The role of *myo*-inositol in G-protein coupled receptor-mediated sub-cellular transduction mechanisms in neuronal cell lines.

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Abstract

The role of *myo*-inositol in G-protein coupled receptor-mediated sub-cellular transduction mechanisms in neuronal cell lines

myo-Inositol (*m*I), a simple polyol isomer of glucose, is an important osmolyte in the brain and a precursor of the phosphatidyl inositol metabolic pathway. It is known to facilitate various cellular events such as membrane trafficking and regulation of cell death and survival. In addition, high oral doses of *m*I have been reported to be effective in the treatment of various psychiatric disorders such as depression, panic disorders and obsessive compulsive disorder. *m*I is also a natural component of the human diet and is obtained from food sources like fruits and whole wheat grains. There are no serious side effects documented relating to the use of even high doses of *m*I. Changes in the pharmacological management of depression is necessary to develop drugs with less side effects, greater tolerability and better patient compliance and these criteria makes *m*I a potential attractive drug for treatment of these disorders.

The current study aimed to investigate the mechanism of action of *m*I at molecular level, specifically by investigating a possible modulatory role of *m*I on 5-HT_{2A} receptor (5-HT_{2A}-R) function and number, as expressed in transfected human neuroblastoma (5-HT_{2A}-SH-SY5Y) cells. The effect of *m*I was compared to the effects of two prototype antidepressants, namely

fluoxetine (a selective serotonin re-uptake inhibitor) and imipramine (a tricyclic antidepressant).

To investigate the possible effect of *m*I, fluoxetine and imipramine on 5-HT_{2A}-R function and expression, 5-HT_{2A}-SH-SY5Y cells were pre-treated with different concentrations of these respective substances. Thereafter, functional studies, radio-ligand binding studies and intracellular [^{3}H]-*m*I uptake studies were performed. Membranes were also prepared and [^{35}S]-GTPγS binding studies were performed. Receptor function was measured by second messenger [^{3}H]-IP_x (Inositolphosphates) accumulation and [^{35}S]-GTPγS binding to $G_{\alpha q}$ protein. Relative receptor number was determined from appropriate radio-ligand binding experiments and total [^{3}H]-*m*I uptake into cells was measured directly from cell lysates.

The current study shows that *m*I may exert its therapeutic effect in depression and related anxiety disorders in part by decreasing $5HT_{2A}$-R function and specifically by decreasing the receptor signalling capacity through G_q proteins. *m*I pre-treatments cause a decrease in [^{3}H]-IP_x production and a decrease in [^{3}H]-*m*I uptake without any significant effect on 5-HT_{2A}-R binding. Fluoxetine pre-treatment also significantly decreased [^{3