that have been employed to selectively block clathrin mediated endocytosis include, incubation with hypertonic and hypotonic media (Daukas & Zigmond, 1985; Novak *et al.*, 1988), potassium depletion (Carpentier *et al.*, 1989), cytosol acidification (Sandvig *et al.*, 1987), anti-clathrin antibodies (Doxsey *et al.*, 1987), and expression of a temperature sensitive mutant of dynamin (Damke *et al.*, 1995). Recent studies using dynamin temperature sensitive mutants demonstrated that upon shifting to a non permissive temperature, RME was blocked within five minutes (Damke *et al.*, 1995), with fluid phase marker uptake continuing at a much reduced level. However, if these cells were held at the non-permissive temperature for a further thirty minutes, then fluid phase marker uptake returned to wild type levels while RME uptake remained inhibited. This would suggest the balance between clathrin mediated and nonclathrin mediated endocytosis is fluid with each being able to compensate each other to a large degree.

1.5.2.ii.b. Role of caveolae in endocytosis

Caveolae are regular flask shaped invaginations (50-80nm in diameter) of the cell membranes that provide another possible route of cell entry which is not mediated by clathrin coated pits (Van Deurs *et al.*, 1993). They are known to exist in a large number of different cell types for example, fibroblasts and adipocytes (Goldberg *et al.*, 1987; Severs, 1988). It has been suggested that the resulting sealed vesicle may play a role in membrane transport processes (Palade & Bruns, 1968; Schnitzer *et al.*, 1994). It has been postulated that caveolae selectively endocytose macromolecules in specialised cells such as endothelia (Schnitzer *et al.*, 1995), but this point remains open to debate (Van Deurs *et al.*, 1993; Mayor et al., 1994). The cytoplasmic face of the caveolae are often associated with a protein coat consisting in part of caveolin (Rothberg *et al.*, 1992) and being arranged into striations (Peters *et al.*, 1985).

A number of receptor-ligand complexes in transfected cells have been reported to localise in caveolae, at least in certain cell types, but this may be an artefact of over-expression. Included within this list are insulin receptors (Goldberg *et al.*, 1987) β-adrenergic receptors (Raposo *et al.*, 1989) as well as non-receptor bound ligands such as cholera and tetanus toxins (Montesano *et al.*, 1982; Morris *et al.*, 1993). Caveolae are thought to contain high concentrations of certain lipids such as glycolipids, cholesterol and sphingomyelin, as these remain insoluble when the membrane components are treated with detergents such as Triton X-100 (Brown & Rose, 1992). Signalling molecules such as members of the Src-family, kinases and GTP binding proteins are also known to associate with caveolae invaginations. (Sargiacomo *et al.*, 1993; Stefanova *et al.*, 1991). Proteins and lipids associated with the caveolae have been reported to be specifically excluded from the coated pits and to be endocytosed by alternate mechanisms (Bretscher *et al.*, 1980; Rothberg *et al.*, 1990b). The organisation of the caveolar coat is disrupted by sterol-binding agents (Rothberg *et al.*, 1992) and the number of caveolae is reduced in cholesterol depleted cells (Rothberg *et al.*, 1990a). This evidence demonstrates that cholesterol is found at high concentrations within these structures and may have a functional role. To date, it is not known whether this mechanism is employed by the 5-HT_{1A}R or receptors with high sequence homology to the 5-HT_{1A}R.

1.5.3. GPCR resensitisation

Receptor resensitisation is the process that follows desensitisation and allows receptors to re-establish a response to extracellular stimuli. It is presumed that this process involves the internalisation, dephosphorylation and recycling of the receptor, and as yet, the mechanism(s) involved are poorly understood. One proposed mechanism thought to be important, is receptor endocytosis. This process also known as sequestration or internalisation and is thought to be induced by agonist activation of the receptor. The receptor is relocated to an intracellular environment by endosomes where it is dephosphorylated and recycled back to the surface plasma membrane (Ferguson *et al.*, 1996b). These receptors are then deemed resensitised, and are receptive to extracellular signals again. Evidence suggesting that this is the case has been presented by Pippig *et al.*, (1995) Blockade of β_2AR sequestration with Concanavalin A (a lectin isolated from the jack bean plant which has a high affinity

for terminal α-D-mannosyl and α-D glucosyl residues) or 0.6 M sucrose (both hypertonic sucrose and Concanavalin A prevent the formation of clathrin coated pits and thus inhibit endocytosis) prevented receptor dephosphorylation as well as receptor resensitisation. Inhibition of protein phosphatases with calyculin A (an inhibitor of protein phosphatase types 1 and 2A) caused a similar blockade of resensitisation in the muscarinic M_3 receptor in SH-SY5Y human neuroblastoma cells (Szekeres *et al.*, 1998). Monensin (an inhibitor of endosomal acidification) impaired recycling of desensitised β_2AR to the cell surface and prevented receptor resensitisation. . In the case of the β_2AR, it has been suggested that endosomal vesicle acidification results in a conformational change in the receptor structure that is an essential event in the dephosphorylation and resensitisation of the receptor (Krueger *et al.*, 1997). All of this data would indicate that protein kinases promote desensitisation and phosphatases relieve it.

1.6. RECEPTOR REGULATION AT THE LEVEL OF GENE TRANSCRIPTION

GPCRs have the ability to regulate their own expression, the expression of other genes involved in cellular homeostasis, immunity and genes involved in short term receptor function. As a result of the wide-ranging transcriptional activity mediated by GPCRs, the regulation of transcriptional cascades is an area of keen scientific interest.
The 5-HT_{1A}R has been directly linked to three signalling pathways, which result in the regulation of gene transcription. Firstly the activation of the Extracellular regulated kinase (ERK) family MAPK (Garnovskaya *et al.*, 1996), secondly the transcription factor NF-κB (Cowen *et al.*, 1997), and finally CREB (Nishi & Azmitia 1999).

1.6.1. Kinases

Several groups have demonstrated the role of the 5-HT_{1A}R in activating ERK in CHO cells and have identified several intermediary molecules involved in the pathway. The 5-HT_{1A}R has been documented as using a complex signalling pathway, which is also utilised by growth factor receptors (Cowen *et al.*, 1996; Garnovskaya *et al.*, 1996). Receptor activation results in pertussis toxin-sensitive induction of ERK by a $G_{\beta\gamma}$

protein. This directly leads to Shc phosphorylation, which then in turn recruits a lipid kinase and an adapter protein (Grb2), to the signalling complex. The Grb2 complex binds the Ras activating protein Sos leading to Ras activation and sequentially Raf activation. Raf functions by phosphorylating and activating mitogen and extracellular signal regulated kinase, resulting in the eventual phosphorylation and activation of ERK (**Figure 7**; Garnovskaya *et al.*, 1996). Reactive oxygen intermediates (ROI) also have a proposed role in the activation of ERK by the 5-HT_{1A}R. It is thought that an nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme is probably responsible for the production of ROI but the underlying mechanisms remain unknown (Raymond *et al.*, 1999).

The PLC thought to play a part in mediating 5-HT_{1A}R triggered ERK activation uses phosphatidylcholine (PC) as a substrate (Cowen *et al.*, 1996). Evidence in support of this theory showed that 8-hydroxy-DPAT elicited the release of [^{3}H] choline from preloaded CHO cells. Both the release of [^{3}H] choline and ERK activation was attenuated using a PC-PLC inhibitor (tricyclodecan-9-yl-xanthogenate; Cowen *et al.*, 1996). Based on the available data, it appears that the 5-HT_{1A}R activates ERK, via two separate mechanisms that converge to activate Ras (**Figure 7**). ERK activation results in the activation of a number transcription factors, including NF-κB, AP-1 (Dieter *et al.*, 1999), and SP-1 (Chupreta *et al.*, 2000). Other receptors that couple to G_i (for example the human somatostatin$_4$ receptor and the prostaglandin$_{F2\alpha}$ receptor) have demonstrated a proliferative response upon activation of ERK (Sellers 1999; Caverzasio *et al.*, 2000). However, the exact consequences of ERK activation by the 5-HT_{1A}R have yet to be explored.

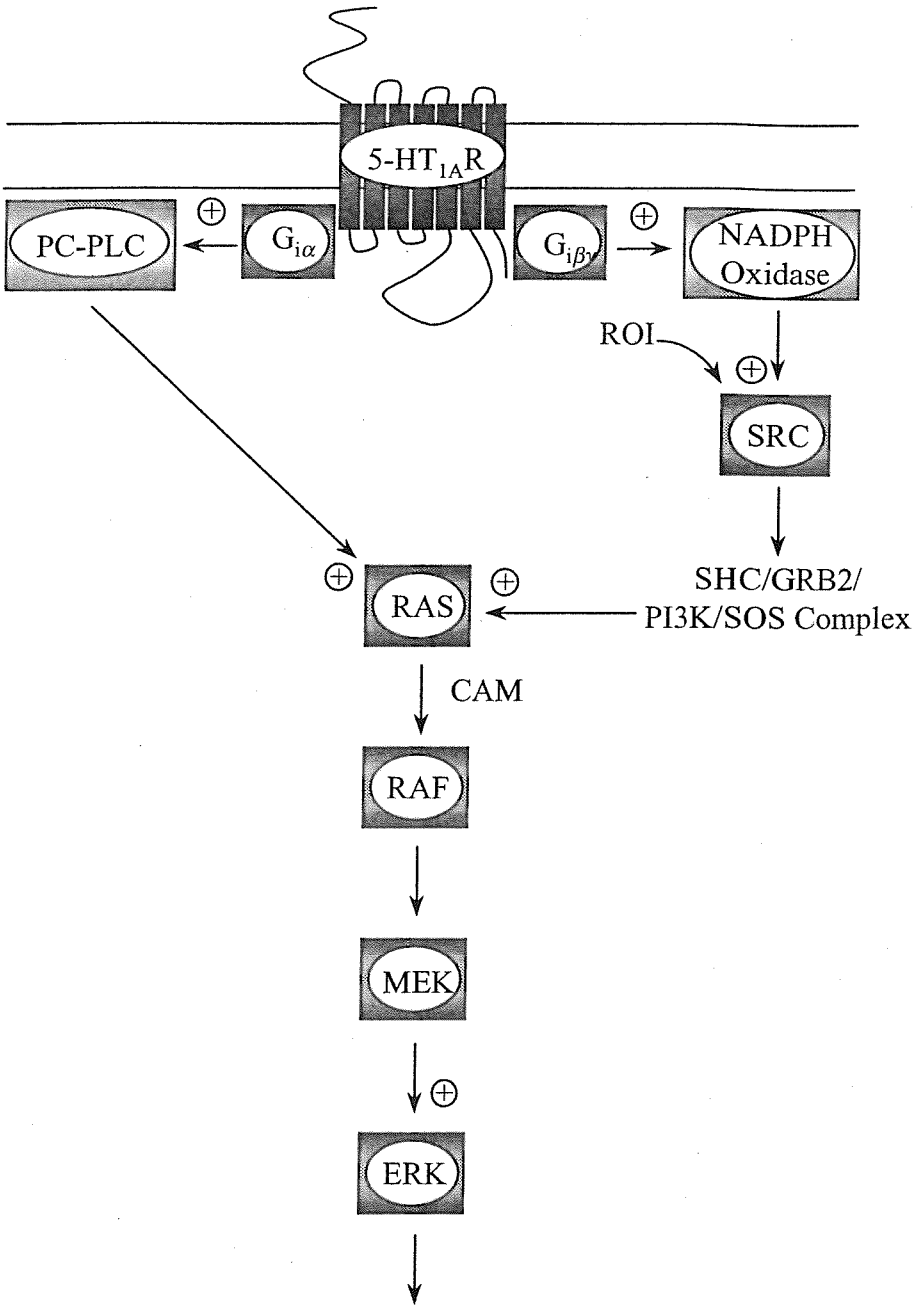


Figure 7: Proposed mechanism of ERK activation mediated by the human 5-HT_{1A}R

Ligand challenge of the 5-HT_{1A}R results in the liberation of $G_{\beta\gamma}$ and $G_{i\alpha}$ which in turn activate NADPH Oxidase and PC PLC. The two separate pathways activated converge on the protein Ras which in turn promotes a chain reaction of phosphorylation ending in the phosphorylation of ERK. ERK in turn is able to activate various transcription factors including NF-κB, SP-1 and AP-1 (Dieter *et al.*, 1999; Chupreta *et al.*, 2000).

1.6.2. NF-κB

NF-κB is a eukaryotic transcription factor that exists in virtually all cell types. It was first described in 1986 as a nuclear factor necessary for transcription of the immunoglobulin kappa light chain in B-lymphocytes (Sen & Baltimore 1986). In mature B cells and plasma cells, NF-κB is localised to the nucleus where it binds a 10 base pair region of the kappa intronic enhancer and activates transcription. NF-κB was initially only thought to exist in mature B cells because sensitive gel-shift assays using the immunoglobulin kappa DNA binding site had failed to bind NF-κB. It was subsequently discovered that the ability of NF-κB to bind DNA was being blocked by an unknown inhibitor (Baeuerle & Baltimore, 1988a; Baeuerle & Baltimore 1988b). It is now known that NF-κB exists in the cytoplasm of most cells in an inactive form bound to the inhibitor protein I-κB. When the complex receives an appropriate signal, NF-κB is unbound from I-κB and translocates to the nucleus where it can influence gene transcription. NF-κB responsive sites have now been found to exist in promoters and enhancers of numerous genes including cytokines, acute phase response proteins and cell adhesion molecules (Grilli *et al.*, 1993; Kopp & Ghosh, 1995). Active NF-κB molecules have been found in mature B cells, plasma cells, macrophages, hepatic stellate cells and CHO cells (Cowen *et al.*, 1997) and some neurones.

1.6.2.i. Structure of NF-κB

NF-κB exists as a dimer and is a member of the "rel" family of proteins. Each family member contains an N-terminal 300 amino acid conserved region known as the rel homology domain (RHD). The region is crucial in DNA binding, dimerisation, nuclear localisation, and interaction with the inhibitory I-κB protein family. The first NF-κB heterodimers described were the p50 and p65 subunits (Kopp & Ghosh, 1995; Verma *et al.*, 1995). These components are still commonly thought of as the archetypal NF-κB despite the diversity of the proteins which belong to the rel family. Two rel proteins make up the motif that contacts with the DNA and it is the slight variations in the 10 base pair consensus sequence, $^{5'}$GGGGYNNCCY$^{3'}$ that is thought to confer selectivity on different rel combinations.

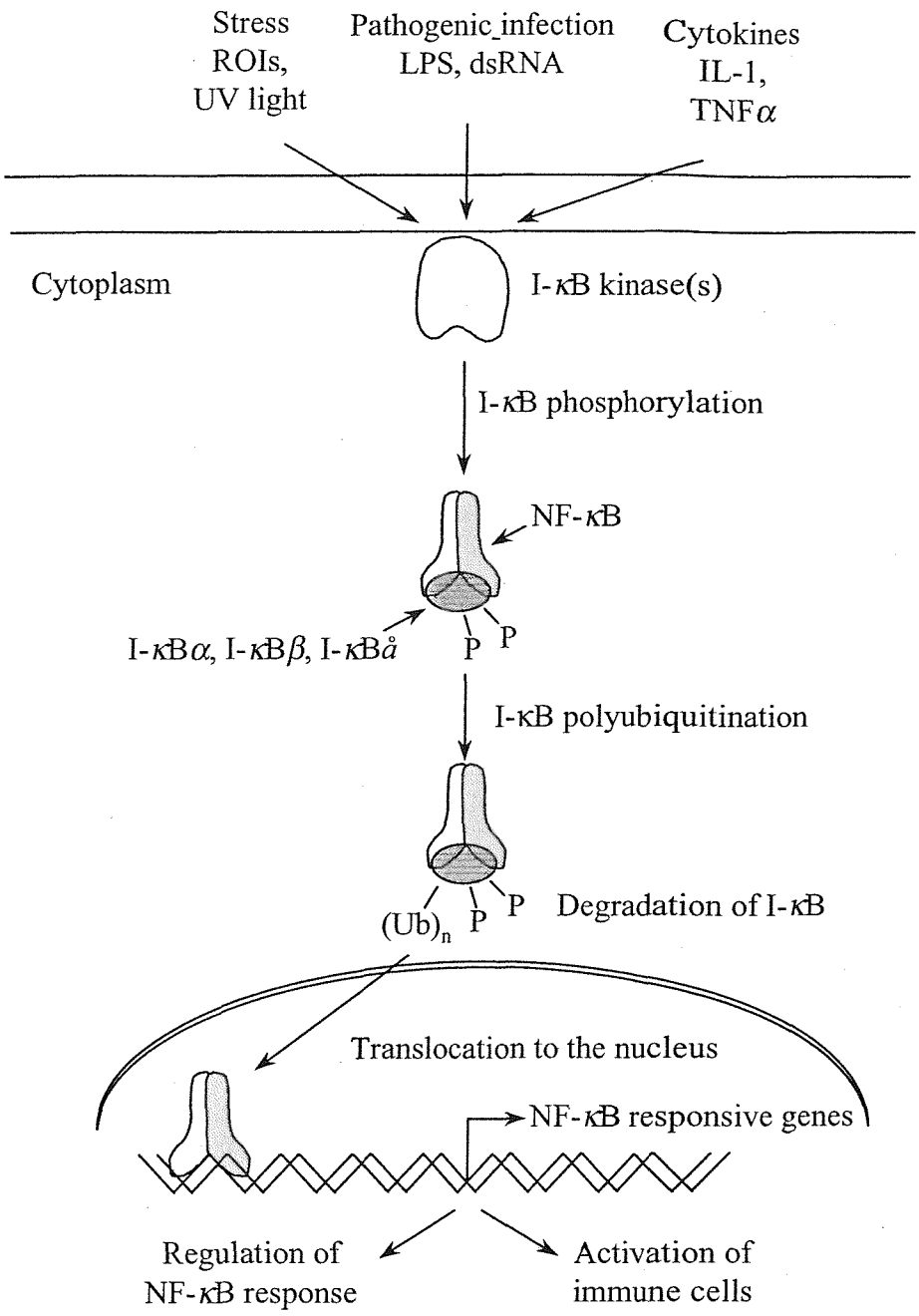


Figure 8: Proposed mechanism for the induction of NF-κB proteins

The NF-κB molecules exist in the cytoplasm in an inactive state, bound to I-κB. Stimulus of an I-κB kinase results in the phosphorlation, polyubiquitionation and eventual break degradation of the I-κB protein. Once activated NF-κB translocates to the nucleus where it can upregulate or downregulate gene transcription depending on the NF-κB dimer involved.

Members of the rel family include, Dif, Dorsal and Relish (all found in Drosophila), v-rel, c-rel, p52 and rel-B. The transcription factor nuclear factor of activated T cells (NFAT) is also homologous in the RHD and is therefore sometimes considered a member of the rel family (Nolan, 1994). Although most of the NF-κB complexes are transcriptionally active (p50/p65, p50/c-rel, p65/p65 and p65/c-rel), some combinations are thought to act as repressors of transcription (p50/p50 and p52/p52). Because p50 and p52 lack a variable C-terminal domain found in the activating rel proteins this domain is suspected to be responsible for transactivation of NF-κB responsive genes.

The first clues as to how rel factors bound DNA was provided by x-ray crystallography (Ghosh *et al.*, 1995; Muller *et al.*, 1995). Each p50 molecule is comprised of two domains linked by a flexible joint. The core of these two domains is made up of β-strands that are similar in pattern to immunoglobulin domains. The p50 subunits dimerise via the C-terminal domain, which has a high number of hydrophobic residues. Both N and C-terminal domains are responsible for DNA binding which is performed by the loops that connect the β-strands. The C-terminal comprises a continuous interface that locks the p50/p50 NF-κB homodimer into the major grove of the DNA (Liu *et al.*, 1994a; Liu *et al.*, 1994b; Toledano *et al.*, 1993).

Recently the DNA binding characteristics and structure of the p65 RHD have also been elucidated. The overall layout is quite similar to that of p50, but the are a few slight differences in that it is not symmetrical. It is also believed that the low sequence specificity of this particular subunit may explain the reason why NF-κB recognises such a wide range of DNA sequences.

1.6.2.ii. Biogenesis

When p50 was eventually cloned, the mRNA was found to encode a much larger protein (approximately 105kDa) than the mature protein. This was also found to be the case with p52, where the mRNA encoded a protein around 100kDa (Bours *et al.*, 1992; Neri *et al.*, 1991; Schmid *et al.*, 1991). Structural analysis of the proteins found that the N terminal regions of the 105kD (p105) and 100kDa (p100) proteins were

identical to p50 and p52 respectively. However the C-terminus was found to contain several ankyrin repeats (areas of protein/ protein interaction) similar to those found in I-κB. It was later demonstrated that p105 and p100 proteins were post-translationally modified in an ATP dependent step to yield p50 and p52 (Blank *et al.*, 1991; Fan & Maniatis, 1991; Mellits *et al.*, 1993; Mercurio *et al.*, 1993). This step may represent another method by which the cell regulates the function of NF-κB, as the immature molecules are held at the cell membrane by the ankyrin repeats until cleaved. At which point they become active and increase the levels of cytosolic p50 and p52 to bind p65 allowing gene transcription to be regulated (Thanos & Maniatis, 1995).

1.6.2.iii. I-κB and its degradation

Activation of NF-κB is largely regulated by the cytoplasmic inhibitor I-κB. I-κB is able to bind to NF-κB and mask its nuclear localisation signal, thus keeping NF-κB in the cytoplasm (Verma *et al.*, 1995; Baldwin 1996). Similar to NF-κB, I-κB is also a member of a larger family of proteins. This family includes I-κBα, I-κBβ, I-κBε and I-κBγ in higher vertebrate cells, and cactus in Drosophila. The I-κB protein subunits are similar to the immature p50 and p52 subunits in that they contain within their sequence, numerous ankyrin repeats. The ankyrin sequences interact with the RHDs to stabilise the complex. Each I-κB differs in the number of ankryin repeats and this number is thought to influence the I-κB selectivity for various RHDs (Gosh *et al.*, 1998).

I-κBα was the first member of the I-κB family to be cloned and the mRNA yielded a protein of approximately 37kDa (Haskill *et al.*, 1991). The amino terminal of the protein is phosphorylated in response to an extracellular signal and the middle portion of the protein consists of a number of ankyrin repeats. The carboxy terminal region of I-κB is involved in the basal turnover of the protein (Verma *et al.*, 1995). As already mentioned the physiological role of I-κB is rapid regulation of NF-κB activity. This is achieved by the participation of I-κB in an autoregulatory feedback loop (Brown *et al.*, 1993). Activation of NF-κB leads to an increase in transcription of a number of genes including the I-κBα gene which has contained within its' own promoter an NF-

κB site (de Martin *et al.*, 1993). This increase in I-κBα levels has the effect of binding any free NF-κB and thus switches off the signal.

I-κBβ was cloned after I-κBα and as a result, less is known about it (Thompson *et al.*, 1995). Immunoprecipitation studies and biochemical purification have shown that the majority of p50/p65 and p50/c-rel complexes are regulated by I-κBα and I-κBβ (Thompson *et al.*, 1995; Whiteside *et al.*, 1997). It was thought that the differences in the two proteins would result in a different selectivity for the NF-κB proteins. I-κBα and I-κBβ were however found to have identical binding characteristics. It was later discovered that the point of selectivity was at the incoming signal and at the timing of the onset and the duration of the response (Thompson *et al.*, 1995). Degradation of I-κB subunits leads to activation of NF-κB, in the case of I-κBα the gene encoding this subunit is also activated and the free NF-κB molecules are bound. I-κBβ on the other hand does not contain an NF-κB site within its promoter which has the effect of prolonging the activity of NF-κB until it is attenuated via another means (Ghosh *et al.*, 1998).

1.6.2.iv. Induction of NF-κB activity

Induction of NF-κB activity is initiated by a wide range of extracellular messengers. As mentioned previously, inducing agents lead directly to the degradation of the inhibitory I-κB molecule, leaving the NF-κB dimer free to interact with the nuclear DNA. This list of inducing agents include those which physically damage the cell, (ROI and ultra violet light) infections (bacterial lipopolysaccaride (LPS), viral transactivating proteins and double stranded RNA) and activators of intracellular signal cascades (e.g. interleukin-1, tumour necrosis factor α (TNFα), sphingomyelin, products of membrane turnover and calcium ionophores).

In the case of the human 5-HT_{1A}R, it has been shown that human T lymphocytes endogenously express the receptor, and the receptor has a demonstrated role in T cell function (Aune *et al* 1993 & 1994). Cowen *et al.*, (1997) demonstrated that agonist activation of the 5-HT_{1A}R in CHO cells resulted in the degradation of I-κBα, but not

I-κBβ leading to an increase in activity of an NF-κB chloramphenicol acetyl transferase (CAT) reporter system (Cowen *et al.*, 1997). To date, no direct link, has yet been shown between NF-κB, the 5-HT_{1A}R and the immune system. Nevertheless, work has been conducted demonstrating that LPS stimulated B lymphocyte proliferation can lead to an increase in the number of plasma membrane 5-HT_{1A}Rs. In turn these newly expressed 5-HT_{1A}Rs can upregulate LPS stimulated B lymphocyte proliferation (Iken *et al.*, 1995). Since LPS is a strong activator of NF-κB activity, this may therefore suggest that 5-HT_{1A}R expression may be regulated by NF-κB. It has been previously shown that CHO cells express endogenous p50 and p65 subunits which functionally bind DNA (Kravchenko *et al.*, 1995).

Protein degradation within a cell, is a very tightly regulated multistage process. It is believed to be initiated and site directed by protein ubiquitination. In the case of I-κBα it is also dependent upon protein phosphorylation. PMA activates PKC which in turn phosphorylates the I-κBα subunit, resulting in the activation of NF-κB (Baeuerle *et al.*, 1988; Sen & Baltimore, 1986b). *In vitro* studies have demonstrated that direct treatment with PKC and PKA resulted in the release of NF-κB (Ghosh & Baltimore, 1990) and translocation of NF-κB to the nucleus (Beg *et al.*, 1993; Miyamoto *et al.*, 1994). Phosphorylation of I-κBα occurs at two serine residues at positions 32 and 36. Site directed mutagenesis studies showed that mutations at these sites prevented protein phosphorylation and subsequent protein break down (Traenckner *et al.*, 1995; Whiteside *et al.*, 1995). The I-κB kinase involved in the phosphorylation processes is as yet, unknown, but PKA, raf-1, PKC and RNA dependant kinase have all been proposed to fill this role as they have been shown to cause dissociation of the NF-κB: I-κB complex *in vitro* (Ghosh & Baltimore, 1990; Diaz-Meco *et al.*, 1994; Kumar *et al.*, 1994; Finco & Baldwin, 1993).

Ubiquitin is a small protein (8.5kDa) present in all eucaryotic cells. This, as already mentioned is a molecular tag for the degradation of proteins. On the I-κBα molecule lysine residues at positions 21 and 22 are ubiquitinated (Chen *et al.*, 1995; Scherer *et al.*, 1995). The actual degradation of ubiquitinated proteins is carried out by a multicatalytic ATP dependent proteasome complex (Ghosh *et al.*, 1998). It is

important to note that phosphorylation and ubiquitination alone are not enough to lead to NF-κB activation, both processes are required for NF-κB activation (Ghosh *et al.*, 1998; see **Figure 8** for overview of NF-κB regulation).

1.6.3. cAMP response element binding protein (CREB)

1.6.3.i. Identification and cloning

The responsiveness of genes to cAMP was discovered in 1986 when Montminy *et al.*, undertook studies examining the regulation of the neuropeptide hormone, somatostatin. Deletion studies in the upstream region of the somatostatin gene identified the 5'-TGACGTCA-3' element as conferring cAMP-inducible transcription. CREB was then purified and identified as a 43kDa cAMP response element (CRE) binding protein (Montminy & Bilezikjian 1987). The isolated CREB protein was able to initiate transcription of somatostatin-CAT fusion gene reporter assay, in rat pheochromocytoma PC12 cells (Andrisani *et al.*, 1989). The findings of Andrisani *et al.*, (1989) provided conclusive proof that the 43kDa CREB protein plays an important role in the activation of the somatostatin gene. CREB is particularly relevant to the study of GPCR regulation as many couple to AC thus each GPCR therefore has an intrinsic ability to regulate CREB activity and hence gene transcription.

Two separate approaches were used to clone CREB. The first reported isolation of the CREB cDNA clone involved the screening of a placental cDNA library using the CRE binding site as a radioactive probe (Hoeffler *et al.*, 1988). The second approach isolated the CREB clone from a PC12 cDNA library. Oligonucleotide probes were synthesised from amino acid sequence information obtained from tryptic digest fragments of purified CREB protein (Gonzalez *et al.*, 1989).

Structural and functional studies performed, demonstrated CREB to be a member of the leucine zipper family of transcription factors (Hoeffler *et al.*, 1988, Gonzalez *et al.*, 1989). The C-terminus of the leucine zipper is involved in homodimerisation, whilst the adjacent basic region is involved in DNA interactions (Santiago-Rivera *et*

al., 1993). The N-terminal domain encodes the transcriptionally active areas of CREB containing two glutamine rich regions (Quinn, 1993). These two regions are contained within an area know as the kinase inducible domain which is the substrate domain for a range of protein kinases (Gonzalez *et al.*, 1989). The glutamine-rich domains are important in basal transcription and interaction with the $hTAF_{II130}$ region of TFIID (Quinn, 1993)

1.6.3.ii. Subcellular localisation of CREB

CREB is found in the nucleus in both the phosphorylated and unphosphorylated states. Transport of CREB into the nucleus is meditated by a nuclear localisation signal (NLS) contained with in the basic region of the protein (Waeber *et al.*, 1991). It is however, unknown if the movement of CREB into and out of the nucleus is in association with other factors. The method of protein degradation would appear to be similar to I-κB in that CREB is first ubiquitinated by the hUBC9 enzyme resulting in S26 proteasome targeting and degradation (Firestein & Feuerstein, 1998; **Figure 9**).

1.6.3.iii. Regulation of CREB DNA binding activity

Within the N-terminus of CREB is the KID domain which allows CREB to be phosphorylated in response to a stimulus. The evidence for CREB phosphorylation in response to a stimulus came from both *in vitro* and *in vivo* studies. Studies using forskolin stimulated PC12 cells demonstrated CREB phosphorylation in the presence of [^{32}P] orthophosphate (Montminy & Bilezikjian 1987). However, CREB phosphorylation was not observed in mutant PC12 cells lacking endogenous PKA activity (Montminy & Bilezikjian, 1987). Studies using CRE affinity purified CREB also showed that CREB was efficiently phosphorylated in vitro by PKA (Montminy & Bilezikjian 1987). The range of protein kinases that are now known to phosphorylate CREB include, GSK-3, (Fiol *et al.*, 1994) Ca^{2+}/ calmodulin dependent protein kinase, types II and IV (Sun *et al.*, 1994) and casein kinase II (deGroot *et al.*, 1993; **Figure 9**).

Relatively few reports exist on the ability of GPCRs to directly modulate CREB activity. However, those that do exist indicate that those receptors coupled to the inhibition and activation of AC both have the ability to regulate CREB. Receptors

that have the ability to regulate AC also have the ability to regulate PKA and therefore CREB. Nishi & Azmitia (1999) demonstrated that pre-treatment of foetal rat hippocampal cell cultures with 8-hydroxy-DPAT blocked the forskolin stimulated increase in phosphorylated CREB immunoreactivity. The results also demonstrated a significant (33%) decrease in 5-HT_{1A}R mRNA levels and protein expression (Nishi & Azmitia, 1999). The results indicated that the decrease in 5-HT_{1A}R mRNA and protein levels were perhaps a direct result of the increase in CREB phosphorylation, suggesting expression of the 5-HT_{1A}R is under the direct regulation of CREB. These changes in receptor mRNA and protein levels could also be reversed by cellular incubation with WAY100635.

Receptors that couple an extracellular stimulus to the activation of PLC like the 5-HT_{2A}R via G_q and the 5-HT_{1A}R via $G_{\beta\gamma}$ also have the potential to modulate CREB activity (Chalecka-Franaszek *et al.*, 1999; Aune *et al.*, 1993). As already mentioned PLC hydrolyses membrane bound PIP_2 to DAG and IP_3. IP_3 acts directly on intracellular calcium stores resulting in an increase in cytosolic Ca^{2+}concentrations. The increased Ca^{2+} concentration activates calmodulin and Ca^{2+}/ calmodulin-dependent kinases. As already mentioned both Ca^{2+}/ calmodulin dependent kinase types II and IV are able to phosphorylate CREB at position Ser_{133}. In the case of the rat 5-HT_{2A}R, incubation with the specific agonist (±)-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane resulted in a time dependent increase in CREB phosphorylation and CREB binding activity (Chalecka-Franaszek *et al.*, 1999). Along with the observed increase in CREB phosphorylation, activity, and binding, an increase in receptor mRNA and protein levels was also noted. These changes could be antagonised by incubating the cells with the 5-HT_{2A}R specific antagonist ketanserin and inhibitors of calmodulin and Ca^{2+}/ calmodulin-dependent kinases (Chalecka-Franaszek *et al.*, 1999).

As well as being regulated by GPCRs as in the case of the 5-HT_{2A}R, CREB is also regulated by mitogen-activated signalling pathways (MASP). These MASP are activated by a number of stimuli, including growth factors, mitogens and cellular stress. Activation of the ERK pathway, the stress activated protein kinase pathway and the p38 stress related pathway (Fanger *et al.*, 1997, Treisman, 1996) are cellular

consequences. The first evidence that CREB was activated by MASP was provided by Ginty *et al.*, (1994). This group demonstrated in PC12 cells that neural growth factor induced c-*fos* transcription as a direct result of CREB phosphorylation at position Ser_{133}. Ginty *et al.*, (1994) also demonstrated a role for a 105kDa CREB kinase in CREB phosphorylation, which was found to be ras dependant. Another protein also found to be important in c-fos activation by neural growth factor was MAPK, this was found to regulate the activity of transcription factors already bound to the serum response element. The 105kDa CREB kinase was discovered to be identical to the ser/thr kinase rsk-2 (Xing *et al.*, 1998; **Figure 9**).

Recent work has also identified a link between CREB activation and cellular stress. Stresses such as TNFα, IL-1, and UV radiation all result in the activation of the p38 pathway (Han *et al.*, 1994, Xing *et al.*, 1998). Studies of CREB phosphorylation found that inhibitors of PKA and expression of mutant forms of Ras did not inhibit the phosphorylation of CREB. The specific inhibitor of the p38 MAPK-activated protein (MAPKAP) kinase-2 pathway, SB203580 was however found to inhibit CREB phosphorylation (Lee *et al.*, 1994). This would suggest that p38 is able to activate a MAPKAP-kinase-2, which is, then in turn responsible for CREB phosphorylation (**Figure 9**).

Evidence for the role of a new 108kDa CREB kinase pathway came from experiments exposing HeLa cells to UV light. Firstly, CREB was identified as activating c-*fos* by mutating the CRE sites in the c-*fos* promoter. The compound SB203580, which blocks CREB phosphorylation, was also found to act on both the MAPKAP-kinase-2 and the 108kDa CREB kinase. It was demonstrated that the MAPKAP-kinase-2 was relatively ineffective at CREB phosphorylation in vitro, but it remains to be shown if the 108kDa CREB kinase can phosphorylate CREB in vivo (Iordanov *et al.*, 1997; **Figure 9**).

1.6.3.iv. The role of CREB in cellular function

CREB is thought to play an important role in cell function because if its ability to regulate the expression of c-*fos* in response to mitogenic activation (Piechaczyk & Blanchard, 1994).

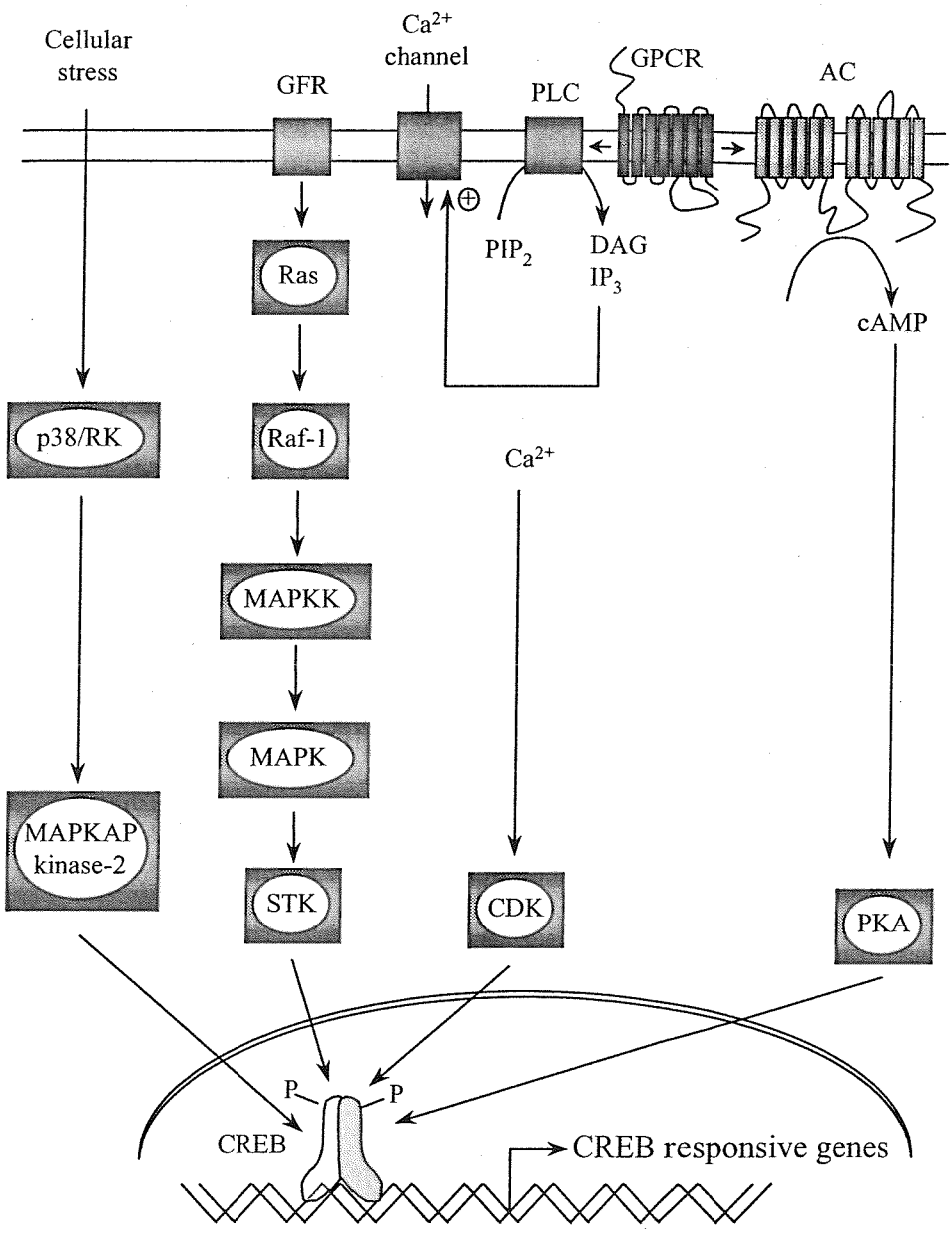


Figure 9: Signalling pathways that activate CREB by phosphorylation at position Ser_{133}

GPCR activation results in either stimulation or inhibition of AC. Changes in cAMP turnover can either activate or inhibit PKA mediated phosphorylation of CREB. Activation of PLC by GPCRs results in an increase in IP_3 turnover which in turn elevates cytosolic Ca^{2+} concentrations, activating Ca^{2+}/ calmodulin dependent kinases, resulting in CREB phosphorylation. Ligand binding to growth factor receptors results in a cascade of protein phosphorylation eventually leading to activation of a serine/threonine kinase , this enzyme is then able to phosphorylate CREB. Cellular stress such as UV light activates p38 kinase and MAPKAP-kinase 2 which eventually phosphorylates CREB.

The role of c-*fos* in cell proliferation and oncogenic transformation is well-documented (Piechaczyk & Blanchard, 1994). Contained within the c-*fos* promoter are three cis acting CRE that are responsive to CREB activation. A mutant form of CREB, that demonstrated no DNA binding characteristics, was able to form heterodimers with wild-type CREB (Ahn *et al.*, 1998). When this mutant form of CREB was transfected into PC12 cells, it attenuated c-*fos* expression. Other mutant forms have also been shown to retard tumour growth and metastatic potential human melanoma cells (Xie *et al.*, 1997). These data suggest that CREB is important in immediate early gene expression. Evidence also exists for the ability of CREB to regulate the expression of various GPCRs. As mentioned previously CREB activity can be regulated by the 5-HT_{1A}R and the 5-HT_{2A}R. In turn, CREB has the ability to regulate receptor transcription (Nishi & Azmitia 1999; Chalecka-Franaszek *et al.*, 1999). The close relationship between receptor and transcription factor may form a negative feedback loop resulting in careful regulation of receptor expression.

1.7. PROJECT AIMS

In 1989 Fargin *et al.*, and subsequently Harrington *et al.*, (1994) explored the intracellular signalling machinery associated with agonist activation of the recombinant human 5-HT_{1A}R receptor. These two studies were a prelude to an investigation into the general principles governing the ways in which "inhibitory" GPCRs might attenuate their signalling processes. Raymond (1991) was the first to show that stimulation of endogenous PKC with phorbol esters could be correlated with phosphorylation of the receptor protein. This result suggested that receptor phosphorylation may represent one mechanism by which the "activity" of this particular receptor could be effectively "switched off". Harrington *et al.*, (1994) took the story one step further by attempting to dissect the signalling pathways responsible for activation of PKC in response to 5-HT_{1A}R agonists. Brief pre-exposure of the recombinant human 5-HT_{1A}R to 8-hydroxy-DPAT had two main consequences: a functional desensitisation characterised by a reduced inhibition of forskolin stimulated AC activity and an apparent decrease in the number of high affinity binding sites (B_{max}). Lembo and Albert (1995) used the recombinant rat 5-HT_{1A}R to explore the mechanistic basis of desensitisation using a site-directed mutagenesis approach. The study employed the use of receptors that had mutations in the putative PKC recognition sites. It was found that the mutations engineered into the 2^{nd} and 3^{rd} intracellular loops of the 5-HT_{1A}R could affect the receptors ability to interact with G proteins and abolished receptor phosphorylation.

1.7.1. Aims 1

- Develop an assay that allows the further study of recombinant 5-HT_{1A}R internalisation characteristics when expressed in a CHO-K1 surrogate cell line.
- To define the response of the 5-HT_{1A}R to short term agonist incubation in terms of changing B_{max} and the cellular pathways involved.
- To define the role of putative phosphorylation sites located on the second and third intracellular loops and 7^{th} transmembrane internalisation motif (NPVIY) in the cellular redistribution of the 5-HT_{1A}R.

It is of course recognised that receptor availability at the plasma membrane may not be due to a transitory receptor redistribution phenomenon. Alterations in gene transcription may reduce the overall pool of receptor protein available to the cell.

1.7.2. Aim 2

- To further define the ability of the unmutated 5-HT_{1A}R expressed in CHO-K1 cells to regulate the activity of the transcription factors NF-κB and CREB in response to short term ligand challenge.

1.8. OBJECTIVES

In order to fulfil Aims part 1, it was important to establish whether the decrease in B_{max} was a consequence of receptor phosphorylation or whether the decrease in number of binding sites occurred via an alternative mechanism. A series of recombinant human 5-HT_{1A}R were generated containing independent mutations in 3 PKC recognition domains. In addition, receptors with residue substitutions in the 7th transmembrane domain were also used in the study since this region of GPCRs has been postulated to play a role in receptor internalisation. It was not known whether the 5-HT_{1A}R could internalise or whether internalisation could occur independently of receptor phosphorylation.

To fulfil aim 2 the approach was to focus on the way in which 5-HT_{1A}R ligands may influence the activity of two ubiquitous transcription factors; NF-κB and CREB. Previous studies have shown the ability of the 5-HT_{1A}R to modulate the activity of the transcription factors NF-κB (Cowen *et al.*, 1997), and CREB (Nishi & Azmitia 1999). However, these studies did not directly link the activation of the receptor to changing transcription factor levels. It was therefore reasoned that the use of 5-HT_{1A}R ligands might help in defining the downstream signalling events that may modulate transcription of the 5-HT_{1A}R gene. In summary, it was hoped that this study would contribute towards an understanding of the mechanisms involved in short and long-term regulation of the cellular availability of 5-HT_{1A}R.

CHAPTER 2

2.1. CELL CULTURE

2.1.1. Maintenance of Chinese hamster ovary cells (CHO-K1) cells expressing recombinant human 5-HT_{1A}Rs

CHO-K1 cells (American Type Culture Collection, USA) were seeded in 90 mm dishes and grown at 37°C in humidified air containing 5% CO_2 under sterile conditions. The adherent cell cultures were grown in Hams F-12 medium supplemented with 5% foetal bovine serum, and 50μg/ ml gentamicin. CHO-K1 cells were stably transfected with plasmid DNA (G-21 clone) encoding both wild type and mutated forms of the human 5-HT_{1A}R (Newman-Tancredi *et al.*, 1992; Page *et al.*, 1996). After the initial transfection of the plasmid cells were grown under high selection pressure (described in the next paragraph) until stably transfected clones remained. The individual clones were isolated with a small rubber ring and picked from the plate and transferred to a separate well and allowed to reach confluence. Radioligand binding analysis was then performed (as described later) to determine the existence of the receptors. Cells were then maintained under a selective pressure to ensure good expression of transfected plasmid DNA.

Each cell line required a separate selection method in order to discriminate between those cells that had been successfully transfected with the foreign DNA and those which had not. The CHO-K1 cells transfected with the unmutated receptors were selected by omission of hypoxanthine and thymidine from the growth medium. The expression plasmid as well as encoding the full-length receptor also encoded the enzyme dihydrofolate reductase (DHFR). This plasmid copy of DHFR is to compensate the CHO-K1 cell line, which has had its endogenous DHFR deleted from the genome. Thus any cell, which has taken up the plasmid encoding DHFR, could grow successfully in medium lacking hypoxanthine and thymidine and was likely to be expressing the 5-HT_{1A}R on the cell surface. The separate selection method employed by Page *et al.*, (1996) involved the use of a toxic eukaryotic antibiotic Geneticin (G418). The expression plasmids incorporating modified forms of the 5-

$HT_{1A}R$ also encoded a gene conferring G418 resistance to those cells that had successfully taken up the plasmid.

In order to allow continued cell growth, cells were diluted and re-seeded through no more than five passages before they were discarded. Cells were re-seeded by first washing the confluent monolayer with 10ml of warmed phosphate buffered saline (PBS, containing in mM: NaCl, 136; KCl, 2.68; Na_2HPO_4, 10; K_2HPO_4, 1.76; pH 7.4) to ensure that all serum bound trypsin inhibitors were removed. 1ml of warmed trypsin-EDTA solution (1X) was added to the dish, which was then placed in a humidified incubator at 37°C. After 2 minutes, the trypsin was promptly deactivated by the addition of 9 volumes of warmed serum-containing growth medium. The cell suspension was then distributed between new 90mm dishes for re-growth.

Several modified forms of the G-21 clone were engineered into CHO-K1 cells; these mutant recombinant receptor DNA were engineered to express receptors containing a single amino acid mutation, or receptors containing mutations of more than one amino acid. The mutations induced were specifically designed to interrupt amino acid sequences thought to be involved with regulation of receptor function or to allow antibody binding (**Table 2**). To generate the mutations in the 5-$HT_{1A}R$ as outlined in **Table 2** the following methods were utilised. Briefly, site directed mutagenesis was performed using a modified, two-step polymerase chain reaction (PCR) method (Page *et al.*, 1996). Primers were used to introduce substitution mutations into the motifs of interest along with a primer designed to flank one of the coding regions of the human 5-$HT_{1A}R$. Reactions were conducted initially with *Taq* DNA polymerase (Promega) and later with *Pfu* DNA polymerase (Stratagene) using conditions recommended by the manufacturers. PCR products were then subjected to a second round of PCR reactions in order to produce full length mutant 5-$HT_{1A}Rs$. Reactions were all performed for 20 cycles at 95°C (1min), 51°C (1min) and 72°C (2min).

The final PCR products were purified, digested and ligated into a eukaryotic expression vector pcDNA3® (Invitrogen). Recombinant constructs were used to transform competent DH5α *E. coli* cells. The entire 5-$HT_{1A}R$ DNA of each positive clone was sequenced in order to verify that the desired mutation had occurred. The

mutated and wild-type receptor constructs were then stably transfected in CHO-K1 cells using the Lipofectin® (Gibco-BRL) transfection system.

2.2. RADIOLIGAND BINDING ANALYSIS

2.2.1. Preparation of whole cell extracts and a membrane enriched fraction.

Each dish of cultured CHO cells (approximately $1x10^6$ cells per dish) was washed with 2x2ml of PBS at room temperature after first having had all growth medium removed. A further 2ml of PBS was added to the dish and the cells were then harvested using a cell scraper. The cell suspension was placed on ice and the dish was washed with a further 2ml of PBS to ensure a maximal cell harvest. The cell suspension could then be used directly or further homogenised. To generate a membrane enriched fraction; the homogenate was centrifuged at 12,000xg for 20 minutes at 4°C. After such treatment, the proteins which were extracted into the supernatant were retained and frozen at -80°C or discarded; the pellets were resuspended in a small volume of ice cold PBS (not more than 2ml) and placed on ice to prevent protein degradation.

2.2.2. Protein estimation

Estimation of the protein concentration of a membrane-enriched fraction was performed using the bicinchoninic acid protein assay (PIERCE, USA). Bovine serum albumin (BSA) of known concentrations (0.2, 0.4, 0.6, 0.8, 1.0mg/ ml) was used to construct a standard curve from which unknown protein concentrations could be estimated. Assays were performed in a final volume of 210μl, at 60°C for 30 minutes according to the manufacturers instructions. The absorbance of samples in the micro-titre plate was read at 562nm and the concentrations of unknowns was extrapolated from the standard curve and expressed in terms of mg protein per ml. After the protein concentration had been estimated, a suitable volume of binding buffer (containing in mM: HEPES, 20; $MgSO_4$, 5; pH 7.4) was added to the protein sample to obtain final protein concentrations of 150μg/ml for [^{3}H] 8-hydroxy-DPAT experiments or in the case of [^{3}H] spiperone binding assays, 500μg/ ml.

Table 2: Location of the modifications to the human 5-HT_{1A}R and their intended purpose (please also refer to Figure 5)

Amino acid sequence and location	Mutation induced	Purpose
K_{147}RTPR (2nd intracellular loop)	T_{149}A	Disrupt phosphorylation of receptor
R_{227}KTVK (3rd intracellular loop)	T_{229}A	Disrupt phosphorylation of receptor
K_{251}KSVN (3rd intracellular loop)	S_{253}G	Disrupt phosphorylation of receptor
R_{340}KTVK (3rd intracellular loop)	T_{342}A	Disrupt phosphorylation of receptor
N_{396}PVIY (7th transmembrane domain	Y_{400}F	Disrupt receptor internalisation
N_{396}PVIY (7th transmembrane domain)	Y_{400}A	Disrupt receptor internalisation
P_{15}PAPFE (N terminal extracellular domain)	A_{17}E, F_{19}E, E_{20}T	Create antibody recognition site

2.2.3. Incubation conditions

750μl (either 150μg total protein in [^{3}H] 8-hydroxy-DPAT assays or 500μg of total protein in [^{3}H] spiperone assays) of a membrane-enriched fraction was incubated in the presence of [^{3}H] 8-hydroxy-DPAT for 1 hour at 30°C or with [^{3}H] spiperone for 2.5 hours at 25°C. The optimal incubation time and temperature was obtained in limited pilot experiments. Non-specific binding was defined by the inclusion of 10μM 5-HT (in incubations using [^{3}H] 8-hydroxy-DPAT) and 100μM unlabelled spiperone (in incubations using [^{3}H] spiperone). For saturation binding analysis [^{3}H] 8-hydroxy-DPAT (125-127Ci/ mmol) was used at final concentrations of 0.625, 1.25, 2.5, 5 and 10nM and [^{3}H] spiperone (15Ci/ mmol) was used at final concentrations of 3.75, 7.5, 15, 30 and 60nM.

2.3. COMPETITION ANALYSIS

Competition analysis was performed in the presence of the non-radiolabelled ligands 8-hydroxy-DPAT, WAY100635, 5-HT and spiperone at final concentrations of $1x10^{-10}$M, $1x10^{-9}$M, $1x10^{-8}$M, $1x10^{-7}$M, $1x10^{-6}$M, $1x10^{-5}$M, $1x10^{-4}$M and $1x10^{-3}$M. Experiments were performed using either [^{3}H] 8-hydroxy-DPAT or [^{3}H] spiperone. During incubations with the non-hydrolysable GTP analogue guanosine 5'-O-(3-thiotriphosphate) (GTPγS), [^{3}H] 8-hydroxy-DPAT was used at a final concentration of 2.5nM.

In experiments using [^{3}H] 8-hydroxy-DPAT as the radioligand, reactions were terminated with 5ml of ice-cold PBS and tubes were then placed on ice. Reaction mixtures were immediately filtered under vacuum using 25mm GF/C filters (Whatman), which had been pre-treated with 0.3% (v/v) polyethylenimine solution for 1 hour to reduce non-specific binding. Filters were subsequently washed three times with 5ml of ice-cold PBS and then placed in scintillation vials with 4ml of liquid scintillation cocktail (toluene containing 0.4% (w/v) PPO and 0.03% (w/v) POPOP). Samples were initially counted for 1 minute in a liquid scintillation counter (1219 Rackbeta, WALLAC) on a suitable tritium programme followed by a repeat count for 5 minutes per sample.

For experiments using [^{3}H] spiperone as the radioligand, a separate method of terminating the reaction was used since it was impractical to filter such a large amount of protein. Microcentrifuge tubes containing the reaction mixture were first centrifuged for 2 minutes at 12,000xg at 4°C. The supernatant was then discarded and the microcentrifuge tubes were then washed twice with ice-cold PBS without disturbing the pellet. This step was to reduce the amount of radioactivity bound to the sides of the microcentrifuge tubes. The bottom of the microcentrifuge tubes (containing the protein pellet) were then cut off and placed in scintillation vials with 4ml of scintillation cocktail. Samples were vortexed then counted for radioactivity in a liquid scintillation counter. Data obtained from the liquid scintillation counter as disintegrations per minute (dpm) were permanently recorded using Microsoft Excel 97 for Windows 98 (Microsoft Corporation).

2.4. RECEPTOR REDISTRIBUTION ANALYSIS

2.4.1. Optimisation of assay conditions

Three separate methods were tested in an attempt to design an appropriate assay to measure receptor redistribution. For initial studies, [^{3}H] 8-hydroxy-DPAT was used at a final concentration of 2.5nM. For the final strategy (see 3.4.1.iii), a range of [^{3}H] 8-hydroxy-DPAT and [^{3}H] spiperone concentrations were used in order to construct complete saturation curves.

2.4.1.i. Method 1

Confluent plates of CHO-K1 cells growing in monolayers were washed in warmed PBS and treated *in situ* with 1μM 8-hydroxy-DPAT, at 37°C, for varying periods of time ranging from 0-60 minutes to attempt to initiate receptor "internalisation" as specified in detail in the legends to **Figure 17**. Reactions were terminated by rapid aspiration of the unlabelled agonist, 8-hydroxy-DPAT followed by incubation in ice to retard the internalisation process. Cells from each plate were then manually harvested and a membrane-enriched fraction was prepared for radioligand binding, as described previously (Sections 2.2).

2.4.1.ii. Method 2

In this case, cells were harvested from confluent plates by trypsinisation (as described previously) and pooled. The intact cell suspension was treated with 1μM 8-hydroxy-DPAT for 1 or 15 minutes to initiate internalisation. At specified time points, aliquots (3ml) were removed and placed on ice prior to membrane preparation and radioligand binding analysis. In some cases, cells were preincubated in the absence or presence of 450mM hypertonic sucrose solution (10ml total volume) for 20 minutes prior to agonist incubation in an attempt to prevent receptor redistribution.

2.4.1.iii. Method 3

This final strategy involved some modifications to Method 2. Cells were first manually harvested using a cell scraper and pooled. Intact cells were then treated with 1μM 8-hydroxy-DPAT for 20 minutes. After this time, the cell suspension was washed twice with 10ml of ice-cold PBS; after each wash, the suspension was centrifuged and the supernatant was discarded. After the final, wash the pellet was resuspended in a small volume of PBS and a membrane fraction was prepared. A full saturation analysis was then performed using either [^{3}H] 8-hydroxy-DPAT or [^{3}H] spiperone. This method was used for all subsequent studies.

2.5. WESTERN ANALYSIS

This technique involves the use of an electrical current to separate proteins on polyacrylamide gels according to their molecular weights and charge. Boiling samples for three minutes in a solution containing Sodium dodecyl sulphate (SDS) and Dithiothreitol (DTT) denatured the proteins. The purpose of SDS is to confer a negative charge onto the proteins whilst DTT breaks disulphide bonds. The resultant polypeptide chains have approximately equal mass to charge ratios and are therefore separated solely based on their molecular weight. Antibodies raised to the specific amino acid sequences of the 5-HT_{1A}R were used to probe the proteins bands following their transfer to nitrocellulose membranes.

Whole cell extracts were prepared from wild type CHO-K1 cells and CHO-K1 cells expressing the unmutated 5-HT_{1A}R by disrupting the cells in "SDS sample buffer" containing (SDS 2% (w/v); DTT 100nM; Tris 60nM; bromophenol blue 0.01%, (v/v); pH6.8). Samples were passed through a 21-gauge needle (in order to disrupt the viscous genomic DNA) and boiled for 3 minutes to break down secondary and tertiary protein structures. Samples (10-30μg protein) were then loaded onto a 4% "stacking gel" (containing 1.2ml 40% acrylamide stock, 3ml 0.5M Tris-HCl (pH 6.8) 0.6 ml 20% SDS 7.26ml H_2O, 100μl 10% APS and 10μl Tetramethylethylenediamine

(TEMED)) and resolved on 9% polyacrylamide gels (6.5x9cm; containing 2.7ml 40% acrylamide stock, 3ml 1.2M Tris-HCl (pH 8.8), 0.6mls 20% SDS, 5.7ml H_2O, 100μl 10% ammonium persulphate (APS) and 10μl TEMED). The protein samples were run under reducing conditions (constant 30mA) using a running buffer (containing in mM: Tris, 27; SDS, 3.8; glycine, 213) until the dye front had reached the bottom of the gel. The stacking gel was cut away and proteins in the resolving gels were transferred to a pre-activated PVDF membrane (Millipore) via a wet transfer process. Non-specific binding sites on the membrane were blocked overnight in "blocking buffer" (containing in mM: NaCl, 150; Tris, 50; Tween, 0.1% (v/v); 3% bovine serum albumin (w/v) pH 7.5) at 4°C.

Polyclonal anti 5-HT_{1A}R antibodies raised in goat (Santa Cruz) were used to probe the proteins on the PVDF membrane, for one hour (diluted to 0.5μg/ml of blocking buffer) at room temperature with gentle agitation. After removal of the primary antibodies, membranes were thoroughly washed for 1 hour with Tris Tween Buffered Saline (TTBS; containing in mM: NaCl, 150; Tris, 50; Tween, 0.1% (v/v); pH 7.5), with changes every 10 minutes. Membranes were then incubated with a secondary antibody (rabbit anti-goat-HRP conjugate; Santa Cruz) diluted 1:8000 in blocking buffer, for 45 minutes at room temperature. The membranes were again thoroughly washed and protein bands were detected using an enhanced chemiluminescent detection system (ECL, Amersham) according to the manufacturers instructions.

2.6. ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

2.6.1. Preparation of nuclear extracts for EMSA

Adherent CHO-K1 cells expressing the unmutated recombinant human 5-HT_{1A}R were washed with 2x2ml ice cold PBS, scraped into 1ml of cold PBS and centrifuged for 1 minute at 10,000 rpm; the supernatants were discarded. Each pellet was then resuspended in 50μl of "Dignam buffer A" (Dignam, 1990; containing in mM HEPES 7.9, $MgCl_2$ 1.5, KCl 10 and DTT 0.5) with 0.2% (v/v) NP40). The pellet was the resuspended by agitating the tube and then microfuged for 30 seconds at 10,000xg. The supernatant was discarded and the pellet was resuspended in 20μl "Dignam buffer C" (Dignam, 1990; containing in mM HEPES 20, NaCl 0.42, $MgCl_2$ 1.5, DTT 0.5, EDTA 0.2, and glycerol 25%) and incubated on ice for 10 minutes, with agitation every 2 minutes during the incubation period. The samples were then microfuged for 30 seconds at 10,000rpm. The supernatant was removed to a clean eppendorf tube, 1μl of the sample was retained separately for protein estimation and the remainder was stored at $^{-}80°$ C.

2.6.2. Preparation of radiolabelled oligonucleotide probes

The oligonucleotides used (NF-κB 5'-AGT TGA GGG GAC TTT CCC AGG-3' and CREB 5'-AGA GAT TGC CTG ACG TCA GAG AGC TAG-3'; Promega) were supplied with the sense strand bound to an antisense strand. The double stranded oligonucletide was 5'-end radiolabelled in a 10μl reaction containing 2.5pmoles oligonucletide, 5μl [γ-^{32}P] ATP (300Ci/ mmol at 10mCi/ ml), 1.6μl 10X forward exchange buffer (containing in mM Tris-HCl 500 pH 7.5, $MgCl_2$ 100, DTT 50 and spermidine 1) and 10 units T4 polynucleotide kinase (Biolabs) incubated at 37°C for 15 minutes. Phenol chloroform extraction and ethanol precipitation were used to purify radiolabelled oligonucleotide. The probe was diluted to 0.1ng/ μl with distilled water and stored at $^{-}20°$ C for up to two weeks.

2.6.3. EMSA incubation conditions and separation of protein-DNA complexes

The electrophoretic mobility shift assay is used to determine the relative concentrations of DNA binding proteins specific to the radiolabelled oligonucleotide used. A radioactive double-stranded oligonucleotide probe containing, a consensus protein-binding motif is incubated with nuclear extract to allow proteins to interact with the probe. The DNA-protein complexes are then resolved by electrophoresis in a non-denaturing polyacrylamide gel to ensure that the complexes remain intact. The bound protein retards the migration of the probe in the gel and the radioactive intensity of each band is proportional to the amount of DNA binding protein.

Nuclear extract of a known protein concentration was diluted in "Dignam C buffer" such that the sample contained approximately 5μg of protein; control samples contained no protein. A mix of 4μl of water and 1μl non-specific DNA competitor (poly dI-dC) for each sample was prepared and added to the nuclear extracts and control sample. Each sample was incubated on ice and after 15 minutes 2μl of radiolabelled oligonucleotide probe (10pg/ ml) was added. All tubes were incubated for a further 15 minutes on ice. 3μl of 6X DNA loading dye (containing 0.25% Bromophenol blue (w/v), 0.25% xylene cyanol FF (w/v), 0.25% Orange G (w/v) and 15% Ficoll type 400 (w/v), was added to each sample and they were loaded onto an 8% non-denaturing gel acrylamide gel (15cm x 15cm).

50ml of 8% gel mix was prepared (containing 10 ml 40% acrylamide stock, 2.5ml 5X TBE 37.5ml distilled water, 500μl 10% APS and 50μl of stock TEMED) and samples were resolved using a 1X Tris-Boric acid-EDTA (TBE) running buffer (containing in mM Tris base 450, boric acid 450 and EDTA 10). Gels were pre-run for 20 minutes under a constant current of 20mA. Samples were loaded and resolved under the same conditions until the dye front had reached the bottom of the gel.

Gels were mounted on Whatman blotting paper and dried for one hour at 80°C under vacuum. Dried gels were exposed to X-ray film and left overnight at -80°C. This first exposure gave an indication of the optimum time needed for subsequent exposures.

2.6.4. *Supershift analysis*

In some cases, samples were subject to "supershift analysis" in order to confirm the identity of the protein. This method involves initially incubating the nuclear protein-DNA complexes with a specific antibody. When subject to resolution on polyacrylamide gels, those complexes that have bound the antibody become retarded on the gel due to the relatively high molecular weight. This strategy therefore allows a visual distinction to be made between samples that were incubated in the presence or absence of the antibody. For supershift assays 1μl of antibody (1ng/ ml) was added to the relevant tubes and incubated on ice for 30 minutes.

2.7. DATA ANALYSIS

All results were expressed as a mean ± standard error of the mean (SEM). Where appropriate, statistical significance was calculated using a two-tailed Students t-test. The degree of confidence interval was set to 95 % in all cases and three levels of significance were employed $P<0.05$ (★), $P<0.01$(★★), $P<0.001$ (★★★), where the latter value was considered the most significant. Saturation curves were transformed to scatchard plots using Microsoft Excel 97 (Microsoft Corporation) and from this transformed data, B_{max} (an estimation of the number of receptors expressed as fmol/ mg) and K_d (an estimation of receptor affinity for the radioligand in nM) values were calculated. In assays where unlabelled ligands were competed with [^{3}H] agonists or antagonists an inhibition constant was calculated (K_i). K_i is defined as the concentration of competing ligand in a competition assay, which would occupy 50% of the receptors if no radioligand were present. In assays where GTPγS was used to inhibit 8-hydroxy-DPAT binding, the results were expressed as an EC_{50}. EC_{50} is defined as a concentration of drug that causes 50% of the maximum possible inhibition of a response of a given drug. Data obtained using the EMSA was analysed visually and therefore, non-quantitatively.

CHAPTER 3

3.1. OPTIMISATION OF CONDITIONS FOR STANDARD RADIOLIGAND BINDING ANALYSIS

3.1.1. Introduction

After the wild-type and mutated variants (see **Table 2** for exact details) of the G-21 clone had been transfected into CHO-K1 cells (as discussed in the methods section 2.1.1.) the characteristics of the 5-HT_{1A}Rs in this particular expression system needed to be assessed and experimental conditions optimised. In order to assess the expression of the various receptor types (including wild-type) at least 10 cell colonies (growing under high selection pressure i.e. G418 sulphate) for each receptor type were rapidly screened for their ability to bind the radioligand, [^{3}H] 8-hydroxy-DPAT (2.5nM). The presence of the receptor type under scrutiny in the CHO-K1-expression system was accepted if the observed non-specific radioligand binding was less than 10% of the observed total ligand binding. This process allowed for an assessment of the success of the transfection process in terms of receptor expression at the plasma membrane. Observations indicated that the level of receptor expression between cell populations exposed to the same construct varied markedly as did the level of expression between cell populations exposed to different constructs. Despite screening over 20 cell colonies, ligand binding could not be detected following transfection with the construct engineered to generate the $T_{342}A$ mutation. Once receptor expression had been established, conditions for further receptor study were optimised (ligand concentrations, incubation time periods and protein concentrations) and the receptor was also characterised in terms of its pharmacological profile and G protein effector coupling.

3.1.2. Optimisation of initial radioligand binding conditions

The aim of the first experiment was to determine the concentration of protein in each assay that would give a suitably high number of counts for a minimal concentration of protein.

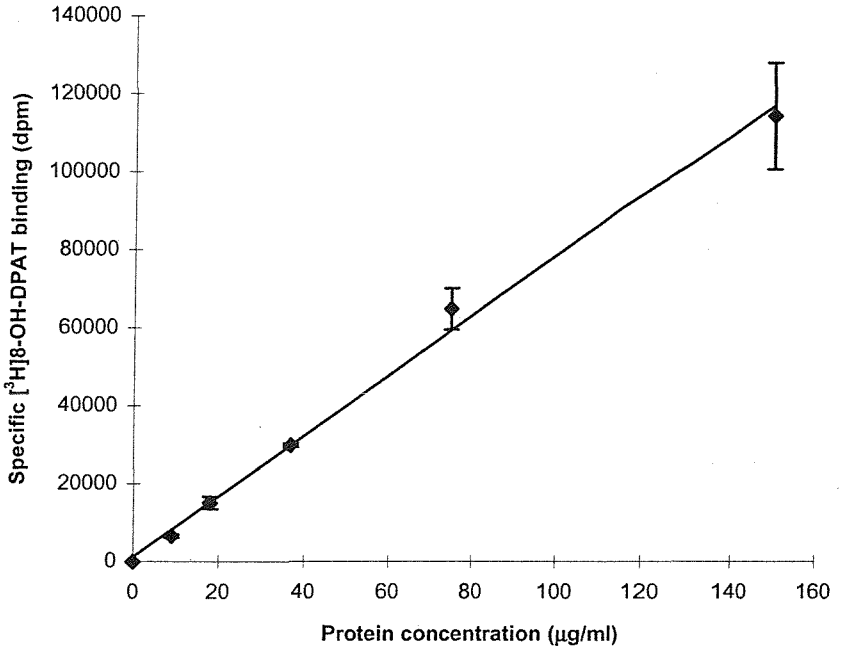


Figure 10: Effect of protein concentrations on binding of [^{3}H] 8-hydroxy-DPAT to the unmutated human 5-HT$_{1A}$R

Whole cell extracts were prepared from CHO-K1 cells expressing the unmutated human 5-HT$_{1A}$R. The specific binding of 2.5nM [^{3}H] 8-hydroxy-DPAT was assessed in the presence of increasing protein concentration. Non specific binding was defined with 10μM 5-HT. Extracts were subjected to radioligand binding analysis as described in detail in section 2.2. Data points are mean values of three separate experiments ± SEM.

An increase in the concentration of CHO-K1 cell protein (10μg/ ml to 150μg/ ml) in the binding assay resulted in an increase in [^{3}H] 8-hydroxy-DPAT binding in a linear manner (**Figure 10**). The protein concentration, which provided approximately 100,000dpm specific binding, was considered reasonable. Consequently, future-binding assays for this receptor employed 100-200μg/ml protein. Once the optimal protein concentration was calculated it was then used in all future experiments including further optimisation experiments. With the optimal protein concentration (giving the most counts per minute for an economically viable amount of protein) known the next experiment performed was aimed at investigating the optimal incubation time period with [^{3}H] 8-hydroxy-DPAThydroxy-DPAT (i.e. that time taken to give suitably high counts per minute in the shortest possible time).

The optimal incubation period was assessed by taking whole cell extract of CHO-K1 cells expressing the human 5-HT_{1A}R (150μg/ ml total protein) and incubating them in the presence of [^{3}H] 8-hydroxy-DPAT for differing time periods (as indicated in the legend to **Figure 11**). Unlabelled 8-hydroxy-DPAT (10μM) was used to define non-specific binding.

During the shorter incubation periods (up to 10 minutes), specific binding increased with time in a linear fashion. During longer periods of incubation (15, 30 and 60 minutes) binding equilibrium was reached. The 30 minute time point was noted as the threshold incubation period required for equilibrium binding (**Figure 11**). With both the optimal protein concentration and optimal incubation period known we were then able to proceed with saturation binding studies to determine receptor expression levels and ligand binding affinity.

3.1.3. Determination of B_{max} and K_d values for unmutated receptors under optimal binding conditions

Receptor expression and ligand affinity can be assessed using a simple radioligand saturation experiment where eventually the amount of radioligand bound does not increase upon further ligand concentration increases.

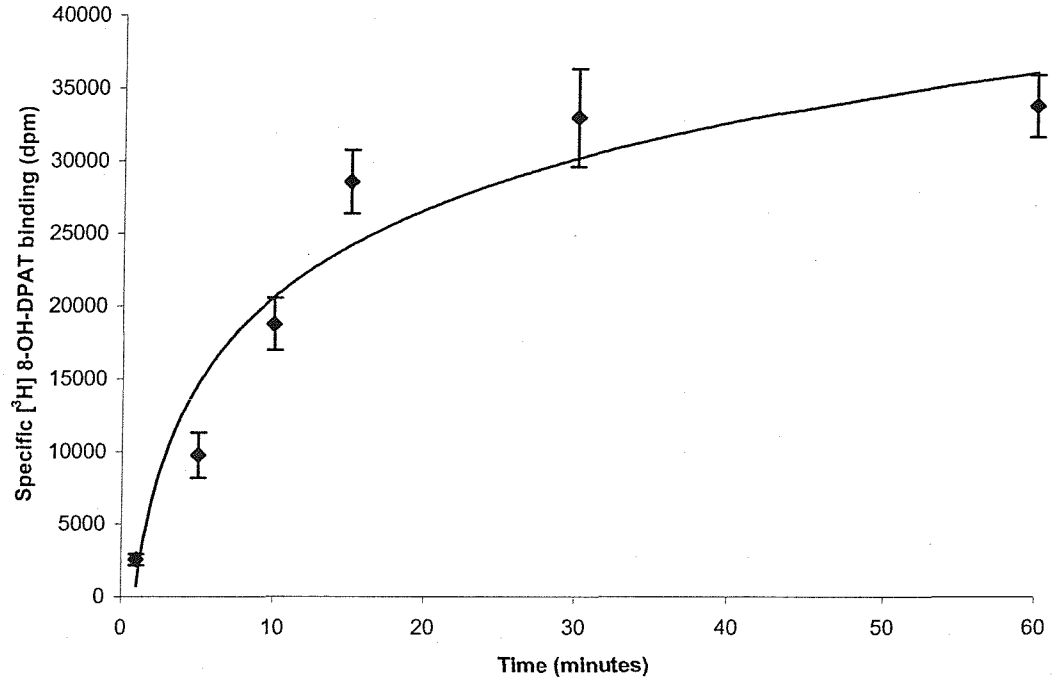


Figure 11: Effect of incubation time on binding of [^{3}H] 8-hydroxy-DPAT to the unmutated human 5-HT$_{1A}$R

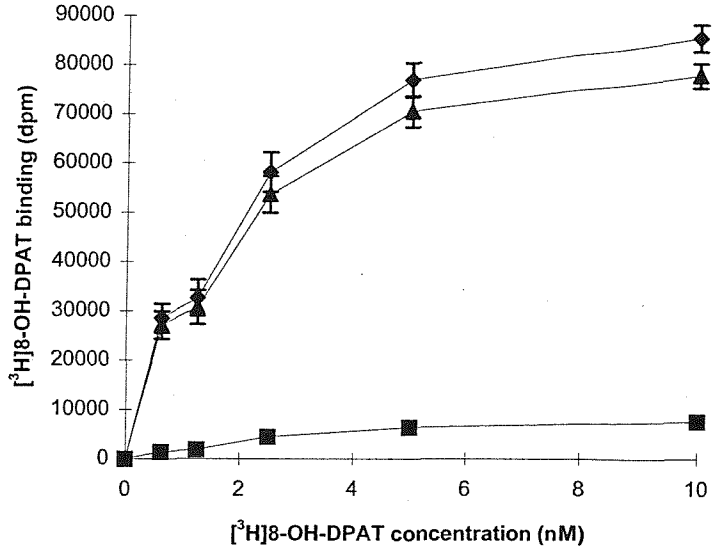
150μg/ ml of whole CHO-K1 cell extracts expressing the unmutated human 5-HT$_{1A}$R was incubated with 2.5nM [^{3}H] 8-hydroxy-DPAT for increasing periods of time. 10μM 8-hydroxy-DPAT was used to define non-specific binding. Extracts were subjected to radioligand binding analysis according to section 2.2. Each data point is the mean value of three separate experiments ± SEM.

As mentioned previously and reported by (Sundaram *et al.*, 1993) whole cell extracts of CHO-K1 cells (150μg/ ml) expressing the full length unmutated human 5-HT_{1A}R demonstrated binding site saturation at final [^{3}H] 8-hydroxy-DPAT concentrations of between 5 and 10nM (**Figure 12**). Non-specific binding (as defined by 10μM 5-HT) was never more than 10% of total binding. Subtracting non-specific [^{3}H] 8-hydroxy-DPAT from total [^{3}H] 8-hydroxy-DPAT at each concentration point giving an indication of specific receptor binding levels. The calculated B_{max} and K_d (B_{max} = 2027.9 ± 149.0fmol/ mg and K_d = 1.89 ± 0.14nM) values indicated a high level of expression in the presence of agonist [^{3}H] 8-hydroxy-DPAT (**Figure 12**). The results also indicated that an adequate range of [^{3}H] 8-hydroxy-DPAT concentrations was employed this particular saturation analysis.

Using the 5-HT_{1A}R antagonist spiperone, whole cell extracts of CHO-K1 cells (500μg/ ml) expressing the full length, unmutated human 5-HT_{1A}Rs were again subjected to radioligand binding analysis. This was deemed and important experiment as in order to be thought of as fully functional the 5-HT_{1A}R expressed in the CHO-K1 surrogate cell system must also be able to bind antagonsists. Using a range of [^{3}H] spiperone concentrations between 3.125 and 50nM as described previously (Methods section 3.2.). The results obtained, demonstrated binding site saturation at [^{3}H] spiperone concentrations of between 25 and 50nM (**Figure 13**). Non-specific binding (as defined by 100μM unlabelled spiperone) was never more than 10% of total binding. The calculated B_{max} and K_d values were 3316.5 ± 239.8fmol/ mg and 15.3 ± 1.5nM respectively (**Figure 13**).

These initial observations that the 5-HT_{1A}R expressed in the CHO-K1 surrogate system bound the agonist, 8-hydroxy-DPAT and the antagonist, spiperone with high affinity the way was paved for the next round of more detailed experiments investigating the pharmacological profile and effector coupling of the 5-HT_{1A}R.

A



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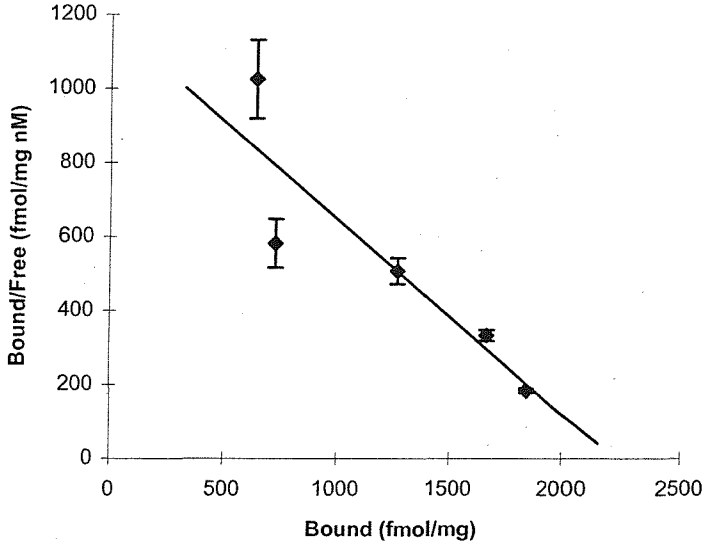
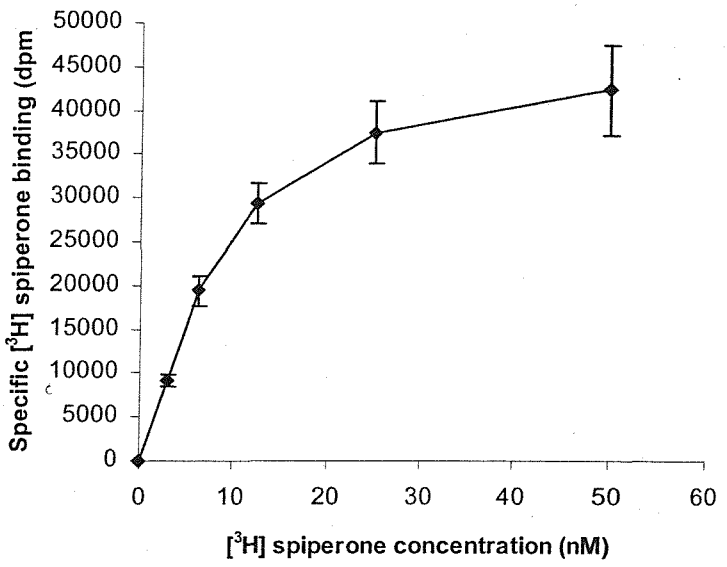


Figure 12: (A); Saturation plot for binding of [^{3}H] 8-hydroxy-DPAT to CHO-K1 cells transfected with the unmutated 5-HT$_{1A}$R and (B); scatchard transformation

(A) Cells transfected with the human 5-HT$_{1A}$R were incubated in the presence of [^{3}H] 8-hydroxy-DPAT (0.625-10nM) as described in the Methods section 2.2. Specific [^{3}H] 8-hydroxy-DPAT binding (●) was calculated by subtracting non-specific [^{3}H] 8-hydroxy-DPAT binding (■ defined using 10μM 5-HT) from total [^{3}H] 8-hydroxy-DPAT binding (◆). All data points are mean values of three separate experiments ± SEM. **(B)** The saturation plot was then linearly transformed (where line gradient represents receptor Kd (nM) and line X-axis intercept represents receptor B_{max} (fmol/mg)). B_{max} = 2027.9 ± 147.0fmol/mg, and K_d = 1.89 ± 0.14nM

A



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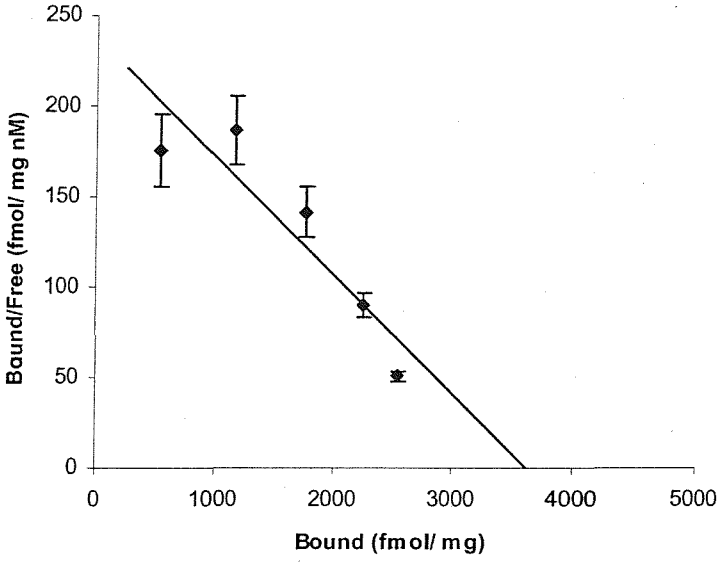


Figure 13: (A); Saturation plot for binding of [^{3}H] spiperone to CHO-K1 cells transfected with the unmutated 5-HT$_{1A}$R and (B); scatchard transformation

(A) Cells transfected with the unmutated 5-HT$_{1A}$R were incubated in the presence of [^{3}H] spiperone (3.125-50nM) as described in the Methods section 2.2. Non-specific [^{3}H] spiperone binding was defined with 100μM cold spiperone. All data points are the average value of three separate experiments ± SEM. **(B)** The saturation plot was then linearly transformed (where line gradient represents receptor K_d (nM) and line X-axis intercept represents receptor B_{max}). B_{max} was calculated to be 3316.5 ± 239.7fmol/ mg and K_d = 15.2 ± 1.5nM.

3.1.4. Pharmacological profiling of the unmutated 5-HT$_{1A}$R

A range of unlabeled 5-HT$_{1A}$R ligands (5-HT (agonist), 8-hydroxy-DPAT (agonist), and WAY100635 (antagonist)) were found to have a concentration dependent, competitive effect on the binding of 2.5nM [^{3}H] 8-hydroxy-DPAT to the full length unmutated 5-HT$_{1A}$R (**Figure 14**). For all three ligands, complete abolition of [^{3}H] 8-hydroxy-DPAT binding was achieved at concentrations above 1μM. No competitive effect was observed on [^{3}H] 8-hydroxy-DPAT binding at concentrations of 1nM and below for WAY100635 or 100pM and below for both 5-HT and 8-hydroxy-DPAT (**Figure 14**). The agonists tested were found to have almost identical K_i values 1.29nM ($\pm$ 0.15nM for 5-HT and $\pm$ 0.22nM for 8-hydroxy-DPAT). The affinity of WAY100635 for the 5-HT$_{1A}$R, was found to be 10 fold less with a calculated K_i value of 13.80 $\pm$ 3.00nM (**Figure 14**).

Unlabelled spiperone was found to have a concentration dependent, competitive effect on the binding of 2nM [^{3}H] spiperone to recombinant 5-HT$_{1A}$R (**Figure 15**). At higher concentrations, (10μM) spiperone was found to exert its maximal competitive effect on [^{3}H] spiperone binding. However, [^{3}H] spiperone binding was never abolished even when the unlabelled spiperone concentration was 10μM. No competitive effect was observed on [^{3}H] spiperone binding at concentrations of 1nM and below for unlabelled spiperone (**Figure 15**). The calculated K_i for unlabelled spiperone was 28.27 $\pm$ 3.00nM.

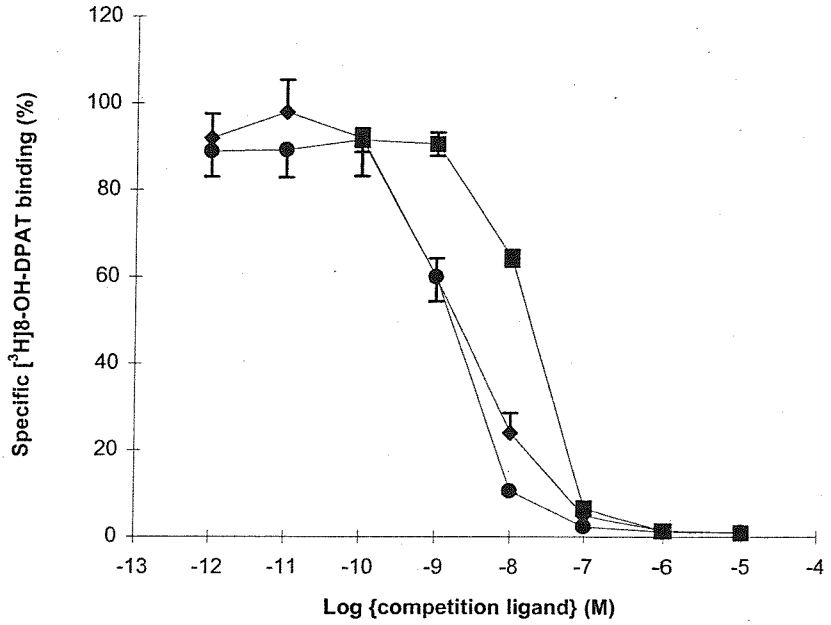


Figure 14: Pharmacological characterisation of recombinant unmutated 5-HT_{1A}R expressed in CHO-K1 cells

A range of unlabeled 5-HT_{1A}R ligands 8-hydroxy-DPAT (●), 5-HT (◆), WAY100635 (■), at various concentrations (1pM-10μM), were competed with [^{3}H] 8-hydroxy-DPAT (2.5nM) for 5-HT_{1A}R binding sites in the standard binding assay described in Methods section 2.3. K_i values were 1.29nM (5-HT ± 0.15nM, unlabelled 8-hydroxy-DPAT± 0.22nM) and 13.80nM (WAY100635 ± 3.00nM). Data points are the mean values of three separate experiments ± SEM.

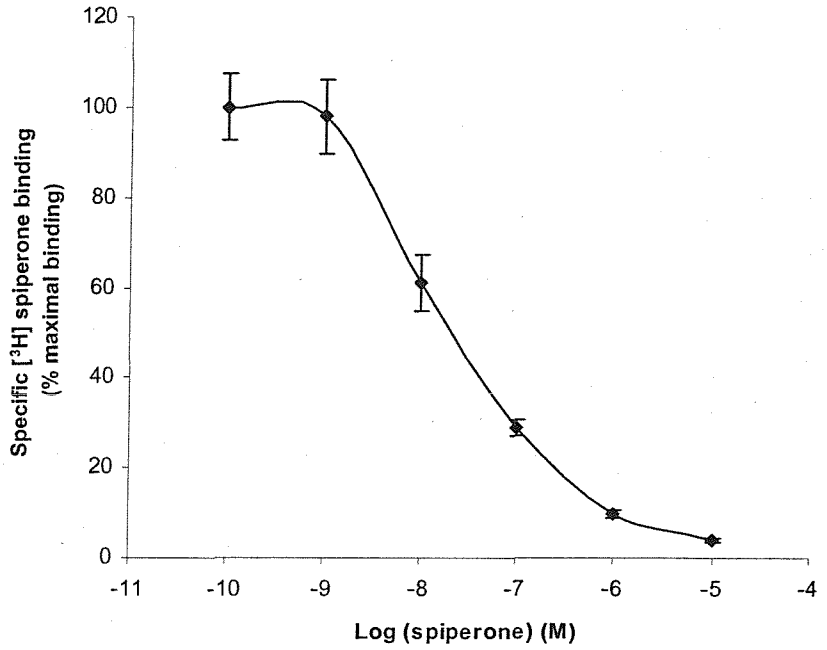


Figure 15: Pharmacological characterisation of unlabelled spiperone competition kinetics on recombinant unmutated 5-HT_{1A}R expressed in CHO-K1 cells

Unlabelled spiperone (100pM-10μM) was competed with [^{3}H] spiperone (2nM) on whole CHO-K1 cell extract, expressing recombinant 5-HT_{1A}R as described in the Methods section 2.3. The calculated $K_i = 28.27 \pm 3.00$nM. All data points are mean values of three separate experiments ± SEM.

3.1.5. Analysis of coupling of the 5-HT$_{1A}$R, to heterotrimeric G proteins

The aim of this experiment was to determine whether the 5-HT$_{1A}$R was able to couple to intracellular heterotrimeric (effector) G proteins. We hypothesised that mutations engineered into intracellular regions would have no effect on receptor/ G protein coupling although they may have an important role in any desensitisation event that occurs after receptor ligand binding and G protein uncoupling. The epitope-tagged receptor was also presumed not to interfere with receptor/ G protein coupling and receptor desensitisation, as the mutations were located extracellularly. It was important to assess the G protein binding characteristics of the unmutated and mutated forms of the receptor to ensure the receptor was functioning in a physiologically relevant manner.

For all three receptor types, the non-hydrolysable GTP analogue, GTPγS appeared to have a competitive effect upon specific [^{3}H] 8-hydroxy-DPAT binding which decreased in a manner that was dependent on GTPγS concentration (**Figure 16**). In the case of unmutated and Y_{400}F mutated 5-HT$_{1A}$R, specific binding was maximally reduced at 10 and 1μM respectively (**Figure 16**), whereas the epitope-tagged 5-HT$_{1A}$R demonstrated no abolition of [^{3}H] 8-hydroxy-DPAT binding at higher GTPγS concentrations (>100μM). The calculated EC_{50} values for each receptor type was as follows: wild type 0.223 ± 0.003μM, Y_{400}F internalisation mutant 0.117 ± 0.005μM and epitope-tagged 1.841 ± 0.195μM (**Figure 16**).

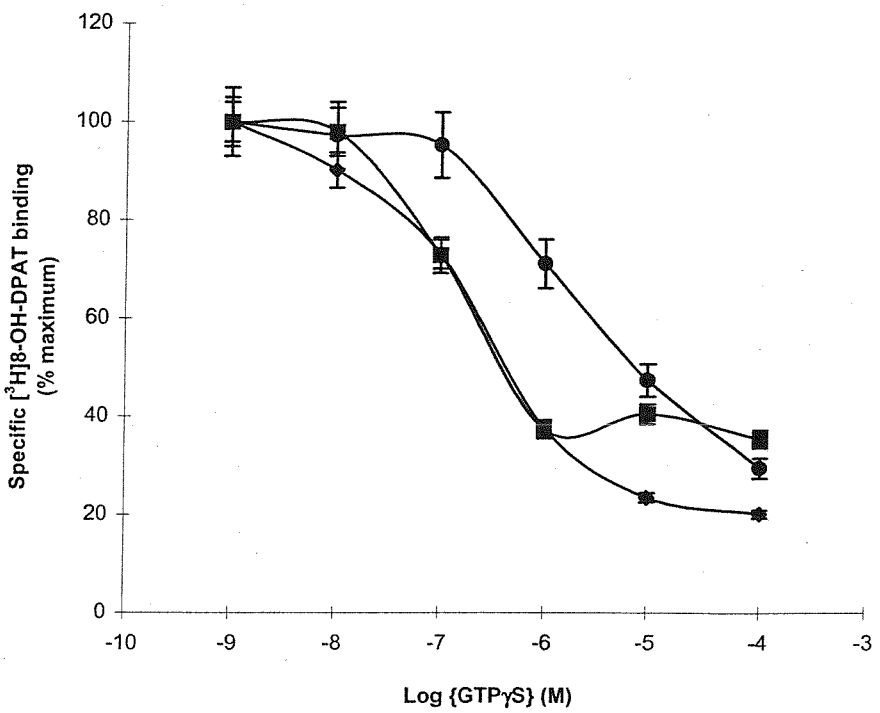


Figure 16: Pharmacological effects of GTPγS on [^{3}H] 8-hydroxy-DPAT binding to the unmutated or epitope-tagged 5-HT$_{1A}$R expressed in CHO-K1 cells

The specific binding of [^{3}H] 8-hydroxy-DPAT (2.5nM) to unmutated (◆), internalisation mutant $Y_{400}A$ (■) and epitope-tagged mutant (●) 5-HT$_{1A}$R was determined in the presence of GTPγS (1nM-100μM). Non specific binding was defined using 10μM 5-HT. The data points are the mean values of three separate experiments ± SEM.

3.2. DETERMINATION OF B_{max} AND K_d VALUES FOR MUTATED RECEPTORS UNDER OPTIMAL BINDING CONDITIONS

3.2.1. Epitope-tagged 5-HT_{1A}R

Site directed Mutagenesis of the G-21 clone was performed at multiple sites in order to obtain a receptor that expressed an epitope-tag ($A_{17}E$, $F_{19}E$, $E_{20}T$). The substitutions were made in the extracellular amino terminal domain ($PPA_{17}PF_{19}E_{20}$) in order to provide for an antibody recognition site. The reason for selecting such an epitope was because of the availability of a rabbit monoclonal antibody produced within the University of Southampton (Dr H. Barton) being supplied at no extra cost. This region of the receptor has no known function in terms of its ability to respond to agonist challenge and it was therefore hypothesised that mutating the receptor at these sites would have a limited effect on receptor characteristics.

Radioligand binding analysis was performed exactly as described in Methods section 2.2. on membrane enriched fractions of CHO-K1 cells expressing the epitope-tagged 5-HT_{1A}R ($A_{17}E$, $F_{19}E$, $E_{20}T$). Linear transformation of saturation data enabled a calculation of B_{max} and K_d. For cells treated with PBS alone (control), the B_{max} was estimated at 155.8 ± 8.3fmol/ mg. The K_d was calculated to be 12.1 ± 0.6nM (**Table 3**).

3.2.2. "Phosphorylation mutants"

Site directed mutagenesis of the G-21 clone was performed in order to obtain a receptor that expressed an amino acid substitution in the second intracellular loop ($T_{149}A$). The substitution was made in one of the domains ($KRT_{149}PR$) recognised as a substrate for PKC.

Radioligand binding analysis was performed exactly as described in Methods section 3.2. on membrane enriched fractions of CHO-K1 cells expressing the mutated ($T_{149}A$) 5-HT_{1A}R. Linear transformation of the saturation data enabled a calculation of B_{max} and K_d. For cells treated with PBS alone (control), the B_{max} was estimated at 303.0 ± 10.6fmol/ mg. The K_d was calculated to be 0.9 ± 0.1nM.

A second independent mutation resulted in a receptor that expressed an amino acid substitution in the third intracellular loop ($T_{229}A$). The substitution was made in one of the domains ($RKT_{229}VK$) recognised as a substrate for PKC (and possibly PKA). Radioligand binding analysis was performed exactly as described in Methods section 3.2. on membrane enriched fractions of CHO-K1 cells expressing the mutated ($T_{229}A$) 5-HT_{1A}R. Linear transformation of saturation data enabled a calculation of B_{max} and K_d. For cells treated with PBS alone (control), the B_{max} was estimated at 384.5 ± 26.5fmol/ mg. The K_d was calculated to be 2.0 ± 0.5nM.

A third, independent mutation gave rise to a receptor that expressed an amino acid substitution in the third intracellular loop ($S_{253}G$). The substitution was made in one of the domains ($KS_{253}L$) recognised as a substrate for PKC.

Radioligand binding analysis was performed exactly as described in Methods section 3.2. on membrane enriched fractions of CHO-K1 cells expressing the mutated ($S_{253}G$) 5-HT_{1A}R. Linear transformation of saturation data enabled a calculation of B_{max} and K_d. For cells treated with PBS alone (control), the B_{max} was estimated at 545.8 ± 31.0fmol/ mg. The K_d calculated to be 7.9 ± 2.4nM. A summary of all values obtained using the phosphorylation mutants can be seen in **Table 3**.

3.2.3. "Internalisation mutants"

Site directed mutagenesis of the G-21 clone was performed in order to obtain a receptor that expressed a conservative amino acid substitution in the seventh transmembrane domain ($Y_{400}F$). The substitution was made in one of the domains ($NPVIY_{400}$) recognised as a putative internalisation motif (NPX_nY).

Radioligand binding analysis was performed exactly as described in Methods section 3.2. Using membrane enriched fractions of CHO-K1 cells expressing the mutated ($Y_{400}F$) 5-HT_{1A}R. Linear transformation of saturation data enabled a calculation of B_{max} and K_d. For cells treated with PBS alone (control), the B_{max} was estimated at 104.5 ± 6.4fmol/mg. The K_d was calculated to be 2.7 ± 0.3nM.

A second independent mutagenesis of the G-21 clone was performed in order to obtain a receptor that expressed as non-conservative amino acid substitution in the seventh transmembrane domain ($Y_{400}A$). The substitution was made in the same domain as the conservative mutation in the putative internalisation motif ($NPVIY_{400}$). Radioligand binding analysis was performed exactly as described in section 3.2. using membrane enriched fractions of CHO-K1 cells expressing the mutated ($Y_{400}A$) 5-$HT_{1A}R$. Linear transformation of saturation data enabled a calculation of B_{max} and K_d. For cells treated with PBS alone (control), the B_{max} was estimated at 78.6 ± 0.5fmol/ mg. The K_d was calculated to be 5.8 ± 0.6nM. A summary of all values obtained using "internalisation defective" receptors is provided in **Table 3**.

3.3. DISCUSSION

To allow us to study functional characteristics of the 5-$HT_{1A}R$ and the mechanism involved in its regulation it was necessary to optimise the experimental conditions for use of the CHO-K1 cell line. It should be noted that the CHO-K1 cell line has been previously used for studies of 5-$HT_{1A}R$ signalling mechanisms (Fargin *et al.*, 1989) and their associated desensitisation characteristics (Raymond, 1991). However, it is important to note that these data provide a framework for understanding whether receptor-based systems undergo desensitisation rather than the receptor themselves. Moreover, a valid reason for the cautious interpretation of early receptor signalling data is the important finding that CHO-K1 cells also express endogenous 5-$HT_{1B}Rs$ which adds a further level of complexity to data interpretation (Kemp *et al.*, 1999; George *et al.*, 1997).

Since one of the objectives of this study was to determine the mechanistic basis of a decrease in B_{max} following pre-exposure to agonist, strategies were employed which would allow for quantification of residual receptors at the plasma membrane and possibly visualisation of redistributed receptors after exposure to agonist. In this way, we hoped to assess the contribution of special intra-receptor amino acid sequences to their agonist-mediated fate.

Table 3: A summary of B_{max} and K_d values calculated from [^{3}H] 8-hydroxy-DPAT binding studies

Mutation type	B_{max} (fmol/mg)	K_d (nM)
Unmutated	2027.9 ± 147.0	1.9 ± 0.1
Phosphorylation Loop 2 $T_{149}A$	303 ± 10.6	0.9 ± 0.1
Phosphorylation Loop 3i $T_{229}A$	384.5 ± 26.5	2.0 ± 0.5
Phosphorylation Loop 3ii $S_{253}G$	545.8 ± 31.0	7.9 ± 2.4
Internalisation $NPVIY_{400}F$	104.5 ± 6.4	2.7 ± 0.3
Internalisation $NPVIY_{400}A$	78.6 ± 0.5	5.8 ± 0.6
Epitope-Tagged	155.8 ± 8.2	12.1 ± 0.6

3.3.1. Determination of B_{max} and K_d for full length recombinant 5-HT_{1A}Rs

The initial experiments using the radioligands [^{3}H] 8-hydroxy-DPAT and [^{3}H] spiperone both demonstrated receptor saturation kinetics, when using optimal protein concentrations and incubation times, as concluded from other experiments. These findings provided further proof that the receptor expressed in the CHO-K1 cell line is expressed at acceptable concentrations (high B_{max}) for this study and with good ligand affinities (K_d in low nanomolar range).

3.3.2. Competition analysis

Using the non-hydrolysable analogue of GTP (GTPγS) we were also able to demonstrate the coupling of the unmutated 5-HT_{1A}R, the epitope tagged receptor, and the Y_{400}F mutant receptor to heterotrimeric G proteins. As the concentration of GTPγS increased it directly inhibited the binding of [^{3}H] 8-hydroxy-DPAT to the 5-HT_{1A}R by occupation of the G protein GTP binding pocket. GTPγS causes the uncoupling of the receptor from its target G protein, mimicking receptor desensitisation resulting in decreased affinity of the receptor for the ligand (Sundaram *et al.*, 1993). In this study, the exact nature of the receptor/ G protein coupling was unknown but it is likely to be G_i/ G_o based on the evidence presented by numerous research groups (Newman-Tancredi *et al.*, 1992; Harrington *et al.*, 1994; Albert *et al.*, 1996). The involvement of the putative $G_{i/o}$ heterotrimer could be further investigated by testing the ability of the 5-HT_{1A}R to inhibit forskolin stimulated cAMP. If the inhibition were indeed sensitive to pertussis toxin, the coupling of the 5-HT_{1A}R to $G_{i/o}$ would be confirmed. Further analysis of the receptors pharmacological profile also provided further evidence of receptor functionality.

Competition of unlabelled ligands with both [^{3}H] 8-hydroxy-DPAT and [^{3}H] spiperone demonstrated that the receptor was able to bind a range of ligands with nanomolar affinities for both agonists and antagonists. The high receptor affinity for the ligands indicates that the receptor was likely to be correctly inserted in the plasma membrane resulting in the correct structure of the ligand-binding pocket. These findings presented are not novel in terms of scientific discoveries but it was vital to establish that they were in agreement with data already published on the

characteristics of the 5-HT_{1A}R (Sundaram *et al.*, 1993; El Mestikawy *et al.*, 1991; Newman-Tancredi *et al.*, 1992). The results demonstrate clearly the ability of the receptor to bind a range of ligands including agonist and antagonist to saturation and that the 5-HT_{1A}R also is able to couple to G proteins to modulate an intracellular effector system. The early experiments also allowed familiarity with culture techniques and cell growth periods. The latter was important in planning the experiments, i.e. the number confluent dishes of cells that were required for each experiment.

It is important to note that large cell yields were required for each experiment and since cells were grown through a limited number of passages, every experiment involved considerable planning. With the experimental conditions optimised and the CHO-K1 cell line expressing the 5-HT_{1A}R at high levels and with acceptable binding characteristics, we could begin to look in detail at the regulation of availability of receptors at the plasma membrane following short-term agonist pre-incubation.

CHAPTER 4

4.1. DEVELOPMENT OF AN ASSAY TO MEASURE RECEPTOR REDISTRIBUTION

4.1.1. Introduction

With the initial experiments aimed at optimising experimental condition for the study and characterising the recombinant 5-HT_{1A}R expressed in CHO-K1 cells complete, the early forays into the study of receptor regulation could begin. Previous studies by Raymond (1991) and Harrington *et al.*, (1994) suggested that stimulation of endogenous PKC by phorbol esters resulted in 5-HT_{1A}R phosphorylation and consequently desensitisation, blockading further signalling activities. Harrington *et al.*, (1994) also showed an apparent loss of high affinity [^{3}H] 8-hydroxy-DPAT binding sites upon agonist incubation. The putative PKC and PKA residues thought to play a role in receptor desensitisation as demonstrated by Raymond (1991) and Harrington *et al.*, (1994) were investigated further by Lembo & Albert (1995). They demonstrated using site-directed mutagenesis, a role for putative PKC and PKA sites (located in the 2nd and 3rd intracellular sequences of the 5-HT_{1A}R), in receptor/ G protein interaction. The compelling evidence presented by these three studies warranted further study of the 5-HT_{1A}R. In this study we set out to investigate the role of the aforementioned PKA and PKC sites in receptor regulation especially internalisation. In order to successfully complete this task, an accurate assay to measure the redistribution of the 5-HT_{1A}Rs upon agonist activation needed to be devised. This chapter is concerned with the early attempts to log receptor redistribution. Exact methodological details can be found in sections 3.2. and 3.4.1.i-ii.

4.1.2. Method 1

Initial experiments were aimed at testing the effectiveness of the first receptor internalisation assay for a range of receptor types (unmutated, epitope-tagged and $Y_{400}F$ internalisation mutant) as outlined in the methods section 3.4.1.i. Briefly, confluent plates of CHO-K1 cells growing in monolayers were washed in warmed PBS and treated *in situ* with 1μM 8-hydroxy-DPAT, at 37°C, for varying periods of

time ranging from 0-60 minutes. This agonist incubation step was performed in order to initiate receptor "internalisation". Reactions were terminated by rapid aspiration of the unlabelled agonist, 8-hydroxy-DPAT followed by incubation on ice to retard the internalisation process. Cells from each plate were then manually harvested and a membrane-enriched fraction was prepared for radioligand binding, as described in section 3.2. Membrane-enriched fraction of CHO-K1 cells expressing unmutated or epitope-tagged modifications of the 5-HT_{1A}R demonstrated a significant time-dependent decrease in [^{3}H] 8-hydroxy-DPAT binding to 51.4 ± 4.6 % (P<0.05) and 28.8 ± 2.7 % (P<0.001) of control, in response to 1μM 8-hydroxy-DPAT preincubation (**Figures 17A** and **17B** respectively) after 5 minutes. These two receptor types reached a maximum decrease in specific [^{3}H] 8-hydroxy-DPAT binding within 15 minutes (19 0 ± 9.3 %, P<0.05 and 15.3 ± 8.1%, P<0.05; of control respectively). In contrast for those cells expressing the Y_{400}F internalisation mutant 5-HT_{1A}R, the time taken for [^{3}H] 8-hydroxy-DPAT to decrease by 30% was approximately 20 minutes, while the maximum decrease in specific binding was achieved after a 60 minute incubation period (35 ± 12.8%; P<0.05; **Figure 17C**).

It proved necessary to modify the method outlined in section 3.4.1.i. as the standard error of the mean observed at each data point was greater than 20% in most cases hence the lack of error bars in **Figure 17A, B, and C**. However, the initial trend lines did indicate a redistribution of receptor proteins from the cell surface to an intracellular compartment. Due to the large standard error, modifications were made to Method 1 (3.4.1.i.) resulting in the evolution of Method 2 (3.4.1.ii.)

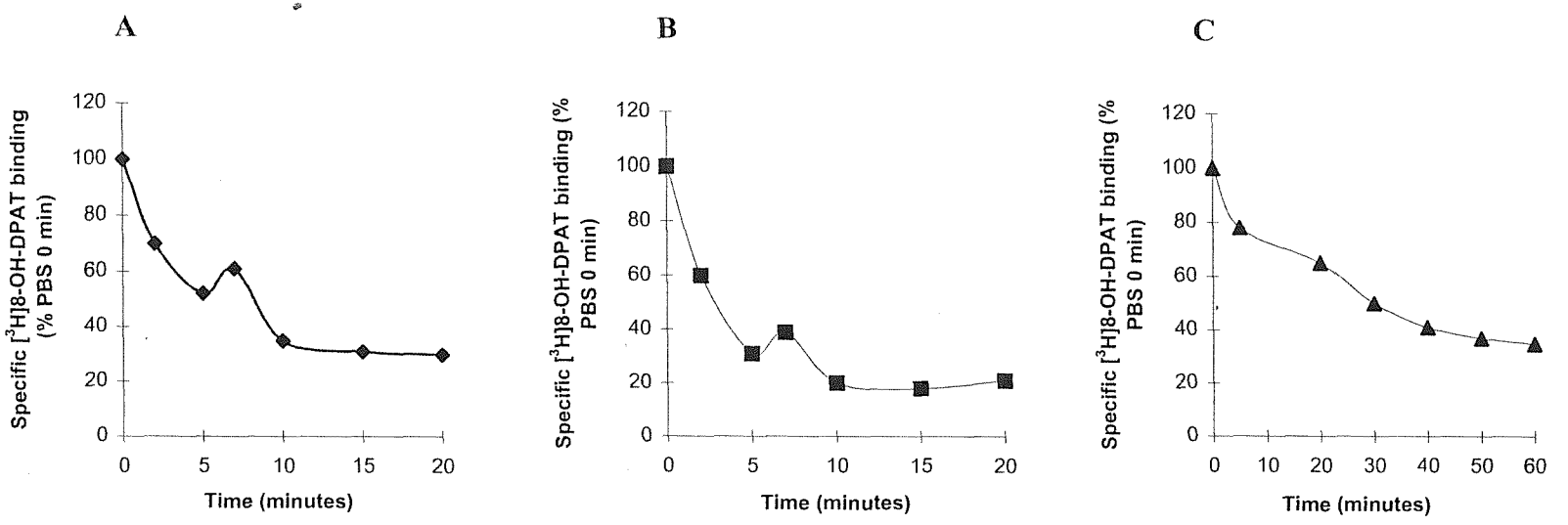


Figure 17: Time dependent decrease in specific binding of [^{3}H] 8-hydroxy-DPAT to the 5-HT$_{1A}$R expressed in CHO-K1 cells, in response to preincubation with 8-hydroxy-DPAT

CHO-K1 cells expressing unmutated (**A**), epitope-tagged (**B**) or putative "internalisation defective" (**C**) receptors were first preincubated *in situ* with 1μM 8-hydroxy-DPAT for periods up to 60 minutes. Cell extracts were subjected to binding analysis as described in section 3.2. Data were normalised a percentage of control values (cells incubated with PBS for the exact same time course) and are expressed as mean values of three separate experiments.

4.1.3. Method 2

In this case, cells were harvested from confluent plates by trypsinisation (as described previously) and pooled. The intact cell suspension was treated with 1μM 8-hydroxy-DPAT for 1 or 15 minutes to initiate internalisation. At specified time points, aliquots (3ml) were removed and placed on ice prior to membrane preparation and radioligand binding analysis. In some cases, cells were preincubated in the absence or presence of 450mM hypertonic sucrose solution (10ml total volume) for 20 minutes prior to agonist incubation in an attempt to prevent receptor redistribution. Antagonism of receptor redistribution was deemed an important experiment, as it would provide further evidence in the case for receptor internalisation.

4.1.3.i. "Pooled" preincubation of CHO-K1 cells expressing the unmutated 5-HT_{1A}R (Method 2)

As with experiments described earlier preincubation of CHO-K1 cells expressing the unmutated 5-HT_{1A}R with 1μM 8-hydroxy-DPAT resulted in a significant decrease in specific binding at both 1 (to 37.8% of control) and 15 (to 38.7% of control) minute time periods ($P \leq 0.001$ and $P \leq 0.001$ respectively; **Figure 18**). The decrease in [^{3}H] 8-hydroxy-DPAT binding upon short-term agonist incubation was thought to represent receptor redistribution. Hypertonic sucrose solutions were then used in an attempt to reverse the observed decrease in specific [^{3}H] 8-hydroxy-DPAT receptor binding in response to agonist preincubation.

Hypertonic sucrose solutions are thought to elicit their effect due to an increased external solute concentration, disrupting the osmotic potential of the cell causing a net loss of water and therefore disrupting of subcellular machinery. In experiments looking at the effects of hypertonic sucrose in conjunction with agonist challenge on the unmutated 5-HT_{1A}R no significant difference in specific [^{3}H] 8-hydroxy-DPAT binding was observed when cells were incubated with PBS (control) for either 1 or 15 minutes (in the absence or presence of 450mM sucrose solution). These data were therefore normalised with respect to specific [^{3}H] 8-hydroxy-DPAT binding with PBS (in the absence or presence 450mM sucrose) 1 minute. All absolute control values were set to 100% ± SEM and the remaining results could be expressed as a percentage

of this allowing direct comparison of various cell treatments. This was the case for all receptor types (unmutated, epitope-tagged and the $Y_{400}F$ mutant 5-$HT_{1A}R$).

For those cells preincubated in the presence of 450mM sucrose the specific [^{3}H] 8-hydroxy-DPAT binding significantly decreased to 43.4 % ($P \leq 0.01$) at 1 minute and 48.6 % ($P \leq 0.001$) at 15 minutes compared to control (**Figure 18**). These values were higher and significantly different ($P \leq 0.05$ and $P \leq 0.01$ respectively) from those obtained for 8-hydroxy-DPAT preincubations alone (**Figure 18**). Decreases in specific binding for those cells preincubated with 8-hydroxy-DPAT in the presence of sucrose were not significantly different from each other at 1 and 15 minutes. In conclusion, it was found that sucrose significantly attenuated the decrease in [^{3}H] 8-hydroxy-DPAT binding in response to preincubation with the unlabelled agonist (**Figure 18**).

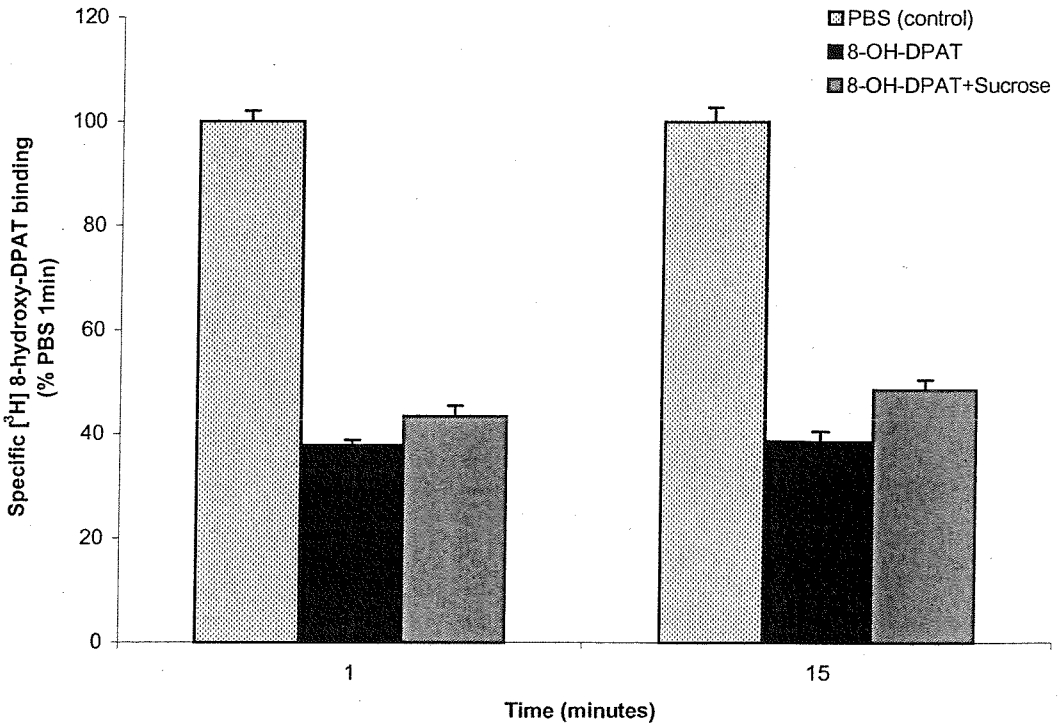


Figure 18: Effect of unlabelled agonist on time-dependent redistribution of the unmutated 5-HT_{1A}R

CHO-K1 cells expressing the unmutated 5-HT_{1A}R were treated with PBS in the absence or presence of 450mM sucrose for 20 minutes. Cells were then incubated with PBS in the absence or presence of 1μM 8-hydroxy-DPAT to stimulate receptor redistribution and in the absence or presence of 450mM sucrose for 1 or 15 minutes. After such time, washing the cells with ice cold PBS stopped the reaction and specific [^{3}H] 8-hydroxy-DPAT binding was determined as described in Methods section 3.2. Data were normalised by expressing them as a percentage of the corresponding PBS control value. The data points are mean values of three separate experiments ± SEM.

4.1.3.ii. "Pooled" preincubation of CHO-K1 cells expressing the epitope-tagged 5-HT_{1A}R (Method 2)

Preincubation with 1μM 8-hydroxy-DPAT resulted in a significant decrease in specific binding at both 1 (46.8 %) and 15 (29.1 %) minute time periods ($P \leq 0.001$ and $P \leq 0.001$ respectively; **Figure 19**), when compared to control (PBS for 1 minute). A significant decrease in [^{3}H] 8-hydroxy-DPAT binding (17.8%) between 1 and 15 minutes was also noted upon 8-hydroxy-DPAT incubation ($P<0.05$). For those cells preincubated in the presence of 450mM sucrose the specific [^{3}H] 8-hydroxy-DPAT binding significantly decreased at 1 minute (42.3 %) and at 15 minutes (47.1 %; compared to control; $P<0.05$). The value obtained at 15 minutes (in the presence of 450mM sucrose) was significantly higher (39.3 %; $P \leq 0.05$) than that obtained for cells incubated with 8-hydroxy-DPAT alone for 15 minutes (**Figure 19**). The increase in specific binding for those cells preincubated with 450mM and 8-hydroxy-DPAT was not significantly different from each other at 1 and 15 minutes. In summary, it was found that sucrose preincubation significantly attenuated the decrease in [^{3}H] 8-hydroxy-DPAT binding in response to 1μM 8-hydroxy-DPAT incubation for 15 minutes (**Figure 19**).

4.1.3.iii. "Pooled" preincubation of CHO-K1 cells expressing the Y_{400}F mutant 5-HT_{1A}R (Method 2)

Preincubation of Y_{400}F mutant 5-HT_{1A}Rs with 1μM 8-hydroxy-DPAT resulted in a significant decrease in specific binding for both 1 (53.2%) and 15 (41.8%) minute time periods ($P \leq 0.01$ and $P \leq 0.001$ respectively; **Figure 20**), when compared to control (PBS 1 minute). A significant decrease in [^{3}H] 8-hydroxy-DPAT binding (11.4 %) between 1 and 15 minutes was also noted upon 8-hydroxy-DPAT treatment ($P<0.05$).

For those cells preincubated in the presence of 450mM sucrose, the specific [^{3}H] 8-hydroxy-DPAT binding significantly decreased to 51.8 % of control ($P<0.001$) at 1 minute and 62.1 % of control ($P<0.001$) at 15 minutes (**Figure 20**). Only the value obtained at 15 minutes from cells treated with both sucrose and 8-hydroxy-DPAT was significantly different from those cells treated for 15 minutes with 8-hydroxy-DPAT alone ($P \leq 0.05$; **Figure 20**).

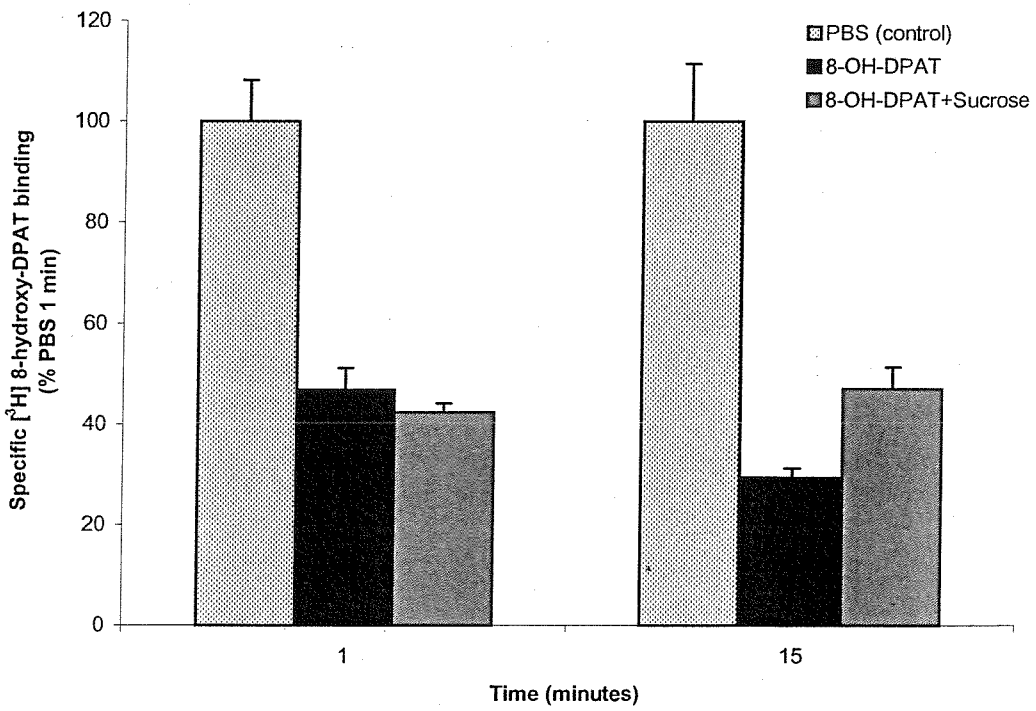


Figure 19: Time dependent effects of specific [^{3}H] 8-hydroxy-DPAT binding to epitope-tagged 5-HT_{1A}R expressed in CHO-K1 cells in response to 8-hydroxy-DPAT in the absence or presence of hypertonic sucrose solution

Initially CHO-K1 cells expressing epitope-tagged 5-HT_{1A}R were treated with PBS in the absence or presence of 450mM sucrose for 20 minutes. Cells were then incubated in the presence or absence of 1μM 8-hydroxy-DPAT to stimulate receptor internalisation and in the absence or presence of 450mM sucrose for 1 or 15 minutes. After such time internalisation was stopped, using ice cold PBS and specific [^{3}H] 8-hydroxy-DPAT binding was determined as described in the Methods section 3.2. Data were normalised by expressing them as a percentage of the corresponding PBS control value. The data points are mean values of three separate experiments ± SEM.

The increase in specific binding between 1 and 15 minutes (an increase of 10.3%) for those cells preincubated with 450mM and 8-hydroxy-DPAT was found to be significantly different ($P<0.01$). In summary, it was found that sucrose preincubation significantly attenuated the decrease in [^{3}H] 8-hydroxy-DPAT binding in response to 1μM 8-hydroxy-DPAT incubation after 15 minutes (**Figure 20**).

In general it was found that cell preincubation with 450mM sucrose significantly attenuated the decrease in [^{3}H] 8-hydroxy-DPAT binding, upon treatment with 1μM 8-hydroxy-DPAT for 15 minutes (this was the case for all receptor variants). Mutation of the 5-HT_{1A}R (Y_{400}F) also significantly ($P<0.05$) attenuated the decrease in [^{3}H] 8-hydroxy-DPAT instigated by treatment with 8-hydroxy-DPAT for 1 minute. The combination of both the Y_{400}F mutation and 450mM sucrose treatment significantly attenuated the decrease in [^{3}H] 8-hydroxy-DPAT binding instigated by 8-hydroxy-DPAT at both 1 and 15 minutes time points. The unmutated and epitope-tagged 5-HT_{1A}R were found to undergo significantly different rates of internalisation upon treatment with 8-hydroxy-DPAT during a 1 minute period. Moreover there was no significant difference between wild type and epitope-tagged receptors upon treatment with PBS in the presence of 450mM sucrose compared to 1μM 8-hydroxy-DPAT. A direct comparison of all three 5-HT_{1A}R types can be see in **Figure 21**, indicating relative levels of receptor redistribution in the presence or absence of 450mM sucrose.

4.1.4. Effect of CHO-K1 cell harvesting methods on receptor binding.

Cells harvested using trypsin EDTA (1X) were found to undergo a significant decrease (75%; $P<0.001$) in specific [^{3}H] 8-hydroxy-DPAT binding when compared to those cells harvested by manual cell scraping (**Figure 22**).

These data indicated that to obtain a maximum protein yield and reproducible results, all future "redistribution/ internalisation" assays omitted trypsin from the harvesting method.

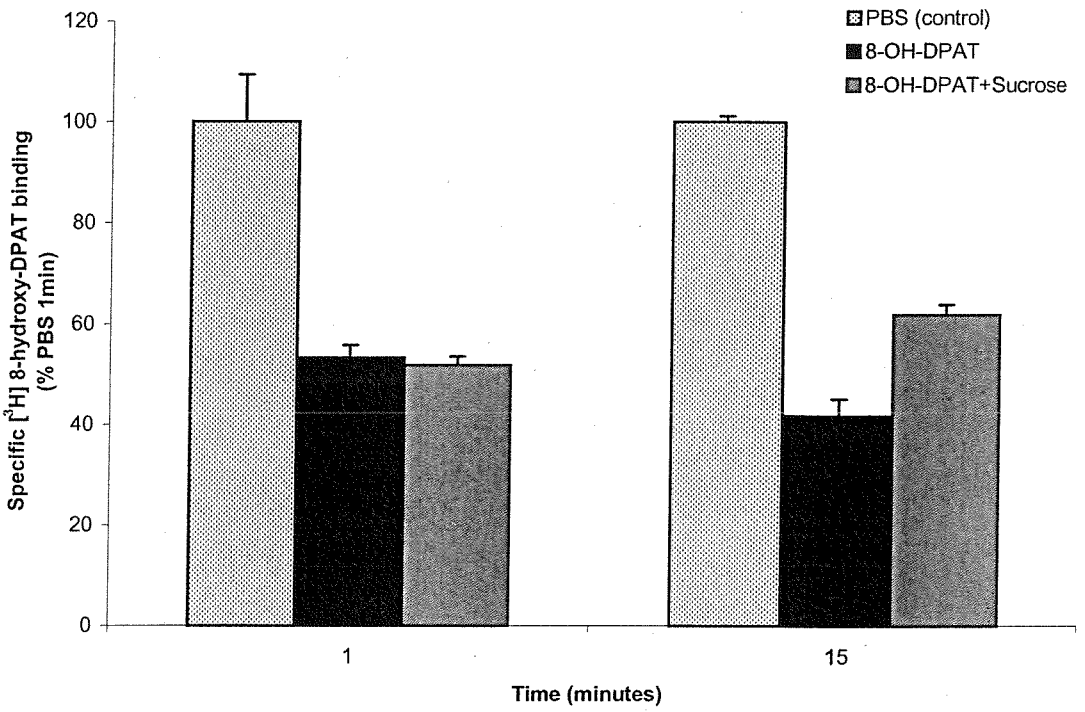
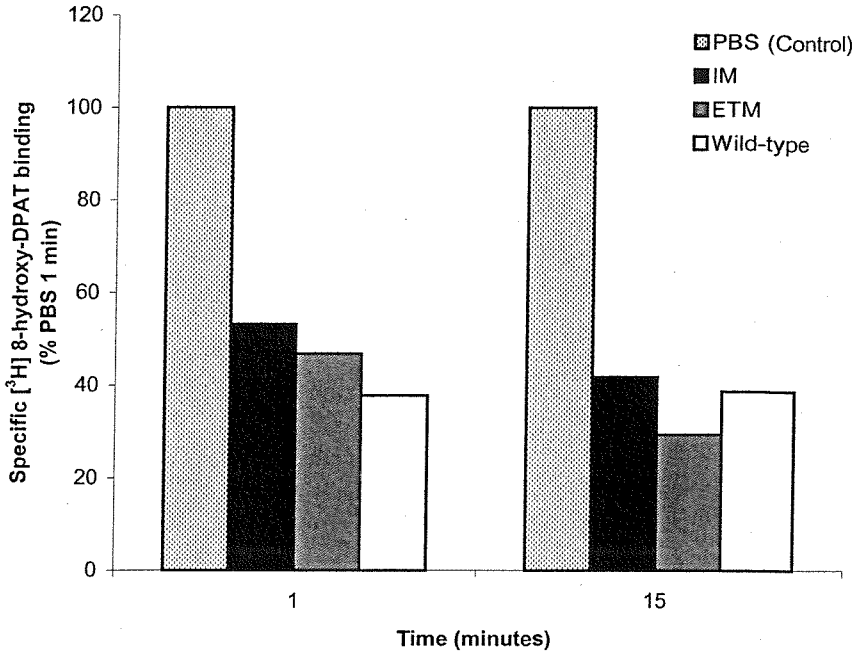


Figure 20: Time dependent decrease in specific [^{3}H] 8-hydroxy-DPAT binding to Y_{400}F internalisation mutant 5-HT_{1A}R expressed in CHO-K1 cells in response to 8-hydroxy-DPAT, in the absence or presence of hypertonic sucrose solution

Initially CHO-K1 cells expressing the Y_{400}A internalisation mutant 5-HT_{1A}R were treated with PBS in the absence or presence of 450mM sucrose for 20 minutes. Cells were then incubated in the presence or absence of 1μM 8-hydroxy-DPAT to stimulate receptor internalisation and in the presence or absence of 450mM sucrose for 1 or 15 minutes. After such time internalisation was stopped, using ice cold PBS and specific [^{3}H] 8-hydroxy-DPAT binding was determined as described in Methods section 3.2. Data was normalised by expressing it as a percentage of the corresponding PBS control value. Each data point is the mean value of three separate experiments ± SEM.

A



B

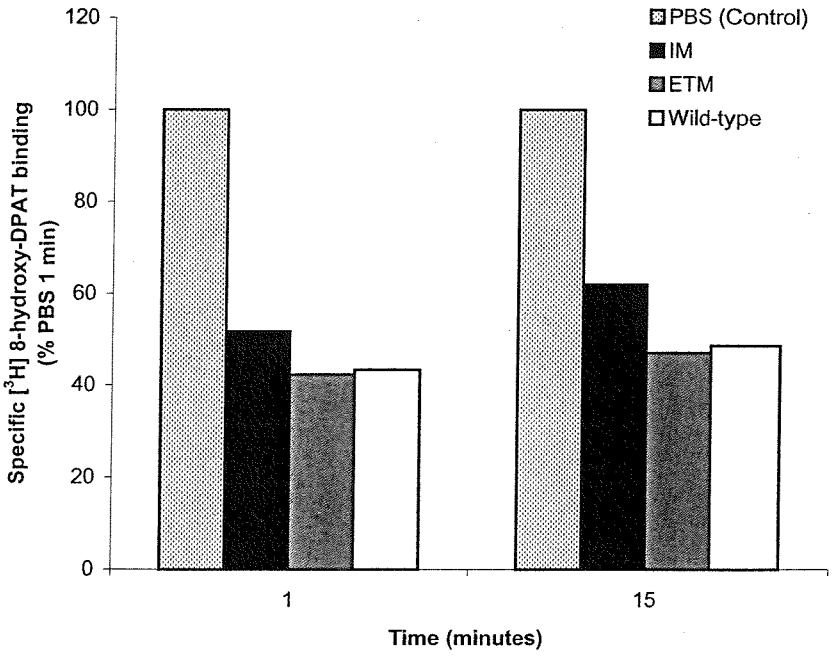


Figure 21: Direct comparison of the effects of 5-HT_{1A}R mutations (PBS = control; IM = internalisation mutant $Y_{400}F$; ETM = epitope-tagged receptor; wild-type = unmutated receptor) incubation with 450mM sucrose, on the decrease in [^{3}H] 8-hydroxy-DPAT binding upon activation with 1μM 8-hydroxy-DPAT

In absence of hypertonic sucrose solution (**A**) or in the presence of hypertonic sucrose solution (**B**). All data points are mean values of three separate experiments ± SEM.

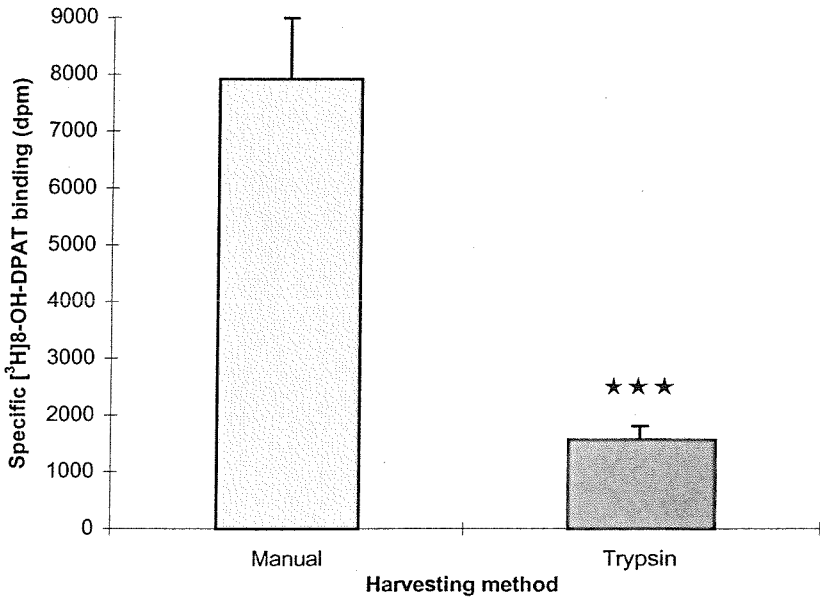


Figure 22: Effects of trypsinisation on specific [^{3}H] 8-hydroxy-DPAT binding to CHO-K1 cells expressing the recombinant 5-HT$_{1A}$R

Cells grown in 100mm culture dishes were harvested using trypsin a standard trypsinisation procedure (Section 3.1.) or a manual cell scraper. The cell suspension was then incubated in a reaction that included 2.5nM [^{3}H] 8-hydroxy-DPAT and specific binding was determined by liquid scintillation counting. All data points are mean values of three separate experiments ± SEM.

4.2. DISCUSSION

4.2.1. Development of an assay to measure receptor redistribution events

The development of an internalisation assay was an important task in helping to address the aims and objectives set out in section 2.1. There are relatively few tools available that allows the study of receptor redistribution *in vivo*. Those that do exist often require expensive equipment. The initial receptor engineering and transfection into CHO-K1 cells was performed in 1994. Although not a vital part of the discussion, it is important to map the development of the internalisation assay and why each change was undertaken. The following sections will be dedicated to the earlier research undertaken with emphasis being placed on results obtained and why these led to assay changes.

4.2.1.i. Internalisation method 1

The first internalisation assay employed demonstrated that internalisation of the unmutated receptor was stimulated by incubation in the presence of the specific agonist 8-hydroxy-DPAT for 20 minutes at 37 °C. For those cells transfected with the unmutated and epitope-tagged receptor, the rates of receptor sequestration appeared to be almost identical. This was demonstrated by a time dependent increase in the specific binding of the radioligand [^{3}H] 8-hydroxy-DPAT to CHO-K1 plasma membranes after agonist incubation. These finding also provided an early indication that the epitope-tag engineered in to the amino extracellular terminus of the receptor had in no way affected the receptors ability to internalise. Conversely, the conservative $Y_{400}F$ mutation engineered into the seventh transmembranous region of the 5-$HT_{1A}R$ had greatly slowed the rate of internalisation by 50%. This was demonstrated by a much-reduced rate of decreasing [^{3}H] 8-hydroxy-DPAT binding triggered by 8-hydroxy-DPAT. After an incubation period of 20 minutes, the $Y_{400}F$ mutation had attenuated receptor sequestration to 66% where as the wild type and epitope-tagged receptors had fallen to 33 and 21% respectively, of original binding.

This initial method indicated that the receptor types studied were able to respond to agonist incubation and that the assay used was partially successful in quantifying

receptor internalisation. However the standard error of the mean at each data point was too large to acceptable (hence the lack of error bars in **Figure 17**). Each dish of cells demonstrated a different B_{max} that varied by as much as 10% per dish. The variation of B_{max} from dish to dish led to a wild difference in the levels of specific [^{3}H] 8-hydroxy-DPAT binding recorded. Although there was a recognisable trend in the decrease in [^{3}H] 8-hydroxy-DPAT binding the error bars proved to be very large. These findings led to the pooling of cell populations prior to agonist incubation in an attempt to reduce the variation observed when plates of cells were analysed separately. Attempts at attenuating the internalisation of the 5-HT_{1A}R using hypertonic sucrose solutions were also unsuccessful. This again was deemed an important experiment, as it would help to further prove and define actual receptor internalisation, and will be discussed in more depth in a later section. In summary the early attempts at quantifying receptor internalisation using this first method were partially successful and led to the pooling of cell populations in order to reduce the observed B_{max} variation observed between individual cell dishes.

4.2.1.ii. Internalisation method 2

The minor adjustments made to strategy 1 as described above led to the development of strategy 2. The results obtained using this method again demonstrated internalisation of three types of 5-HT_{1A}R (unmutated, epitope-tagged and Y_{400}F) upon agonist incubation. As with strategy one receptor internalisation was represented by a time dependent decrease in [^{3}H] 8-hydroxy-DPAT binding to the isolated CHO-K1 plasma membranes. In addition to pooling cells to reduce to error of the results, the specific binding of [^{3}H] 8-hydroxy-DPAT to CHO-K1 plasma membrane preparations was only defined at 1 and 15 minutes. These two time points were selected because at these time points in strategy one the 1 minute time point represented a small decrease in receptor number where as at 15 minutes maximal receptor loss had occurred. It was also at this point that it was realised that the main aim if these experiments was not absolute definition of receptor internalisation but the development of a strategy to allow study of other receptor types.

The unmutated receptor demonstrated the most rapid kinetics of all the three-receptor types studied, with a 62.2% decrease in specific [^{3}H] 8-hydroxy-DPAT binding within

1 minute of 8-hydroxy-DPAT incubation. In contrast, the $Y_{400}F$ mutant 5-$HT_{1A}R$ again demonstrated the slowest kinetics with a 46.8% decrease at 1 minute and 58.2% decrease after 15 minutes of agonist incubation. It should be noted that even after 15 minutes the $Y_{400}F$ internalisation mutant 5-$HT_{1A}R$ did not reach the degree of internalisation achieved by the wild type receptor after 1 minute of agonist incubation. In the case of the epitope-tagged receptor, similar internalisation kinetics was observed to those demonstrated by the unmutated receptor. The only slight differences observed was the slightly lower rate of internalisation after 1 minute of agonist incubation when compared to the unmutated rate of internalisation and the continued internalisation after 15 minutes of agonist incubation.

4.2.2. Attenuation of unmutated, epitope-tagged and $Y_{400}F$ muatant 5-$HT_{1A}R$ internalisation using hypertonic sucrose solution (Method 2)

In an attempt to attenuate receptor internalisation, CHO-K1 cells were incubated in PBS containing 450mM sucrose for 20 minutes prior to agonist incubation. Here preincubation of CHO-K1 cells expressing mutated and wild type versions of the 5-$HT_{1A}R$ with a hypertonic sucrose solution significantly attenuated receptor sequestration. This was demonstrated by an increase in [^{3}H] 8-hydroxy-DPAT binding when compared to cells treated with 8-hydroxy-DPAT alone. The effect elicited by hypertonic sucrose being more apparent at 15 minutes. Experiments performed on various GPCRs (for example, the M_4 muscarinic acetylcholine receptors) have demonstrated that pre-treatment of cells with a hypertonic sucrose buffer prior to agonist incubation totally abolished receptor internalisation (Bogatkewitsch *et al.*, 1996). Our findings were in conflict with these data, which reported the complete blockade of RME by hypertonic sucrose (Bogatkewitsch *et al.*, 1996). The 5-$HT_{1A}R$ may be internalising via a pathway that is only partially sensitive to hypertonic sucrose preincubation at this concentration. However, the effects of sucrose and other agents used to attenuate receptor internalisation will be covered in greater depth in a latter section.

Whilst performing the experiments described above one important flaw in the experimental design became clear. The flaw being that the 8-hydroxy-DPAT used to trigger receptor internalisation had not been washed from the cell incubation medium.

As a result of this finding an extra wash step was included in the protocol that allowed the cell suspension to be washed with PBS in an attempt to remove all unbound 8-hydroxy-DPAT. The theory behind this step was that in the subsequent radioligand-binding assay the unlabelled 8-hydroxy-DPAT would act as a competitor to [^{3}H] 8-hydroxy-DPAT resulting in a phantom decrease in specific radioligand binding. Another important observation was the effect of trypsin on receptor [^{3}H] 8-hydroxy-DPAT binding after cell harvesting. Trypsin was found to significantly decrease the radioligand binding observed, when compared to those cells that had been manually harvested. Thus the cells were no longer harvested by trypsinisation and manual harvesting was included in the experimental protocol.

Again, these results were similar to those obtained using strategy one in that they demonstrated the ability of various receptor types to internalise in response to agonist challenge. Two important steps were added to the experimental protocol and the initial studies using this completed method suggested it was ready for use in more detailed studies. Thus, the next strategy (3) was employed to help address the aims given in section 1.7.1.

CHAPTER 5

5.1. DEVELOPMENT OF AN INTERNALISATION ASSAY: METHOD 3

5.1.1. Introduction

The culmination of all the methodology development led to the third and final strategy aimed at quantification of 5-HT_{1A}R redistribution in response to agonist pre-incubation. Briefly, cells were first manually harvested using a cell scraper and pooled. Intact cells were then treated with 1μM 8-hydroxy-DPAT for 20 minutes. After this time, the cell suspension was washed twice with 10ml of ice-cold PBS; after each wash, the suspension was centrifuged and the supernatant was discarded. After the final wash the pellets was resuspended in a small volume of PBS and a membrane fraction was prepared. A full saturation analysis was then performed using either [^{3}H] 8-hydroxy-DPAT or [^{3}H] spiperone. This method was used for all subsequent studies.

5.1.2. Pre-incubation of cells in vitro (Method 3)

In order to gauge the degree of unmutated receptor redistribution upon agonist incubation a standard internalisation assay was used as described briefly above. The degree of unmutated receptor redistribution was then used as a comparison for all resulting internalisation assays using various receptor mutants. A decrease in specific radioligand binding at plasma membrane bound receptors in response to incubation with unlabelled agonist was assumed to be an indication of redistribution of the 5-HT_{1A}Rs to an intracellular compartment. Membrane enriched fractions were prepared as described previously in section 2.2. and then subjected to radioligand binding analysis (**Figure 23i**). Linear transformation of saturation data for both control and agonist-treated samples (**Figure 23ii**) enabled a calculation of B_{max} in terms of fmol radioligand bound per mg protein and K_d in terms of nM. For host cells treated with PBS alone (control), the B_{max} was estimated at 805.0 ± 22.7fmol/mg.

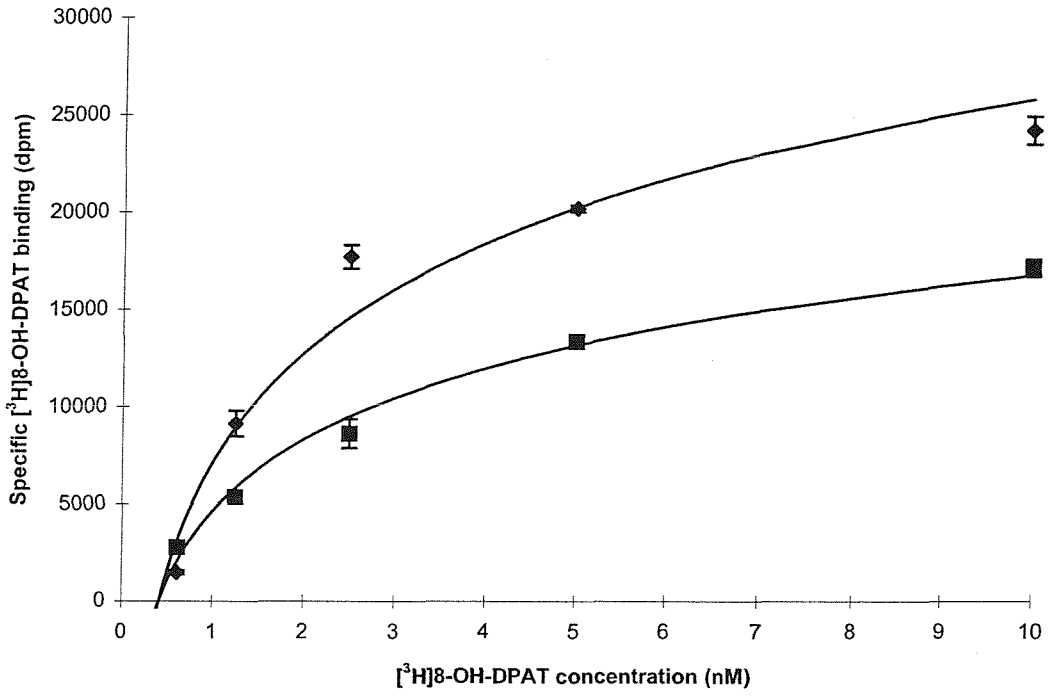


Figure 23i: Saturation analysis of specific [^{3}H] 8-hydroxy-DPAT binding to the unmutated human 5-HT$_{1A}$R expressed in CHO-K1 cells

CHO-K1 cells expressing the unmutated 5-HT$_{1A}$R were incubated for 20 minutes in the absence (◆) or presence (■) of 1μM 8-hydroxy-DPAT. Membrane enriched fractions were subject to radioligand binding analysis in the presence of [^{3}H] 8-hydroxy-DPAT as described in detail in sections 2.2. Each data point is the mean value of three separate assays ± SEM.

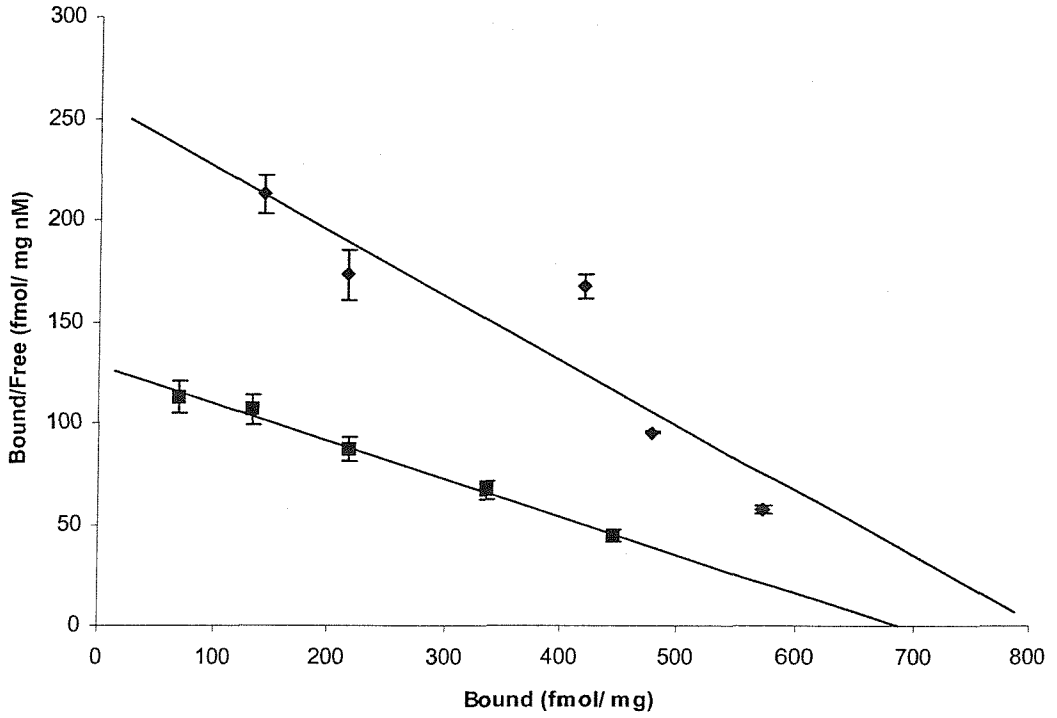


Figure 23ii: Scatchard analysis of saturation data for the unmutated human 5-HT_{1A}R expressed in CHO-K1 cells

Key; cells incubated in the absence (◆) or presence (■) of 1μM 8-hydroxy-DPAT. The X-axis represents the conversion of [^{3}H] 8-hydroxy-DPAT specific binding in dpm to fmol/ mg protein. The Y-axis is a ratio; this is calculated as bound radioligand as a function of the original "free" radioligand concentration. The B_{max} (fmol/ mg) was calculated from the X-axis intercept; the K_d (nM) was derived from the gradient of each line. Data points are the mean values of three separate assays ± SEM.

The K_d was calculated to be 2.5 ± 0.7nM. For those cells treated with 8-hydroxy-DPAT (1μM) the B_{max} was estimated at 663.4 ± 19fmol/mg while the K_d was calculated to be 5.5 ± 0.3nM (**Figure 23ii**). The decrease in B_{max} and increase in K_d for agonist treated cells were found to be significant (P<0.05) compared to control.

Results presented by Sundaram *et al.*, (1993) demonstrated the ability of [^{3}H] spiperone to recognise receptors, which were both coupled to and uncoupled from their G protein. Hence, any decrease in [^{3}H] spiperone binding after the incubation of CHO-K1 cells with 1μM 8-hydroxy-DPAT would represent a true decrease in the number of receptors from the plasma membrane and not just the transition of the receptor to a lower agonist affinity state. In the context of this type of information, this experiment was considered to be a definitive test of whether the receptors were redistributing away from the plasma membrane following pre-exposure to agonist.

CHO-K1 cells expressing the unmutated 5-HT_{1A}R were incubated (for 20 minutes at 37°C) in the absence or presence of unlabelled agonist 8-hydroxy-DPAT (1μM). Membrane enriched fractions were prepared as described previously (Methods section 2.2.) and then subjected to radioligand binding analysis in this case [^{3}H] spiperone was the radioligand used at concentrations between 3.125nM and 50nM. This range was pre-determined in pilot experiments (**Figure 24i**).

Linear transformation of the saturation data for both control and agonist-treated samples enabled the calculation of B_{max} in terms of the number of fmol of radioligand bound per mg of protein and receptor K_d in nM (**Figure 24ii**). For host cells treated with PBS (control) alone the B_{max} was estimated as 2927.1 ± 79.6fmol/ mg. The K_d was calculated to be 8.9 ± 0.6nM. For those cells treated with 1μM 8-hydroxy-DPAT the B_{max} was calculated to be 2128.4 ± 213.4fmol/ mg and the K_d as 8.7 ± 0.7nM (**Figure 24ii**). The decrease in specific ligand binding observed when cells were incubated in the presence of 1μM 8-hydroxy-DPAT when compared to those incubated in the absence of 1μM 8-hydroxy-DPAT was found to be significant (P<0.05).

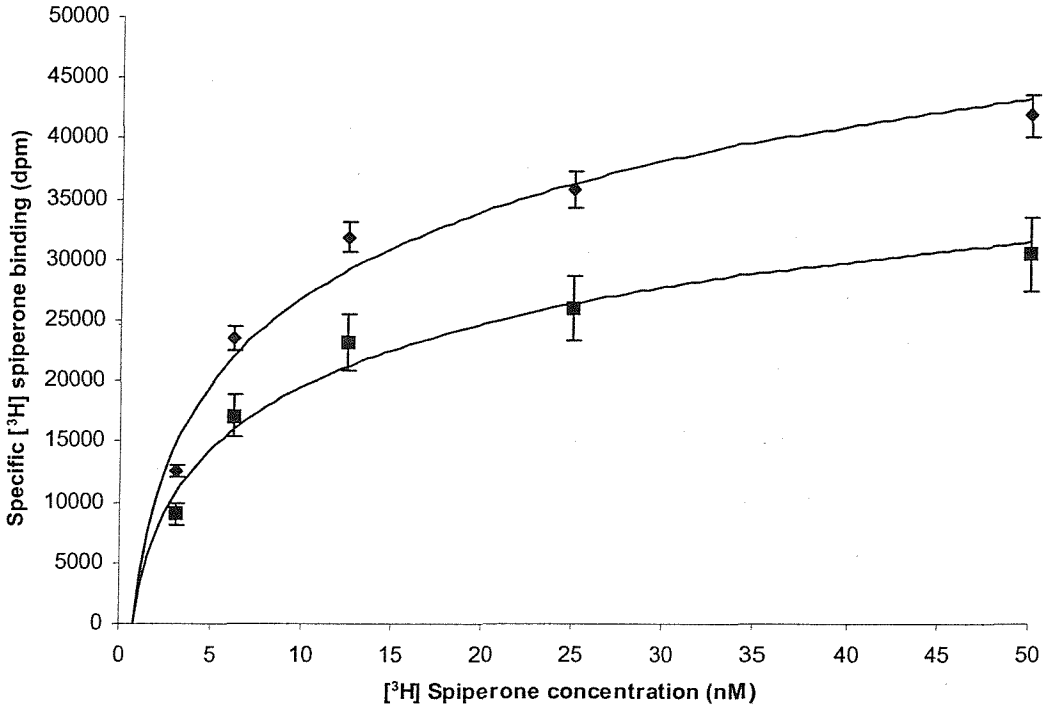


Figure 24i: Saturation analysis of specific [^{3}H] spiperone binding to the unmutated 5-HT$_{1A}$R expressed in CHO-K1 cells

CHO-K1 cells expressing the unmutated 5-HT$_{1A}$R were incubated for 20 minutes in the absence (◆) or presence (■) of 1μM 8-hydroxy-DPAT. Membrane enriched fractions were then subjected to radioligand binding analysis in the presence of [^{3}H] spiperone as described in detail in sections 2.2. Each data point is the mean value of three separate assays ± SEM.

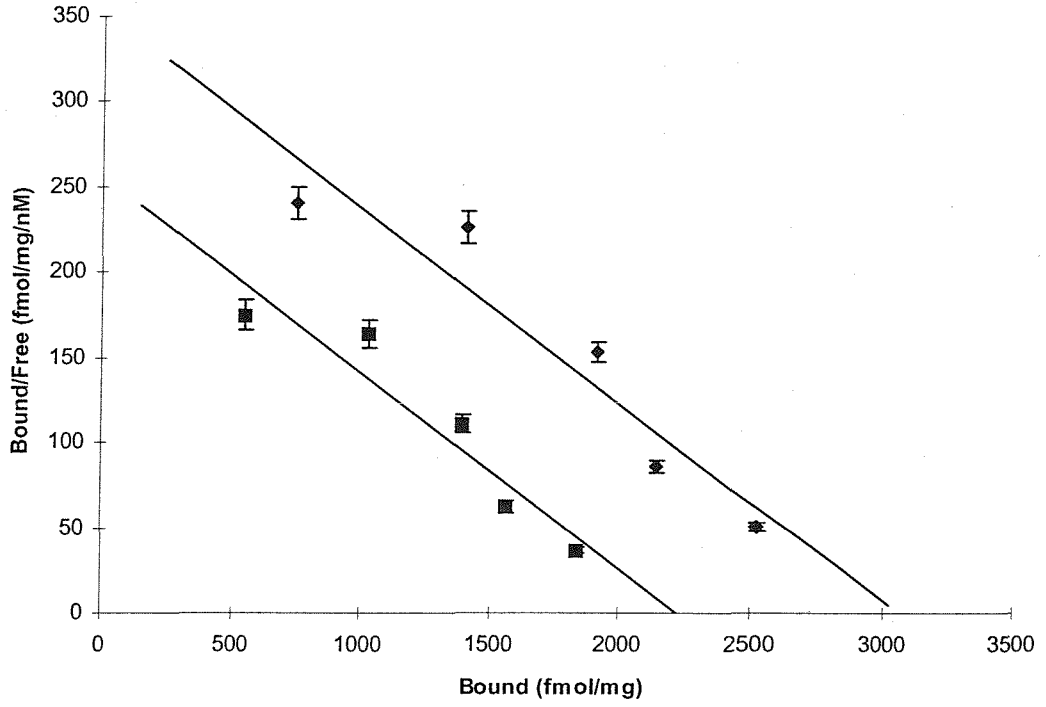


Figure 24ii: Scatchard analysis of saturation data for the unmutated 5-HT_{1A}R receptor expressed in CHO-K1 cells

Key; cells incubated in the absence (◆) or presence (■) of 1μM 8-hydroxy-DPAT. The X-axis represents the conversion of [^{3}H] spiperone specific binding in dpm to fmol/ mg protein. The Y-axis is a ratio; this is calculated as bound radioligand as a function of the original "free" radioligand concentration. The B_{max} was calculated from the X-axis intercept (fmol/mg); and the K_d (nM) is derived from the gradient of each line. Data points are the mean values of three separate assays ± SEM.

In summary, both ligands used to define receptor redistribution ([^{3}H] 8-hydroxy-DPAT and [^{3}H] spiperone) demonstrated a comparable decrease in specific binding upon agonist challenge. However the relative change in K_d (in the case of spiperone incubation it stayed the same and in the case of 8-hydroxy-DPAT it increased) upon agonist incubation may reflect the ability of the ligands to bind 5-HT_{1A}Rs' in a phosphorylated form.

5.2. USING MODIFIED RECOMBINANT 5-HT_{1A}Rs TO STUDY RECEPTOR REDISTRIBUTION

The level of receptor expression and the receptor-ligand affinity were established for all transfected cell lines under optimal radioligand conditions (Chapter 3). Thereafter, it was possible to begin to look at whether the B_{max} and K_d of each modified receptor could be influenced by preincubation of intact cells with the unlabelled agonist 8-hydroxy-DPAT. The proposed role of each individual receptor modification will be outlined and investigated below.

5.2.1. Epitope-tagged receptors

As previously mentioned the epitope-tag mutation ($A_{17}E$, $F_{19}E$, $E_{20}T$) was engineered into the amino terminus of the 5-HT_{1A}R in order to allow antibody recognition and binding to an extracellular site of the receptor using an antibody already in existence. It was therefore deemed necessary to investigate the validity and characteristics of such an epitope-tagged (mutated receptor) in future internalisation studies using antibodies conjugated to fluorescent labels. In order to generate the specified mutations site directed mutagenesis of the G-21 clone (as described in the Methods section 2.1.1.) was performed. The substitution was made in the N-terminus extracellular domain ($PPA_{17}PF_{19}E_{20}$) which was proposed to have no important role in receptor function.

Incubations in the absence or presence of 1μM 8-hydroxy-DPAT were performed exactly as described in section 2.4.1.iii. Membrane enriched fractions of CHO-K1 cells expressing the epitope-tagged 5-HT_{1A}R ($A_{17}E$, $F_{19}E$, $E_{20}T$) were subjected to radioligand binding analysis (**Figure 25i**) as described in section 2.2.

Linear transformation of saturation data for both control and agonist-treated samples (**Figure 25ii**) enabled a calculation of B_{max} and K_d. For host cells treated with PBS alone (control), the B_{max} was estimated at 155.8 ± 8.2fmol/ mg. The K_d was calculated to be 12.1 ± 0.6nM.

For those cells treated with 8-hydroxy-DPAT (1μM) the B_{max} was estimated at 127.8 ± 1.5fmol/mg while the K_d was calculated to be and 9.9 ± 0.6nM (**Figure 25ii**). The change in B_{max} for agonist treated cells were found to be significant (P<0.05) when compared to control. These data suggested that this series of modifications to the amino terminal region did not compromise the ability of the receptor to undergo agonist promoted redistribution.

The levels of the epitope-tagged receptor expression (B_{max}) were observed to be much lower than those recorded for unmutated receptor. The receptor was expressed at almost the limits of ligand binding study sensitivity however the receptor was expressed at a much more physiologically relevant levels. The observed K_d was also considerably higher than that observed for the unmutated receptor, however it was in the low nanomolar range and deemed acceptable. The increase in K_d or reduction in epitope-tagged receptors' affinity for 8-hydroxy-DPAT may also reflect the limitations of the ligand binding studies at such low receptor concentrations.

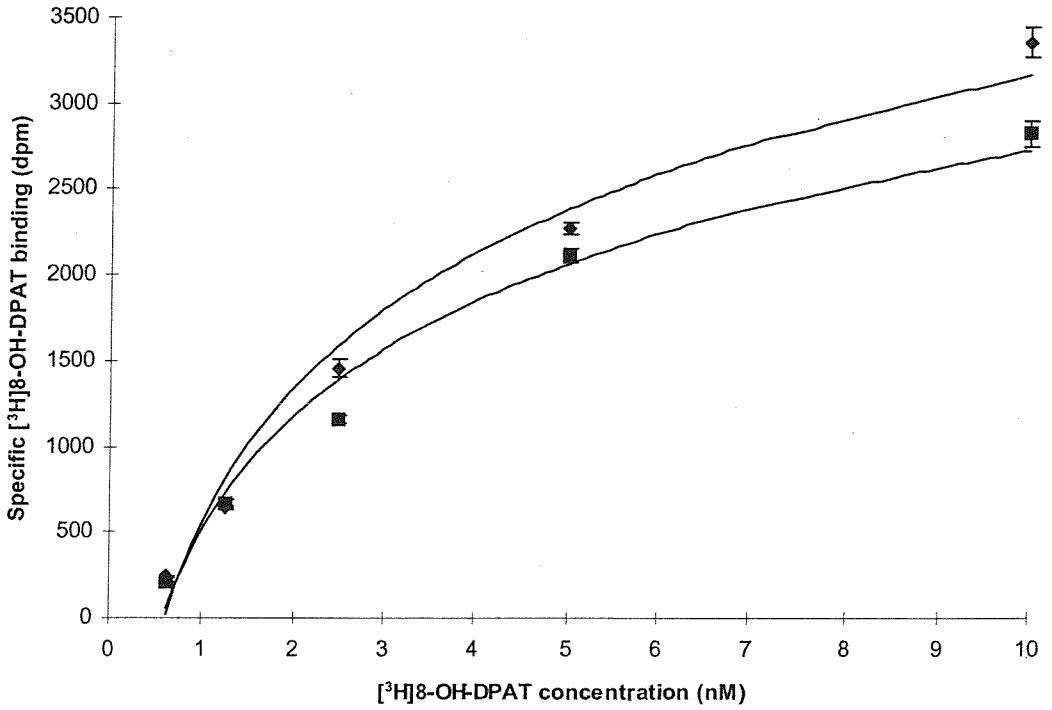


Figure 25i: Saturation analysis of specific [^{3}H] 8-hydroxy-DPAT binding to the epitope-tagged 5-HT$_{1A}$R expressed in CHO-K1 cells

5-HT$_{1A}$Rs expressing an N-terminus epitope-tag were incubated for 20 minutes in the absence (◆) or presence (■) of 1μM 8-hydroxy-DPAT. Membrane enriched fractions were subject to radioligand binding analysis in the presence of [^{3}H] 8-hydroxy-DPAT as described in detail in Method section 2.2. Each data point represents the mean values of three separate assays ± SEM.

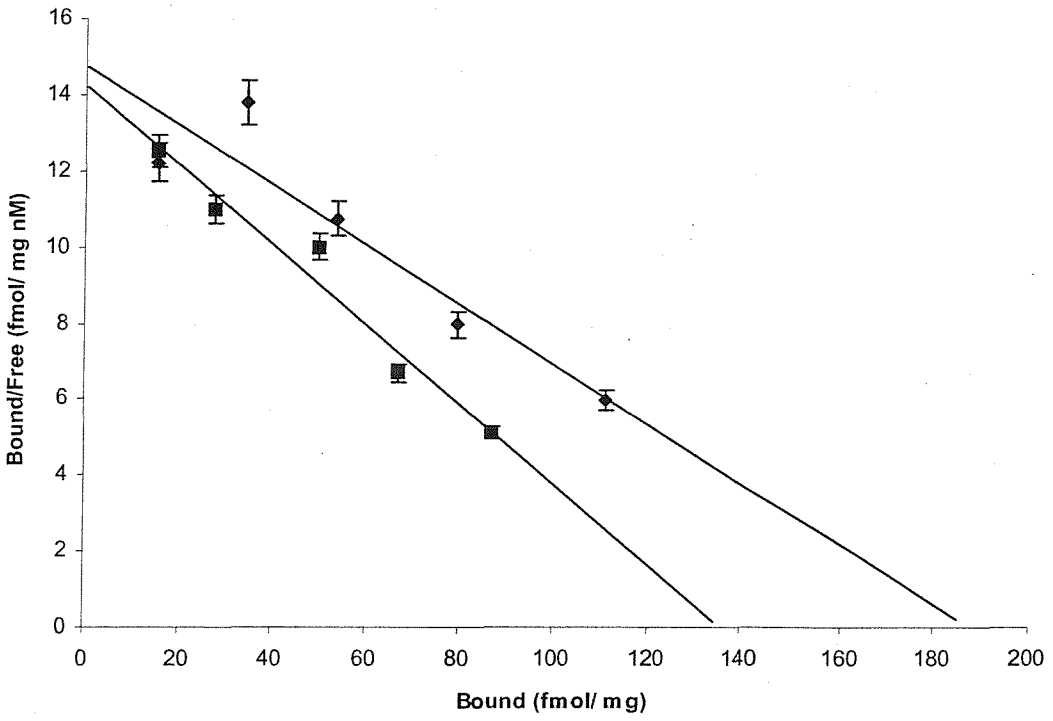


Figure 25ii: Scatchard analysis of saturation data for the epitope-tagged 5-$HT_{1A}R$ expressed in CHO-K1 cells

Key; cells incubated in the absence (◆) or presence (■) of 1μM 8-hydroxy-DPAT. The X-axis represents the conversion of [^{3}H] 8-hydroxy-DPAT specific binding in dpm to fmol/ mg protein. The Y-axis is a ratio; this is calculated as bound radioligand as a function of the original "free" radioligand concentration. The B_{max} (fmol/ mg) was calculated from the X-axis intercept. The K_d (nM) was derived from the gradient of each line. Data points are the mean values of three separate assays ± SEM.

5.2.2. Phosphorylation defective mutants

5.2.2.i. Effect of agonist pre-incubation on the $T_{149}A$ phosphorylation mutant

As previously mentioned the $T_{149}A$ mutation was engineered into the second intracellular loop of the 5-$HT_{1A}R$ ($KRT_{149}PR$) in one of the domains recognised as a substrate for PKC. The disruption of the putative phosphorylation site at this position was proposed to play a role in the short-term regulation of receptor function in response to agonist incubation, therefore helping us to address one of the aims of this project outlined in section 1.7.1. In order to generate the specified mutations site directed mutagenesis of the G-21 clone (as described in the Methods section 2.1.1.) was performed.

Incubations in the absence or presence of 1μM 8-hydroxy-DPAT were performed exactly as described in the Method section 2.4.1.iii. Membrane enriched fractions of CHO-K1 cells expressing the mutated ($T_{149}A$) 5-$HT_{1A}R$ were subjected to radioligand saturation binding analysis (**Figure 26i**) as described in Methods section 2.2.

Linear transformation of the saturation data (**Figure 26i**) for both control and agonist-treated samples enabled a calculation of B_{max} and K_d (**Figure 26ii**). For cells treated with PBS alone (control), the B_{max} was estimated at 303.0 ± 10.6fmol/mg. The K_d was calculated to be 0.9 ± 0.1nM. For those cells treated with 8-hydroxy-DPAT (1μM) the B_{max} was estimated at 330.1 ± 34.4fmol/mg while the K_d was calculated to be 1.7 ± 0.7nM (**Figure 26ii**). The changes in B_{max} and K_d were both found not to be significant when compared to the control values suggesting that this mutation attenuated the ability of the receptor to undergo redistribution.

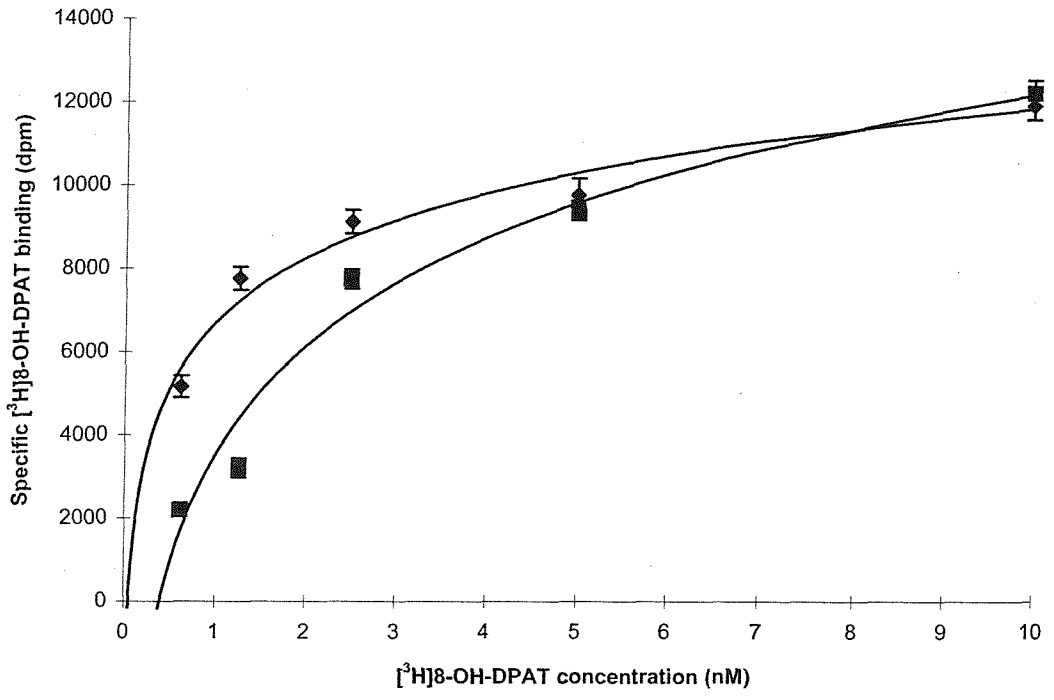


Figure 26i: Saturation analysis of specific [^{3}H] 8-hydroxy-DPAT binding to the $T_{149}A$ phosphorylation mutant 5-$HT_{1A}R$ expressed in CHO-K1 cells

5-HT_{1A}Rs expressing the $T_{149}A$ mutation were incubated for 20 minutes in the absence (◆) or presence (■) of 1μM 8-hydroxy-DPAT. Membrane enriched fractions were subjected to radioligand binding analysis in the presence of [^{3}H] 8-hydroxy-DPAT as described in detail in Method section 2.2. Each data point is the mean values of three separate assays ± SEM.

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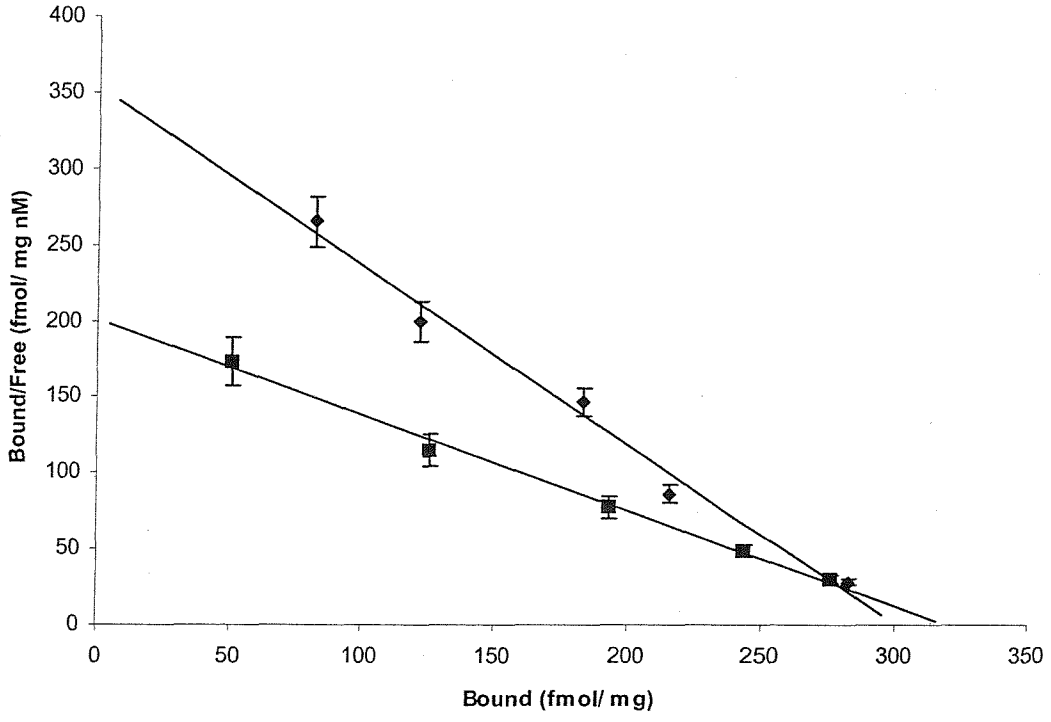

Figure 26ii: Scatchard analysis of saturation data for the $T_{149}A$ phosphorylation defective 5-$HT_{1A}R$ expressed in CHO-K1 cells

Key; cells incubated in the absence (◆) or presence (■) of 1μM 8-hydroxy-DPAT. The X-axis represents the conversion of [^{3}H] 8-hydroxy-DPAT specific binding in dpm to fmol/ mg protein. The Y-axis is a ratio; this is calculated as bound radioligand as a function of the original "free" radioligand concentration. The B_{max} (fmol/ mg) was calculated from the X-axis intercept. The K_d (nM) was derived from the gradient of each line. Data points are the mean values of three separate assays ± SEM.

5.2.2.ii. Effect of agonist pre-incubation on the $T_{229}A$ phosphorylation mutant

As previously mentioned the $T_{229}A$ mutation was engineered into the third intracellular loop of the 5-$HT_{1A}R$ ($RKT_{229}VK$) in one of the domains recognised as a substrate for PKC/ PKA. The disruption of the putative phosphorylation site at this position was proposed to play a role in the short-term regulation of receptor function in response to agonist incubation, therefore helping us to address one of the aims of this project outlined in section 1.7.1. In order to generate the specified mutations site directed mutagenesis of the G-21 clone (as described in the Methods section 2.1.1.) was performed.

Incubations in the absence or presence of 1μM 8-hydroxy-DPAT were performed exactly as described in the sections 2.4.1.iii. Membrane enriched fractions of CHO-K1 cells expressing the mutated ($T_{229}A$) 5-$HT_{1A}R$ were subjected to radioligand saturation binding analysis (**Figure 27i**) as described in section 2.2.

Linear transformation of saturation data for both control and agonist-treated samples (**Figure 27ii**) enabled a calculation of B_{max} and K_d. For host cells treated with PBS alone (control), the B_{max} was estimated at 384.5 ± 26.5fmol/mg. The K_d was calculated to be 2.0 ± 0.5nM. For those cells treated with 8-hydroxy-DPAT (1μM) the B_{max} was estimated at 380.9 ± 36.3fmol/mg while the K_d was calculated to be 2.2 ± 0.5nM (**Figure 27ii**). The changes in B_{max} and K_d were not significant when compared to the control values suggesting that this mutation attenuated the ability of the receptor to undergo redistribution.

5.2.2.iii. Effect of agonist pre-incubation on the $S_{253}G$ phosphorylation mutant

As previously mentioned the $S_{253}G$ mutation was engineered into the third intracellular loop of the 5-$HT_{1A}R$ ($KKS_{253}VN$) in one of the domains recognised as a substrate for PKC/ PKA. The disruption of the putative phosphorylation site at this position was proposed to play a role in the short-term regulation of receptor function in response to agonist incubation, therefore helping us to address one of the aims of this project outlined in section 1.7.1.

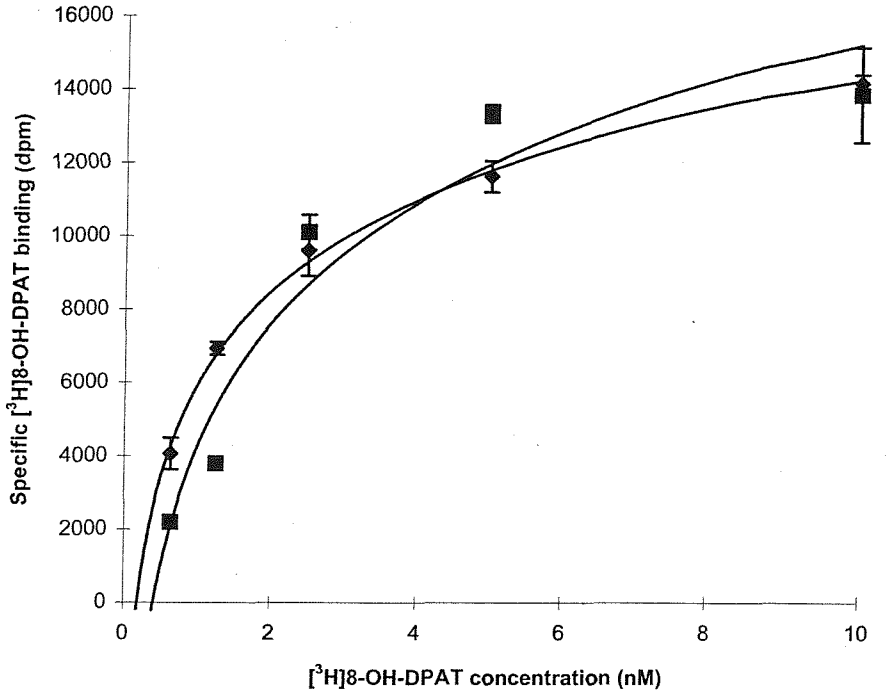

Figure 27i: Saturation analysis of specific [^{3}H] 8-hydroxy-DPAT binding to the T_{229}A phosphorylation defective 5-HT_{1A}R expressed in CHO-K1 cells

5-HT_{1A}Rs expressing the T_{229}A mutation were incubated for 20 minutes in the absence (◆) or presence (■) of 1μM 8-hydroxy-DPAT. Membrane enriched fractions were subject to radioligand binding analysis in the presence of [^{3}H] 8-hydroxy-DPAT as described in detail in sections 2.2. Each data point represents the mean of three separate assays ± SEM.

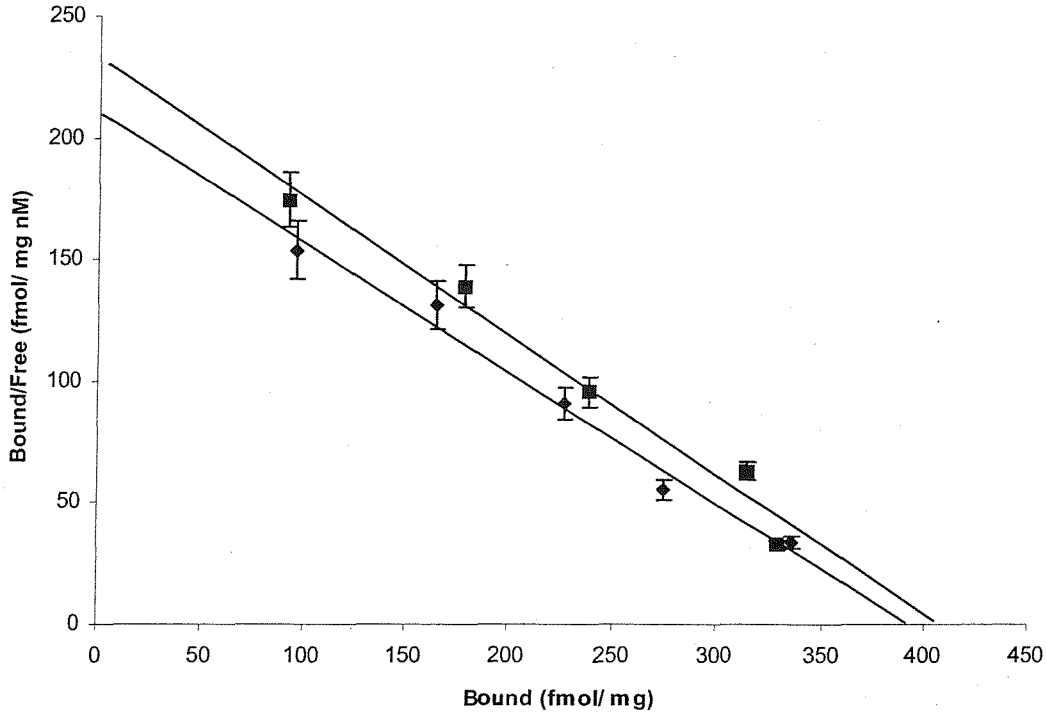

Figure 27ii: Scatchard analysis of saturation data for the $T_{229}A$ phosphorylation defective 5-$HT_{1A}R$ expressed in CHO-K1 cells

Key; cells incubated in the absence (◆) or presence (■) of 1μM 8-hydroxy-DPAT. The X-axis represents the conversion of [^{3}H] 8-hydroxy-DPAT specific binding in dpm to fmol/ mg protein. The Y-axis is a ratio; this is calculated as bound radioligand as a function of the original "free" radioligand concentration. The B_{max} was calculated from the X-axis intercept. The K_d (nM) was derived from the gradient of each line. Data points are the mean values of three separate experiments ± SEM.

In order to generate the specified mutations site directed mutagenesis of the G-21 clone (as described in the Methods section 2.1.1.) was performed. Incubations in the absence or presence of 1μM 8-hydroxy-DPAT were performed exactly as described in the sections 2.4.1.iii. Membrane enriched fractions of CHO-K1 cells expressing the mutated ($S_{253}G$) 5-$HT_{1A}R$ were subjected to radioligand binding analysis (**Figure 28i**) as described in section 2.2.

Linear transformation of saturation data for both control and agonist-treated samples (**Figure 28ii**) enabled a calculation of B_{max} and K_d. For cells treated with PBS alone (control), the B_{max} was estimated at 545.8 ± 31.0fmol/mg. The K_d was calculated to be 7.9 ± 2.4nM. For those cells treated with 8-hydroxy-DPAT (1μM) the B_{max} was estimated at 131.2 ± 24.3fmol/mg while the K_d was calculated to be and 4.4 ± 1.8nM (**Figure 28ii**). The decrease in B_{max} for agonist treated cells was found to be highly significant (P<0.001) compared to control values suggesting that the mutation promoted receptor redistribution. The increase in receptor affinity (represented by a decrease in K_d) was not statistically significant. A summary of the data obtained when the phosphorylation mutants were incubated in the presence of 1μM 8-hydroxy-DPAT is included in **Table 4** (page 126).

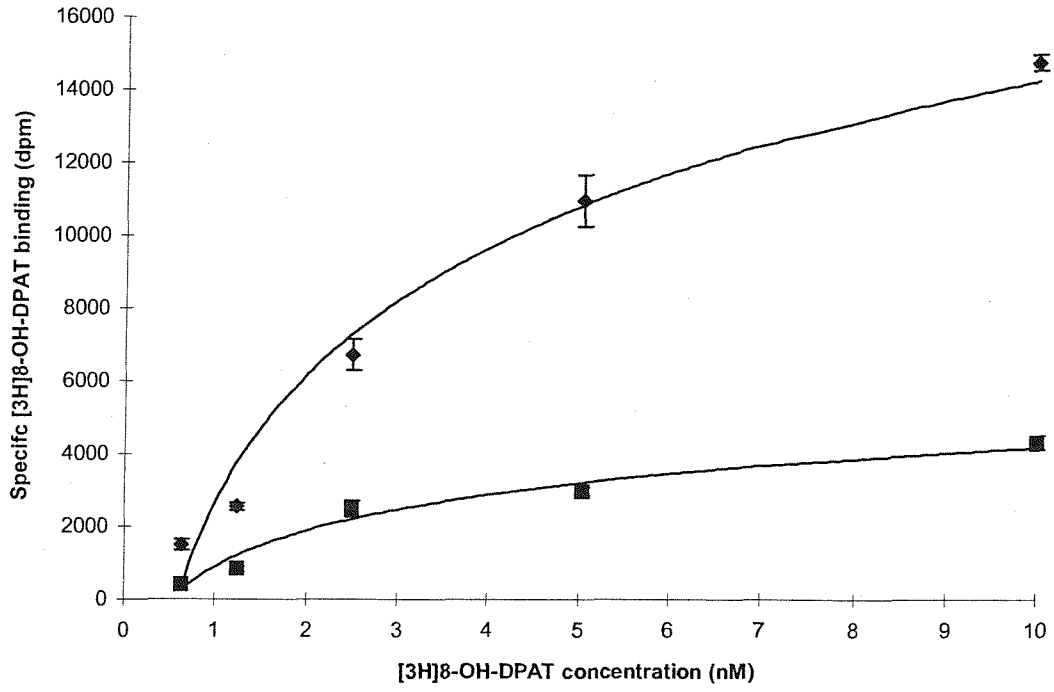

Figure 28i: Saturation analysis of specific [^{3}H] 8-hydroxy-DPAT binding to the $S_{253}G$ phosphorylation defective 5-$HT_{1A}R$ expressed in CHO-K1 cells

5-HT_{1A}Rs expressing the $S_{253}G$ mutation were incubated for 20 minutes in the absence (◆) or presence (■) of 1μM 8-hydroxy-DPAT. Membrane enriched fractions were subjected to radioligand binding analysis in the presence of [^{3}H] 8-hydroxy-DPAT as described in detail in sections 2.2. Each data point represents the mean of three separate assays ± SEM.

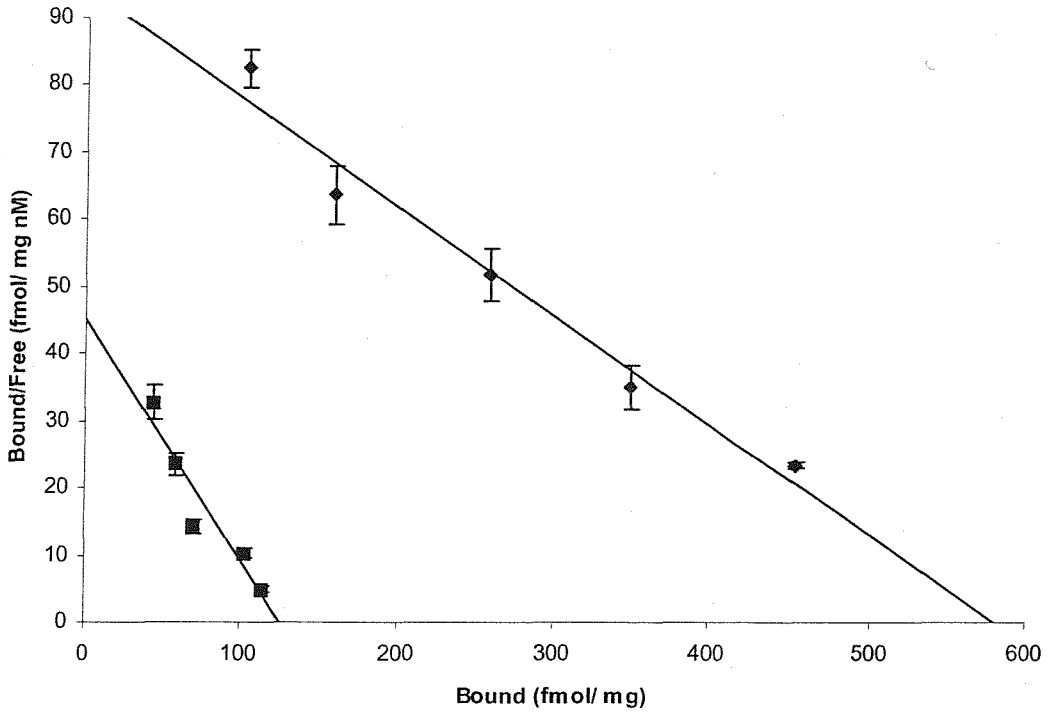

Figure 28ii: Scatchard analysis of saturation data for the $S_{253}G$ phosphorylation defective 5-$HT_{1A}R$ expressed in CHO-K1 cells

Key; cells incubated in the absence (◆) or presence (■) of 1μM 8-hydroxy-DPAT. The X-axis represents the conversion of [^{3}H] 8-hydroxy-DPAT specific binding in dpm to fmol/ mg protein. The Y-axis is a ratio; this is calculated as bound radioligand as a function of the original "free" radioligand concentration. The B_{max} (fmol/ mg) was calculated from the X-axis intercept. The K_d (nM) was derived from the gradient of each line. Data points are the mean values of three separate assays ± SEM.

5.2.3. "Internalisation motif" mutants

5.2.3.i. Effect of agonist preincubation on the $Y_{400}F$ "internalisation motif" mutant

As previously mentioned the $Y_{400}F$ conservative mutation was engineered into the seventh membrane spanning domain of the 5-$HT_{1A}R$ ($NPVIY_{400}$) in a domain recognised as a putative internalisation signal. The disruption of the internalisation motif at this position was proposed to interrupt receptor redistribution in response to agonist incubation. Therefore any observable changes in receptor redistribution rates in response to agonist would help us to address one of the aims of this project outlined in section 1.7.1., that being "To define the role of putative phosphorylation sites located on the second and third intracellular loops and 7^{th} transmembrane internalisation motif (NPVIY) in the cellular redistribution of the 5-$HT_{1A}R$". In order to generate the specified mutations site directed mutagenesis of the G-21 clone (as described in the Methods section 2.1.1.) was performed. Incubations in the absence or presence of 1μM 8-hydroxy-DPAT were performed exactly as described in the sections 2.4.1.iii. Membrane enriched fractions of CHO-K1 cells expressing the mutated ($Y_{400}F$) 5-$HT_{1A}R$ were subjected to radioligand binding analysis (**Figure 29i**) as described in section 2.2.

Linear transformation of saturation data for both control and agonist-treated samples (**Figure 29ii**) enabled a calculation of B_{max} and K_d in terms of nM. For host cells treated with PBS alone (control), the B_{max} was estimated at 104.5 ± 6.4fmol/mg. The K_d was calculated to be 2.7 ± 0.3nM. For those cells treated with 8-hydroxy-DPAT (1μM) the B_{max} was estimated at 99.7 ± 31.9fmol/mg while the K_d was calculated to be and 5.7 ± 2.9nM (**Figure 29ii**). The changes in B_{max} and K_d were not significant when compared to the control values suggesting that this mutation attenuated the ability of the receptor to undergo redistribution.

5.2.3.ii. Effect of agonist pre-incubation on the $Y_{400}A$ "internalisation motif" mutant

As previously mentioned the $Y_{400}A$ non-conservative mutation was engineered into the seventh membrane spanning domain of the 5-$HT_{1A}R$ ($NPVIY_{400}$) in a domain recognised as a putative internalisation signal.

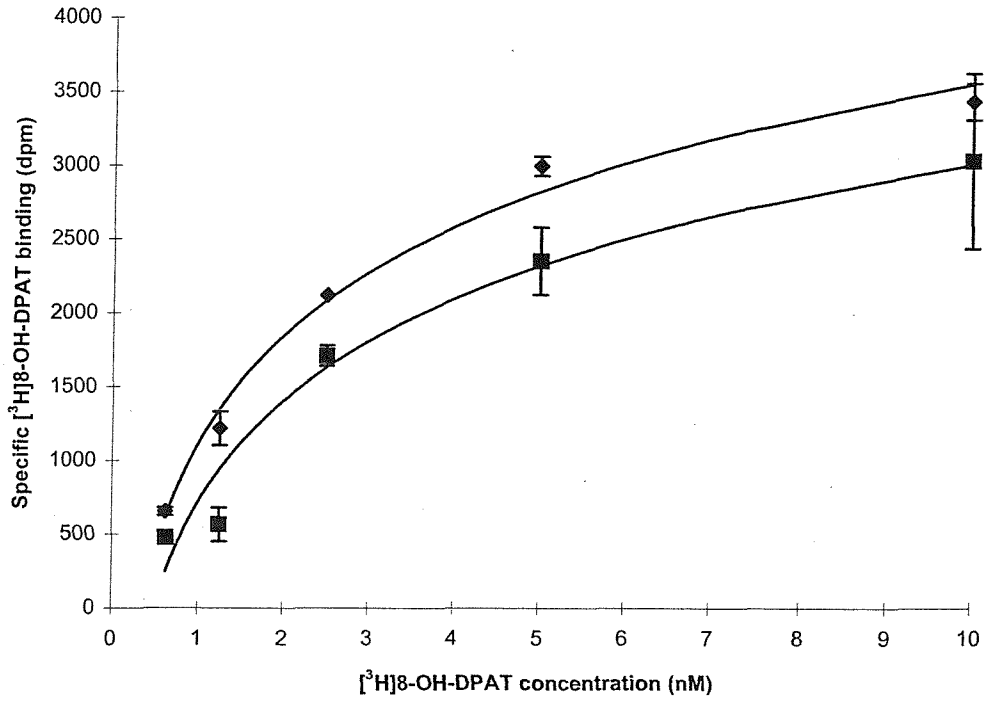

Figure 29i: Saturation analysis of specific [^{3}H] 8-hydroxy-DPAT binding to the $Y_{400}F$ internalisation motif mutant 5-$HT_{1A}R$ expressed in CHO-K1 cells

5-HT_{1A}Rs expressing the $Y_{400}F$ mutant were incubated for 20 minutes in the absence (◆) or presence (■) of 1μM 8-hydroxy-DPAT. Membrane enriched fractions were subject to radioligand binding analysis in the presence of [^{3}H] 8-hydroxy-DPAT as described in detail in sections 2.2. Each data point represents the mean of three separate assays ± SEM.

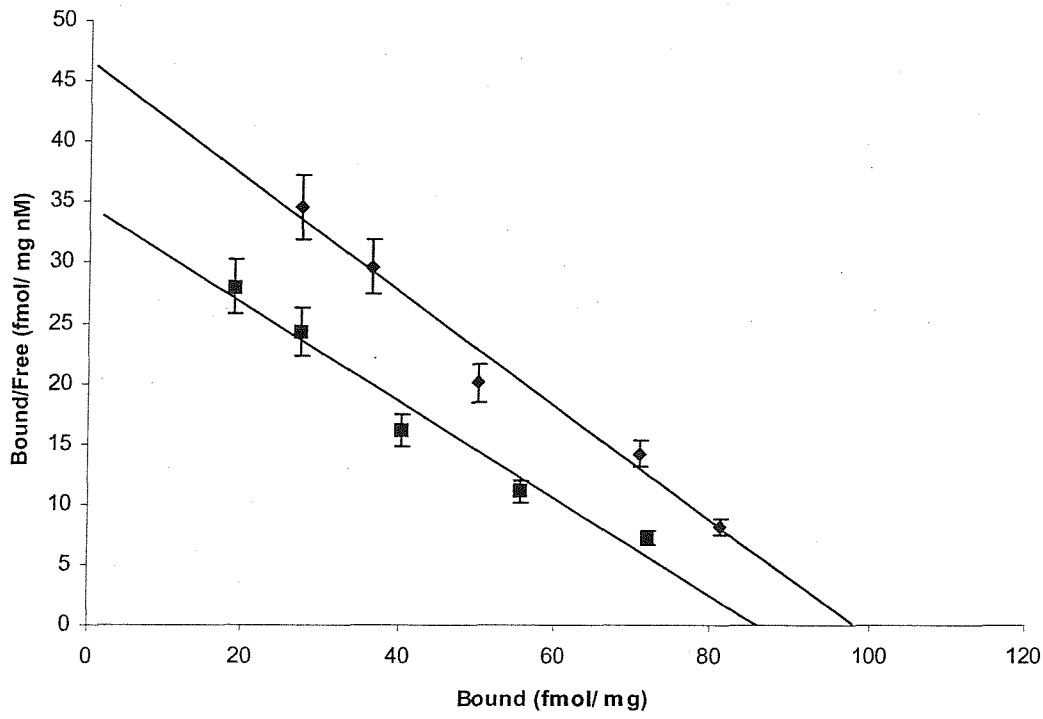

Figure 29ii: Scatchard analysis of saturation data for the $Y_{400}F$ internalisation defective 5-$HT_{1A}R$ expressed in CHO-K1 cells

Key; cells incubated in the absence (◆) or presence (■) of 1μM 8-hydroxy-DPAT. The X-axis represents the conversion of [^{3}H] 8-hydroxy-DPAT specific binding in dpm to fmol/ mg protein. The Y-axis is a ratio; this is calculated as bound radioligand as a function of the original "free" radioligand concentration. The B_{max} (fmol/ mg) was calculated from the X-axis intercept. The K_d (nM) was derived from the gradient of each line. Data points are the mean values of three separate assays ± SEM.

The disruption of the internalisation motif at this position was proposed to interrupt receptor redistribution in response to agonist incubation. Therefore any observable changes in receptor redistribution rates in response to agonist would help us to address one of the aims of this project outlined in section 1.7.1., that being "To define the role of putative phosphorylation sites located on the second and third intracellular loops and 7th transmembrane internalisation motif (NPVIY) in the cellular redistribution of the 5-HT_{1A}R". In order to generate the specified mutations site directed mutagenesis of the G-21 clone (as described in the Methods section 2.1.1.) was performed. Incubations in the absence or presence of 1μM 8-hydroxy-DPAT were performed exactly as described in the sections 2.4.1.iii. Membrane enriched fractions of CHO-K1 cells expressing the mutated ($Y_{400}A$) 5-HT_{1A}R were subjected to radioligand binding analysis (**Figure 30i**) as described in section 2.2.

Linear transformation of saturation data for both control and agonist-treated samples (**Figure 30ii**) enabled a calculation of B_{max} and K_d in terms of nM. For host cells treated with PBS alone (control), the B_{max} was estimated at 78.6 ± 0.5fmol/ mg. The K_d was calculated to be 5.8 ± 0.6nM. For those cells treated with 8-hydroxy-DPAT (1μM) the B_{max} was estimated at 94.3 ± 3.0fmol/mg while the K_d was calculated to be and 8.7 ± 0.5nM (**Figure 30ii**). Changes in B_{max} and K_d for agonist treated cells were found to be significant ($P<0.05$ and $P<0.05$ respectively) when compared to control. These data suggest that the substitution of an aromatic residue for a non-aromatic amino acid at this site had the effect of increasing the availability of receptors at the plasma membrane whilst maintaining a low nanomolar affinity for the agonist.

A summary of the values obtained for all "internalisation motif" mutant receptor types is included (**Table 4;** page 126).

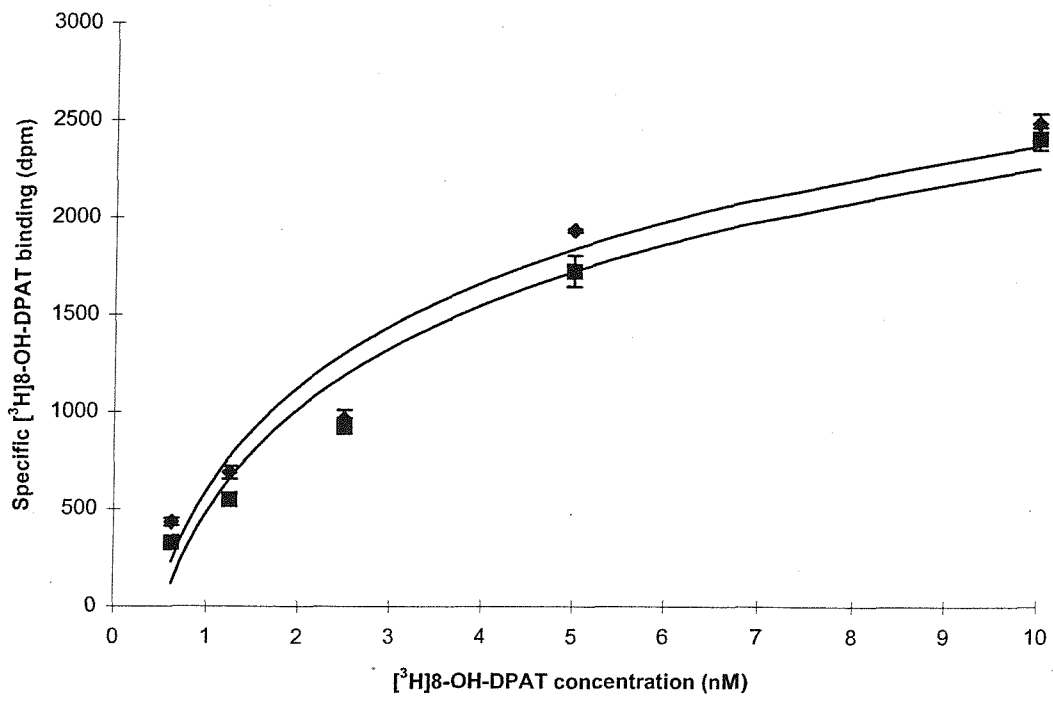

Figure 30i: Saturation analysis of specific [^{3}H] 8-hydroxy-DPAT binding to the Y_{400}A internalisation defective 5-HT_{1A}R expressed in CHO-K1 cells

5-HT_{1A}Rs expressing the Y_{400}A mutation were incubated for 20 minutes in the absence (◆) or presence (■) of 1μM8-hydroxy-DPAT. Membrane enriched fractions were subject to radioligand binding analysis in the presence of [^{3}H] 8-hydroxy-DPAT as described in detail in sections 2.2. Each data point represents the mean of three separate assays ± SEM.

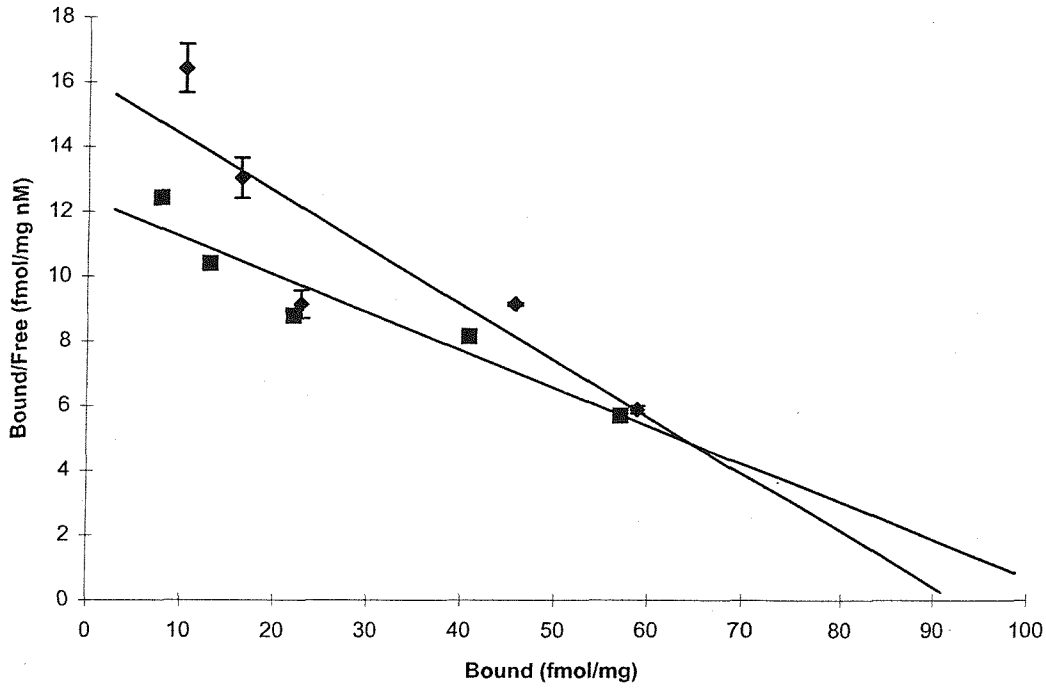

Figure 30ii: Scatchard analysis of saturation data for the $Y_{400}A$ internalisation defective 5-$HT_{1A}R$ expressed in CHO-K1 cells

Key; cells incubated in the absence (◆) or presence (■) of 1μM 8-hydroxy-DPAT. The X-axis represents the conversion of [^{3}H] 8-hydroxy-DPAT specific binding in dpm to fmol/ mg protein. The Y-axis is a ratio; this is calculated as bound radioligand as a function of the original "free" radioligand concentration. The B_{max} (fmol/ mg) was calculated from the X-axis intercept. The K_d (nM) was derived from the gradient of each line. Data points are the mean values of three separate assays ± SEM.

Table 4: Summary of calculated B_{max} and K_d values for 5-HT_{1A}Rs incubated in the presence of the specific agonist 8-hydroxy-DPAT

Receptor Mutation	8-hydroxy-DPAT B_{max} (fmol/ mg)	8-hydroxy-DPAT K_d (nM)	8-hydroxy-DPAT B_{max} (% PBS)	8-hydroxy-DPAT K_d (% PBS)
Unmutated	663.4 ± 19.0	5.5 ± 0.3	82.4 ± 0.3 ★	220 ± 5.4 ★
Phosphorylation Loop 2 $T_{149}A$	330 ± 37.4	1.7 ± 0.7	109 ± 11.3	189 ± 40
Phosphorylation Loop 3i $T_{229}A$	380.9 ± 36.3	2.2 ± 0.5	99 ± 9.5	100 ± 22.7
Phosphorylation Loop 3ii $S_{253}G$	131.2 ± 24.3	4.4 ± 1.8	24 ± 18.5 ★★★	56 ± 40
Internalisation $NPVIY_{400}F$	99.7 ± 31.9	5.7 ± 2.9	95 ± 32	211 ± 50
Internalisation $NPVIY_{400}A$	94.3 ± 3.0	8.7 ± 0.3	120 ± 3.0 ★	150 ± 0.3 ★
Epitope Tagged	127.8 ± 1.5	9.9 ± 0.6	80.3 ± 0.1 ★	81.4 ± 4.5

★ Indicates level of data significance using students t-test.

★ $=P<0.05$
★★ $=P<0.01$
★★★ $=P<0.001$

5.3. INHIBITION OF RECEPTOR REDISTRIBUTION

5.3.1. Introduction

The experimental data presented in this section provide further evidence for the phenomenon of receptor internalisation. To help us address this question it was hypothesised that compounds shown to prevent receptor internalisation in the hands of other authors (see for example Pippig *et al.*, 1995) such as hypertonic sucrose solutions and concanavalin A, may in our system also be effective in attenuating receptor redistribution. To test this idea various concentrations of sucrose and Concanvalin A were used to pre-treat CHO-K1 cells expressing the human 5-HT_{1A}R prior to agonist exposure.

5.3.2. Effect of hypertonic sucrose

As already discussed in earlier chapters hypertonic sucrose is thought to exert its effect due to an increased external solute concentration, disrupting the osmotic potential of the cell causing a net loss of water. Exposure to hypertonic medium and intracellular potassium depletion has also been shown to induce the loss of plasma membrane-associated clathrin lattices and disappearance of clathrin-coated pits (Hansen *et al.*, 1993). The experimental methods used here were very similar to those outlined in section 2.4.1.iii. except that cells were incubated in the absence or presence of the specified compound whilst still adherent to the culture dish. The cells were then washed with PBS in a similar fashion to those described in section 2.4.1.iii. and then pooled prior to [^{3}H] 8-hydroxy-DPAT binding studies. In this case pre-incubation of CHO-K1 cells expressing the 5-HT_{1A}R with 1μM 8-hydroxy-DPAT resulted in a significant decrease (72.5 %; $P<0.05$) in 2.5nM [^{3}H] 8-hydroxy-DPAT binding when compared to the control cells incubated in PBS indicating the levels of receptor redistribution under normal conditions. CHO-K1 cells were briefly pre-incubated (for 20 minutes at 37°C) in a range of hypertonic sucrose solutions (from 0.4M to 0.8M) prior to incorporation of unlabelled agonist in the reaction mixture.

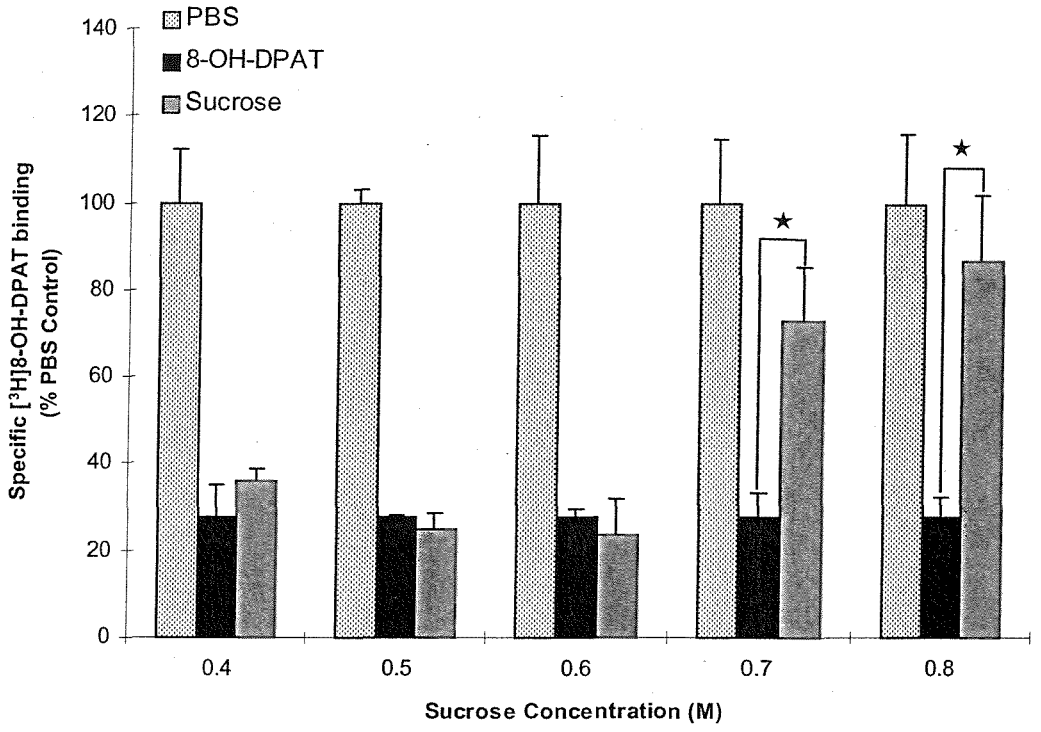

Figure 31: Effect of pre-incubation with hypertonic sucrose on the specific binding of [^{3}H] 8-hydroxy-DPAT to CHO-K1 cells expressing the unmutated 5-HT_{1A}R

CHO-K1 cells expressing the human 5-HT_{1A}R were incubated in the absence or presence of hypertonic sucrose solutions (0.4-0.8M) for 20 minutes prior to incubation with PBS in the absence or presence 1μM 8-hydroxy-DPAT. Key; PBS = cells treated with PBS alone, 8-hydroxy-DPAT = cells treated with PBS in the presence of 1μM 8-hydroxy-DPAT and Sucrose = cells treated with PBS in the presence of 1μM 8-hydroxy-DPAT and hypertonic sucrose at specified concentrations. All reactions were then terminated using an ice-cold PBS wash and membrane fractions were prepared as described previously (Methods section 2.2.). Radioligand binding was performed using 2.5nM [^{3}H] 8-hydroxy-DPAT. All data points represent the mean value of three separate assays ± SEM. ★ indicates level of significance (P<0.05) for results which were significantly different from each other.

Following radioligand binding analysis, the results showed a significant inhibition in the decrease of [^{3}H] 8-hydroxy-DPAT specific binding induced by 1μM 8-hydroxy-DPAT. It was however apparent that at all sucrose concentrations tested, specific binding of [^{3}H] 8-hydroxy-DPAT was submaximal when compared to control cells. At the two highest concentrations tested (0.7 and 0.8M) a significant (P<0.05) attenuation of the decrease in [^{3}H] 8-hydroxy-DPAT initiated by 1μM 8-hydroxy-DPAT was observed (72.9 ±12.7 and 87.0 ± 15.1% respectively; **Figure 31**). When lower concentrations of sucrose were used (0.4, 0.5 and 0.6M) reduction in specific binding (35.9 ± 2.7, 24.8 ± 4.1 and 23.3 ±8.7% respectively; **Figure 31**) were observed but the changes were not significant upon analysis. Incubation of the CHO-K1 cells with PBS in the presence of all hypertonic sucrose solutions had no observable effect on the specific binding of [^{3}H] 8-hydroxy-DPAT (results not shown).

5.3.3. Effects of Concanavalin A (Con A)

As already discussed in previous chapters Con A is a lectin isolated from the jack bean plant which has a high affinity for terminal α-D-mannosyl and α-D glucosyl residues found within surface glycoproteins and impairs their mobility within the membrane bilayer (Luttrell *et al.*, 1997). The experimental methods used here were very similar to those outlined in section 2.4.1.iii. except that cells were incubated in the absence or presence of the specified compound whilst still adherent to the culture dish. The cells were then washed with PBS in a similar fashion to those described in section 2.4.1.iii. and then pooled prior to [^{3}H] 8-hydroxy-DPAT binding studies. In this case incubation of CHO-K1 cells expressing the unmutated 5-HT_{1A}R with 1μM 8-hydroxy-DPAT resulted in a significant mean decrease (79.3 ± 5.38%, P<0.01) in [^{3}H] 8-hydroxy-DPAT binding when compared to the control cells incubated with PBS alone (control values were set to100%).

CHO-K1 cells were pre-treated with Concanavalin A (Con A) within the range 50 μg/ ml to 1mg/ ml for 20 minutes at 37°C prior to incorporation of unlabelled agonist in the reaction mixture.

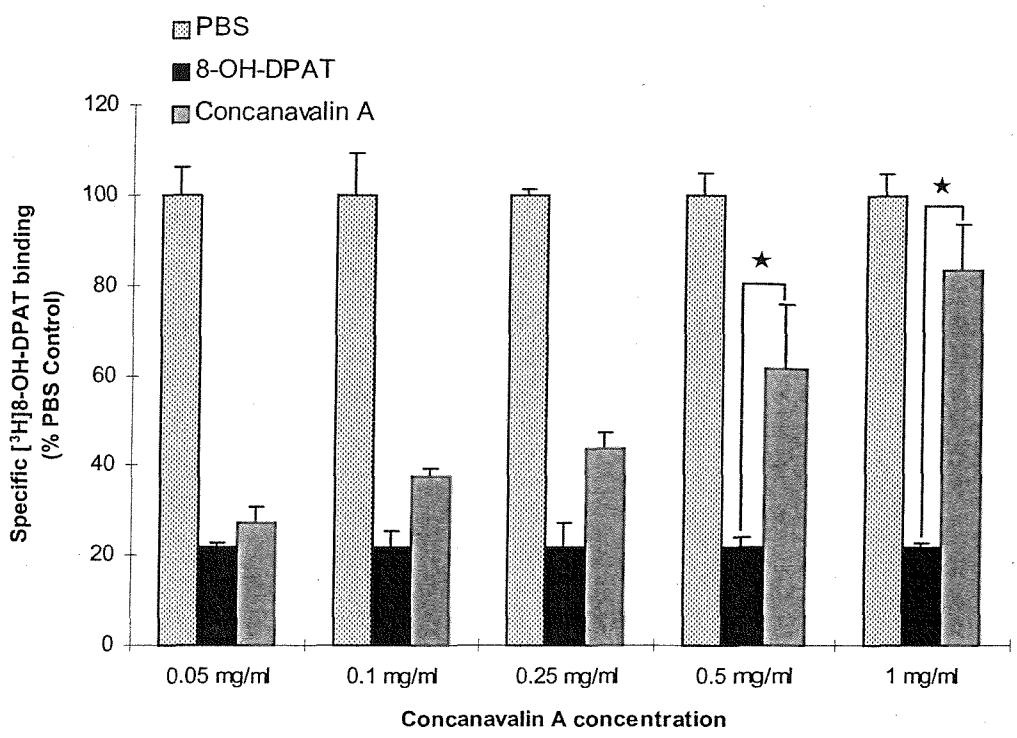

Figure 32: Effect of pre-incubation with Concanavalin A (Con A) on specific binding of [^{3}H] 8-hydroxy-DPAT to CHO-K1 cells expressing the unmutated 5-HT_{1A}R

CHO-K1 cells expressing the unmutated human 5-HT_{1A}R were incubated in the absence or presence of Concanavalin A (50 μg/ ml-1mg/ ml) for 20 minutes prior to incubation in the absence or presence 1μM 8-hydroxy-DPAT. Key; PBS = cells treated with PBS alone, 8-OH-DPAT = cells treated with PBS in the presence of 1μM 8-hydroxy-DPAT, Concanavalin A = cells treated with PBS in the presence of 1μM 8-hydroxy-DPAT plus Concanavalin A at specified concentrations. All reactions were then terminated using an ice-cold PBS wash and membrane fractions were prepared as described previously (Methods section 2.2). Radioligand binding analysis was performed in the presence of 2.5nM [^{3}H] 8-hydroxy-DPAT. All data points are the mean value of three separate assays ± SEM. ★ indicates level of significance (P<0.05) for results which were significantly different.

With all Con A concentrations tested, [^{3}H] 8-hydroxy-DPAT specific binding was found to be submaximal (i.e. less than 100% of the control cell binding) when compared to those cells incubated with PBS alone. At the two highest concentrations of Con A used (500μg/ ml and 1mg/ ml) a significant (P<0.05) attenuation of the decrease in [^{3}H] 8-hydroxy-DPAT triggered by 1μM 8-hydroxy-DPAT was observed (61.4 ± 14.7% and 83.6 ± 10.3% respectively; **Figure 32**). The lower concentrations of Con A (50μg/ ml 100μg/ ml and 250μg/ ml) tested, also resulted in an increase of [^{3}H] 8-hydroxy-DPAT specific binding but when these results were compared to those obtained following treatment with 1μM unlabelled 8-hydroxy-DPAT they were not significant. Incubation of the CHO-K1 cells with PBS in the presence of all Con A concentrations had no observable effect on the specific binding of [^{3}H] 8-hydroxy-DPAT (results not shown).

5.3.4. Receptor recycling and the effects of cycloheximide

In these experiments we aimed to demonstrate that once the 5-HT$_{1A}$R had internalised upon agonist incubation, it was able to recycle to the cell surface even in the presence of the protein synthesis inhibitor cycloheximide (Obrig *et al.*, 1971). We reasoned that if, after agonist challenge an increase in [^{3}H] 8-hydroxy-DPAT was recorded even in the presence of cycloheximide, that the increase in specific binding could be uncoupled from new receptor synthesis and therefore could only be receptor recycling. All experiments were performed as outlined in section 2.4.1.iii. except that instead of full receptor saturation analysis being performed, only the binding of 2.5nM [^{3}H] 8-hydroxy-DPAT was defined.

The intact CHO-K1 cells expressing the unmutated 5-HT$_{1A}$R were incubated in the absence or presence of the protein synthesis inhibitor cycloheximide (20μg/ ml) for1 hour followed by incubation for 20 minutes in the absence or presence of 1μM 8-hydroxy-DPAT at 37°C. Membrane enriched fractions were then prepared and subjected to radioligand binding analysis in the presence of 2.5nM [^{3}H] 8-hydroxy-DPAT as previously described (Methods section 2.2).

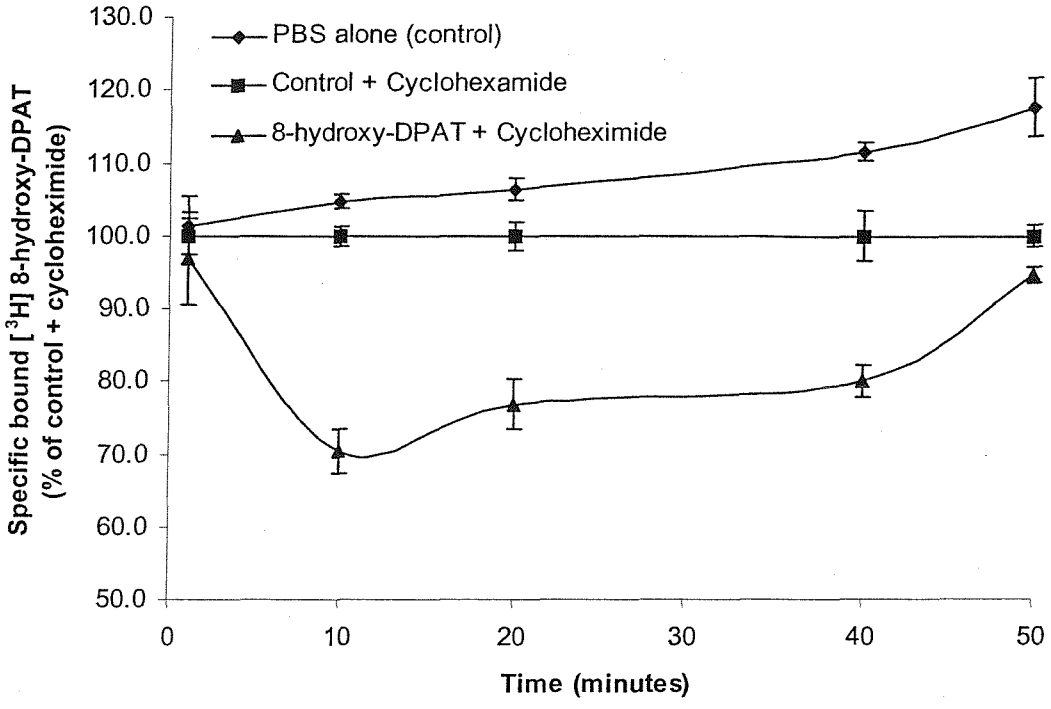

Figure 33: Effect of the protein synthesis inhibitor; cycloheximide on time-dependent redistribution of the unmutated 5-HT_{1A}R

Intact CHO-K1 cells expressing the unmutated 5-HT_{1A}R were incubated in the absence or presence of 20μg/ml cycloheximide for 1 hour before incubation in the absence or presence of 1μM 8-hydroxy-DPAT (at 37°C for time periods indicated above). At 1, 10 and 20-minute intervals, aliquots of cell suspension were removed from all reaction tubes and divided in half. One half an aliquot was washed with room temperature PBS, and returned to the incubator in the absence of 8-hydroxy-DPAT for periods up to 50 minutes. The remaining half aliquots were used to prepare a membrane enriched fraction which was subjected to radioligand binding in the presence of [^{3}H] 8-hydroxy-DPAT (2.5nM). All data points are the mean values of three separate assays ± SEM.

There was an observed decrease in specific [^{3}H] 8-hydroxy-DPAT binding for cells incubated with PBS in the presence of cycloheximide and 1μM 8-hydroxy-DPAT of 29.6% to 70.4 ± 2.9% that of the control values (PBS in the presence of cycloheximide) and 23.1% to 76.9 ± 3.5% that of the control values at time points 10 and 20 minutes respectively. All values were found to be significant when compared to cells incubated with PBS in the presence of cycloheximide alone (in all cases $P<0.05$; **Figure 33**).

Early indications also suggested that the $S_{253}G$ mutant version of the 5-$HT_{1A}R$ was able to internalise upon agonist challenge. This led us to question if the receptor mutant was also able to recycle in the presence of cycloheximide. In order to test this hypothesis intact CHO-K1 cells expressing the $S_{253}G$ mutant form of the 5-$HT_{1A}R$ were preincubated with PBS in the absence or presence of cycloheximide (20μg/ ml) for 1 hour prior to incubation for 20 minutes in the absence or presence of 1μM 8-hydroxy-DPAT at 37°C. Membrane enriched fractions were prepared and then subjected to radioligand binding analysis with 2.5nM [^{3}H] 8-hydroxy-DPAT as described previously (2.2.).

The observed decrease in specific [^{3}H] 8-hydroxy-DPAT binding for cells incubated in the presence of cycloheximide and 1μM 8-hydroxy-DPAT was 28.0 ± 3.6% and 15.3 ± 3.2% at time points 10 and 20 minutes respectively. All values were found to be significant when compared to the specific [^{3}H] 8-hydroxy-DPAT binding of those cells incubated in the presence of cycloheximide alone (in all cases $P<0.01$; **Figure 34**).

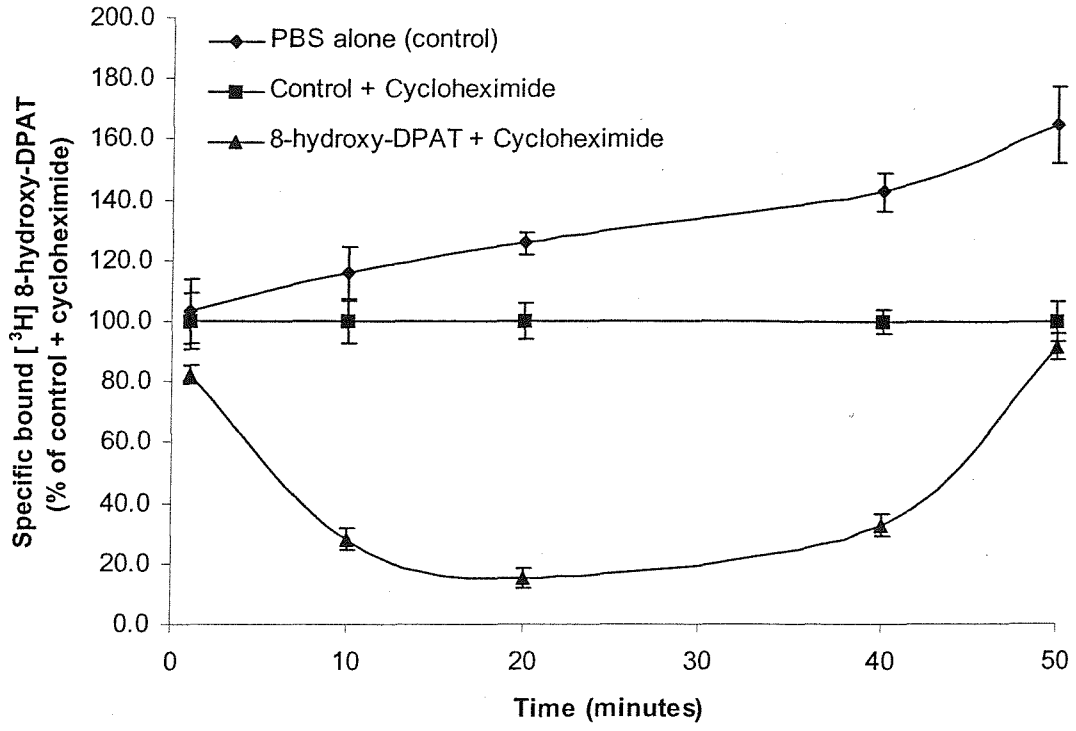

Figure 34: The effect of the protein synthesis inhibitor, cycloheximide on time dependent redistribution of the $S_{253}G$ mutant 5-$HT_{1A}R$

CHO-K1 cells expressing the $S_{253}G$ mutant 5-$HT_{1A}R$ were incubated in the absence or presence of 20μg/ml cycloheximide for 1 hour before incubation in the absence or presence of 1μM 8-hydroxy-DPAT (at 37°C for time periods indicated above). At periods of 1, 10 and 20-minutes, aliquots of cell suspension were removed from all reaction tubes and divided in half. One half an aliquot was washed with room temperature PBS, and returned to the incubator in the absence of 8-hydroxy-DPAT for periods up to 50 minutes. The remaining half aliquots were used to prepare a membrane enriched fraction which was subjected to radioligand binding in the presence of 2.5nM [^{3}H] 8-hydroxy-DPAT. All data points are the mean values of three separate assays ± SEM.

5.4. DISCUSSION

5.4.1. Internalisation strategy 3

This section of the discussion will be entirely dedicated to reviewing the evidence provided suggesting that the 5-HT_{1A}R is subject to endocytosis using the third internalisation protocol. This first section will be concerned with the unmutated 5-HT_{1A}R and will cover findings indicating that the receptor is able to internalise, recycle and the pathways involved. Once covered it should provide a basis for the comparison of results obtained using mutated variants of the 5-HT_{1A}R. The comparison of unmutated and mutated receptor variants will help to address the hypothesis that mutations created in consensus phosphorylation and internalisation motif (NPVIY) would abolish agonist-mediated internalisation.

5.4.2. Definition of the unmutated 5-HT_{1A}R internalisation characteristics

After 20 minutes of agonist incubation at 37°C, approximately 20% of unmutated 5-HT_{1A}Rs were observed to internalise to an intracellular compartment of the CHO-K1 cell. Again as with other experiments described earlier, receptor internalisation was defined by the loss of specific [^{3}H] 8-hydroxy-DPAT and [^{3}H] spiperone from isolated CHO-K1 plasma membranes. However, in these experiments rather than just the specific binding of one [^{3}H] 8-hydroxy-DPAT concentration (i.e. 2.5nM) being defined, complete saturation analysis was performed on the isolated cell plasma membranes. Complete saturation analysis allowed the definition of the actual loss of receptors in terms of B_{max} and the change in ligand/ receptor affinity (K_d) if any.

Unmutated 5-HT_{1A}Rs were observed to internalise after 20 minutes of incubation with 8-hydroxy-DPAT at 37°C. Except in this experiment internalisation was defined by the loss of specific [^{3}H] spiperone binding to isolated CHO-K1 plasma membranes. The specific binding of [^{3}H] spiperone was observed to decrease by 28% when compared to CHO-K1 cells incubated with PBS (control). This was an important experiment because it demonstrated that the decrease in receptor [^{3}H] 8-hydroxy-DPAT binding observed for all other experiments was not due to receptor

desensitisation and receptor/ G protein uncoupling. Results presented by Sundaram *et al.*, (1993) demonstrated the decrease in specific [^{3}H] 8-hydroxy-DPAT binding and the increase of [^{3}H] spiperone binding upon receptor activation. In other words activation of the receptor by 8-hydroxy-DPAT leads to the desensitisation of the receptor decreasing the receptor affinity for [^{3}H] 8-hydroxy-DPAT and increasing the affinity for [^{3}H] spiperone. Thus, the decrease in [^{3}H] 8-hydroxy-DPAT specific binding upon agonist treatment was not just the transition of the receptor to a lower agonist affinity state (desensitised) but an actual loss of receptors from the cell plasma membrane.

Comparison of this unmutated rate of internalisation (20-28% receptor internalisation after 20 minutes) with rates reported by other research groups for other receptor types demonstrated the rate presented here to be considerably slower. Ten minutes after agonist addition >50% of β_2-ARs expressed in HEK 293 cells were internalised (Moore *et al.*, 1995). Internalisation of 80-90% of human muscarinic$_2$ (M_2) receptors expressed in CHO-K1 cells occurred within 19 minutes (Tsuga *et al.*, 1998). Results presented by Hasbi *et al.,* (2000) also demonstrated that after 15 minutes of agonist incubation, 60 to 70% of human δ-opioid receptors (also coupled to G_i and G_o) had undergone internalisation. The findings presented here were in agreement with those presented by Harrington *et al.*, (1994), regarding the 5-HT_{1A}R expressed in HeLa cells. They were able to demonstrate an 80% decrease in receptor numbers in response to short term agonist incubation. However, data published by Cowen *et al.*, (1997), suggested that the 5-HT_{1A}R was unable to internalise in CHO cells after 10 minutes of agonist incubation (or at any time). There is no obvious reasons why the results of Cowen *et al.*, (1997) were so different from these presented here and by Harrington et al., (1994). Possible explanations for the difference in results might be the use of a different radioligand ([^{3}H]-MPPI) to define receptor binding and slightly different methods for membrane preparation employed in their study.

The unmutated 5-HT_{1A}R was also observed to recycle from an intracellular compartment to the plasma membrane once the agonist had been removed from the incubation medium. When the CHO-K1 cells were incubated with the agonist, the receptors were observed to be maximally internalised within 10-20 minutes. The

internalisation and recycling of the receptor was indicated by an initial decrease in [^{3}H] 8-hydroxy-DPAT binding followed by a recovery in the amount of radioligand binding. To ensure that the recovery of radioligand binding was not due to the synthesis of new receptors, the cells were incubated for 1 hour in the presence of the protein synthesis inhibitor, cycloheximide. Unmutated 5-HT$_{1A}$Rs were still observed to recycle in the presence of cycloheximide, consequently, new receptor synthesis could be divorced from the observed increase in [^{3}H] 8-hydroxy-DPAT binding suggesting an actual recycling event.

Recycling has been described for a number of receptors and has been suggested as a mechanism by which the cell can regulate the function of a specific receptor. As with receptor internalisation and blockade of receptor internalisation, recycling of receptors is a well-documented event. The list of receptors that are known to recycle include, the M_3 muscarinic receptor (Szekeres *et al.*, 1998), the β_2AR (Pippig *et al.*, 1995; Morrison *et al.*, 1996.), the δ-opioid receptor (Hasbi *et al.*, 2000) and the μ-opioid receptor (Segredo *et al.*, 1997).

The internalisation of the 5-HT$_{1A}$R was also susceptible to the actions of Con A and hypertonic sucrose solutions. Both Con A and hypertonic sucrose solutions were observed to block receptor internalisation in a dose dependent manner. This blockade of receptor internalisation was represented by a gradual attenuation of the decrease in [^{3}H] 8-hydroxy-DPAT binding triggered by the unlabelled agonist. In other words, at the higher concentrations of Con A and sucrose used, almost complete blockade of receptor internalisation was observed.

Hypertonic sucrose solutions and Con A are compounds that are commonly used to block the internalisation of a wide range of receptor types. The blockade of receptor internalisation by hypertonic sucrose and Con A have been reported for among others the δ-opioid receptor (Law *et al.*, 2000; Hasbi *et al.*, 2000), the angiotensin$_2$ type 1 receptor (Tang *et al.*, 2000), the M_3 muscarinic receptor (Edwardson & Szekeres, 1999) and the β_2AR (Pippig *et al.*, 1995).

The regulation of surface bound receptors is an important function in cell homeostasis and as presented here CHO-K1 cells demonstrate the ability to regulate 5-HT_{1A}R function through internalisation. Internalisation of the receptor is an integral part of the desensitisation process involved in the short-term regulation of receptor function. Initially receptor desensitisation occurs, which can be defined as the addition of phosphate groups to serine and threonine residues within consensus sequences, in an intracellular environment, although this is not an absolute requirement. The three proteins that are implicated in 5-HT_{1A}R phosphorylation are, PKC (Raymond, 1991), PKA (Harrington *et al.*, 1994) and GRKs (Nebigil *et al.*, 1995). Four putative PKC phosphorylation sites, two putative PKA sites and 17 potential substrate residues for GRKs (Raymond *et al.*, 1999) are contained within the 2nd and 3rd intracellular loops of the receptor (**Figure 5**). The phosphorylation of the receptor is associated with uncoupling of several signals in several cell types including AC and PLC (Harrington *et al.*, 1994; Raymond, 1991). After the receptor has been phosphorylated the next proposed step is association of the receptor with arrestin proteins and eventual receptor internalisation (Premont *et al.*, 1995; Law *et al.*, 2000). Arrestins are known to bind the receptor with high affinity but Lee *et al.*, (2000) proposed that receptor phosphorylation "facilitates the removal of an inhibitory constraint that precludes receptor-arrestin association in the absence of receptor phosphorylation". The arrestin proteins block the ability of the receptor to continue activating intracellular cascades (Alberts *et al.*, 1994). Phosphorylation of the GPCR at the consensus phosphorylation sequences is thought to be the precursor for receptor internalisation. Further evidence for the requirement of receptor phosphorylation in internalisation was provided by Hipkin *et al.*, (2000). Results presented by Hipkin *et al.*, (2000) showed that activation of PKC by PMA led to the phosphorylation and internalisation of the somatostatin$_{2A}$ receptor GPCR by a mechanism, which was sensitive to PKC inhibitors (GF109203X) but insensitive to hypertonic sucrose.

The recycling and resensitisation of several receptors has been reported, but perhaps the best characterised of these are the β_2AR (Ferguson *et al.*, 1996b, Krueger *et al.*, 1997) and the muscarinic M_3 receptor expressed in SHSY5Y neuroblastoma cells (Szekeres *et al.*, 1998). Of the various internalisation pathways utilised by GPCRs it is unclear as to what factors regulate the exact pathway of receptor internalisation. It

has been reported that GPCRs can use a number of internalisation pathways including the clathrin-mediated pathway (β_2AR; Zhang *et al.*, 1996), caveolae mediated pathway (βAR; Raposo *et al.*, 1989) and non-clathrin mediated pathway (Damke *et al.*, 1995). The list of agents used to study receptor internalisation, resensitisation and recycling included; inhibitors of protein phosphatases, (calyculin), agents that disrupt endosomal vesicle acidification (NH_4Cl), agents that prevent receptor recycling (nigericin) and agents that prevented receptor endocytosis (hypertonic sucrose solution and concanavalin A). The use of such compounds has showed that receptor internalisation is important in the function of resensitisation. Resensitisation can be defined as the dephosphorylation of an internalised receptor resulting in receptor reactivation. Resensitisation is known to occur in intracellular vesicles and is performed by protein phophatases (Ferguson *et al.*, 1996b). The actual resensitisation of a receptor also precedes receptor recycling (Innamorati *et al.*, 1999). Another function of the endosomal vesicle is thought to be the uncoupling of the receptor from its ligand which occurs when the pH of the vesicle is lower by a proton pump (Ferguson *et al.*, 1996b). Once both processes of receptor dephosphorylation and ligand uncoupling have finished, the receptor is free to recycle to the cell surface in a functional state.

The function of receptor internalisation has therefore been proposed as a mechanism by which the cell can dephosphorylate/ resensitise the receptor or in the case of prolonged receptor activity (>3hrs), downregulate receptor protein (Hasbi *et al.*, 2000). In the case of the 5-HT_{1A}R, desensitisation and phosphorylation has been reported (Harrington *et al.*, 1994; Raymond, 1991; Nebigil *et al.*, 1995) and internalisation and recycling of the 5-HT_{1A}R has been shown in this study. It is therefore reasonable to assume judging by the evidence presented by other authors that 5-HT_{1A}R phosphorylation is an important precursor to the internalisation of the receptor.

The data shown in **Figures 31 and 32** show the ability of hypertonic sucrose and Con A to interfere with 5-HT_{1A}R redistribution in response to agonist incubation, suggesting that the 5-HT_{1A}R is indeed undergoing internalisation. The exact mechanism by which hypertonic sucrose solutions act to disrupt cellular

internalisation is unknown, but it is thought to induce the loss of plasma membrane-associated clathrin lattices and disappearance of clathrin-coated pits (Hansen *et al.*, 1993). This loss and disruption of the cells internalisation machinery is thought to retard receptor redistribution. Concanavalin A is also a specific antagonist of endocytosis, specifically clathrin-mediated endocytosis. Again relatively little is known of its mechanism of action but it is thought that its high affinity for terminal α-D-mannosyl and α-D glucosyl residues found within surface glycoproteins, impairs their mobility within the membrane bilayer (Luttrell *et al.*, 1997). The loss of membrane fluidity caused by Con A is thought to inhibit clathrin-dependent endocytic vesicles formation and thus clathrin mediated endocytosis. The ability of both compounds to specifically inhibit clathrin mediated endocytosis pathway indicates that one route of 5-HT_{1A}R internalisation in CHO-K1 cells is via the clathrin-mediated pathway. These findings present here are in agreement with results presented for the human δ-opioid receptor (Hasbi *et al.*, 2000) the human M_3 muscarinic receptor (Edwardson & Szekeres 1999) and the human β_2AR (Pippig *et al.*, 1995) and the μ-opioid receptor (Segredo *et al.*, 1997).

In summary, the 5-HT_{1A}R has been shown in this study to internalise and recycle via a clathrin mediated pathway. Phosphorylation of the receptor has been implicated in receptor desensitisation. To investigate if these phosphorylation sequences are as important in regulation of receptor internalisation a series of mutation were engineered in to the intracellular loops of the 5-HT_{1A}R. In work presented here and by other groups the NPXY motif found in the seventh transmembrane domain of the 5-HT_{1A}R and other receptors has also been implicated in receptor internalisation. Two mutations were engineered into the receptor at position 400 to see if the NPX_nY motif also had a role in 5-HT_{1A}R internalisation. With these mutated variants of the 5-HT_{1A}R it is hoped that we can address the hypothesis, which suggests the important role for consensus phosphorylation sequences and the NPX_nY motif in 5-HT_{1A}R internalisation.

5.4.3. Characteristics of the epitope-tagged 5-HT_{1A}R in terms of internalisation

An epitope-tag was engineered into the extracellular amino-terminus of the 5-HT_{1A}R and the response of this modified receptor to radioligand binding (section 3.2.1.) and GTPγS (section 3.1.5.) were described earlier in discussion section 3.3. In brief, the epitope tagged receptor was able to interact with G proteins (showed using GTPγS), and was, able to bind [^{3}H] 8-hydroxy-DPAT with an affinity in the low nM range.

The epitope-tagged 5-HT_{1A}R internalised at a rate that was comparable to the unmutated receptor. The internalisation of the receptor was indicated by a 20% decrease in specific [^{3}H] 8-hydroxy-DPAT binding to isolated CHO-K1 plasma membranes after agonist incubation when compared to control. This would indicate that the engineered epitope-tag had not affected the receptor conformation and hence the ability to interact with cellular internalisation apparatus. Pharmacological analysis suggested a normal coupling to G proteins. The results presented here were in agreement with the literature. Several groups have successfully engineered epitope tags into the amino terminal of the μ-opioid receptor (Segredo *et al.*, 1997; Arden *et al.*, 1995), and the amino-terminal of the β_2AR (Morrison *et al.*, 1996; Von Zastrow & Kobilka, 1992). All of which reported that the mutations induced did not affect receptor characteristics, as assessed by tracer binding studies in the case of the μ-opioid receptor and flow cytometry and radioligand binding assays in the case of the β_2AR.

Since the epitope-tagged receptor was found to display similar characteristics to the unmutated receptor, it was deemed suitable for use in studies aimed at, visually tracking receptor internalisation in a time dependent fashion. During this study, the epitope-tagged receptors were subjected to a limited feasibility test using FITC conjugated antibodies (data not shown), time constraints precluded a thorough study. That said, real time visualisation of receptor movement in response to agonist exposure would be an interesting and useful subject for further study.

5.4.4. Mutant 5-HT_{1A}Rs displaying accelerated rates of internalisation

Perhaps the most novel finding from this investigation was the discovery that replacement of $serine_{253}$ by glycine located within a consensus PKC phosphorylation site in the 3rd intracellular loop facilitated 5-HT_{1A}R internalisation. The exact aim of this mutation engineered into the 5-HT_{1A}R was to disrupt a putative PKC phosphorylation thought to be important in receptor function and signalling. The S_{253}G mutant variant of the receptor was observed to internalise by 75% upon agonist incubation (i.e. 75% of receptors were absent from the isolated CHO-K1 plasma membrane) when compared to control. The mutation did not significantly affect ligand binding affinity as it was observed to be in the low nanomolar range, an observation that was corroborated by Lembo & Albert, (1995). Upon incubation of the receptor with unlabelled ligand the receptors' affinity for 8-hydroxy-DPAT did increase (approximately 44%, from 7.9 to 4.4nM) this may have been an artefact of the experimental methods used as it was not a significant increase. However, there is a possibility that at this consensus phosphorylation site (serine253), phosphorylation results in a desensitisation effect present in the unmutated receptor but absent in the S_{253}G mutant 5-HT_{1A}R.

The S_{253}G mutant 5-HT_{1A}R was also shown to recycle from an intracellular compartment once the agonist had been removed. When the CHO-K1 cells expressing the mutant variant of the 5-HT_{1A}R were incubated with the agonist 8-hydroxy-DPAT, the receptors were maximally internalised within 10-20 minutes. When the unlabeled agonist was removed, the receptors were observed to recycle back to the cell surface. Both receptor internalisation and recycling were defined by the decrease (within 10 to 20 minutes) upon agonist incubation and then increase (after 40 to 50 minutes) of specific [^{3}H] 8-hydroxy-DPATspecific binding after the cells were washed with PBS. To ensure that the recovery of radioligand binding was not due to the synthesis of new receptors, the cells were incubated for 1 hour in the presence of the protein synthesis inhibitor, cycloheximide. S_{253}G mutants were found to recycle in the presence of cycloheximide and consequently, the observed increase in specific [^{3}H] 8-hydroxy-DPAT binding suggested a recycling event, which could be dissociated from the synthesis of new receptor protein.

The literature regarding the increased internalisation and recycling of a receptor, upon mutation of putative phosphorylation sequence, is limited. Most literature reports the lack of internalisation of phosphorylation mutants (for example Lee *et al.*, 2000). However, Wolf *et al.*, (1999) were able to show that mutation of threonine$_{394}$ to alanine (a putative AEPT$_{394}$AP phosphorylation motif located within the carboxy-terminus) facilitated internalisation and resensitisation (i.e. recycling) of the rat μ-opioid receptor (which also couples to G_i). They suggested that T_{394} represented a negative regulatory signal for μ-opioid receptor internalisation (Wolf *et al.*, 1999). Other authors have also provided evidence suggesting that phosphorylation of the rat μ-opioid receptor by PKC is detrimental to receptor internalisation (Ueda *et al.*, 2001). In the absence of any specific PKC inhibitors, morphine was unable to trigger internalisation, however, in the presence of specific inhibitors of PKC $\alpha\beta$ and γ isoforms (calphostin C, Go6967 and HBDDE) morphine was able to initiate receptor internalisation. These findings suggested that PKC isoforms $\alpha\beta$ and γ prevent μ-opioid receptor internalisation and eventually resensitisation via a recycling event (Ueda *et al.*, 2001). Ueda *et al.*, also reported that these events were also specific to PKC as pre-treatment of cells expressing the μ-opioid receptor with specific inhibitors of PKA and calcium/ calmodulin-dependent protein kinase II (KT5720 and KN93 respectively) had no effect on the dynamics of receptor internalisation upon morphine incubation. These results are significant to this study because they again provide corroborating evidence suggesting that certain consensus PKC phosphorylation sites may act as attenuating signals retarding receptor internalisation. These results also indicate that the mutation in the 5-HT$_{1A}$R may be having its effect on receptor internalisation by preventing PKC phosphorylation rather than by an alteration of receptor characteristics (i.e. conformation, ligand affinity etc.). However, the reason why this point mutation in the 5-HT$_{1A}$R ($S_{253}G$) resulted in such an increased rate of receptor internalisation is unknown. The 2nd and portions of the 3rd intracellular loops of the 5-HT$_{1A}$R are also proposed to form an amphipathic α-helix with a positively charged face and hydrophobic face forming an intracellular binding pocket for interaction with G proteins (Albert *et al.*, 1998). Our findings may also suggest that the mutation may have disrupted the proposed G protein binding pocket. This process

may result in an increased affinity of the receptor for protein kinases or arrestins, to facilitate receptor internalisation.

In the case of the $S_{253}G$ phosphorylation mutant 5-$HT_{1A}R$ it can be argued that not all mutations have a negative effect on receptor internalisation. As Wolf *et al.*, (1999) proposed, it may be a negative regulatory signal for 5-$HT_{1A}R$ internalisation. Furthermore it could prolong the ability of the receptor to activate G proteins even when phosphorylated at an alternative site(s). Further phosphorylation of the receptor in response to continued intracellular signalling might then result in a full receptor desensitisation and internalisation event. A definitive approach to studying the general contribution of phosphorylation to the internalisation process, would be to engineer multiple mutations into the same receptor sequence (i.e. effectively disrupt all putative phosphorylation sites.

In summary the serine located at position 253 in the human full length 5-$HT_{1A}R$ represents an amino acid residue that is a component of a putative PKC phosphorylation site, which acts as an internalisation-attenuating signal (as indicated by mutagenesis studies). These findings are in contrast to observations presented by Hosey *et al.*, (1999), Lee *et al.*, (2000), Law *et al.*, (2000) and Ferguson *et al.*, (1995), who reported a decreased rate of receptor internalisation upon putative phosphorylation site mutation for a range of other receptors (see below). The results are however in agreement with Wolf *et al.*, (1999) and Ueda *et al.*, (2001) who also showed an increased rate of receptor internalisation upon mutation of a putative phosphorylation site or by treatment with specific inhibitors of PKC.

5.4.5. Mutations which attenuate receptor redistribution

5.4.5.i. Mutations within putative phosphorylation sites

Mutations were engineered into putative phosphorylation sites (K_{147}RTPR and R_{227}KTVK) in the 2nd ($T_{149}A$) and 3rd ($T_{229}A$) intracellular loops of the 5-$HT_{1A}R$. These engineered mutations were designed to examine the role of each respective residue in receptor redistribution. Both mutations were found to significantly attenuate internalisation of the 5-$HT_{1A}R$. The attenuation of receptor internalisation

was represented by the absence of any change in [^{3}H] 8-hydroxy-DPAT specific binding to isolated CHO-K1 plasma membranes in response to agonist incubation. These mutations were aimed at disrupting consensus phosphorylation sequences found within the second and third intracellular loops, thought to be important in receptor phosphorylation and regulating receptor function. As discussed earlier it is thought that receptor phosphorylation (desensitisation) is a prerequisite for receptor internalisation. In the case of these two putative phosphorylation motifs K_{147}RTPR and R_{227}KTVK it would appear that, they have an important role in 5-HT_{1A}R internalisation since T_{149}A and T_{229}A were found to inhibit internalisation. That said, the mutant receptors displayed agonist affinities that were in the low nanomolar range similar to that observed for the unmutated receptor. This would indicate that the T_{149}A and T_{229}A mutations had not, themselves, altered the receptor redistribution characteristics (Due to altered 3 dimensional characteristics), rather the lack of phosphorylation at these residues is vital for normal kinetics of internalisation to occur.

The use of phosphorylation mutants to define the role of phosphorylation in receptor function is a common theme in the literature. Site directed mutagenesis has been widely employed to investigate the role phosphorylation in the function of the M_2 muscarinic receptor (Hosey *et al.*, 1999; Lee *et al.*, 2000), the δ-opioid receptor (Law *et al.*, 2000), parathyroid hormone receptor (Malecz *et al.*, 1998), the β_2AR (Ferguson *et al.*, 1995), the dopamine D_2 receptor (Itokawa *et al.*, 1996) and the μ-opioid receptor (Wolf *et al.*, 1999; Ueda *et al.*, 2001). Most of the investigations into receptor phosphorylation have reported that phosphorylation of the receptor is vital for internalisation to occur (Ferguson *et al.*, 1995; Itokawa *et al.*, 1996; Hosey *et al.*, 1999; Lee *et al.*, 2000 and Law *et al.*, 2000). However, there are exceptions to this general pattern as in the case of the parathyroid hormone receptor (Malecz *et al.*, 1998) and as previously described, in the case of the rat μ-opioid receptor, where phosphorylation is detrimental to receptor internalisation (Wolf *et al.*, 1999).

Analysis of receptor function has also been performed on the rat 5-HT_{1A}R (Albert *et al.*, 1999; Albert *et al.*, 1998; Lembo & Albert, 1995; Lembo *et al.*, 1997). The main

thrust of the work was to define the role of these sequences in desensitisation of the rat 5-HT_{1A}R through their ability to influence G protein coupling. Lembo *et al.*, (1997) initially showed that the mutation T_{149}A of the rat 5-HT_{1A}R did not affect ligand binding affinities, and these data were confirmed by the present study on the human 5-HT_{1A}R. However when expressed in Ltk^- fibroblasts, the mutated receptor displayed a reduced ability to inhibit the accumulation of cAMP and an inability to mediate calcium mobilisation. They concluded that the T_{149} residue is important in mediating G protein coupling (via $G_{\beta\gamma}$) to calcium mobilisation and has a less important influence on coupling $G_{i\alpha}$ to inhibition of cAMP turnover (Lembo *et al.*, 1997). In our hands, the mutation in the second intracellular loop was found to cause a 100 fold decrease in IC_{50} when forskolin-induced cAMP accumulation was inhibited by 8-hydroxy-DPAT (Brown & Shaw, 1998, unpublished undergraduate dissertation). These data together with those presented in this study provide further evidence for the importance of the T_{149} residue in regulating receptor function.

Lembo & Albert (1995) also provided evidence suggesting that the T_{229}A mutant 5-HT_{1A}R shared similar characteristics to the T_{149}A mutant. They showed that the T_{229}A mutation did not alter receptor ligand binding affinities when compared to the unmutated receptor; this result was similar to our findings. Unlike the T_{149}A mutation, the T_{229}A mutation had a relatively small effect on receptor coupling to AC via $G_{i\alpha}$. In our laboratory this was represented as a 5 fold decrease in IC_{50} when 8-hydroxy-DPAT was used to inhibit forskolin-stimulated accumulations in cAMP (Brown & Shaw unpublished data) suggesting a significant effect upon signalling through inhibition of AC. In addition, the T_{229}A mutation did result in uncoupling of the receptor from calcium mobilisation mediated by $G_{\beta\gamma}$ in a similar manner to the T_{149}A mutation (Lembo & Albert, 1995). Treatment of the Ltk^- cells with an activator of PKC, 12-O-tetradecanoylphorbol-13-acetate (TPA) result in the decrease of both T_{149}A and T_{229}A mutants ability to mobilise intracellular calcium stores (Lembo & Albert, 1995). As with the T_{149} residue the T_{229} residue is also hypothesised to be an integral part of the amphipathic α-helix, that forms a G protein binding pocket (Albert *et al.*, 1998).

In summary, the findings of Albert *et al.*, 1999; Albert *et al.*, 1998; Lembo & Albert, 1995; Lembo *et al.*, 1997 indicate that the T_{149} and T_{229} residues did not alter receptor ligand binding affinities but they are important in regulating PLC via $G_{\beta\gamma}$. Moreover, there appears to be some debate as to their relative importance in regulating cAMP turnover (via $G_{i\alpha}$ and AC). Results would indicate that in response to agonist activation, both residues are phosphorylated by PKC. Structurally both the T_{149} and T_{229} residues are proposed to contribute to the formation an amphipathic α-helix important in G protein binding. These findings lend weight to our results that report the importance of residues T_{149} and T_{229} in receptor regulation.

Other authors have also been able to show receptor internalisation in response to PKC activation rather than by direct ligand challenge (Hipkin *et al.*, 2000). Using PMA and bombesin Hipkin *et al.*, (2000) were able to show internalisation of the somatostain$_{2A}$ receptor, which is dependent upon receptor phosphorylation. PMA activation of PKC and subsequent internalisation of the somatostain$_{2A}$ receptor was blocked by the PKC inhibitor GF109203X suggesting that in this example at least receptor phosphorylation is a prerequisite for receptor internalisation (Hipkin *et al.*, 2000). In contrast to these findings Smyth *et al.*, (2000) reported that in the case of the human prostacyclin receptor (also a GPCR), using the PKC inhibitor GF109203X and consensus phosphorylation site mutant receptors, that internalisation of the prostacyclin receptor occoured via a PKC independent pathway distinct from desensitisation.

In addition to internalisation triggered by receptor agonist incubation and PKC activation there is also evidence to suggest that receptor internalisation can be triggered by the activation of other GPCRs. One such report suggests that stimulation of the G_q-coupled α_{1A} adrenergic receptor with the specific agonist A61603 lead to the phosphorylation (serine$_{344}$) and internalisation of the mouse δ-opioid receptor (Xiang *et al.*, 2001). The mechanism involved in the internalisation of the δ-opioid receptor triggered by α_{1A} adrenergic receptor stimulation is thought to involve activation of PKC which then promiscuously phosphorylates the δ-opioid receptor (Xiang *et al.*, 2001). There is also evidence suggesting that persistent activation of the β_2AR by

isoproterenol leads to the desensitisation and internalisation of the M_1 muscarinic receptor (Lee & Fraser, 1993). It should however be noted that the internalisation response in both cases is comparable to native ligand stimulation but is smaller in magnitude. A newly discovered assembly protein that is thought to target and promote both PKA and PKC mediated receptor phosphorylation is the A-kinase-anchoring protein (AKAP79/150; Fraser *et al.*, 2000). AKAP79/150 is thought to interact directly and constitutively with the β_2AR promoting phosphorylation by anchoring PKA and PKC in a membrane proximal position thus enhancing receptor phosphorylation.

The role of PKA in the regulation of short-term receptor function has also been explored by several authors (see for example Van Koppen *et al.*, 1995 and Lee *et al.*, 1993). Using site directed mutagenesis techniques Van Koppen *et al.*, (1995) observed that M_4 muscarinic receptor internalisation was independent of PKA and was unhindered by incubation with the PKA inhibitor H-8. In the case of the M_1 muscarinic receptor Lee & Fraser (1993) were able to show that activation of PKA and subsequent internalisation of the M_1 receptor by the β_2AR was blocked by H-8. However, the ability of H-8 to block internalisation of the M_1 receptor when stimulated by the M_1 specific agonist carbachol was absent, suggesting PKA may only be important in heterologous receptor regulation (Lee & Fraser, 1993).

There may also be a role for GRKs' in the mediation of receptor internalisation. As yet a direct link between GRK activation and receptor internalisation remains to be proved but it is well documented that GRKs' mediate receptor phosphorylation in a range of GPCRs' (for example see Pronin & Benovic, 1997 (rhodopsin), Tang *et al.*, 1998 (Type 1A angiotensin II receptor), Ferguson *et al.*, 2000 (adenosine A1 and A3 receptors) and Aiyar *et al.*, 2000 (calcitonin gene related peptide receptor). The regulation of receptor function is further complicated by the ability of PKC to phosphorylate GRK5 indicating that feedback loops may exist within various protein kinases (Pronin & Benvic, 1997).

In summary the role of phosphorylation as a precursor to receptor internalisation is uncertain to say the least, a review of the literature also indicates that receptor phosphorylation does not always result in receptor internalisation. Taking into account the various kinases involved in the phosphorylation process adds a further level of complexity to the equation. PKC has been observed to inhibit (μ-opioid receptor), promote (somatostatin2A receptor) and play no part (prostacyclin receptor) in the internalisation of a variety of GPCRs (Ueda *et al.*, 2001; Hipkin *et al.*, 2000 and Smyth *et al.*, 2000; respectively). PKC may also have a role to play in the heterologous regulation of receptor function as in the case of the δ-opioid receptor and α_{1A} adrenergic receptor (Xiang *et al.*, 2001). On the other hand PKA has been shown not to promote receptor internalisation and is also thought to play a role in heterologous receptor regulation (Van Koppen *et al.*, 1995; Lee & Fraser, 1993). For GRKs it is still unproven if they do indeed have a role in regulating receptor internalisation but their ability to phosphorylate GPCRs is well documented.

5.4.5.ii. Mutations within the NPVIY internalisation motif

The 5-HT_{1A}R contains a highly conserved NPX_nY motif located at positions 396 to 400 in the 7th transmembrane domain. This motif is highly conserved amongst GPCRs but it is also found in the sequences of other receptor superfamilies including the tyrosine kinase receptor family (see Dawidowicz, 1993, for example). In the human 5-HT_{1A}R the sequence of the motif is N_{396}PVIY and is thought to be an important constituent of the internalisation machinery. NPVIY has been proposed as a motif that is essential for receptor internalisation so the experiments presented here were aimed at addressing this question by mutating the 5-HT_{1A}R at position Y_{400}. The receptor sequence was mutated, such that tyrosine was replaced by phenylalanine (Y_{400}F); the latter was deemed similar in structure and charge to tyrosine (a so-called "conservative" substitution). In addition a non-conservative mutation was generated (Y_{400}A) where alanine was deemed very different to tyrosine in terms of structure and charge.

Both mutations significantly attenuated radioligand binding following pre-exposure to the agonist. However, it should be noted that the conservative mutation resulted in a

small degree of internalisation (approximately 5% decrease in [^{3}H] 8-hydroxy-DPAT binding), where as the non-conservative mutation demonstrated no internalisation characteristics. These mutants displayed agonist affinities that were in the low nanomolar range similar to that of the unmutated receptor suggesting that as predicted the Y_{400} residue was not critical for agonist binding. These findings were also in concurrence with results presented for the α_{1b}AR (Y_{348}A) and for the angiotensin II type1 receptor (Y_{302}F/A) (Wang *et al.*, 1997; Laporte *et al.*, 1996).

The function of the NPX_nY motif has been explored across several receptor types. Mutagenesis studies performed on the angiotensin II type 1 receptor demonstrated that the tyrosine residue was important in receptor G protein coupling however it was not vital for receptor internalisation (Laporte *et al.*, 1996). The same trend was observed in studies performed on the α_{1B} adrenergic receptor by Wang *et al.*, (1997), who showed that mutation of the tyrosine residue at position 348 to an alanine resulted in complete G protein uncoupling without effect on receptor sequestration. Studies performed on the β_2AR demonstrated that mutation of $tyrosine_{326}$ to alanine in the NPX_nY motif resulted in normal receptor ligand affinities (Barak *et al.*, 1994), normal G protein coupling, an ability to desensitise and an ability to down-regulate in response to agonist exposure. However, there was a complete lack of receptor internalisation or an ability to resensitise (Barak *et al.*, 1994). Bohm *et al.*, (1997) engineered both conservative (Y_{305}F) and non-conservative (Y_{305}A) mutations into the $neurokinin_1$ receptor to investigate the function of $tyrosine_{305}$. They observed that the conservative mutation demonstrated normal signalling characteristics and impaired internalisation, whereas the radical mutation demonstrated impaired signalling and compromised expression at the cell plasma membrane (Bohm *et al.*, 1997). In contrast, mutagenesis of the conserved tyrosine residue in the $cholecystokinin_A$ receptor and gastrin-releasing peptide receptor had no effect on G protein coupling or receptor internalisation (Go *et al.*, 1998; Slice *et al.*, 1994). These complex and often contradictory findings indicate that each GPCR may have evolved its own combinations of mechanisms to regulate its availability at the cell surface and hence its ability to respond to ligands. Therefore it would seem that the function and mechanism of action of the NPX_nY motif has to be defined for each receptor and cannot be termed a generic internalisation motif. That said, it would appear that the

NPX_nY motif certainly has an important role in the regulation of the human 5-HT_{1A}R. It is perhaps important to note that although the tyrosine residue has been the subject of a few limited studies, none of the residues within the motif have been under as much scrutiny and these should be explored in future studies.

CHAPTER 6

6.1. INDUCTION OF TRANSCRIPTION FACTOR ACTIVITY IN CHO-K1 CELLS EXPRESSING THE UNMUTATED HUMAN 5-HT_{1A}R

6.1.1. NF-κB Introduction

NF-κB was first described in 1986 as a nuclear factor necessary for transcription of immunoglobulin kappa light in B-lymphocytes (Sen & Baltimore, 1986). NF-κB can translocate to the nucleus upon receipt of an appropriate signal where it binds a consensus 10 base pair sequence ($^{5'}$GGGGYNNCCY$^{3'}$). However, NF-κB is maintained in the cytoplasm in an inactive form bound to an inhibitory protein (I-κB) that effectively masks' NF-κBs' nuclear localisation signal. NF-κB activity is induced by a range of factors (see **Figure 8** for overview of NF-κB regulation) which trigger I-κB degradation thus allowing NF-κB to translocate to the nucleus where it can influence gene transcription. In the case of the 5-HT_{1A}R it has been shown that ligand challenge can result in I-κBα degradation (Cowen *et al.*, 1997). However, a direct link between 5-HT_{1A}R challenge and NF-κB has not been reported and it was the aim of the next three experiments to try and show a link using the electrophoretic mobility shift assay (see section 2.6. for method details).

6.1.1.i. Effect of murine TNFα

TNFα is a cytokine secreted by macrophages and mast cells in response to bacterial infection. It is known to be highly cytotoxic for some tumor cells as well as targeting cells involved in the inflammatory response, resulting in an upregulation of other cytokine production including IL-6 and IL-8. Two receptors have been shown to bind TNFα and these are termed TNF receptor 1 (p55) and 2 (p75). Acting via these two receptors TNFα has been shown to activate NF-κB (Mohan *et al.*, 2000). TNFα was therefore used as a positive control in these experiments to ensure NF-κB was at least possible in this system.

We hypothesised that incubation of CHO-K1 cells expressing the unmutated human 5-HT_{1A}R with increasing concentrations of murine TNFα would result in the induction of NF-κB activity. Before the start of each experiment CHO-K1 cells growing in monolayers were incubated for 40 minutes in the absence or presence of TNFα (1ng/ ml to 100ng/ ml) at 37°C. Incubations were terminated and washed with ice cold PBS. Nuclear extracts were prepared from each individual group of cells as described in the Method section 2.6.1. and stored until required (-80°C). Nuclear extracts were incubated with a [^{32}P] radiolabelled NF-κB oligonucleotide probe and complexes were resolved under non-reducing conditions as described in section 2.6.3. In some cases, extracts were also incubated in the presence of commercially prepared antibodies (Santa Cruz) specific to p50 and p65 NF-κB subunits or control antiserum raised against Fra-2 (**Figure 35**). The results showed the ability of CHO-K1 cells to respond in a dose dependent manner to incubation with TNFα.

All TNFα concentrations tested increased the amount of NF-κB binding to the NF-κB probe suggesting that CHO-K1 cells possess TNFα receptors and that this system could therefore be used as a positive control for NF-κB stimulation in CHO-K1 cells. The effects of the specific 5-HT_{1A}R agonist (8-hydroxy-DPAT; **Lane 7**) and antagonist (WAY100635; **Lane 8**) on NF-κB activity were also subjected to investigation. Preliminary data suggested that both compounds may exert an effect over NF-κB DNA binding activity thus warranting further investigation. However the results observed when CHO-K1 cells expressing the 5-HT_{1A}R were incubated in the presence of WAY100635 proved not to be significant (discussed later).

Nuclear extracts that were incubated with the p50 and p65 antibodies (**Lanes 5 and 9**) showed a "supershift" effect (**Figure 35**). That is to say that the antibody specifically retarded the complexes in the polyacyrlamide gel. The supershift effect was found to be absent from those extracts incubated with Fra-2 antibodies (**Lane 6 and 10**).

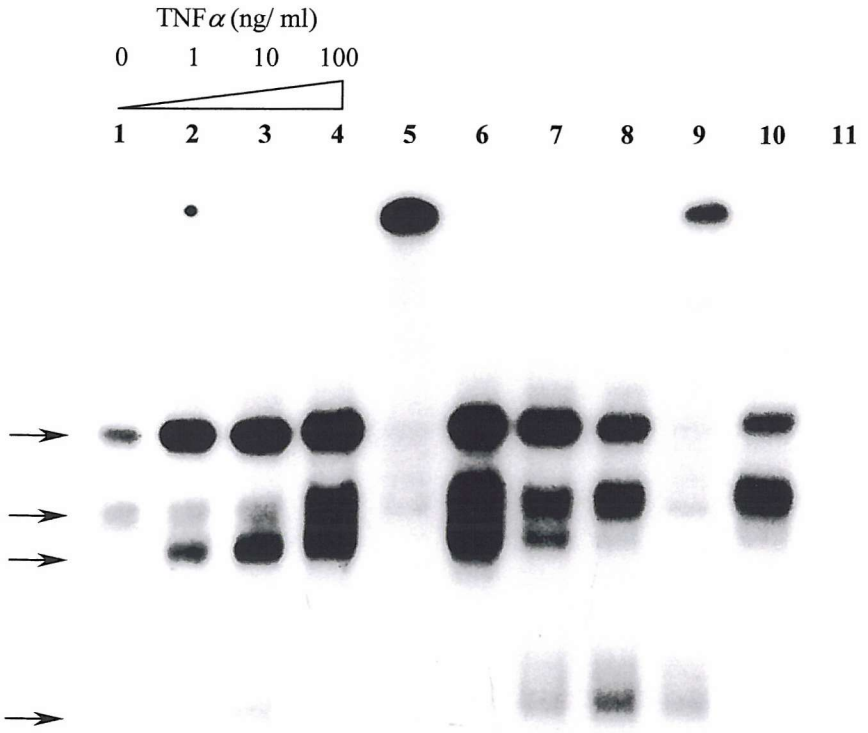

Figure 35: Effect of murine TNFα on NF-κB activity in CHO-K1 cells expressing the full length unmutated human 5-HT_{1A}R

Cells were grown in six well plates and cultured in serum free medium for 2 days before use. All cells were incubated in the absence or presence of effectors for 40 minutes at 37°C. Nuclear extracts were then prepared as previously described (methods section 2.6.1.) and stored until required (-80°C). Nuclear extracts were incubated with a [^{32}P] NF-κB oligonucleotide probe and resolved on an 8% non-denaturing polyacrylamide. To confirm the identity of the p50 and p65 NF-κB subunits supershift analysis was performed (methods section 2.6.4.). Photographs were taken from an autoradiograph exposed for at least 12 hours. Similar results were observed in the three separate experiments performed.

Lane plan from left to right; **Lane 1**: growth medium alone (control). **Lane 2-4**: as indicated. **Lane 5**: as for lane 4 plus p50/ p65 antibodies. **Lane 6**: as for lane 4 plus Fra-2 antibodies. **Lane 7**: as for Lane 1 plus 10μM 8-hydroxy-DPAT. **Lane 8**: as for Lane 1 except cells plus 10μM WAY100635. **Lane 9**: as for Lane 8 plus p50/ p65 antibodies. **Lane 10**: as for Lane 9 plus Fra-2 antibodies. **Lane 11**: [^{32}P] NF-κB probe only. Arrows indicated bands of interest.

6.1.1.ii. Effect of 8-hydroxy-DPAT

In this experiment we hypothesised that incubation of CHO-K1 expressing the unmutated 5-HT_{1A}R with 8-hydroxy-DPAT would induce NF-κB activity. Before use, CHO-K1 cells were incubated in serum free medium for two days. All cells were then incubated in the absence or presence of 8-hydroxy-DPAT (10nM-100μM) for 40 minutes at 37°C. Reactions were terminated by washing cell monolayers with ice cold PBS. Nuclear extracts were prepared and radiolabelled complexes resolved as described previously (Methods section 2.6.).

In contrast to incubation in the presence of growth medium alone (**Figure 36, Lane 6**) treatment of CHO-K1 cells expressing the full length 5-HT_{1A}R with 8-hydroxy-DPAT gave rise to a concentration dependent induction effect on four discernible DNA-protein complexes (indicated by arrows in **Figure 36**). The DNA-protein complexes were correlated with activation of NF-κB. Only the three least mobile complexes were exclusively associated with exposure to agonist. Moreover, the increase in "amount" of these three complexes was optimal when cells were exposed to 1μM 8-hydroxy-DPAT. These results suggest that the human 5-HT_{1A}R at least when expressed in the surrogate environment of CHO-K1 cells, is coupled in some way to the degradation of I-κB and activation of endogenous NF-κB which is then able to translocate to the nucleus.

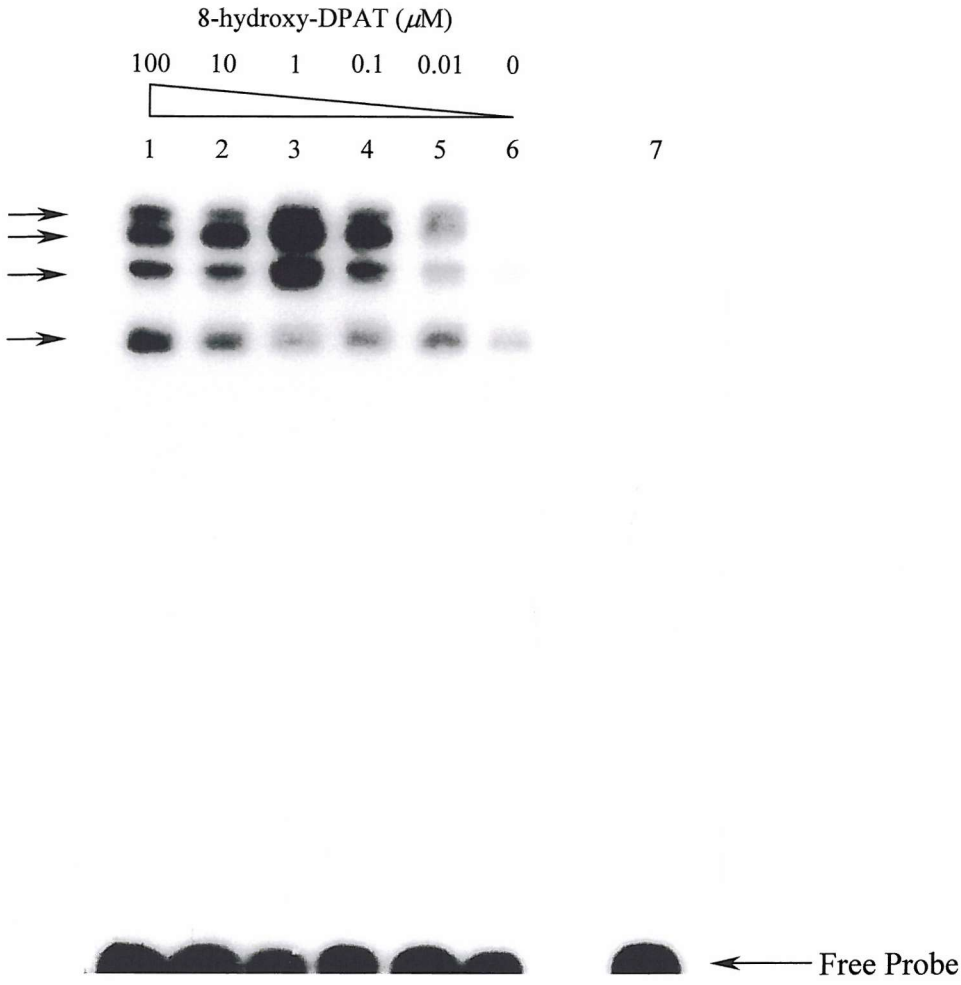

Figure 36: Effect of 8-hydroxy-DPAT on NF-κB activity in CHO-K1 cells expressing the full length human 5-HT_{1A}R

Cells were grown in six well plates and cultured in serum free medium for 2 days before use. All cells were incubated in the absence or presence of 8-hydroxy-DPAT (as indicated in above) for 40 minutes at 37°C. Nuclear extracts were prepared as described in the sections 2.6.1. and stored until required (˜80°C). Extracts were then incubated with a [^{32}P] NF-κB oligonucleotide probe and resolved on an 8% acrylamide gel as described in section 2.6.3.. Photographs were taken from an autoradiograph exposed for at least 12 hours. Similar results were observed in the three separate experiments performed. **Lane 1-5**: 8-hydroxy-DPAT as indicated; **Lane 6**: growth medium alone (control); **Lane 7**: [^{32}P] NF-κB probe alone. Arrows indicate bands of interest relative to control.

6.1.1.iii. Effect of WAY100635

WAY100635 is thought to act as a true antagonist at the 5-HT_{1A}R. In this regard, we hypothesised that it would have no independent receptor-mediated effect upon NF-κB activity in CHO-K1 cells expressing the 5-HT_{1A}R. All cells were then incubated in the absence or presence of WAY100635 (10nM-100μM) for 40 minutes at 37°C. Reactions were terminated by washing cell monolayers with ice cold PBS. Nuclear extracts were prepared and radiolabelled complexes resolved as described previously (section 2.6.).

Incubations with WAY100635 in the nanomolar concentration range, seemed to have no visible effect upon NF-κB activity, even when higher concentrations of WAY100635 (10 and 100μM) were used no effect was observed. Comparison of cells incubated WAY100635 with cells incubated with growth medium alone indicated that the specific antagonist was having little to no effect on the DNA binding activity of NF-κB.

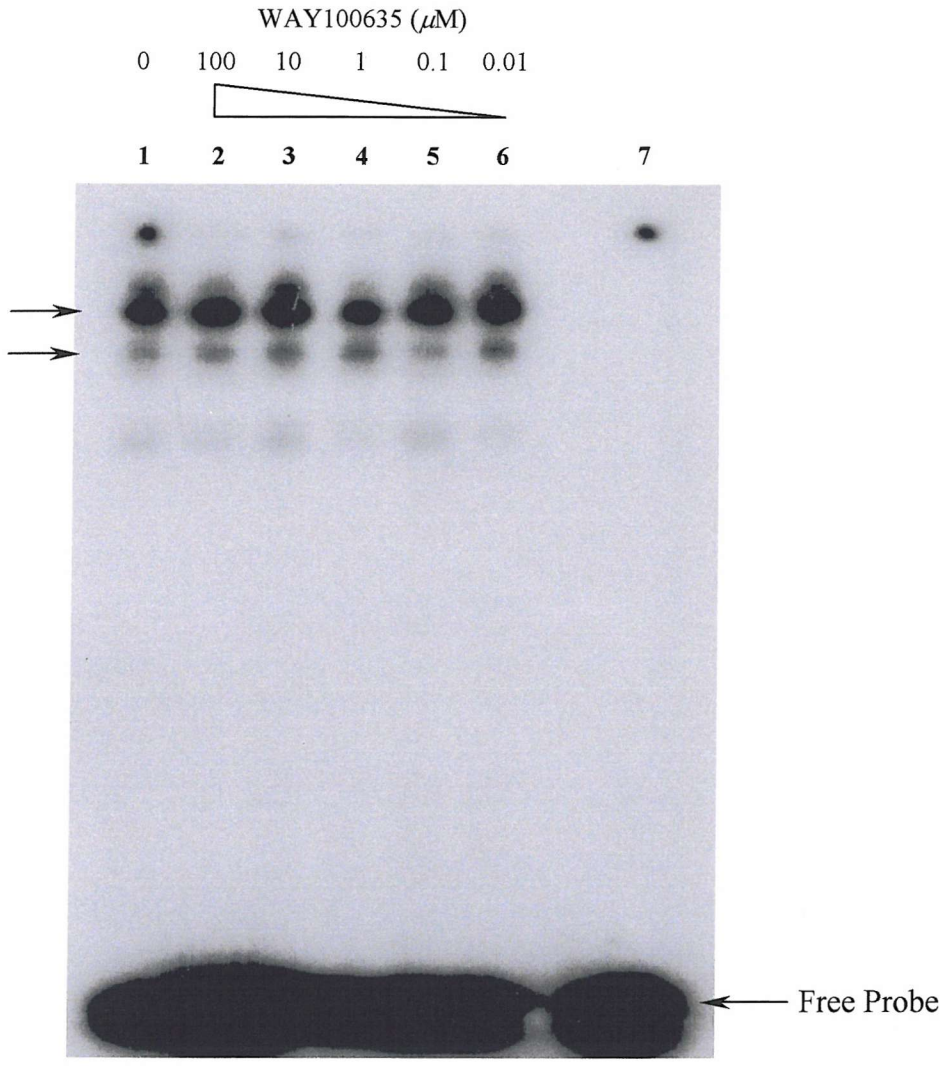

Figure 37: Effect of WAY100635 on induction of NF-κB activity in CHO-K1 cells expressing the unmutated human 5-HT_{1A}R

Cells were grown in six well plates and cultured in serum free medium for 2 days before use. All cells were incubated in the absence or presence of WAY100635 (as indicated above) for 40 minutes at 37°C. Nuclear extracts were then prepared as described in the section 2.6.1. and stored until required (-80°C). Extracts were incubated with a [^{32}P] NF-κB oligonucleotide probe and resolved on an 8% acrylamide gel as described in the section 2.6.3. Photographs were taken from an autoradiogarph exposed for at least 12 hours. Similar results were observed in the three separate experiments performed. **Lane 1**: growth medium alone (control); **Lane 2-6**: WAY100635 as indicated; **Lane 7** [^{32}P] NF-κB probe alone. Arrows indicate bands of interest relative to control.

6.1.2. CREB Introduction

CREB was discovered as a vital component in the regulation of the somatostatin gene expression (Montminy *et al.*, 1986; Andrisani *et al.*, 1989). It is known to exist in the nucleus and is activated by phosphorylation of the kinase inducible domain (Ser_{133}) resulting in an ability to bind the cAMP response element ($^{5'}$TGACGTCA$^{3'}$). CREB has been reported (*in vivo*) to be regulated by the 5-HT_{1A}R agonist 8-hydroxy-DPAT and antagonist WAY100635. Incubation with 8-hydroxy-DPAT also resulted in a 33% inhibition of 5-HT_{1A}R mRNA levels and protein expression suggesting the receptor is also under the control of CREB (Nishi & Azmitia, 1999). It was proposed that the effect on CREB might be due to the ability of the receptor to inhibit AC thus decreasing cAMP turnover. However, as with NF-κB no direct link has been shown between the 5-HT_{1A}R and actual CREB regulation. Thus, the aim of this next set of experiments was to see if there is indeed a link and if so how was the 5-HT_{1A}R able to affect CREB activity.

6.1.2.i. Effect of forskolin

CREB transcription activity is linked to the intracellular concentration of cAMP and thus any proteins that alter the concentration of cAMP also have in theory the ability to alter CREB activity. The 5-HT_{1A}R is one such protein, the coupling of the receptor to cAMP turnover inhibition is demonstrated by agonist (8-hydroxy-DPAT) stimulated inhibition of forskolin induced cAMP production (Harrington *et al.*, 1994). It was therefore reasoned that in this system the 5-HT_{1A}R might be able to alter the DNA binding properties of CREB. Forskolin achieves the increase in cAMP accumulation by directly activating AC, the increase in cAMP leads to the activation of PKA and the phosphorylation of CREB.

Forskolin (a plant alkaloid) directly activates AC in the absence of receptors. In this experiment it was hypothesised that forskolin would likely influence endogenous CREB activity. CHO-K1 cells expressing the unmutated 5-HT_{1A}R were treated with a range of forskolin concentrations (10nM to 100μM) for 40 minutes at 37°C. Nuclear extracts and incubation with [^{32}P] CREB probe were then performed exactly as described in section 2.6.

Basal CREB activity appeared to be significant in CHO-K1 cells as indicated by the presence of radiolabelled complexes associated with samples which had not been exposed to forskolin (**Figure 38, Lane 1**). Forskolin appeared to have a concentration-dependent effect on CREB activity in CHO-K1 cells (**Lanes 2 to 6**). Between 10nM and 100nM forskolin, the banding pattern of four DNA-protein complexes was similar to control. At the higher concentrations tested (1 to 100μM), an additional (lower mobility) complex appeared as indicated by the position of the arrow. These data suggested that forskolin at sufficient concentrations was able to promote the binding of CREB to nuclear proteins presumably through activation of AC.

6.1.2.ii. Effect of 5-HT_{1A}R ligands

In this experiment we hypothesised that as a result of the predominant coupling of the 5-HT_{1A}R to an inhibitory G protein (G_i), application of agonist may inhibit CREB activity. Since basal CREB activity was high (relative to endogenous levels of NF-κB for example), it was reasoned that pre-treatment of the cells with forskolin may not be necessary in order to show an agonist-induced inhibition of CREB.

Following growth in serum free growth medium for two days, CHO-K1 cells expressing the full length 5-HT_{1A}R were treated in the absence or presence of 8-hydroxy-DPAT or WAY100635 (10nM to 100μM) for 40 minutes at 37°C. Nuclear extracts and incubations with [^{32}P] CREB probe were performed exactly as described in section 2.6.

Cells that were incubated in the presence 8-hydroxy-DPAT showed a concentration dependent increase in CREB activity when compared to cells incubated with serum free growth medium alone (**Lane 6**; **Figure 39**). At the lowest and highest concentration range used, (10nM and 100μM) the observed change in CREB binding activity were comparable with each other. The mid ranges of the 8-hydroxy-DPAT concentrations tested, resulted in an increase in active CREB present within the nuclear extracts tested which was dose dependent. This change was most intense between the concentrations of 100nM, 1μM and 10μM with the band intensity decreasing at 100μM (**Figure 39**).

By comparison, WAY100635 appeared to have no clear effect on CREB activity. At the concentration ranges tested (10nM to 100μM) no dose dependent response was observed that was consistent throughout the three separate experiments performed. In **Figure 39, lane 7** and **10** there was an observable decrease in the CREB binding activity when compared to the control (Lane 12), however these findings were not consistent over the three experiments performed. These data would suggest that in the CHO-K1 surrogate cell system acting via the unmutated 5-$HT_{1A}R$, WAY100635 is unlikely to alter endogenous CREB DNA binding activity (**Figure 39**).

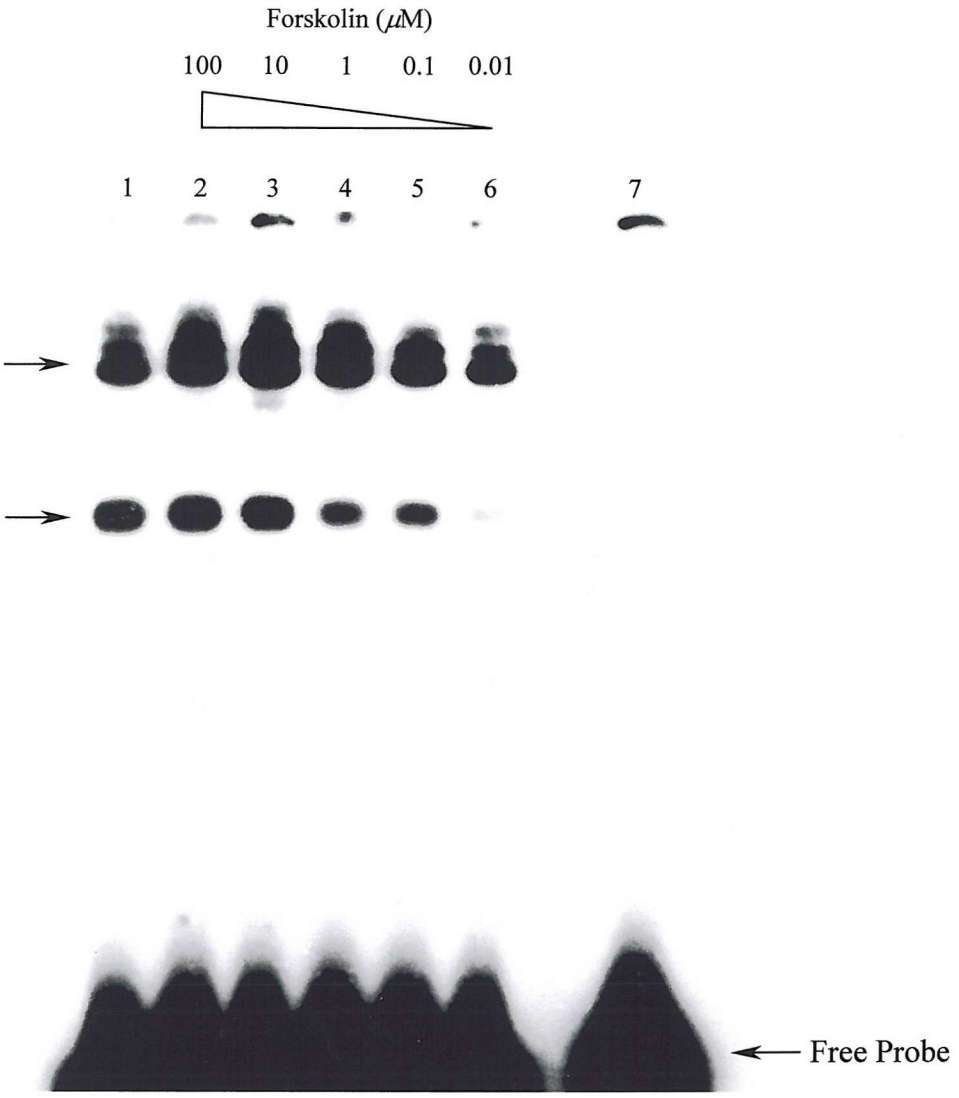

Figure 38: Effect of forskolin on induction of putative CREB activity in CHO-K1 cells expressing the unmutated human 5-HT_{1A}R

Cells were grown in six well plates and cultured in serum free medium for 2 days before use. All cells were incubated in the absence or presence of forskolin (as indicated above) for 40 minutes at 37°C. Nuclear extracts were prepared as described in section 2.6.1. and stored until required (-80°C). Extracts were incubated with a [^{32}P] CRE oligonucleotide probe and resolved on a 8% acrylamide gel as described in section 2.6.3. Photographs were taken from an autoradiograph exposed for at least 12 hours. Similar results were observed in the three separate experiments performed. **Lane 1**: growth medium only (control); **Lane 2 to 6**: cells treated with forskolin as indicated; Lane 7: [^{32}P] oligonucleotide probe alone. Arrows indicates bands of interest relative to control.

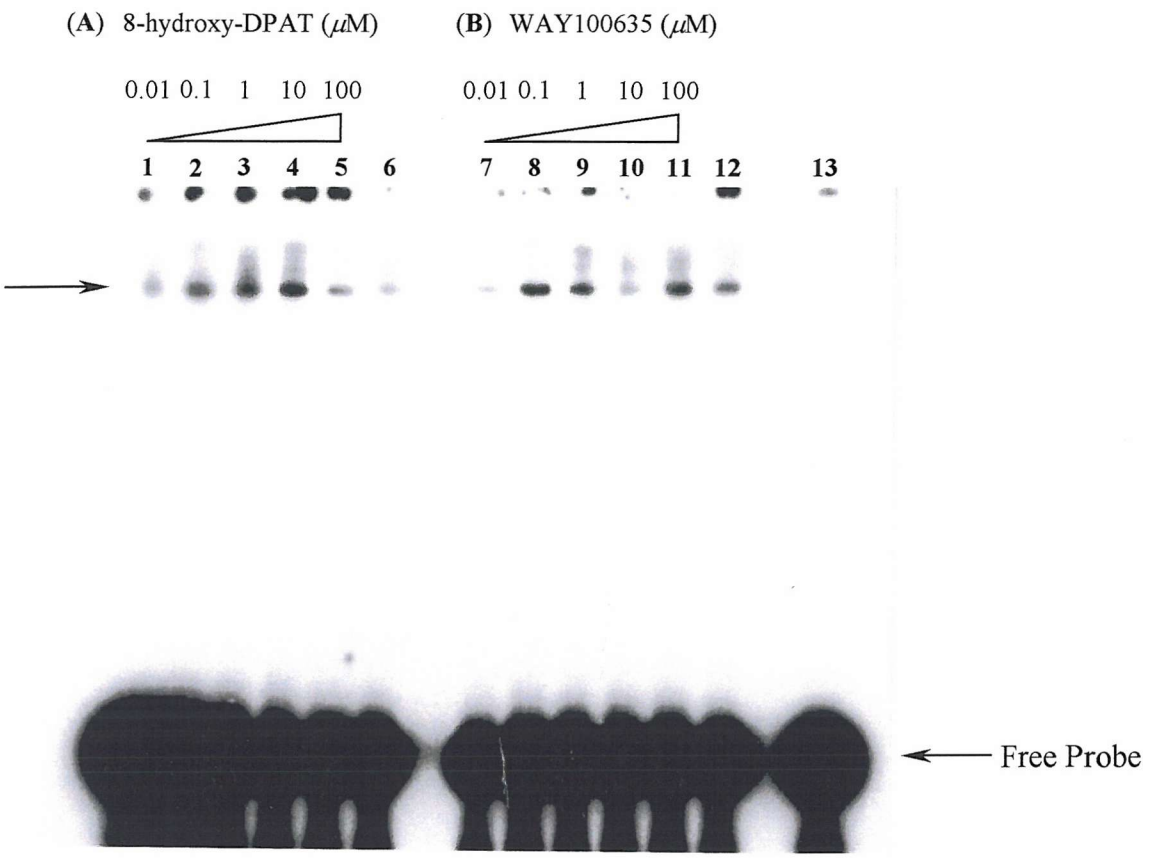

Figure 39: Effect of 5-HT_{1A}R ligands on CREB activity in CHO-K1 cells expressing the unmutated 5-HT_{1A}R

Cells were grown in six well plates and cultured in serum free medium for two days before use. All cells were incubated in the absence of effectors for 40 minutes at 37°C. Nuclear extracts were then prepared as described in section 2.6.1. and stored until required (-80°C). Extracts were incubated with a [^{32}P] CRE oligonucleotide probe and resolved on an 8% acrylamide gel as described in section 2.6.3. Photographs were taken from an autoradiograph exposed for at least 12 hours. Similar results were observed in the three separate experiments performed. (**A**) **Lane 1-5**: 8-hydroxy-DPAT as indicated above; **Lane 6**: growth medium alone (control). (**B**) **Lanes 7-11**: WAY100635 as indicated; **Lane 12**: growth medium alone (control); **Lane 13**: [^{32}P] oligonucleotide probe alone. Arrows indicate bands of interest relative to controls.

6.2. Characterisation of goat anti-5-HT$_{1A}$R polyclonal antibodies (Santa Cruz)

Whole cell extracts were prepared from CHO-K1 cells expressing the unmutated human 5-HT$_{1A}$R as described in detail in "methods" (section 3.5.). Proteins were then resolved on a 9% polyacrylamide gel under reducing conditions after having been loaded into a 4% stacking gel. Western analysis indicated that an immunoreactive band was present in CHO-K1 cells expressing the unmutated 5-HT$_{1A}$R. Based on comparison with standard low molecular weight rainbow markers (biorad) of known molecular weight, the immunoreactive band was estimated to be a protein with a molecular weight of approximately 52kDa. This band was not detectable in extracts of wild-type CHO-K1 cells or rat hepatic stellate cells (**Figure 40**).

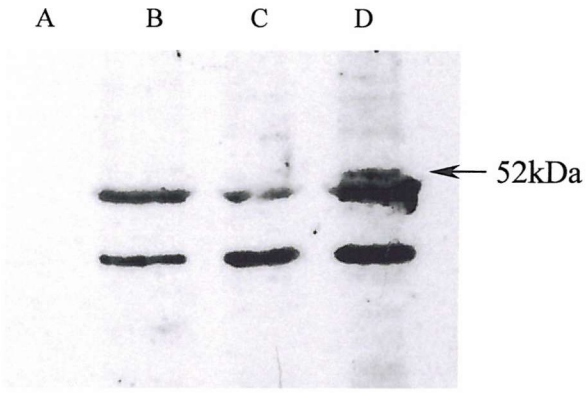

Figure 40: Characterisation of a Goat anti-5-HT_{1A}R polyclonal antibody

Whole cell extracts were prepared from quiescent rat hepatic stellate cells (lane A), activated rat hepatic stellate cells (lane B), wild-type CHO-K1 (lane C) and CHO-K1 cells expressing the unmutated 5-HT_{1A}R (lane D). Proteins were resolved on a 9% SDS polyacrylamide gel under reducing conditions and transferred to PVDF membranes. The band representing the unmutated human 5-HT_{1A}R is highlighted with an arrow giving approximate molecular weight (52kDa). Identical results were observed in three separate experiments.

6.3. MODULATION OF TRANSCRIPTION FACTOR ACTIVITY IN CHO-K1 CELLS EXPRESSING THE UNMUTATED HUMAN 5-HT_{1A}R

6.3.1. Confirmation of protocol sensitivity

In order to study the role of the 5-HT_{1A}R in the regulation of gene expression the activity of two transcription factors (NF-κB and CREB) were studied. Attempts were made to induce and modulate CREB and NF-κB transcription factors using a range of compounds, these included; 8-hydroxy-DPAT, WAY100635, forskolin and TNFα. Had more time been available, experiments looking at the time course of transcription factor induction would have been performed.

The initial experiments were performed to test whether the CHO-K1 system was adequately sensitive to detect the changes in transcription factor activity. Murine TNFα and forskolin were selected because of their proven ability to induce NF-κB and CREB activity respectively (Ghosh *et al.*, 1998; Montminy & Bilezikjian 1987) and consequently served as "positive controls". Both forskolin and murine TNFα demonstrated a positive effect on CREB and NF-κB activity respectively (when incubated at 37°C for 40 minutes), which was observed to be dose dependent (see legends to **Figures 35** and **38**). Not only was forskolin observed to activate CREB it also had a positive effect on NF-κB activity leading to an observable change in band density on the autoradiograph. The mechanisms behind the activation of CREB and NF-κB by forskolin and TNFα are well defined (see Montminy & Bilezikjian, 1987; Ghosh *et al.*, 1998 respectively). Forskolin acts directly upon AC resulting in an increased turnover of ATP to cAMP, the increase in cAMP levels results in the activation of PKA followed by phosphorylation of CREB. On the other hand TNFα (acting through membrane bound TNF receptors) activates NF-κB by first activating an I-κB kinase, resulting in the phosphorylation and degradation of I-κB and the release of activated NF-κB. The induction of NF-kB activity by forskolin is thought to occur via a combination of both pathways. Similar to the initial stages of CREB activation forskolin activates AC increasing intracellular cAMP levels, which in turn activates PKA which is in turn thought to phosphorylate I-κB resulting in I-κB degradation and activation of NF-κB (Koh *et al.*, 1997).

6.3.2. Effects of the specific 5-HT$_{1A}$R ligands 8-hydroxy-DPAT and WAY100635 on the activity of NF-κB

8-hydroxy-DPAT was found to increase the amount of NF-κB present in the nuclear extract of CHO-K1 cells (**Figure 36**). The increase in NF-κB activity stimulated by 8-hydroxy-DPAT was dose dependent and maximal at 1μM with a desensitisation effect noted at higher concentrations (100-10μM). The 5-HT$_{1A}$R specific antagonist WAY100635 demonstrated no effect on the activity of NF-κB in CHO-K1 cells. These findings presented here are in agreement with those presented by Cowen *et al.*, (1997) who reported the ability of 1μM 8-hydroxy-DPAT to stimulate the degradation of I-κB and the inability of the antagonist 4-(2'-methoxyphenyl)-1-[2'-[N-(2'-pyridinyl)-p-iodobenzamido] ethyl] piperazine (MPPI) to stimulate the degradation of I-κB in CHO-K1 cells expressing 5-HT$_{1A}$Rs. Cowen *et al.*, (1997) also reported that treatment of CHO-K1 cells expressing 5-HT$_{1A}$R with 1μM 8-hydroxy-DPAT resulted in the enhanced transcription of a CAT reporter gene, indicating that certain factors essential for reporter gene transcription had been activated. Contained within the transfected plasmid encoding the CAT reporter gene were two copies of the HIV long terminal repeat (LTR) sequences containing tandem consensus NF-κB binding sites (Cowen *et al.*, 1997). Cowen *et al.*, (1997) had therefore proposed that the enhanced transcription of the CAT reporter plasmid was attributed to the degradation of I-κBα and subsequent activation of NF-κB mediated by the 5-HT$_{1A}$R. It is worthy of note that contained within the HIV LTR are other elements that confer responsiveness to other transcription factors. Included in those response elements found within the HIV LTR are, Sp1, CREB and chicken ovalbumin upstream promoter transcription factor (COUP-TF; Schwartz *et al.*, 2000; Rohr *et al.*, 1999). This variety of transcriptional regulation elements contained within the HIV LTR means that no direct link can be made between the human 5-HT$_{1A}$R expressed in CHO-K1, NF-κB and the activation of the CAT reporter assay used by Cowen *et al.*, (1997). However, the EMSA technique used in this study showed a direct link between activation of the 5-HT$_{1A}$R and translocation of NF-κB to the nucleus in an active form.

The proposed mechanisms involved in NF-κB activation by the 5-HT$_{1A}$R are thought to include the activation of a PC-PLC (Phosphatidylcholine specific-Phospholipase C). Treatment with the specific inhibitor of PC-PLC, tricyclodecan-9-yl-

xanthogenate potassium (D609) resulted in the abolition of 8-hydroxy-DPAT induced NF-κB activation (Cowen *et al.*, 1997). In the experiments where 8-hydroxy-DPAT and WAY100635 have been used to induce NF-κB, pre-treatment of CHO-K1 cells with D609 suggests that in this system NF-κB activation may be mediated by PC-PLC (Cowen *et al.*, 1997).

In the current study, antibodies were also used in some experiments in an attempt to identify the specific NF-κB subunits activated by the 5-HT_{1A}R. The antibodies used were specific to NF-κB subunits p50 and p65 which as already mentioned combine to make up the archetypal NF-κB hetrodimer. In these experiments intracellular components induced by 5-HT_{1A}R specific ligands were supershifted (i.e. further retarded in the EMSA) by antibodies specific to NF-κB subunits p50 and p65. In the case of NF-κB induction, all attempts to supershift the most mobile band were unsuccessful. At present further work is continuing to try to identify the more mobile NF-κB subunits involved and is currently thought to be CCAAT-Binding Factor 1 (CBF1; Oakley and Mann, unpublished findings).

6.3.3. Effects of specific 5-HT_{1A}R ligands 8-hydroxy-DPAT and WAY100635 on the activity of CREB

Data presented in this study showed the ability of 8-hydroxy-DPAT to stimulate the activity of CREB in CHO-K1 cells in a dose dependent manner (**Figure 39**). These findings are in contrast to those presented by Nishi & Azmitia (1999) who documented the ability of the 5-HT_{1A}R agonist 8-hydroxy-DPAT (in vitro) to inhibit forskolin stimulated increase in CREB immunoreactivity. CHO-K1 cells that were incubated with WAY100635 elicited relatively little change in the activity of CREB (**Figure 39**). The exact pathway by which the 5-HT_{1A}R regulates the activity of CREB is unknown but it is thought there are two possible pathways. The first of these involves the enzyme PKA; -activation of the 5-HT_{1A}R leads in turn to the inhibition of AC and a decrease in the turnover of cAMP. The resulting decrease in intracellular cAMP results in the inhibition of PKA, which in turn is thought to decrease the phosphorylation of CREB (Montminy & Bilezikjian 1987), these changes would manifest as a decrease in band intensity on an EMSA. The second pathway via which the 5-HT_{1A}R is thought to regulate CREB activity involves the

membrane bound enzyme PLC. The pathway involving PLC appears to have a positive effect upon CREB, as the activation of PLC by the 5-HT_{1A}R eventually results in an increase in intracellular Ca^{2+} concentrations. The increase in intracellular Ca^{2+} is then thought to activate Ca^{2+}/ calmodulin dependent kinases resulting in CREB phosphorylation and activation (Chalecka-Franaszek *et al.*, 1999). In CHO and HeLa cells the 5-HT_{1A}R has also been shown to activate both PI-PLC and PC-PLC (Fargin *et al.*, 1989; Cowen *et al.*, 1997 respectively). It is therefore logical, that as both pathways are activated by the 5-HT_{1A}R it is possible to both activate and inhibit CREB DNA binding characteristics. However, current evidence would suggest that in true physiological systems such as rat foetal hippocampal neurones, the activation of the 5-HT_{1A}R results in a decrease in CREB activity (Nishi & Azmitia 1999). Arresting the activity of CREB resulted in a long-term decrease in 5-HT_{1A}R mRNA and an eventual decrease in receptor protein (Nishi & Azmitia 1999). The discovery that activation of the 5-HT_{1A}R has the ability to regulate the expression of the 5-HT_{1A}R gene is not novel but it may provide a research focus for future treatment of various clinical disorders related to 5-HT_{1A}R dysfunction. However, the data presented here suggests the 5-HT_{1A}R has the ability up regulate the activity of CREB. Further studies need to be performed to look at how the 5-HT_{1A}R, PC-PLC and CREB interact in neurones in order to determine if the activation of CREB in CHO-K1 cells is a real event and not an artefact of receptor expression in a surrogate system.

As the factors regulating the expression of the 5-HT_{1A}R become better defined, modulators of transcription factors such as NF-κB and CREB may become useful targets for compounds used in the treatment of psychiatric disorders. Compounds that may prove useful in the treatment of various psychiatric disorders could be targeted at regulating the transcription activities of CREB. Such compounds used to regulate CREB and NF-κB would need to be highly specific; perhaps unattainably so, as unregulated gene expression is of far greater risk to the patient.

CHAPTER 7

7.1. GENERAL DISCUSSION

The recombinant human 5-HT_{1A}R was expressed in CHO-K1 cells and initial experiments were aimed at the evaluation of this surrogate system as a tool for assessment of the behaviour of the 5-HT_{1A}R. In order to characterise the expression system; ligand saturation analysis, competition experiments and GTPγS competition experiments were performed. Results from these experiments were compared and contrasted with data from the literature (see for example El Mestikawy *et al.*, 1991; Newman-Tancredi *et al.*, 1992 and Sundaram *et al.*, 1993).

The findings presented in this study show that complete receptor saturation could be achieved using [^{3}H] 8-hydroxy-DPAT and [^{3}H] spiperone. Non-specific binding (as defined by 5-HT) in experiments using [^{3}H] 8-hydroxy-DPAT (and spiperone) in experiments using [^{3}H] spiperone was never greater than 10%. Initial results showed that the recombinant 5-HT_{1A}R could be expressed at high levels in CHO-K1 cells with a K_d in the low nanomolar range (1.89 ± 0.14). Western blotting techniques provided further evidence for the expression of the human 5-HT_{1A}R at the plasma membrane in CHO-K1 cells where it had an approximate molecular weight of 52kDa. Reports regarding the molecular weight of the 5-HT_{1A}R indicate some conflicting findings. Chanda *et al.*, (1993) used western techniques to report an immunoreactive band with a molecular weight of 51kDa (COS-1 cells) whereas Zhou *et al.*, (1999) reported an immunoreactive band indicating a molecular weight of 67kDa (hippocampus, amygdala, entorhinal cortex, septum and raphe nuclei). The differences in observed molecular weights may reflect differences in the site of expression and the ability of the cell/ tissue to post-translationally modify the receptor. However, the data presented by Chanda *et al.*, (1993) are in some agreement with data presented in this study. The pharmacological profile of the receptor show 5-HT, 8-hydroxy-DPAT, and WAY100635 to have inhibition constants in the low nanomolar range (K_i = 1.29 ± 0.15nM for 5-HT and 1.29 ± 0.22nM for 8-hydroxy-DPAT and 13.80 ± 3.00nM for WAY100635). In competition experiments where [^{3}H] spiperone was used to define

receptor binding, unlabelled spiperone demonstrated a low inhibition constant also in the nanomolar range (28.27 ± 3.00nM).

The unmutated, epitope tagged and $Y_{400}F$ mutant receptors were all found to couple to an unknown G protein that appeared to be disrupted by GTPγS a non-hydrolysable analogue of GTP. The specific G protein alpha subunit to which the 5-HT_{1A}R couples to in CHO-K1 cells was not explored, but the literature speculates that it is likely to be $G_{\alpha i}$ and $G_{\alpha o}$. All of these data suggest that attempts made to express functional 5-HT_{1A}Rs in CHO-K1 cells were successful and further investigations into the mechanisms of receptor function and regulation could be pursued.

The evaluation and development of three similar experimental protocols with distinct nuances lead to the adoption of the final method used to define the receptor response to agonist challenge. The recombinant unmutated 5-HT_{1A}R underwent a reduction in B_{max} when both adherent and suspended CHO-K1 cells were incubated in the presence of 1μM 8-hydroxy-DPAT. The net loss of receptors from the cell surface membrane (defined with [^{3}H] 8-hydroxy-DPAT and [^{3}H] spiperone) was approximately 20% after 20 minutes of agonist incubation. The net loss of receptors recorded when using [^{3}H] spiperone to define specific binding was approximately 28%. Experiments using [^{3}H] spiperone were important since [^{3}H] spiperone is able to bind receptors which are uncoupled from their G protein perhaps via phosphorylation, whereas [^{3}H] 8-hydroxy-DPAT has been observed to have a far lower affinity for desensitised 5-HT_{1A}R's (Sundaram *et al.*, 1993). Thus it was reasoned that the decrease in specific [^{3}H] 8-hydroxy-DPAT observed after 1μM 8-hydroxy-DPAT challenge was not due to a decrease in receptor/ ligand affinity, but an absolute decrease in receptors present at the plasma membrane.

Con A and hypertonic sucrose solutions also successfully blocked internalisation of the unmutated 5-HT_{1A}R. Since Con A specifically blocks clathrin mediated endocytosis these data indicate that internalisation of the receptor triggered by 8-hydroxy-DPAT is via a clathrin-mediated route of endocytosis. Following internalisation in the presence of 1μM 8-hydroxy-DPAT, the unmutated 5-HT_{1A}R was able to recycle back to the cell surface in the presence of the protein synthesis

inhibitor cycloheximide. Since receptor recycling took place in the presence of cycloheximide, the recovery of [^{3}H] 8-hydroxy-DPAT binding with time can be reasonably divorced from new protein synthesis. It is likely however, that *in vivo*, repeated or long-term exposure to agonists induces changes in the availability of new receptor protein. With the knowledge that the 5-HT_{1A}R gene contains sites for modulation and appears to be under the control of numerous transcription factors (Cowen *et al.*, 1997; Nishi & Azmitia, 1999; Parks & Shenk, 1996 Storring *et al.*, 1999 and Ou *et al.*, 2000), the relationship between the 5-HT_{1A}R response to agonist and the behaviour of two ubiquitous transcription factors was explored (as discussed below).

The reasons for receptor internalisation and recycling are by no means clear since not all GPCRs undergo internalisation and not all respond to specific agonist challenge by internalising. In this case it would appear that the unmutated 5-HT_{1A}R redistributes from the plasma membrane to an intracellular compartment. The proposed reasons for receptor internalisation are three fold; to uncouple the receptor from the specific ligand (in this case 8-hydroxy-DPAT) in a process known as CURL (compartment uncoupling of receptor and ligand), which involves acidification of the internalisation vesicle and subsequent receptor/ ligand dissociation (Alberts *et al.*, 1994); to allow dephosphorylation of the receptor by intracellular phosphatases which also occurs in acidified vesicles. The protein phosphatases known to dephosphorylate GPCRs include the G protein-coupled receptor phophatase (GRP) which is a member of the protein phosphatase type IIA family which is inhibited by okadaic acid and NH_4Cl which has been shown to inhibit vesicle acidification (Pitcher *et al.*, 1995b; Krueger *et al.*, 1997). The third proposed reason for GPCR internalisation is degradation as in the case of the PAR-1 (Trejo *et al.*, 2000). Protease degradation has the effect of permanently removing GPCRs from the plasma membrane as a form of receptor downregulation, where only new protein synthesis will replenish receptors at the cell surface. Data presented in this study suggest that the unmutated 5-HT_{1A}R is able to internalise via a clathrin-mediated pathway resulting in the recycling of the receptor in a state where ligand binding is again possible.

Site directed mutagenesis of the G-21 clone at position 253 resulted in the mutation of a serine residue to a glycine residue in a motif recognised as a substrate for PKC.

This mutation was intended to disrupt receptor phosphorylation at this site, indicating whether phosphorylation had a role in internalisation of the 5-HT_{1A}R. Upon stable transfection of the plasmid encoding the S_{253}G mutant receptor into the CHO-K1 surrogate expression system, radioligand-binding studies indicated expression of the receptor at the plasma membrane (B_{max} 545.8 ± 31.0fmol/ mg K_d 7.9 ± 2.4nM). The S_{253}G mutant 5-HT_{1A}R was observed to undergo internalisation at an accelerated rate (approximately 80% net loss receptor binding after 20 minutes). The rapid kinetics would indicate that serine at position 253 may have an important role in regulating receptor endocytosis. Recycling of the S_{253}G mutant receptor was also observed even in the presence of the protein synthesis inhibitor, cycloheximide, indicating that the return of receptors to the plasma membrane was not due to the production of new receptor protein. It has been shown that mutation of a putative consensus sequence ($AEPT_{394}AP$) within the rat μ-opioid receptor (also known to couple to G_i) and pre-treatment with PKC inhibitors facilitated receptor internalisation and recycling (Wolf *et al.*, 1999; Ueda *et al.*, 2001). These novel findings help corroborate and put the data presented here into context since it would appear that receptor phosphorylation is not a prerequisite to receptor internalisation and in some cases it is clearly detrimental. These data also suggest that the S_{253}G mutation may have its resultant effect on receptor internalisation by preventing PKC mediated phosphorylation. In this study, this manifested in a greater degree of receptor internalisation, suggesting that in the unmutated 5-HT_{1A}R, phosphorylation of the serine residue at position 253 impedes receptor internalisation.

Mutations which attenuated or abolished receptor internalisation were engineered into locations thought to be consensus substrate motifs for PKC in the 2nd and 3rd intracellular loops (T_{149}A, PKC and T_{229}A, PKC), and in the putative internalisation motif located in the seventh transmembrane domain (Y_{400}F and Y_{400}A).

Site directed mutagenesis of the G-21 clone at position 149 resulted in the mutation of a threonine residue to an alanine residue in a motif recognised as a substrate for PKC. This mutation was intended to disrupt receptor phosphorylation at this site, indicating whether phosphorylation of this residue had a role in internalisation of the human 5-HT_{1A}R. Upon stable transfection of the plasmid encoding the T_{149}A mutant receptor

into the CHO-K1 surrogate expression system, radioligand-binding studies indicated expression of the receptor at the plasma membrane (B_{max} 303 ± 10.6fmol/ mg, K_d 0.9 ± 0.1nM). The T_{149}A mutant 5-HT_{1A}R was observed not to undergo internalisation upon receptor challenge with 1μM 8-hydroxy-DPAT. The lack of receptor internalisation would indicate that threonine at position 149 may have an important role in regulating receptor endocytosis. Results presented here regarding the unchanged expression and ligand binding properties of the T_{149}A mutant 5-HT_{1A}R were corroborated by Lembo *et al.*, (1997). Initially they reported that the T_{149}A mutation of the rat 5-HT_{1A}R did not affect ligand-binding affinities. However, when expressed in Ltk^- fibroblasts, the mutated receptor displayed a reduced ability to inhibit the accumulation of cAMP and an inability to mediate calcium mobilisation. They concluded that the T_{149} residue is important in mediating G protein coupling (via $G_{\beta\gamma}$) to calcium mobilisation and has a less important influence on coupling $G_{i\alpha}$ to inhibition of cAMP turnover (Lembo *et al.*, 1997). Our novel findings suggest that the threonine residue at position 149 is also essential for receptor internalisation, in this study, this manifested in a lesser degree of receptor internalisation. The exact mechanism by which T_{149} mediates receptor internalisation is unknown, however, the T_{149} residue is hypothesised to be an integral part of the amphipathic α-helix, that forms a G protein binding pocket which may mediate interactions with cellular internalisation machinery (Albert *et al.*, 1998). Alternatively the T_{149} residue may be an integral part of an internalisation pathway mediated by PKC phosphorylation and not related to any tertiary receptor structure involvement.

Site directed mutagenesis of the G-21 clone at position 229 resulted in the mutation of a threonine residue to an alanine residue in a motif recognised as a substrate for PKC. This mutation was intended to disrupt receptor phosphorylation at this site, indicating whether phosphorylation of this residue had a role in internalisation of the human 5-HT_{1A}R. Upon stable transfection of the plasmid encoding the T_{229}A mutant receptor into the CHO-K1 surrogate expression system, radioligand-binding studies indicated expression of the receptor at the plasma membrane (B_{max} 384.5 ± 26.5fmol/ mg, K_d 2.0 ± 0.5nM). The T_{229}A mutant 5-HT_{1A}R was observed not to undergo internalisation upon receptor challenge with 1μM 8-hydroxy-DPAT. The lack of receptor internalisation would indicate that threonine at position 229 may have an

important role in regulating receptor endocytosis. Results presented here regarding the unchanged expression and ligand binding properties of the $T_{229}A$ mutant 5-$HT_{1A}R$ were corroborated by Lembo & Albert (1995). Unlike the $T_{149}A$ mutation, the $T_{229}A$ mutation had a relatively small effect on receptor coupling to AC via $G_{i\alpha}$. Our novel findings suggest that the threonine residue at position 229 is also essential for receptor internalisation, in this study, this manifested in a lesser degree of receptor internalisation. The exact mechanism by which T_{229} mediates receptor internalisation is unknown, however, the T_{229} residue is hypothesised to be an integral part of the amphipathic α-helix, that forms a G protein binding pocket which may mediate interactions with cellular internalisation machinery (Albert *et al.*, 1998). Alternatively the T_{229} residue may be an integral part of an internalisation pathway mediated by PKC phosphorylation and not related to any tertiary receptor structure involvement.

Site directed mutagenesis of the G-21 clone at position 400 resulted in the conservative mutation of a tyrosine residue to a phenylalanine residue (similar in charge and size) in a highly conserved motif recognised as a putative internalisation signal. This mutation was intended to disrupt receptor internalisation indicating whether this motif does actually play a role in 5-$HT_{1A}R$ internalisation. Upon stable transfection of the plasmid encoding the $Y_{400}F$ mutant receptor into the CHO-K1 surrogate expression system, radioligand-binding studies indicated expression of the receptor at the plasma membrane (B_{max} 104.5 ± 6.4fmol/ mg, K_d 2.7 ± 0.3nM). The $Y_{400}F$ mutant 5-$HT_{1A}R$ was observed to undergo a small but insignificant degree of internalisation (approximately 5%) upon challenge with 1μM 8-hydroxy-DPAT. The retarded rate of receptor internalisation would indicate that tyrosine at position 400 does have an important role in regulating receptor endocytosis. Mutation of the tyrosine residue at position 400 is a novel approach to the study of the 5-$HT_{1A}R$ as is the data presented here indicating a role for Y_{400} in the regulation of receptor internalisation. However, the role of the putative NPX_nY internalisation motif in regulating receptor redistribution is well documented. The $Y_{400}F$ mutant displayed agonist affinities that were in the low nanomolar range similar to that of the unmutated receptor suggesting that the Y_{400} residue was not critical for agonist binding. These findings were also in concurrence with results presented for the

α_{1b}AR (Y_{348}A) and for the angiotensin II type1 receptor (Y_{302}F/A) (Wang *et al.*, 1997; Laporte *et al.*, 1996) who also reported that in each case the NPX_nY motif was not essential for agonist binding.

Site directed mutagenesis of the G-21 clone at position 400 resulted in the non-conservative mutation of a tyrosine residue to an alanine residue (very different in terms of size and charge) in a highly conserved motif recognised as a putative internalisation signal. This mutation was intended to disrupt receptor internalisation indicating whether this motif does actually play a role in 5-HT_{1A}R internalisation. Upon stable transfection of the plasmid encoding the Y_{400}A mutant receptor into the CHO-K1 surrogate expression system, radioligand-binding studies indicated expression of the receptor at the plasma membrane (B_{max} 78.6 ± 0.5fmol/ mg, K_d 5.8 ± 0.6nM). The Y_{400}A mutant 5-HT_{1A}R was observed to totally abolish receptor internalisation upon agonist challenge with 1μM 8-hydroxy-DPAT. The abolition of receptor internalisation mediated by the non-conservative receptor mutation lends further weight to the findings suggesting that tyrosine at position 400 has a vital role in regulating receptor endocytosis. Mutation of the tyrosine residue at position 400 is a novel approach to the study of the 5-HT_{1A}R as is the data presented here indicating a role for Y_{400} in the regulation of receptor internalisation. However, the role of the putative NPX_nY internalisation motif in regulating receptor redistribution is well documented. The Y_{400}A mutant displayed agonist affinities that were in the low nanomolar range similar to that of the unmutated receptor suggesting that the Y_{400} residue was not critical for agonist binding. These findings were also in concurrence with results presented for the α_{1b}AR (Y_{348}A) and for the angiotensin II type1 receptor (Y_{302}F/A) (Wang *et al.*, 1997; Laporte *et al.*, 1996) who also reported that in each case the NPX_nY motif was not essential for agonist binding.

The role of the NPX_nY motif appears to be receptor specific, even though the motif is a highly conserved aspect of GPCR structure it does not necessarily have a role to play in receptor internalisation. In contrast to the 5-HT_{1A}R, those receptors in which the NPX_nY motif has been shown as not having a role in receptor internalisation include the angiotensin II type 1 receptor and the α_{1B} adrenergic receptor (Laporte *et al.*, 1996; Wang *et al.*, 1997). However, in these receptor types it was reported that

the NPX_nY motif did have an important role in receptor/ G protein coupling. Studies performed on the β_2AR showed that mutation of $tyrosine_{326}$ to alanine in the NPX_nY motif resulted in normal receptor ligand affinities (Barak *et al.*, 1994), normal G protein coupling, an ability to desensitise and an ability to down-regulate in response to agonist exposure. However, there was a complete lack of receptor internalisation or an ability to resensitise (Barak *et al.*, 1994). Bohm *et al.*, (1997) engineered both conservative ($Y_{305}F$) and non-conservative ($Y_{305}A$) mutations into the $neurokinin_1$ receptor to investigate the function of $tyrosine_{305}$. They observed that the conservative mutation exhibited normal signalling characteristics and impaired internalisation, whereas the radical mutation exhibited impaired signalling and compromised expression at the cell plasma membrane (Bohm *et al.*, 1997). In contrast, mutagenesis of the conserved tyrosine residue in the $cholecystokinin_A$ receptor and gastrin-releasing peptide receptor had no effect on G protein coupling or receptor internalisation (Go *et al.*, 1998; Slice *et al.*, 1994). From these contradictory reports it can be concluded that as previously suggested the role of the NPX_nY motif is specialised to suit the requirements of each receptor. This study reports that in the case of the 5-HT_{1A}R the NPVIY motif located within the seventh transmembranous domain plays an important role in regulating and facilitating receptor endocytosis, it would however appear that it does not have a role in either ligand binding or G protein coupling (shown using the GTPγS competition studies). It is also unclear how the NPVIY motif exerts its effects on receptor internalisation but it is known that the proline residue (which again is highly conserved) is an important structural aspect of the motif. It has been postulated that NPX_nY motif may also target GRK mediated receptor phosphorylation as in the case of the β_2AR (Ferguson *et al.*, 1995). What is however clear from these data is that further investigation into the role of the NPVIY in regulating 5-HT_{1A}R redistribution is warranted.

In summary, these findings suggest that phosphorylation of the receptor at position 149 and 229 leading to desensitisation of the 5-HT_{1A}R are a prerequisite for internalisation. These data also suggest that the putative NPVIY motif located in the seventh transmembrane domain plays an important role in regulating receptor internalisation.

The ability of the activated 5-HT_{1A}R to regulate the activity of the transcription factors NF-κB and CREB were explored. In initial experiments, it was observed that treatment of CHO-K1 cells expressing the 5-HT_{1A}R with murine TNFα and forskolin resulted in the dose dependent induction of NF-κB and CREB respectively. 8-hydroxy-DPAT was also found to induce NF-κB activity in a concentration dependent manner; the response was desensitised with higher concentrations of agonist. Analysis of the protein subunits involved revealed that the NF-κB subunits induced via the activation of the 5-HT_{1A}R were p50 and p65. The mechanism by which 8-hydroxy-DPAT activates NF-κB is thought to be via the activation of PC-PLC leading to the activation of Ca^{2+}/ calmodulin dependent kinases and the eventual phosphorylation and degradation of I-κBα (Cowen *et al.*, 1997). The consequences of NF-κB activation are numerous when the number of genes with NF-κB responsive elements within the promoter are considered (see Ghosh *et al.*, 1998 for review). It has however, been reported that the 5-HT_{1A}R gene is included amongst these NF-κB responsive genes (Cowen *et al.*, 1997). Cowen and colleagues were able to show that upon stimulation of the 5-HT_{1A}R by both 8-hydroxy-DPAT and spiperone the expression of receptor protein was almost tripled. It has been suggested therefore that 5-HT_{1A}R mediated NF-κB activation results in a positive feedback effect resulting in an increase in plasma membrane bound receptors. Novel results presented in this study illustrate the ability of the 5-HT_{1A}R to activate p50 and p65 NF-κB subunits indicating that in tissues/ cells that naturally express the 5-HT_{1A}R autoregulation is a possibility. Bearing this in mind, our data and those data presented by Cowen and colleagues (1997) suggest that downregulation of the human 5-HT_{1A}R in response to long term drug therapies is an unlikely scenario. As with most biological cascades, the activation of NF-κB and the resultant increase in receptor protein concentration may not be so simple. When one considers the role of numerous other transcription factors, repressor protein and the fact that there is no literature demonstrating the existence of an NF-κB binding site upstream of the human and rat gene (Parks & Shenk 1996; Ou *et al.*, 2000) it is possible that activation and upregulation of the 5-HT_{1A}R expression may occur via a separate pathway.

Activation of the 5-HT_{1A}R with 8-hydroxy-DPAT also lead to the induction of CREB. The mechanism by which the 5-HT_{1A}R modulates CREB activity is thought to be

through the inhibition of AC and PKA (which would decrease CREB activity; Nishi & Azmitia, 1999). However, in these studies CREB was activated by agonist incubation leading to the possibility that in this cell system the 5-HT_{1A}R may couple to a positive signalling pathway (possibly via PLC) resulting in CREB phosphorylation. When the array of genes that contain cAMP responsive elements are considered, then the consequences of 5-HT_{1A}R activation could be diverse. However, very little is known about 5-HT_{1A}R mediation of gene transcription via CREB, except that stimulation of the 5-HT_{1A}R *in vivo* results in a decrease of receptor gene expression via a CREB mediated pathway (Nishi & Azmitia, 1999). These data suggest, that as with the NF-κB that the 5-HT_{1A}R and CREB are intrinsically linked, except this time possibly via a negative feedback loop. However, in the system described here where the 5-HT_{1A}R is expressed at supraphysiological levels in a surrogate cell line direct activation of CREB by the receptor has been shown. The ability of the receptor to stimulate CREB may be an artefact of the high levels of receptor expression and the ability to promiscuously couple to abnormal signalling pathways (in this case possibly PLC).

The numerous signalling cascades associated with the 5-HT_{1A}R and the complex nature of their interactions suggests that activation of NF-κB and CREB by the receptor and the resultant alterations in gene transcription maybe multi-faceted (See **Figure 41** for overview). Colocalisation of the 5-HT_{1A}R with other GPCRs and other receptor types (e.g. tyrosine kinase receptor) may have a role in regulating the sensitivity of one or both receptors to their respective ligands resulting in enhanced or reduced transcription factor activity. Activation of a separate signalling pathways coupled to another GPCR may also prime/ desensitise the signalling pathway associated with the 5-HT_{1A}R. The signalling systems linked with the 5-HT_{1A}R could feasibly lead to the activation or inhibition of both NF-κB and CREB signalling activity. This could be achieved by the inhibition of PKA activity (via AC inhibition) and activation of PLC activity, both of which have been shown to mediate the phosphorylation of I-κB and CREB. The exact I-κB subtype involved in this example remains unknown however, the 5-HT_{1A}R has been to cause phosphorylation and subsequent degradation of I-κBα (Cowen *et al.*, 1997).

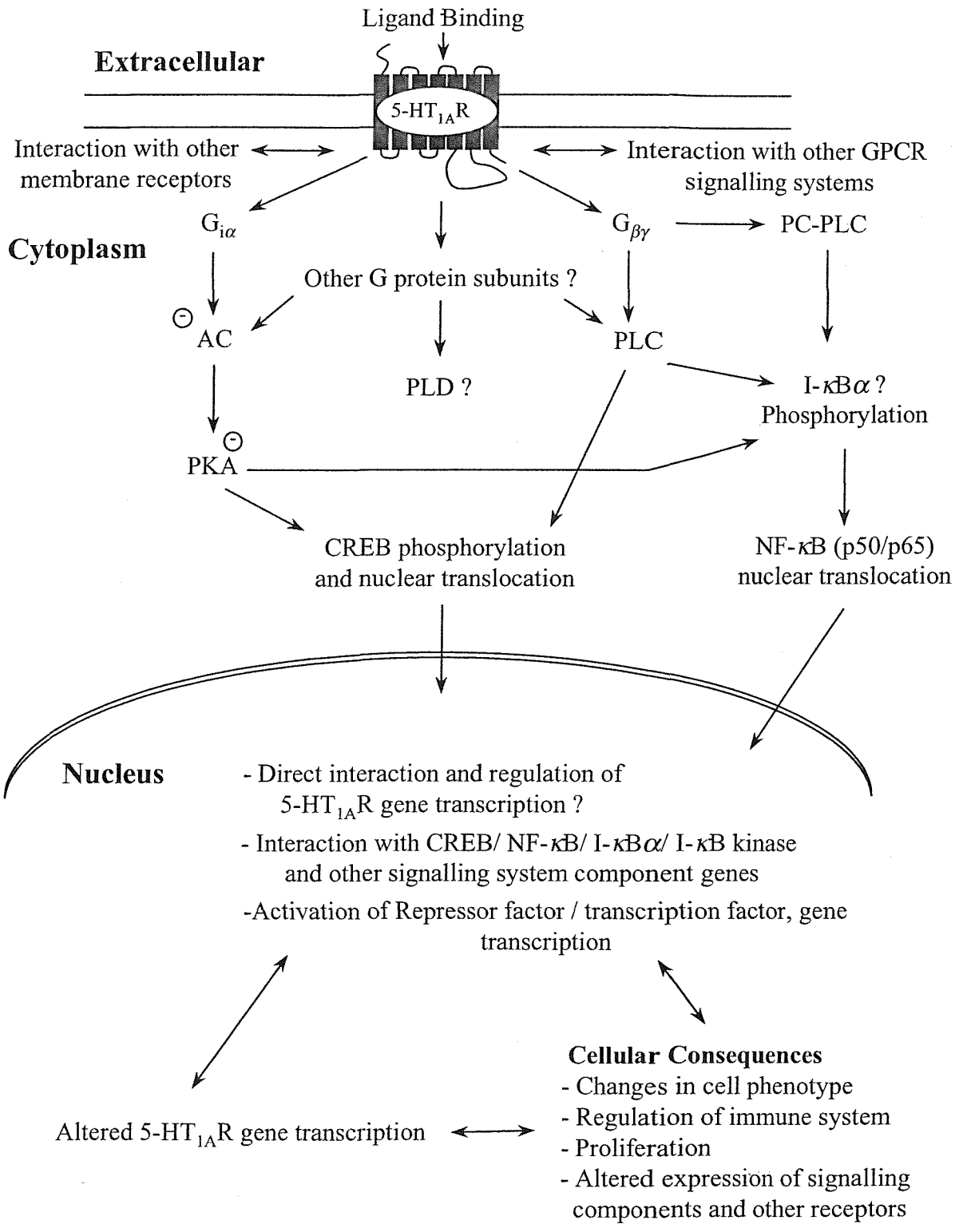

Figure 41: The postulated interactions of the 5-HT_{1A}R with intracellular signalling machinery resulting in an altered rate 5-HT_{1A}R gene transcription.

Once translocated to the nucleus the targeting of CREB and NF-κB to specific DNA sequences within target genes adds a further level of complexity to the equation. This study was able to show that activation of the 5-HT_{1A}R with 8-hydroxy-DPAT resulted in the translocation of the p50/ p65 NF-κB subunits to the nucleus. However, it was observed on the EMSA autoradiographs that other bands (with both NF-κB and CREB) did become evident upon receptor activation. These extra observed bands could in part help to explain the diversity of genes targeted by both NF-κB and CREB. CREB and NF-κB could also help to enhance the transcription of each other respectively and other repressor/ activator proteins indicating that the link between the 5-HT_{1A}R and gene transcription may not be very clear cut. With each layer of complexity added another regulatory step comes into play helping to further regulate the transcription of genes under the regulation of the 5-HT_{1A}R.

In conclusion, data in the present studies reports the ability of the unmutated human 5-HT_{1A}R to undergo internalisation via the clathrin-mediated pathway. The threonine residues at positions 149 and 229 in the putative PKC phosphorylation sequences are vital for receptor internalisation, as is the tyrosine at position 400 within the putative NPVIY internalisation motif. In contrast, the serine at position 253 would appear to be acting as a negative regulatory signal, attenuating receptor internalisation. The residues all represent vital components in the mechanisms involved in short term receptor function as it responds to agonist challenge. The 5-HT_{1A}R has also been shown to couple via unknown G proteins to the activation of putative CREB and NF-κB mediated gene expression. Providing a possible link between the 5-HT_{1A}R and mechanism involved in its long-term regulation.

7.2. FUTURE WORK

Data presented in this study have led to further questions and possible experiments, which would give a deeper insight into short-term receptor 5-HT_{1A}R regulation.

7.2.1. 5-HT_{1A}R regulation

1. Regarding receptor internalisation, elegant experiments that would allow real time visualisation of receptor trafficking would been an approach that would be of value. This could be achieved using a fusion protein technologies where the

unmutated receptor is fused to green florescent protein allowing eppifluorescent and confocal visualisation.

2. The role of PKC, PKA and GRKs in mediating receptor internalisation is also worthy of further investigation. A range of protein kinase activators/ inhibitors could be used to see if the redistribution of the 5-HT_{1A}R is due to protein kinase activation and if so, what specific enzyme subtypes are mediating the effect. The role of each putative protein kinase motif mutant could also be investigated indicating further the role of each motif in internalisation.
3. The internalisation motif located in the seventh transmembranous region of the receptor also warrants further investigation. The role of other amino acid residues within the NPVIY sequence could be investigated using mutagenesis techniques and also the proximity of the motif to the membrane could be investigated further.
4. Recycling and receptor resensitisation could be studied to elucidate the role of protein phosphatases in mediating receptor resensitisation. This could be simply achieved by pre-treating cells with a range of protein phosphatase inhibitors such as Okadaic acid, phenylarsine oxide, NH_4Cl and Calyculin A.

7.2.2. Regulation of putative transcription factor activity

1. Further investigation of the ability of the 5-HT_{1A}R to regulate both CREB and NF-κB activity would be a pertinent course of further investigation. The use of reporter plasmids encoding CAT under the control of CREB and NF-κB binding domains would indicate further the ability of the 5-HT_{1A}R to activate CREB and NF-κB. Compounds that inhibit various signalling pathways could also be used to show which signalling cascades result in the activation of each putative transcription factor.
2. Using differential display techniques, differences in mRNA populations that arise upon 5-HT_{1A}R challenge could be scrutinised. The mRNA populations could then be sequenced and characterised indicating genes that are responsive to 5-HT_{1A}R activation.

This study provides an insight into the mechanisms involved in the long and short-term regulation of the human 5-HT_{1A}R function. Novel and significant contributions made by this study that help to address the project aims and objectives outlined in sections 1.7. and 1.8. include; the 5-HT_{1A}R is able to undergo internalisation via

clathrin-mediated endocytosis and recycle back to the plasma membrane in response to short term agonist incubation. Contained within the receptor amino acid sequence are putative PKC phosphorylation sites that have been shown to attenuate (S_{253}) and facilitate (T_{149} and T_{229}) receptor internalisation. The putative internalisation motif ($NPVIY_{400}$) located in the seventh transmembranous domain is involved in facilitating receptor internalisation. In our hands the 5-HT_{1A}R was shown to couple positively to the putative transcription factor CREB and NF-κB p50 and p65 subunits. However, one should be cautious when interpreting these results as data was gathered from an expression system that is artificially expressing the receptor and therefore cannot be applied directly to the *in vivo* situation. However, these data do indicate that important contributions have been made to the understanding of the behaviour of the 5-HT_{1A}R in response to short term agonist challenge.

REFERENCES

Ahn, S., Olive, M., Aggarwal, S., Krylov, D., Ginty, D.D. and Vinson, C. Dominant-negative inhibitor of CREB reveals that it is a general meditor of stimulus-dependent transcription of c-fos. *Mol. Cell. Biol.* **18**: 967-977, 1998.

Aiyar, N., Disa, J., Dang, K., Pronin, A.N., Benovic, J.L. and Nambi, P. Involvement of G protein-coupled kinase-6 in desensitisation of CGRP receptors. *Eur. J. Pharmacol.* **403**: 1-7, 2000.

Albert, P.R., Lembo, P., Storrin, J.M., Charest, A. and Saucier, C. The 5-HT_{1A} receptor: signalling, desensitisation and gene transcription. *Neuropsychopharmacol.* **14**: 19-25, 1996.

Albert, P.R., Morris, S.J., Ghahremani, M.H., Storring, J.M. and Lembo, P.M. A putative α-helical $G_{\beta\gamma}$-coupling domain in the second intracellular loop of the 5-HT_{1A} receptor. *Ann. N. Y. Acad. Sci.* **15**: 146-161, 1998.

Albert, P.R., Sajedi, N., Lemonde, S. and Ghahremani, M.H. Constiutive $G_{(i2)}$-dependent activation of adenylyl cyclase type II by the 5-HT_{1A} receptor. Inhibition by anxiolytic partial agonists. *J. Biol. Chem.* **274**: 35469-35474, 1999.

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K. and Watson, J.D. Molecular biology of the cell. Garland Publishing, Inc. New York & London. 3rd edit. 1994.

Alhaider, A.A. and Wilcox, G.L. Differential roles of 5-hydroxytryptamine$_{1A}$ and 5-hydroxytryptamine$_{1B}$ receptor subtypes in modulating spinal nociceptive transmission in mice. *J. Pharmacol. Exp. Ther.* **265**: 378-85, 1993.

Anderson, R.G.W., Brown, M.S. and Goldstein, J.L. Role of the coated endoctic vesicle in the uptake of receptor-bound low density lipoprotein in human fibroblasts. *Cell* **10**: 351-264, 1977.

Anderson, R.G.W., Brown, M.S. Beisiegel, U., and Goldstein, J.L. Surface distribution and recycling of the low density lipoprotein receptor as visualized with antireceptor antibodies. *J. Cell. Biol.* **93**: 523-531, 1982.

Andrade, R. and Nicol, R.A. Pharmacologically distinct actions of serotonin on single pyramidal neurones of the rat hippocampus rcorded *in vitro*. *J. Physiol.* **394**: 99-124. 1987.

Andrisani, O.M., Zhu, Z., Pot, D.A. and Dixon, J.E. *In vitro* transcription directed from the somatostatin promoter is dependent upon a purified 43kDa DNA-binding protein. *Proc. Natl. Acad. Sci. USA.* **86**: 2181-2185. 1989.

Arden, J.R., Segredo, V., Wang, Z., Lameh, J. and Sadee, W. Phosphorylation and agonist-specific intracellular trafficking of an epitope-tagged μ-opiod receptor expressed in HEK 293 cells. *J. Neurochem.* **65**: 1634-1645, 1995.

Asano, M. and Hidaka, H. Potentiation of the contractile response to acetylcholine in aortic strips by low concentrations of vascular contractile agents. *Br. J. Pharmacol* **69**: 639-646, 1980.

Aune, T.M., Golden, H.W. and McGrath, K.M. Inhibitors of serotonin synthesis and antagonists of serotonin$_{1A}$ receptors inhibit T lymphocyte function *in vitro* and cell mediated immmunity *in vivo*. *J. Immunol.* **153**: 489-498, 1994.

Aune, T.M., McGrath, K.M., Sarr, T., Bombara, M.P. and Kelley, K.A. Expression of 5-HT$_{1A}$ receptors on activated human T cells. Regulation of cyclic AMP levels and T cell proliferation by 5-hydroxtryptamine. *J. Immunol.* **151**: 1175-1183, 1993.

Baeuerle, P.A. and Baltimore, D. Activation of DNA-binding activity in apparently cytoplasmic precursor of the NF-κB transcription factor. *Cell*, **53**: 211-217, 1988a.

Baeuerle, P.A. and Baltimore, D. I-κB: a specific inhibitor of the NF-κB transcription factor. *Science*, **242**: 540-546, 1988b.

Baeuerle, P.A., Lenardo, M., Pierce, J.W. and Baltimore, D. Phorbol-ester-induced activation of the NF-κB transcription factor involves dissociation of an apparently cytoplasmic NF-κB/ inhibitor complex. *Quant. Biol.* **53**: 789-798, 1988.

Baldwin, A.S. The NF-κB and I-κB proteins: new discoveries and insights. *Annu. Rev. Immunol.* **14**:649-681, 1996.

Baltensperger, K., Karoor, V., Paul, H., Ruoho, A., Czech, M.P. and Malbon, C.C. The β adrenergic receptor is a substrate for the insulin receptor tyrosine kinase. *J. Biol. Chem.* **271**: 1061-1064, 1996.

Barak, L.S. Tiberi, M., Freedman, N.J., Kwatra, M.M. Lefkowitz, R.J. and Caron, M.G. A highly conserved tyrosine residue in G protein-coupled receptors is required for agonist-mediated β_2-adrenergic receptor sequestration. *J. Biol. Chem.* **269**: 2790-2795, 1994.

Barak, L.S., Menard, L., Ferguson, S.S.G., Colapietro, A.M. and Caron, M.G. The conserved 7-transmembrane sequence NP(X)(2,3)Y of the G protein-coupled receptor superfamily regulates multiple properties of the β_2-adrenergic receptor. *Biochem.* **34**: 15407-15414, 1995.

Beg, A.A., Finco, T.S., Nantermet, P.V. and Baldwin, A.S. Tumor necrosis factor and interleukin-1 lead to phosphorylation and loss of I-κBα: a mechanism for NF-κB activation. *Mol. Cell. Biol.* **13**: 3301-3310, 1993.

Blank, V., Kourilsky, P. and Israel, A. Cytoplasmic retention, DNA binding and processing of the NF-κB p50 precursor are controlled by a small region in its C-terminus. *EMBO. J.* **10**: 4159-4167, 1991.

Bogatkewitsch, G.S., Lenz, W., Jacobs, K.H. and Van Koppen, C.J. Receptor internalisation delays M_4 muscarinic acetylcholine receptor resensitisation at the plasma membrane. *Mol. Pharmacol.* **50**: 424-429, 1996.

Bohm, S.K., Khitin, L.M., Smeekens, S.P., Grady, E.F., Payan, D.G. and Bunnett, N.W. Identification of potential tyrosine-containing endoctic motifs in the carboxy-tail and seventh transmembrane domain of the neurokinin$_1$ receptor. *J. Biol. Chem.* **272**: 2363-2372, 1997.

Bours, V., Burd, P.R., Brown, K., Villalobos, J., Park, S., Ryseck, R.P., Bravo, R., Kelly, K. and Siebenlist, U. A novel mitogen –inducible gene product related to p50/ p105-NF-κB participates in transactivation through a κB site. *Mol. Cell. Biol.* **12**: 685-695, 1992.

Bradley, P.B., Engel, G., Feniuk, W., Fozard, J.R., Humphery, P.P.A., Middlemiss, D.N., Mylecharane, E.J., Richardson, B.P. and Saxena, P.R. Proposals for the classification and nomenclature of functional receptors for 5-hydroxytryptamine. *Neuropharmacology* **25**. 563-576, 1986.

Bretscher, M.S., Thomson, J.N. and Pearse, B. M. F. Coated pits as molecular filters. *Proc. Natl. Acad. Sci. USA* **77**: 4156-4159 1980.

Brown, D.A. and Rose, J.K. Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. *Cell* **68**: 533-544, 1992.

Brown, K., Park, S., Kanno, T., Franzoso, G. and Siebenlist, U. Mutual regulation of the transcriptional activator NF-κB and its inhibitor, I-κB-α. *Proc. Natl. Acad. Sci. USA*, **90**: 2532-2536, 1993.

Burnstock, G. Do some nerve cells release more than one transmitter. *Neuroscience* **1**: 239-248, 1976.

Cadwallader, K., Beltman, J., McCormick, F. and Cook, S. Differential regulation of extracellular signal-regulated protein kinase 1 and Jun N-terminal kinase 1 by Ca^{2+} and protein kinase C in endothelin-stimulated Rat-1 cells. *Biochem. J.* **321**: 795-804, 1997.

Carpentier, J.L., Brown, D., Iacopetta, B. and Orci, L. Detection of surface-bound ligands by freeze fracture autoradiography. *J. Cell. Biol.* **101**: 887-890, 1985.

Carpentier, J.L., Sawano, F. Geiger, D., Gorden, P., Perrelet, A. and Orci, L. Potassium depletion and hypertonic medium reduce "non-coated" and clathrin-coated pit formation, as well as endocytosis through these two gates. *J. Cell. Physiol.* **138**: 519-526 1989.

Caverzasio, J., Palmer, G., Suzuki, A. and Bonjour, J.P. Evidence for the involvement of two pathways in activation of extracellular signal-regulated kinase

(ERK) and cell proliferation by G_i and G_q protein-coupled receptors in osteoblast-like cells. *J. Bone. Miner. Res.* **15**: 1697-1706, 2000.

Chalecka-Franaszek, E., Chen, H. and Chuang, D.M. 5-Hydroxytryptamine$_{2A}$ receptor stimulation induces activator protein-1 and cyclic AMP-responsive element binding with cyclic AMP-responsive element-binding protein and Jun D as common components in cerebellar neurons. *Neuroscience.* **88**: 885-98, 1999.

Chanda, P.K., Minchin, M.C., Davis, A.R., Greenberg, L., Reilly, Y., McGregor, W.H., Bhat, R., Lubeck, M.D., Mizutani, S. and Hung, P.P. Identification of residues important for ligand binding to the human 5-hydroxytryptamine$_{1A}$ serotonin receptor. *Mol. Pharmacol.* **43**: 516-20, 1993.

Chen, W.J., Goldstein, J.L. and Brown, M.S. NPXY, a sequence often found in cytoplasmic tails, is required for coated pit-mediated internalization of the low density lipoprotein receptor. *J. Biol. Chem.* **265**: 3116-3123, 1990.

Chen, Z.J., Hagler, J., Palombello, V., Melandri, F., Scherer, D., Ballard, D. and Maniatis, T. Signal induced site-specific phosphorylation targets I-$\kappa B\alpha$ to the ubiquitin-proteasome pathway. *Genes Dev.* **9**: 1586-1597, 1995.

Chojnackawojoik, E., Klodzinska, A., Drabozynska, A., Pawlowski, M., Charakchievaminol, S., Chlon, G. and Gorezyca, M. A new putative 5-HT$_{1A}$ receptor antagonist of the 1-arylpiperazine class of ligands. *Euro. J. Med. Chem.* **30**: 587-592, 1998.

Chopin, P. and Briley, M. Animal models of anxiety: the effects of compounds that modify 5-HT transmission. *TIPS.* **8**: 383-388, 1987.

Chuang, P.I., Young, B.A., Thiagarajan, R.R., Cornejo, C., Winn, R.K. and Harlan, J.M. Cytoplasmic domain of E-selectin contains a non-tyrosine endocytosis signal. *J. Biol. Chem.* **272**: 24813-8, 1997.

Chupreta, S., Du, M., Todisco, A. and Merchant. J.L. EGF stimulates gastrin promoter through activation of Sp1 kinase activity. *Am. J. Physiol. Cell. Physiol.* **278**: C697-708, 2000.

Ciechanover, A. The ubiquitin-proteasome proteolytic pathway. *Cell.* **79**: 13-21, 1994.

Claustre, Y. Benavides, J. and Scatton, B. Potential mechanisms involved in the negative coupling between serotonin 5-HT$_{1A}$ receptors and carbachol-stimulated phosphoinositide turnover in the rat hippocampus. *J. Neurochem.* **56**: 1276-1285, 1991.

Claustre, Y., Benavides, J. and Scatton, B. 5-HT$_{1A}$ receptor agonists inhibit carbachol-induced stimulation of phosphinositide turnover in the rat hippocampus. *Euro. J. Pharmacol.* **149**: 149-153, 1988.

Coleman, D.E. and Sprang, S.R. How G proteins work: a continuing story. *TIBS.* **21**: 41-43, 1996.

Colino, A. and Halliwell, J.V. Differential Modulation of three separate K^+ Conductances in hippocampal CA1 neurones by serotonin. *Nature*, **326**: 73-77. 1987.

Collawn J.F., Lai A., Domingo D., Fitch M., Hatton S., and Trowbridge I.S. YTRF is the conserved internalisation signal of the transferrin receptor, and a second YTRF signal at position 31-34 enhances endocytosis. *J Biol. Chem.* **268**: 21686-92, 1993.

Collawn, J.F., Stangel, M., Kuhn, L.A., Esekogwu, V., Jing, S.Q., Trwbridge, I.S. and Tainer, J.A. Transferrin receptor internalisation sequence YXRF implicates a tight turn as the structural recognition motif for endocytosis. *Cell.* **63**: 1061-1072, 1990.

Cowen, D.S., Malinoff, P.B. and Manning, D.R. 5-Hydroxytryptamine$_{1A}$ receptor-mediated increases in receptor expression and activation of nuclear factor-κB in transfected chinese hamster ovary cells. *Mol. Pharmacol.* **52**: 221-226, 1997.

Cowen, D.S., Sowers, R.S., and Manning, D.R. Activation of a mitogen-activated protein kinase (ERK2) by the 5-hydroxytryptamine$_{1A}$ receptor is sensitive not only to inhibitors of phosphatidylinositol 3-kinase, but to an inhibitor of phosphatidylcholine hydrolysis. *J. Biol. Chem.*, **271**: 22297-22300, 1996.

Crespo, P., Xu, N., Daniotti, J.L., Troppmair, J., Rapp, U.R. and Gutkind, J.S. Signalling through transforming G protein-coupled receptors in NIH 3T3 cells involves c-Raf activation. Evidence for a protein kinase C-independent pathway. *J. Biol. Chem.* **269**: 21103-21109, 1994.

Curzon,G. Serotonin and appetite. *Annals. New York. Acad. Sci.* **600**: 521-531, 1990.

Damke, H., Baba, T., Van Der Bliek, A.M. and Schmid, S.L. Clathrin-independent pinocytosis is induced in cells overexpressing a temperature-sensitive mutant of dynamin. *J. Cell. Biol.* **131**: 69-80, 1995.

Daukas, G. and Zigmond, S.H. Inhibition of receptor-mediated but not fluid-phase endocytosis in polymorphonuclear leukocytes. *J. Cell. Biol* **101**: 1673-1679 1996.

Daval, G., Verge, D., Basbaum, A.I., Bourgoin, S. and Hamon, M. Autoradiographic evidence of serotonin 1 binding sites on primary afferent fibres in the dorsal hornof the rat spinal cord. *Neurosci. Letters* **83**: 71-76, 1987.

Dawidowicz, E.A. Lipid sorting and trafficking in cells. *Curr. Opin. Sttruct. Biol.* **3**: 495-498, 1993.

De Camilli, P., Takei, K. and McPhercon, P.S. The function of dynamin in endocytosis. *Curr. Opin. Neurobiol.* **5**: 559-565, 1995.

De Groot, R.P., den Hertog, J., Vandenhee, J.R., Goris, J. and Sassone-Corsi, P. Multiple and co-operative phosphorylation events regulate the CREM activation factor. *EMBO. J.* **12**: 3903-3911, 1993.

De Martin, R., Vanhove, B., Cheng, Q., Hofer, E., Csizmadia, V., Winkler, H. and Bach, F. Cytokine-inducible expression in endothelial cells of an I-κB-α-like gene isregulated by NF-κB. *EMBO. J.* **12**: 2773-2779, 1993.

De Vivo, M. and Maayani, S. Characterisation of the 5-hydroxytryptamine $_{1A}$ receptor-mediated inhibition of forskolin-stimulated adenylate cyclase activity in guinea pig and rat hippocampal membranes. *J. Pharmacol. Exp. Ther.* **238**: 248-253, 1986.

De Vivo, M. and Maayani, S. Stimulation and inhibition of adenylyl cyclase by distinct 5-hydroxtryptamine receptors. *Biochem. Pharmacol.* **40**: 1551-1558. 1990.

Diaz-Meco, M.T., Dominguez, I., Sanz, L., Dent, P., Lozano, J., Municio, M.M., Berra, E., Hay, R.T., Sturgil, T.W. and Moscat, J. ζPKC induces phosphorylation and inactivation of I-κBα *in vitro*. *EMBO. J.* **13**: 2842-2848, 1994.

Dickenson, J.M. and Hill, S.J. Interactions between adenosine A (1) receptor and Histamine H1 receptor. *Int. J. Biochem.* **26**: 959-969, 1994.

Dickenson, J.M. and Hill, S.J. Transfected adenosine A (1) receptor-mediated modulation of thrombin-stimulated phospholipase C and phospholipase A (2) activity in CHO cells. *Euro. J. Pharmacol.* **321**: 77-86, 1997.

Dieter. P., Hempel. U., Kamionka. S., Kolada. A., Malessa. B., Fitzke. E. and Tran-Thi. T.A. Prostaglandin E2 affects differently the release of inflammatory mediators from resident macrophages by LPS and muramyl tripeptides. *Mediators. Inflamm.* **8**: 295-303, 1999.

Dietrich, G., Gaibelet, G., Capeyrou, R., Butour, J.L., Pontet, F. and Emorine, L.J. Implication of the first and third extracellualr loops of the μ-opioid receptor in the formation of the ligand binding site: A study using chimeric μ-opioid/angiotensin receptors. *J. Neurochem.* **70**: 2106-2111, 1998.

Dignam, J.D. Preparation of extracts from higher eukaryotes. *Methods. Enzymol.* **182**: 194-203, 1990.

Dourish, C.T., Athlenius, S. and Huston, P.H. Brain 5-HT$_{1A}$ receptors, Chichester: Ellis Horwood series in biomedicine Ltd, 1987.

Doxsey, S.J., Brodsky, F.M., Blank, G.S. and Helenius, A. Inhibition of endocytosis by anti-clathrin antibodies. *Cell,* **50**: 453-463, 1987.

Dumartin, B., Jaber, M., Gonon, F., Caron, M.G., Giros, B. and Bloch, B. Dopamine tone regulates D_1 receptor trafficking and delivery in striatal neurons in dopamine transporter-deficient mice. *Proc. Natl. Acad. Sci. USA.* **97**: 1879-84, 2000.

Dunn, W.A. and Hubbard, A.L. Receptor mediated endocytosis of epidermal growth factor by hepatocytes in the perfused rat liver: ligand and receptor dynamics. *J. Cell. Biol.* **98**: 2148-2159, 1984.

Eason, M.G., Moreira, S.P. and Liggett, S.B. Four consecutive serines in the third intracellular loop are the sites for β-adrenegic receptor kinase-mediated phosphorylation and desensitisation of the α_{2A}-adrenergic receptor. *J. Biol. Chem.* **270**: 4681-8, 1995.

Edvinsson, L., Ekblad, E., Hakanson, R. and Wahlestedt, C. Neuropeptide-Y potentiates the effect of various vasoconstrictor agents on rabbit-blood vessels. *Br. J. Pharmacol.*, **83**: 519-25, 1984.

Edwardson, J.M. and Szekeres, P.G. Endocytosis and recycling of muscarinic receptors. *Life Sci.*, **64**: 487-94, 1999.

Eide, P.K., Hole, K. and Broch, O.G. 5-HT depletion with 5,7-DHT, PCA and PCPA in mice: differential effects on the sensitivity to 5-MeODMT, 8-OH-DPAT and 5-HTP as measured by two nociceptive tests *Brain Res.*, **440**: 42-52, 1988.

El Mansoury, A.M. and Morgan, N.G. Activation of protein kinase C modulates α_2-adrenergic signalling in rat pancreatic islets. *Cell. Signal.* **10**: 637-43, 1998.

El Mestikawy, S., Fargin, A., Raymond, J.R., Gozlan, H. and Hnatowich, M. The 5-HT_{1A} receptor: an overview of recent advances. *Neurochem. Res.* **16**: 1-10, 1991.

Erlander, M.G., Lovenberg, T.W., Baron, B.M., DeLecea, L., Danielson, P.E., Racke, M., Slone, A.L., Siegel, B.W., Foye, P.E., Cannon, K. and Burns, J.E. Two members of a distinct subfamily of 5-hydroxytryptamine receptors differentially expressed in rat brain. *Proc. Natln. Acad. Sci. USA.* **90**: 3452-3456, 1993.

Fan, C.M. and Maniatis, T. Generation of p50 subunit of NF-κB by processing of p105 through an ATP-dependent pathway. *Nature*, **354**: 395-398, 1991.

Fanger, G.R., Gerwins, P., Widmann, C., Jarpe, M.B. and Johnson, G.L. MEKKs, GCKs, MLKs, PAKs, TAKs and TPLS: upstream regulators of the c-Jun amino-terminal kinases? *Curr. Opin. Genet. Dev.* **7**: 67-74, 1997.

Fargin, A., Raymond, J.R., Lohse, M., Kobilka, B.K., Caron, M.G. and Lefkowitz, R.J. A genomic clone which resembles a β-adrenergic receptor sequence encodes the 5-HT_{1A} receptor. *Nature*, **335**: 358-360, 1988.

Fargin, A., Raymond, J.R., Regan, J.W., Cotecchia, S., Lefkowitz, R.J. and Caron, M.G. Effector coupling mechanisms of the cloned 5-HT_{1A} receptor. *J. Biol. Chem.* **264**: 14848-14852, 1989.

Faure, M., Voynoyasenetskaya, T.A. and Bourne, H.R. cAMP and $\beta\gamma$subunits of heterotrimeric G proteins stimulate the mitogen-activated protein kinase pathway in COS-7 cells. *J. Biol. Chem.* **269**: 7851-7854, 1994.

Felder, C.C., Williams, H.L. and Axelrod, J. A transduction pathway associated with receptors coupled to inhibitory guanine nucleotide binding protein G_i that amplifies ATP mediated arachidonic acid release. *Proc. Natl. Acad. Sci. USA.* **88**: 6477-6480, 1991.

Ferguson, G., Watterson, K.R. and Palmer, T.M. Subtype-specific kinetics of inhibitory Adenosine receptor internalisation are determined by sensitivity to Phosphorylation by G protein-coupled receptor kinases. *Mol. Pharmacol.* **57**: 546-552, 2000.

Ferguson, S.S.G., Downey, W.E., Colapietro, A.M., Barak, L.S., Menard, L. and Caron, M.G. Role of β-arrestin in mediating agonist promoted G protein-coupled receptor internalisation. *Science.* **271**: 363-366, 1996(a).

Ferguson, S.S.G., Menard, L., Barak, L.S., Koch, W.J., Colapietro, A.M. and Caron, M.G. Role of phosphorylation in agonist-promoted $\beta_{(2)}$-adrenergic receptor sequestration; resucue of a sequestration defective mutant by βARK1. *J. Biol. Chem.* **270**: 24782-24789, 1995.

Ferguson, S.S.G., Zhang, J., Barak, L.S. and Caron, M.G. G protein-coupled receptor kinases and arrestins; Regulators of G protein-coupled receptor sequestration. *Biochem. Soc. Transactions.* **24**: 953-959, 1996(b).

Finco, T. and Baldwin, A. κB site-dependent induction of gene expression by diverse inducers of nuclear factor κB requires Raf-1*. *J. Biol. Chem.* **268**: 17676-17679, 1993.

Firestein, R. and Feurestein, N. Association of activating transcription factor 2 (ATF2) with the ubiquitin-conjugating enzyme hUBC9. *J. Biol. Chem.* **273**: 5892-5902, 1998.

Flavahan, N.A. and Vanhoutte, P.M. Threshold phenomena and interactions between receptors. *J. Cardiovas. Pharmacol.* **11**: S67-S72, 1988.

Fletcher, A., Bill, D.J., Bill, S.J., Cliffe, I.A., Dover, G.M., Forster, E.A., Hskins, J.T., Jones, D., Mansell, H.L. and Reilly, Y. WAY100135: a novel, selective antagonist at presynaptic and postsynaptic 5-HT_{1A} receptors. *Eur. J. Pharmacol.* **237**: 283-291, 1993.

Fornal, A.A., Marrosu, F., Metzler, C.W., Tada, K. and Jacobs, B.L. Effects of the putative 5-hydroxytryptamine$_{1A}$ antagonist BMY378, NAN 190 and (-)-propranolol

on serotoninergic dorsal raphe unit activity in behaving cats. *J. Pharmacol. Exp. Therapu.* **270**: 1359-1366, 1994.

Fraser, I.D., Cong, M., Kim, J. Rollins, E.N., Daaka, Y., Lefkowitz, R.J. and Scott, J.D. Assembly of an A-kinase-anchoring protein-β_2-adrenergic receptor complex facilitates receptor phosphorylation and signalling. *Curr. Biol.* **10**: 9-12, 2000.

Gabilondo, A.M., Hegler, J., Krasel, C., Boivin-Jahns, V., Hein, L. and Lohse, M.J. A diluecine motif in the C terminus of the β2-adrenergic receptor is involved in receptor internalisation. *Proc. Natl. Acad. Sci. USA*. **94**:12285-12290, 1997.

Gaddumm, J.H and Picarrelli, Z.P. Two kinds of tryptamine receptors. *Br. J. Pharmacol.* **12**: 323, 1957.

Garcia-Sevilla, J.A., Escriba, P.V., Ozaita, A. La Harpe, R., Walzer, C., Eytan, G. and Guimon, J. Up-regulation of immunolabeled α_{2A}-adrenoceptors, G_i coupling proteins, and regulatory receptor kinases in the prefrontal cortex of depressed suicides. *J. Neurochem.* **72**: 282-91, 1999.

Gardner, B.R., Hall, D.A. and Strange, P.G. Agonist action at D_2(short) dopamine receptors determined in ligand binding and functional assays. *J. Neurochem.* **69**: 2589-98, 1997.

Garnovskaya, M.N., Getty, T.W., Van Biesen, T., Prpic, V., Chuprun, J.K. and Raymond, J.R. 5-HT_{1A} receptor activates Na^+/ H^+ exchange in CHO-K1 cells through $G_{i\alpha2}$ and $G_{i\alpha3}$. *J. Biol. Chem.* **272**: 7770-7776, 1997.

Garnovskaya, M.N., Mukhin, Y. and Raymond, J.R. Rapid activation of sodium-proton exchange and extracellular signal-regulated protein kinase in fibroblasts by G protein-coupled 5-HT_{1A} receptor involves distinct signalling cascades. *Biochem. J.* **330**: 489-495, 1998.

Garnovskaya, M.N., Van Biesen, T., Hawes, B., Ramos, S.C., Lefkowitz, R.J. and Raymond, J.R. Ras-dependant activation of fibroblast mitogen activated protein-kinase by 5-HT_{1A} receptor via a G protein $\beta\gamma$-subunit-initiated pathway. *Biochem.*, **35**: 13716-13722, 1996.

George, S.E., Bungay, P.J. and Naylor, L.H. Functional coupling of endogenous serotonin (5-HT_{1B}) and calcitonin (C1a) receptors in CHO cells to a cyclic AMP-responsive luciferase reporter gene. *J. Neurochem.*, **69**: 1278-1285, 1997.

Ghosh, G., van Duyne, G., Ghosh, S. and Sigler, P.B. Structure of NF-κB p50 homodimer bound to a κB site. *Nature* **373**: 303-310, 1995

Ghosh, S. and Baltimore, D. Activation *in vitro* of NF-κB by phosphorylation of its inhibitor I-κB. *Nature*, **344**: 678-682, 1990.

Ghosh, S., May, M.J. and Kopp, E.B. NF-κB and Rel proteins: Evolutionarily conserved mediators of immune responses. *Annu. Rev. Immunol.* **16**: 225-260, 1998.

Gilman, A.G. G proteins, transducers of receptor-generated signals. *Annu. Rev. Biochem.,* **56**: 615-649, 1987.

Ginty, D., Bonni, A. and Greenberg, M. Nerve growth activates a Ras-dependent protein kinase that stimulates c-fos transcription via phosphorylation of CREB. *Cell.* **13**: 777-83

Go, W.Y., Holicky, E.L., Hadac, E.M., Rao, R.V. and Miller, L.J. Identification of a domain in the carboxy terminus of CCK receptor that affects its intracellular trafficking. *Am. J. Physiol.* **275**: G56-62, 1998.

Goldberg, R.I., Smith, R.M. and Jarett, L. Insulin and α_2 macroglobulin-methylamine undergo endocytosis by differentmechanisms in rat adipocytes. I. Comparison of cell surface vents. *J. Cell. Physiol.,* **133**: 203-212, 1987.

Goldstein, J.L., Brown, M.S., Anderson, R.G.W., Russell, D.W. and Schneider, W.J. Receptor-mediated endocytosis: concepts emerging from the LDL receptor system. *Annu. Rev. Cell Biol.* **1**: 1-39, 1985.

Gonzalez, G.A. and Montminy, M.R. Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at Ser_{133}. *Cell.* **59**: 675-680, 1989.

Grilli, M., Chiu, J.J-S. and Lenardo, M. NF-κB and rel: participants in a multiform transcriptional regulatory system. *Int. Rev. Cytol.* **143**: 1-62, 1993.

Guan, X. Miscellaneous receptors. In: Handbook of receptors and channels: G protein-coupled receptors. (Peroutka, S.J. ed.) **1**: 299-328, 1994. CRC Press Inc., Boca Raton.

Han, J., Lee, J.D., Bibbs, L. and Ulevitch, R.J. A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. *Science.* **265**: 808-811, 1994.

Hansen, S.H., Sandvig, K. and van Duers, B. Molecules internalized by clathrin-independent endocytosis are delivered to endosomes containing transferrin receptors. *J. Cell. Biol.* **123**: 89-97, 1993.

Harrington, M.A., Shaw, K., Zhong, P. and Ciaraniello, R.D. Agonist induced desensitisation and loss of high affinity binding sites of stably expressed human 5-HT_{1A} receptors. *J. Pharmacol. Exp. Ther.* **268**: 1098-1106, 1994.

Hasbi, A., Allouche, S., Sichel, F., Stanaila, L., Massotte, D., Landemore, G., Polastron, J. and Jauzac, P. Internalisation and recycling of δ-opioid receptor are dependent on a phosphorylation-dephosphorylation mechanism. *J. Pharmacol. Exp. Ther.* **293**: 237-47, 2000.

Haskill, S., Beg, A.A., Tompkins, S.M., Morris, J.S. Yurochko, A.D., Sampson-Johannes, A., Mondal, K., Ralph, P. and Baldwin, A.S. Jr. Characterisation of an immediate-early gene induced in adherent monocytes that encodes I-κB like activity. *Cell* **65**: 1281-1289, 1991.

Hausdorff, W.P., Bouvier, M., O'Dowd, B.F., Irons, G.P., Caron, M.G. and Lefkowitz, R.J. Phosphorylation sites on 2 domains of the β_2-adrenergic receptor are involved in distinct pathways of receptor desensitisation. *J. Biol. Chem.* **264**: 12657-12665, 1989.

Hawes, B.E., Luttrell, L.M., VanBiesen, T. and Lefkowitz, R.J. Phosphatidylinositol 3-kinase is an early intermediate in the G-$\beta\gamma$-mediated mitogen-activated protein kinase signalling pathway. *J. Biol. Chem.* **271**: 12133-12136, 1996.

Hawes, B.E., VanBiesen, T. Koch, W.J., Luttrell, L.M. and Lefkowitz, R.J. Distinct pathways of G_i mediated and G_q mediated mitogen-activated protein kinase activation. *J. Biol. Chem.* **270**: 17148-17153, 1995.

Hellstrand, K. and Hermodson, S. Role of serotonin in the regulation of human natural killer cell cytotoxicity. *J. Immunol.* **139**: 869-875, 1987.

Hensler, J.G., Cervera, L.S., Miller, H.A. and Corbitt, J. Expression and modulation of 5-hydroxytryptamine$_{1A}$ receptors in P11 cells. *J. Pharmacol. Exp. Ther.* **278**: 1138-1145, 1996.

Hicke, L., and Riezman, H. Ubiquitination of a yeast plasma membrane receptor signals its ligand-stimulated endocytosis. *Cell.* **84**: 277-287, 1996.

Hill, S.J. Receptor Crosstalk: Communication through cell signalling pathways. *Anat. Rec. (New Anat.)* **253**: 42-48, 1998.

Hill, S.J. and Kendall, D.A. Cross-talk between different receptor effector systems in the mammalian CNS. *Cell. Signal.* **1**: 135-141, 1989.

Hipkin, W.R., Wang, Y. and Schonbrunn, A. Protein kinase C activation stimulates the Phoshorylation and internalisation of the sst2A Somatostatin receptor. *J. Biol. Chem.* **275**: 5591-5599, 2000.

Hoeffler, J.P., Meyer, T.E., Yun, Y., Jameson, L.J. and Habener, J.F. Cyclic AMP-responsive DNA-binding protein:structure base on a cloned placental cDNA. *Science.* **242**: 1430-1433, 1988.

Hokfelt, T. Neuropeptides in perspective: the last ten years. *Neuron* **7**: 867-879, 1991.

Hosey, M.M., Pals-Rylaarsdam, R., Lee, K.B., Roseberry, A.G., Bonovic, J.L. Gurevich, V.V. and Bunemann, M. Molecular events associated with the regulation of signaling by M_2 muscarinic receptors. *Life. Sci.* **64**: 363-368, 1999.

Hsu, D., Knidson, P.E., Zapf, A., Rolband, G.C. and Olefsky, J.M. NPXY motif in the insulin-like growth factor receptor is required for efficient ligand-mediated receptor internalization and biological signaling. *Endocrinology.* **134**: 744-50, 1994.

Huang, Z., Chen, Y. and Nissenson, R.A. The cytoplasmic tail of the G protein-coupled receptor parathyroid hormone and parathyroid hormone-related protein contains positive and negative signals for endocytosis. *J. Biol. Chem.* **270**: 151-6, 1995.

Hukovic, N., Panetta, R., Kumar, U., Rochville, M. and Patel, Y.C. The cytoplasmic tail of the human somatostatin receptor type 5 is crucial for interaction with adenylyl cyclase and in mediating desensitization and internalization. *J. Biol. Chem.* **273**: 21416-22, 1998.

Iken, K., Cheng, S., Fargin, A., Goulet, A.C. and Kouassi, E. Serotonin upregulates mitogen-stimulated B lymphocyte proliferation through 5-HT_{1A} receptors. *Cell. Immunol.*, **163**: 1-9, 1995.

Imai, H., Steindler, D.A. and Kitai, S.T. The organistaion of divergent axonal projections from the mid-brain raphe nuclei in the rat. *J. Comp. Neurology* **243**: 263-380, 1986.

Inglese, J., Freedman, N.J., Koch, W.J. and Lefkowitz, R.J. Structure and mechanism of the G protein-coupled receptor kinases. *J. Biol. Chem.* **268**: 23735-23738, 1993.

Innamorati, G., Sadeghi, H. and Birnbaumer, M. Phosphorylation and recycling kinetics of G protein coupled receptors. *J. Recept. Signal. Transduct. Res.* **19**: 315-26, 1999.

Iordanov, M., Bender, K., Ade, T., Schmid, W., Sachsenmaier, C., Engle, K., Gaestel, M., Rahmsdorf, H.J. and Herrlich, P. CREB is activated by UVC through a p38/HOB-1-dependent protein kinase. *EMBO. J.* **16**: 1009-1022, 1997.

Itin, C., Kappeler, F., Linstedt, A.D. and Hauri H.P. A novel endocytosis signal related to the KKXX ER-retrieval siganl. *EMBO J.* **14**: 2250-2256, 1995.

Itokawa, M., Toru, M., Ito, K., Tsuga, H., Kameyama, K., Haga, T., Arinami, T. and Hamaguchi, H. Sequestration of the short and long isoforms of dopamine D-2 receptors expressed in chinese hamster ovary cells. *Mol. Pharmacol.* **49**: 560-566, 1996.

Kanaseki, T. and Kadota, K. The "vesicle in a basket." A morphological study of the coated vesicle isolated from the nerve endings of the guinea pig brain, with special reference to the mechanism of membrane movements. *J. Cell. Biol.* **42**: 202-220, 1969.

Karoor, V., Baltensperger, K., Paul, H., Czech, M.P. and Malbon, C.C. Phosphorylation of tyrosyl residues-350/354 of the β-adrenergic receptor is obligatory for counterregulatory effects of insulin. J. *Biol. Chem.* **43**: 25305-25308, 1995.

Karschin, A, Ho, B.Y., Labarca, C., Elroy-stein, O., Moss, B., Davidson, N. and Lester, H.A. Heterologously expressed serotonin$_{1A}$ receptors couple to muscarinic K_+ channels in the heart. *Proc. Natl. Acad. Sci. USA.* **88**: 5694-5698, 1991.

Kemp, D.M., George, S.E., Bungay, P.J. and Naylor, L.H. Partial agonism at serotonin 5-HT$_{1B}$ and dopamine D_2L receptors using a luciferase reporter gene assay. *Eur. J. Pharmacol.*, **373**: 215-222, 1999.

Kirchhausen, T. Coated pits and coated vesicles-sorting it all out. *Curr. Opin. Struct. Biol.* **3**: 182-188' 1993.

Kitazawa, T., Masuo, M. and Somlyo, A.P. G protein mediated inhibition of myosin light chain phosphatase in vascular smooth muscle. *Proc. Natnl. Acad. Sci. USA.* **88**: 9307-9310, 1991.

Kobilka, B., Frielle, T., Collins, S., Yang-Feng, T., Kobilka, T.S., Francke, U., Lefkowitz, R.J. and Caron, M.G. An intronless gene encoding a potential member of the family of receptors coupled to guanine nucleotide regulatory proteins. *Nature* **329**: 75-70, 1987.

Koella, W.P. Serotonin and sleep. In Neuronal Serotonin (eds N.N. Osbourne and M. Hamon) pp153-170. John Wiley, New York.1988.

Koh, W.S., Jeon, Y.J., Herring, A.C. and Kaminski, N.E. Transient CRE- and kappa B site-binding is cross-regulated by cAMP-dependent protein kinase and a protein phosphatase in mouse splenocytes. *Life Sci.* **60**: 425-432, 1997.

Kopp, E. and Ghosh, S. NF-κB and rel proteins in innate immunity. *Adv. Immunol.* **58**: 1-27, 1995.

Kravchenko, V.V., Pan, Z., Han, J., Herbert, J.M., Ulevitch, R.J. and Ye, R.D. Platelet-activating factor induces NF-κB activation through a G protein-coupled pathway. *J. Biol. Chem.* **270**: 14928-14934, 1995.

Krueger, K.M., Daaka, Y., Pitcher, J.A. and Lefkowitz, R.J. The role of sequestration in the G protein-coupled receptor resensitization. Regulation of β_2-adrenergic receptor dephosphorylation by vesicular acidification. *J. Biol. Chem.* **272**: 5-8, 1997.

Kumar, A., Haque, J., Lacoste, J., Hiscott, J. and Williams, B.R. Double-stranded RNA-dependent protein kinase activates transcription factor NF-κB by phosphorylating I-κB. *Proc. Natl. Acad. Sci. USA.* **91**: 6288-6292, 1994.

Laporte, S.A., Servant, G., Richard, D.E., Escher, E., Guillemette, G. and Leduc, R. The tyrosine within the NPX$_n$Y motif of the human angiotensin II type 1 receptor is

involved in mediating signal transduction but is not essential for internalization. *Mol. Pharmacol.* **49**: 89-95, 1996.

Law, P.Y., Maestri-El kouhen, O., Soberg, J., Wang, W., Erickson, L.J. and Loh, H.H. Deltorphin II-induced rapid desensitisation of δ-opioid receptor requires both phosphorylation and internalisation of the receptor. *J. Biol. Chem.* Jul 11; (epub ahead of print) 2000.

Lazarovits, J. and Roth, M. A single amino acid change in the cytoplasmic domain allows the influenza virus hemagglutinin to be endocytosed through coated pits. *Cell* **53**: 743-752, 1988.

Lee, J.C., Laydon, J.T., McDonnell, P.C., Gallagher, T.F., Kumar, S., Green, D., McNulty, D., Blumenthal, M.J., Hey, J.R. and Landvatter, S.W. A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature.* **273**: 739-746, 1994.

Lee, K.B., Ptasienski, J.A., Pals-Rylaarsdam, R., Gurevich, V.V. and Hosey, M.M. Arrestin binding to the M_2 muscarinic acetylcholine receptor is precluded by an inhibitory element in the third intracellular loop of the receptor. *J. Biol. Chem.* **275**: 9284-9289, 2000.

Lee, N.H. and Fraser, C.M. Cross-talk between m1 muscarinic acetylcholine and β_2 adrenergic receptors. CAMP and the third intracellular loop of m1 muscarinic receptors confer heterologous regulation. *J.Biol. Chem.* **268**: 7949-7957, 1993.

Lembo, P.M. and Albert, P.R. Multiple phosphorylation sites are required for pathway-selective uncoupling of the 5-hydroxtyrptamine$_{1A}$ receptor by protein kinase C. *Mol. Pharmacol.* **48**: 1024-1029, 1995.

Lembo, P.M., Ghahremani, M.H. and Albert, P.R. Receptor selectivity of the cloned opossum G protein-coupled receptor kinase 2 (GRK2) in intact opossum kidney cells: role in desensitization of endogenous α_{2C}-adrenergic but not serotonin$_{1B}$ receptors. *Mol. Endocrinol.* **13**: 138-47, 1999.

Lembo, P.M., Ghahremani, M.H., Morris, S.J. and Albert, P.R. A conserved thronine residue in the second intracellular loop of the 5-hydroxytryptamine$_{1A}$ receptor directs signaling specificity. *Mol. Pharmacol.* **52**: 164-171, 1997.

Liu, J., Fan, Q., Sodoeka, M., Lane, W.S. and Verdine, G.L. DNA binding by an amino acid residue in the C-terminal half of the rel homolgy domain. *Chem. Biol.* **1**: 47-55, 1994.

Liu, J., Sodeoka, M., Lane, W.S. and Verdine, G.L. Evidence for a non-alpha-helical DNA-binding motif in the rel homology region. *Proc. Natl. Acad. Sci. USA*, **91**: 908-12, 1994.

Liu, Y.F. and Albert, P.R. Cell-specific signaling of the 5-HT_{1A} receptor. Modulation by protein kinase C and A. *J. Biol. Chem.* **266**: 23689-23697, 1991.

Lohse, M.J., Benovic, J.L., Caron, M.G. and Lefkowitz, R.J. Multiple pathways of rapid β2-adrenergic receptor desensitisation; deliniation with specific inhibitors. *J. Biol. Chem.* **265**: 3202-3209, 1990.

Lundberg, J.M. and Hokfelt, T. Coexsitence of peptides and classical neurotransmitters. *TINS.* **6**: 325-333, 1983.

Luttrell, L.M., Daaka, Y., Della Rocca, G.J. and Lefkowitz, R.J. G protein coupled receptors mediate two functionally distinct pathways of tyrosine phosphorylation in rat 1a fibroblasts. *J. Biol. Chem.* **50**: 31648-31656, 1997.

Malecz, N., Bambino, T., Bencsik, M. and Nissenson, R.A. Identification of phosphorylation sites in the G protein-coupled receptor for parathyroid hormone. Receptor phosphorylation is not required for agonist-induced internalization. *Mol. Endocrinol.* **12**: 1846-1856, 1998.

Martin, G.R. and Eglen, R.M. 5-hydroxytryptamine receptors, 1998 receptor & ion channel nomenclature supplement. *TiPS*, 1998.

Mayor, S., Rothberg, K.G. and Maxfield F.R. Sequestration of GPI-anchored proteins in caveolae triggered by cross-linking. *Science* **264**: 1948-1951, 1994.

McCall, R.B. and Clement, M.E. Role of $serotonin_{1A}$ and $serotonin_2$ receptors in the central regulation of the cardiovascular system. *Pharmacol. Rev.* **46**: 231-243, 1994.

McKinley, D.N., and Wiley, H.S. Reassessment of fluid-phase endocytosis and diacytosis in monolayer cultures of human fibroblasts. *J. Cell. Physiol.* **136**: 389-397, 1988.

Megson, A.C., Dickenson, J.M., Townsend-Nicholson, A. and Hill, S.J. Synergy between the inositol phosphate responses to transfected human adenosine A1-receptors and constitutive P2-purinoceptors in CHO-K1 cells. *Br. J. Pharmacol.* **115**: 1415-1425, 1995.

Mellits, K.H., Hay, R.T. and Goodbourn, S. Proteolytic degradation of MAD3 (I-$\kappa B\alpha$) and enhanced processing of the NF-κB precursor p105 are obligatory steps in the activation of NF-κB. *Nuceleic Acids Res.* **21**: 5059-5066, 1993.

Mercurio, F., DiDonato, J.A. Rosette, C. and Karin, M. p105 and p98 precursor proteins play an active role in NF-κB-mediated signal transduction. *Genes Dev.* **7**: 705-718, 1993.

Middleton, J.P., Raymond, J.R., Whorton, A.R. and Dennis, V.W. Short term regulation of Na^+/K^+ adenosine triphosphatase by recombinant human serotonin 5-HT_{1A}R receptor expressed in HeLa cells. *J. Clin. Invest.* **86**: 1799-1805, 1990.

Miettinen, H.M., Rose, J.K. and Mellman, I. Fc receptor isoforms exhibit distinct abilities for coated pit localisation as a result of cytoplasmic domain heterogeneity. *Cell* **58**: 317-327, 1989.

Miyamoto, S., Maki, M., Schmitt, M.J., Hatanaka, M. and Verma, I.M. TNFα induced phosphorylation of I-κBα is a signal for its degradation but not dissociation from NF-κB . *Proc. Natl. Acad. Sci. USA.* **91**: 12740-12744, 1994.

Mohan, R.R., Mohan, R.R., Kim, W.J. and Wilson, S.E. Modulation of TNFα-induced apoptosis in corneal fibroblasts by transcription factor NF-κB. *Invest. Opthalmol. Vis. Sci.* **41**: 1327-36, 2000.

Molliver, M.E. Serotonergic neuronal systems: What their anatomic organisation tells us about function. *J. Clin. Psycopharmacology* **7**: 3S-23S 1987.

Montesano, R., Roth, J., Robert, A. and Orci, L. Non-coated membrane invaginations are involved in binding and internalisation of cholera and tetanus toxins. *Nature* **296**: 651-653, 1992.

Montminy, M.R. and Bilezikjian, L.M. Binding of a nuclear protein to the cAMP response element of the somatostatin gene. *Nature.* **328**: 175-178, 1987.

Montminy, M.R., Low, M.J., Tapia-Arancibia, L., Reichlin, S., Mandel, G. and Goodman, R.H. Cyclic AMP regulates somatostatin mRNA accumulation in primary diencephalic cultures in transfected fibroblast cells. *J. Neurosci.* **6**: 1171-1176, 1986.

Moore, R.H., Sadovnikoff, N., Hoffenberg, S., Liu, S., Woodford, P., Angelides, K., Trial, J., Carsrud, V., Dickey, B.F. and Knoll, B. Ligand-stimulated β2-adrenergic receptor internalisation via the constitutive endocytic pathway into rab5-containing endosomes. *J. Cell. Sci.* **108**: 2983-2991, 1995.

Mori, S., Heldin, C.H. and Claesson-Welsh, L. Ligand-induced polyubiquitination of the platelet-derived growth factor β-receptor. *J. Biol. Chem.* **267**: 6429-6434, 1992.

Morris, S.A., Schroder, S., Plessmann, U., Weber, K. and Ungewickell, E. Clathrin assembly protein AP180: primary structure, domain organisation and identification of a clathrin binding site. *EMBO J.* **12**: 667-675, 1993.

Morrison, K.J., Moore, R.H., Carsrud, N.D., Trial, J., Millman, E.E., Tuvim, M., Clark, R.B., Barber, R., Dickey, B.F. and Knoll, B.J. Repetitive endocytosis and recycling of the β_2-adrenergic receptor during agonist-induced steady state redistribution. *Mol. Pharmacol.* **50**: 692-699, 1996.

Muller, C.W., Rey, F.A., Sodeoka, M., Verdine, G.L. and Harrison, S.C. Structure of the NF-κB p50 homodimer bound to DNA. *Nature*, **373**: 311-317, 1995.

Myers, R.D. Serotonin and thermoregulation: Old and new view. *J. Physiol.* (Paris) **77**: 505-513, 1981.

Nathanson, N.M. and Harden, T.K. Structure/ function relationships of Ras and guanosine triphosphate-activating protein. G proteins and signal transduction, New York: the Rockerfeller University Press, 77-79.

Nebigil, C.G., Garnovskaya, M.N., Casañas, S.J., Mulheron, J.G., Parker, E.M., Gettys, T.W. and Raymond, J.R. Agonist-induced phosphorylation and desensitization of 5-HT_{1A} receptor expressed in Sf9 insect cells. *Biochemistry.* **34**: 11954-11962, 1995.

Neri, A., Chang, C.C., Lombardi, L., Salina, M., Corradini, P., Maiolo, A.T., Chaganti, R.S. and Dalla-Favera, R. B cell lymphoma-associated chromosomal translocation involves candidate oncogene lyt-10, homologous to NF-κB p50. *Cell,* **67**: 1075-1087, 1991. *Neuropsychpharmacol.* **14**: 19-25, 1996.

Newman-Tancredi, A., Wootton, R. and Strange, G. High-level stable expression of recombinant 5-hydroxytryptamine receptors in Chinese hamster ovary cells. *Biochem. J.* **285**: 933-938, 1992.

Ni, Y.G., Panicker, M.M. and Miledi, R. Efficient coupling of 5-HT_{1A} receptors to phospholipase C pathway in *Xenopus* oocytes. *Mol. Brain. Res.*, **51**: 115-122, 1997.

Nie, Z., Mei, Y. and Ramkumar, V. Short term desensitization of the $adenosine_{A1}$ receptors in DDT1MF-2 cells. *Mol. Pharmacol.* **52**: 456-64, 1997.

Nilsson, J., Thyberg, J., Heldin, C.H., Westermark, B. and Wasteson, A. Surface binding and internalisation of platelet-derived growth factor in human fibroblasts. *Proc. Natl. Acad. Sci. USA.* **80**: 5592-6, 1983.

Nishi, M. and Azmitia, E.C. Agonist and antagonist-induced plasticity of rat 5-HT_{1A} receptor in hippocampal cell culture. *Synapse.* **31**: 186-95, 1999.

Nolan, G.P. NF-AT-AP-1 and rel bZIP: hybrid vigor and binding under the influence. *Cell*, **77**: 795-798, 1994.

Novak, J.M., Ward, D M., Buys, S.S., and Kaplan, J. Effect of hypo-osmotic incubation on membrane recycling. *J. Cell. Physiol.* **137**: 235-242, 1988.

Obrig, T.G., Culp, W.J., McKeehan, W.L. and Hardesty, B. The mechanism by which cycloheximide and related glutarimide antibiotics inhibit peptide synthesis on reticulocyte ribosomes. *J. Biol. Chem.* **246**: 174-181, 1971

Ou, X.M., Jafar-Nejad, H., Storring, J.M., Meng, J.H., Lemonde, S. and Albert, P.R. Novel dual repressor elements for neuronal cell-specific transcription of the rat 5-HT_{1A} receptor gene. *J. Biol. Chem.* **275**: 8161-8, 2000.

Pagano, R.E. Lipid traffic in eukaryotic cells: mechanisms for intracellular transport and organelle-specific enrichment of lipids. *Curr. Opin. Cell. Biol.* **2**: 652-663, 1990.

Page, N.M., McNab, D. and Shaw, K. *Society for Neuroscience*. **22**: 1352, 1996.

Palade, G.E. and Bruns, R,R. Structural modulations of plasmalemmal vesicles. *J. Cell. Biol.* **37**: 633-649, 1968.

Parks, C.L. and Shenk, T. The serotonin$_{1A}$ receptor gene contains a TATA-less promoter that responds to MAZ and Sp1. *J. Biol. Chem.* **271**: 4417-30, 1996.

Pedigo, N.W., Yamamura, H.I. and Nelson, N. Discrimination of multiple [^{3}H]5-hydroxytryptamine binding sites by the neuroleptic spiperone in rat brain. *J. Neurochem.* **36**: 220-226, 1981.

Penela, P., Ruiz-Gomez, A., Castano, J.G. and Mayor, F. Jr. Degradation of the G protein-coupled receptor kinase 2 by the proteasome pathway. *J. Biol. Chem.* **273**: 35238-44, 1998.

Peroutka, S.J. and Howell, T.A. The molecular evolution of G protein coupled receptors: Focus on 5-hydroxytryptamine receptors. *Neuropharmacol* **33**: 391-397, 1994.

Peroutka, S.J. and Snyder, S.H. Multiple serotonin receptors: Differential binding of [^{3}H] 5-hydroxytryptamine, [^{3}H] lysergic acid diethylamide and [^{3}H] spiroperidol. *Mol. Pharmacol.* **16**: 687-689, 1979.

Peters, K.R., Carley, W.W. and Palade, G.E. Endothelial plasmalemmal vesicles have a characteristic striped bipolar surface structure. *J. Cell. Biol.* **101**: 2233-2238, 1985. *Pharmacol.* **83**: 519-525, 1984.

Piechaczyk, M. and Blanchard, J.M. c-fos protooncogene regulation and function. *Crit. Rev. Oncol. Hematol.* **17**: 93-131, 1994.

Pippig, S., Andexinger, S., and Lohse, M.J. Sequestration and recycling of β(2)-adrenergic receptors permit receptor resensitisation. *Mol. Pharmacol.* **47**: 666-676, 1995.

Pitcher, J.A., Payne, E.S., Csortos, C., DePaoli-Roach, A.A. and Lefkowitz, R.J. The G protein-coupled receptor phosphatase: a protein phosphatase type IIA with a distinct subcellular distribution and substrate specificity. *Proc. Natl. Acad. Sci. U.S.A.* **92**: 8343-8347, 1995b.

Pitcher, J.A., Touhara, K., Payne, E.S. and Lefkowitz, R.J. Pleckstrin homology domain-mediated membrane association and activation of the β-adrenergic receptor kinase requires co-ordinate interaction with G$_{\beta\gamma}$ subunits and lipid. *J. Biol. Chem.* **270**: 11707-11710, 1995a.

Premont, R.T., Inglese, J. and Lefkowitz, R.J. Protein-kinases 3; Protein kinases that phosphorylate activated G protein-coupled receptors. *FASEB.* **9**: 175-182, 1995.

Pronin, A.N. and Benovic, J.L. Regulation of the G protein coupled receptor kinase GRK5 by protein kinase C. *J. Biol. Chem.* **272**: 3806-3812, 1997.

Quinn, P.G. Distinct activation domains within cAMP response element-binding protein (CREB) mediate basal and cAMP stimulate transcription. *J. Biol. Chem.* **268**: 16999-17009, 1993.

Rang, H.P., Dale. M.M. and Ritter, J.M. 5-hydroxytryptamine Pharmacology, 3rd ed, New York: Churchill Livingstone Inc, 177-180, 1995.

Raposo, G., Dunia, I., Delavier-Klutchko, C., Kaveri, S., Strosberg, A.D. and Benedetti, E.L. Internalisation of β-adrenergic receptor in A431 cells involves non-coated vesicles. *Eur. J. Cell. Biol* **50**: 340-352, 1989.

Rapport, M.M., Green, A.A. and Page, I.H. Serum vasoconstrictor (serotonin) IV isolation and characterisation. *J. Chem. Biol.* **178**: 1243-1251, 1948.

Raymond, J.R. Protein kinase C induces phosphorylation and desensitisation of the human 5-HT_{1A} receptor. *J. Biol. Chem.* **266**: 14747-14753, 1991.

Raymond, J.R., Fargin, A., Middleton, P.J., Graff, M.J., Haupt, D.M., Caron, M.G., Lefkowitz, R.J. and Dennis, V.M. The human 5-HT_{1A} receptor expressed in HeLa cells stimulates sodium-dependent phosphate uptake via protein kinase C. *J. Biol. Chem.* **264**: 21943-21950, 1989.

Raymond, J.R., Kim, J., Beach, R.E., Tisher, C.C. Immunohistochemical mapping of cellular and subcellular distribution of 5-HT_{1A} receptors in rat and human kidneys. *Am. J. Physiol.* **264**: F9-19, 1993.

Raymond, J.R., Middleton, J.P. and Dennis, V.W. HeLa cells express cAMP-inhibitable sodium-dependent phosphate uptake. *Am. J. Physiol.* **258**: F433-437, 1990.

Raymond, J.R., Murkin, Y.V., Gettys, T.W. and Garnovskaya, M.N. The recombinant 5-HT_{1A} receptor: G protein couplingand signalling pathways. *Br. J. Pharmacol.* **127**: 1751-1764, 1999.

Robinson, M.J. and Cobb, M.H. Mitogen-activated protein kinase pathways. *Curr. Opin. Cell. Biol.* **9**: 180-186, 1997.

Rohr, O., Schwartz, C., Aunis, D. and Schaeffer, E. CREB and COUP-TF mediate transcriptional activation of the human immunodeficiency virus type 1 genome in Jurkat T cells in response to cyclic AMP and dopamine. *J. Cell. Biochem.* **75**: 404-413, 1999.

Roth, M.G., Doyle, J., Sambrook, J. and Gething, M.J. Heterologous transmembrane and cytoplasmic domains direct functional chimeric influenza virus hemagglutinins into the endocytic pathway. *J. Cell. Biol.* **102**: 1271-1283, 1986.

Roth, T.F. and Porter, K.R. Yolk protein uptake in the oocyte of the mosquito *Aedes aegypti L. J. Cell. Biol.* **20**: 313-332, 1964.

Rothberg, K.G., Heuser, J.E., Donzell, W.C., Ying, Y.S., Glenney, J.R. and Anderson, R.G.W. Caveolin, a protein-component of caveolae membrane coats. *Cell.* **68**: 673-682, 1992.

Rothberg, K.G., Ying, Y.S., Kamen, B.A. and Anderson, R.G.W. Cholesterol controls the clustering of the glycophospholipid-anchored membrane receptor for 5-methyltetrahydrofolate. *J. Cell. Biol.* **111**: 2931-2938, 1990a.

Rothberg, K.G., Ying, Y.S., Kolhouse, J.F., Kamen, B.A. and Anderson, R.G.W. The glycophospholipid-linked folate receptor internalises folate without entering the clathrin-coated pit endoctic pathway. *J. Cell. Biol.* **110**: 637-649, 1990b.

Sanders-Bush, E. and Canton, H. Serotonin receptors: siganal transduction pathways. Psychopharmacology, New York: Raven Press Ltd., chapter 38, 431-440.

Sandvig, K., Olsnes, S., Brown, J.E., Petersen, O.W. and Van Deurs, B. Endocytosis from coated pits of Shiga toxin; a glycolipid-binding protein from *Shigella dysenteriae. J. Cell. Biol.* **108**: 1331-1343, 1989.

Sandvig, K., Olsnes, S., Petersen, O.W. and Van Deurs, B. Acidification of the cytosol inhibits endocytosis from coated pits. *J. Cell. Biol.* **105**: 679-689, 1987.

Sargiacomo, M., Sudol, M., Tang, Z.L. and Lisanti, M.P. Signal-transducing molecules and glycosyl-phosphatidylinositol-linked proteins from a caveolin-rich insoluble complex in MDCK cells. *J. Cell. Biol.* **122**: 789-807, 1993.

Sartania, N. and Strange, P.G. Role of conserved serine residues in the interaction of agonists with D_3 dopamine receptors. *J. Neurochem.* **72**: 2621-2624, 1999.

Saudou, F. and Hen, R. 5-hydroxytryptamine receptor subtypes in vertebrates and invertebrates. *Neurochem. Int.* **25**: 503-532 1994.

Schekman, R. Genetic and biochemical analysis of vesicular traffic in yeast. *Curr. Opin. Cell. Biol.* **4**: 587-592, 1992.

Scherer, D.C., Brockman, J.A., Chen, Z., Maniatis, T. and Ballard, D.W. Signal-induced degradation of I-$\kappa B\alpha$ requires site-specific ubiquitination. *Proc. Natl. Acad. Sci. USA.* **92**: 11259-11263, 1995.

Schmid, R.M., Perkins, N.D., Duckett, C.S., Andrews, P.C. and Nabel, G.J. Cloning of an NF-κB subunit which stimulates HIV transcription in synergy with p65. *Nature*, **352**: 733-736, 1991.

Schmid, S.L. The mechanism of receptor-mediated endocytoisi; more questions than answers. *Bioessays*. **14**: 589-596, 1992.

Schnitzer, J.E., Liu, J. and Oh, P. Endothelia caveolae have the molecular transport machinery for vesicle budding, docking and fussion including VAMP, NSF, SNAP, annexins and GTPases. *J. Biol. Chem.* **270**: 14399-14404, 1995.

Schnitzer, J.E., Oh, P., Pinney, E. and Allard, J. Filipin-sensitive caveolae-mediated transport in endothelium: reduced transcytosis, scavenger endocytosis and capillary permeability of select macromolecules. *J. Cell. Biol.* **127**: 1217-1232, 1994.

Schwartz, C., Catez, P., Rohr, O., Lecestre, D., Aunis, D. and Schaeffer, E. Functional interactions between C/EBP, Sp1 and COUP-TF regulate human immunodeficiency virus type 1 gene transcription in human brain cells. *J. Virol.* **74**: 65-73, 2000.

Segredo, V., Burford, N.T., Lameh, J. and Sadee, W. A constitutive internalising and recycling mutant of the μ-opioid receptor. *J. Neurochem.* **68**: 2395-2404, 1997.

Selbie, L.A. and Hill, S.J. G protein-coupled-receptor cross-talk: the fine-tuning of multiple receptor-signalling pathways. *TiPS.* **19**: 87-93, 1998.

Selbie, L.A., Darby, K. Schmitzpeiffer, C., Browne, C.L., Herzog, H., Shine, J. and Biden, T.J. Synergistic interaction of Y1-neuropeptide-Y and α(1B)-adrenergic receptors in the regulation of phospholipase-C, protein kinase-C, and arachidonic acid production. *J. Biol. Chem.* **270**: 11789-11796, 1995.

Selbie, L.A., King, N.V., Dickenson, J.M. and Hill S.J. Role of G protein beta gamma subunits in the augmentation of P2Y(2) (P-2U) receptor-stimulated responses by neuropeptide Y Y-1 $G_{(i/o)}$-coupled receptors. *Biochem. J.* **328**: 153-158, 1997.

Sellars, L.A. Prolonged activation of extracellular signal-regulated kinase by a protein kinase C-dependent and N17Ras-insensitive mechanisms mediates the proliferative response of $G_{(i/o)}$-coupled somatostatin $(sst)_4$ receptors. *J. Biol. Chem.* **274**: 24280-24288, 1999.

Sen, R. and Baltimore, D. Multiple nuclear factors interact with the immunoglobulin enhancer sequences. *Cell*, **46**: 705-716, 1986a.

Sen, R. and Baltimore, D. Inducibility of κ immunoglobulin enhancer-binding protein NF-κB by a post-translational mechanism. *Cell,* **47**: 921-928, 1986b.

Severs, N.J. Caveolae: static inpocketings of the plasma membrane, dynamic vesicles or plain artifact. *J. Cell. Sci.* **90**: 341-348, 1988.

Slice, L.W., Wong, H.C., Sternini, C., Grady, E.F., Bunnett, N.W. and Walsh, J.H. The conserved NPX_nY motif present in the gastrin-releasing peptide receptor is not a general sequestration sequence. *J. Biol. Chem.* **269**: 21755-21761, 1994.

Smyth, E.M., Austin, S.C., Reilly, M.P. and FitzGerald, G.A. Internalisation and Sequestration of the human Prostacyclin Receptor. *J. Biol. Chem.* **275**: 32037-32045, 2000.

Somlyo, A.P. and Somlyo, A.V. Signal transduction and regulation in smooth muscle. *Nature.* **372**: 231-236, 1994.

Stefanova, I., Horejsi, V., Ansotegui, I.J., Knapp, W. and Stockinger, H. GPI-anchored cell-surface molecules complexed to protein tyrosine kinases. *Science.* **254**: 1016-1019, 1991.

Storring, J.M., Charest, A., Cheng, P. and Albert P.R. TATA-driven transcriptional initiation and regulation of the rat 5-HT_{1A} receptor gene. *J. Neurochem.* 72: 2239-47, 1999.

Strange, P.G. The energetics of ligand binding at catecholamine receptors. *TIPS.* **17**: 238-244, 1996.

Strous, G.J., Vankerkhof, P., Govers, R., Ciechanover, A. and Schwartz, A.L. The ubiquitin conjugation system is required for ligand-induced endocytosis and degradation of the growth-hormone receptor. *EMBO. J.* **15**: 3806-3812, 1996.

Stupecky, G.L., Murray, D.L. and Purdy, R.E. Vasoconstrictor threshold synergism and potentiation in the rabbit isolated thoracic aorta. *J. Pharmacol. Exp. Ther.* **238**: 802-808, 1986.

Sugden, P.H. and Clerk, A. Regulation of the ERK subgroup of MAP kinase cascades through G protein coupled receptors. *Cell. Signal* **9**: 337-351, 1997.

Sun, P., Enslen, H., Myung, P.S. and Maurer, R.A. Differential activation of CREB by Ca^{2+}/ calmodulin-dependent protein kinases type II and type IV involves phosphorylation of a site that negatively regulates activity. *Genes. Dev.* **8**: 2527-2539, 1994.

Sundaram, H., Newman-Tancredi, A. and Strange, P.G. Characterisation of recombinant human serotonin $5HT_{1A}$ receptors expressed in chinese hamster ovary cells [^{3}H] spiperone discriminates between the G protein-coupled and G protein-uncoupled forms. *Biochem. Pharmacol.* **45**: 1003-1009, 1993.

Szekeres, P.G., Koenig, J.A. and Edwardson, J.M. The relationship between agonist intrinsic activity and the rate of endocytosis of muscarinic receptors in a human neuroblastoma cell line. *Mol. Pharmacol.* **53**: 759-765, 1998.

Tang, H., Guo, D.F., Porter, J.P. Wanaka, Y. and Inagami, T. Role of cytoplasmic tail of the Type 1A angiotensin II receptor in agonist and phorbol ester induced desensitisation. *Circ. Res.* **82**: 523-531, 1998.

Tang, H., Nishishita, T., Fitzgerald, T., Landon, E.J. and Inagami, T. Inhibition of AT1 receptor internalisation by Concanavalin A blocks angiotensin II-induced Erk activation in vascular smooth muscle cells. Involvement of epidermal growth factor receptor proteolysis but not AT1 receptor internalisation. *J. Biol. Chem.* **275**: 13420-13426, 2000.

Thanos, D. and Maniatis, T. NF-κB: a lesson in family values. *Cell*, **80**: 529-532, 1995.

Thompson, J., Philips, R., Erdjument-Bromage, H., Tempst, P. and Ghosh, S. I-κBβ regulates the persistent response in biphasic activation of NF-κB. *Cell*, **80**:573-582, 1995.

Toledano, M.B., Ghosh, D., Trinh, F. and Leonardo, W.J. N-terminal DNA-binding domains contribute to differential DNA-binding specificity's of NF-κB p50 and p65. *Mol. Cell. Biol.* **13**: 852-860, 1993.

Traencker, B-M., Pahl, H., Henkel, T., Schmidt, K., Wilk, S. and Baeuerle, P. Phosphorylation of human I-κBα on serines 32 and 36 controls I-κBα proteolysis and NF-κB activation in response to diverse stimuli. *EMBO. J.* **14**: 2876-2883, 1995.

Treisman, R. Regulation of transcription by MAP kinase cascades. *Cell. Biol.* **8**: 205-215, 1996.

Trejo, J., Altschuler, Y., Fu, H.W., Mostov, K.E. and Coughlin, S.R. Protease-activated receptor-1 downregulation: A mutant HeLa cell line suggests novel requirements for PAR1 phosphorylation and recruitment to clathrin-coated pits. *J. Biol. Chem.* (epub ahead of print), July 11, 2000.

Tsuga, H., Kameyama, K., Haga, T., Honma, T., Lameh, J. and Sadee, W. Internalisation and down-regulation of human muscarinic acetylcholine receptor M_2 subtypes - Role f third intracellular M_2 loop and G protein-coupled receptor kinase 2. *J. Biol. Chem.* **273**: 5323-5330, 1998.

Tsuga, H., Kameyama, K., Haga, T., Kurose, H. and Nagao, T. Sequestration of muscarinic acetylcholine-receptor M2 subtypes-facilitation by G protein-coupled receptor kinase (GRK2) and attenuation by a dominant-negative mutant of GRK2. *J. Biol. Chem.* **269**: 32522-32527, 1994.

Tuomisto, J. and Mannisto, P. Neurotransmitter regulation of anterior pituitary hormones. *Pharmacol. Rev.* **37**: 249-332, 1985.

Ueda, H., Inoue, M. and Matsumoto, T. Protein Kinase C-mediated inhibition of μ-Opioid receptor internalisation and its involvement in the Development of acute tolerance to peripheral μ-Agnist analgesia. *J. Neurosci.* **21**: 2967-2973, 2001.

Van Biesen, T., Hawes, B.E., Raymond, J.R., Luttrell, L.M., Koch, W.J. and Lefkowitz, R.J. G_o-protein α-subunits activate mitogen-activated protein kinase via a novel protein kinase C-dependent mechanism. *J. Biol. Chem.* **271**: 1266-1269, 1996.

Van der Bliek, A.M., Redelmeier, T.E., Damke, H., Tisdale, E.J., Meyerowitz, E.M. and Schmid, S.L. Mutations in human dynamin block an intermediate stage in coated vesicle formation. *J. Cell. Biol.* **122**: 553-563, 1993.

Van Deurs, B., Holm, P.K., Sandvig, K. and Hansen, S.H. Are caveolae involved in clathrin-independent endocytosis. *Trends in Cell Biology.* **94**: 24-251, 1993.

Van Koppen, C.J., Lenz, W., Nunes, J.P., Zhang, C., Schmidt, M. and Jakobs, K.H. The role of membrane proximal threonine residues conserved among guanine-nucleotide-bindinG protein-coupled receptors in internalisation of the m4 muscarinic acetylcholine receptor. *Eur. J. Biochem.* **234**: 536-541, 1995.

Van Neuten, J.M., Janssens, W.J. and Vanhoutte, P.M. (1985) in serotonin and the cardiovascular system (Vanhoutte, P.M. ed): 95-105, Raven Press.

Varrault, A., Bockaert, J. and Waeber, C. Activation of 5-HT_{1A} receptors expressed in NIH-3T3 cells induces focus formation and potentiates EGF effect on DNA synthesis. *Mol. Biol. Cell*, **9**: 961-969, 1992.

Verma, I.M., Stevenson, J.K., Schwarz, E.M., Van Antwerp, D. and Miyamoto, S. Rel/ NF-κB/ I-κB family: intimate tales of association and dissociation. *Genes Dev.* **9**: 2723-2735, 1995.

Von Zastrow, M. and Kobilka, B.K. Ligand-regulated internalization and recycling of human β_2-adrenergic receptors between the plasma membrane and endosomes containing transferrin receptors. *J. Biol. Chem.* **267**: 3530-3538, 1992.

Voorhees, P., Deignan, E., VanDonselaar, E., Humphery, J., Marks, M.S., Peters, P.J. and Bonifacino, J.S. An acidic sequence within the cytoplasmic domain of furin functions as a determinant of the trans-golgi network localisation and internalisation from the cell surface. *Embo. J.* **14**: 4961-4975, 1995.

Waeber, G. and Habner, J.F. Nuclear translocation and DNA recognition signals colocalized within the bZIP domain of cyclic adenosine 3'5'-monophosphate response element binding protein CREB. *Mol. Endocrinol.* **262**: 395-399, 1991.

Wang, J.F., Zheng, J.L. Anderson, J.L. and Toews, M.L. A mutation in the hamster alpha (1B)-adrenergic receptor that differentiates two steps in the pathway of receptor internalisation. *Mol. Pharmacol.* **52**: 306-313, 1997.

Wesemann, W. and Weiner, N. Circadian rhythm of serotonin binding in rat brain. *Prog. Neurobiol.* **35**: 405-428, 1990.

Whiteside, S.T. Ernst, M.K., LeBail, O., Laurent-Winter, C., Rice, N. and Israel, A. N- and C-terminal sequences control degradation of MAD3/ I-κBα in response to inducers of NF-κB activity. *Mol. Cell. Biol.* **15**: 5339-5345, 1995.

Whiteside, S.T., Epinat, J.C., Rice, N.R. and Israel, A. I-κBε, a novel member of the I-κB family, controls RelA and c-Rel NF-κB activity. *EMBO. J.* **16**: 1413-1426, 1997.

Willingham, M.C., Pastan, I.H., Sahagian, G.G., Jourdian, G.W. and Neufeld, E.F. Morphologic study of the internalisation of a lysosomal enzyme by the mannose 6-phosphate receptor in cultured chinese-hamster ovary cells. *Proc. Natnl. Acad. Sci. USA.* **78**: 6967-6971, 1981.

Wilson, J.A., Shahkolahi, A.M., Abbassi, M. and Donahue, M.J. Identification of a novel 5-HT_N (Nematoda) receptor from Ascaris suum muscle. *Biochem. Physiol.* **101C**: 469-474, 1973.

Wolf, R., Kock, T., Schulz, S., Klutzny, M., Schroder, H., Raulf, E., Bushling, F. and Hollt, V. Replacement of thronine 394 by alanine facilitates internalisation and resensitisation of the rat μ-opioid receptor. *Mol. Pharmacol.* **55**: 263-268, 1999.

Xiang, B., Yu, G., Guo, J., Chen, L., Hu, W., Pei, G. and Ma, L. Heterologous activation of protein kinase C stimulates phosphorylation of the δ-Opioid receptor at Serine 344, resulting in β-Arrestin and clathrin-meditated receptor internalisation. *J. Biol. Chem.* **276**: 4709-4716, 2001.

Xie, H., Price, J.E., Luca, M., Jean, D., Ronai, Z. and Bar-Eli, M. Dominant-negative CREB inhibits tumour growth and metastasis of human melanoma cells. *Oncogene.* **15**: 2069-2075, 1997.

Xing, J., Kornhauser, J.M., Xiz, Z., Thiele, E.A. and Greenberg, M.E. Nerve growth factors activates extracellular signal-regulated kinase and p38 mitogen-activated protein kinase pathways to stimulate CREB Ser_{133} phosphorylation. *Mol. Cell. Biol.* **18**: 1946-1955, 1998.

Yee, N.S., Hsiau, C.M., Serve, H., Voseller, K. and Besmer, P. Mechanism of down-regulation of c-kit receptor: roles of receptor tyrosine kinase, PI kinase and protein kinase C. *J. Biol. Chem.* **269**: 31991-31998, 1994.

Zhang, J., Ferguson, S.S.G., Barak, L.S. Menard, L. and Caron, M.G. Dynamin and β-arrestin reveal distinct mechanisms for G protein-coupled receptor internalisation. *J. Biol. Chem.* **271**: 18302-18305, 1996.

Zhang, J., Ferguson, S.S.G., Barak, L.S., Aber, M.J., Giros, B., Lefkowitz, R.J. and Caron, M.G. Molecular mechanisms of G protein coupled receptor signalling: Role of

G protein coupled receptor kinases and arrestins in receptor desensitisation and resensitisation. *Receptors and Channels*, **5**: 193-199, 1997.

Zhou, F.C., Patel, T.D., Swartz, D., Xu, Y. and Kelley, M.R. Production and characterisation of an anti-serotonin 1A receptor antibody which detects functional 5-HT_{1A} binding sites. Brain. Res. Mol. Brain. Res. 69: 186-201, 1999.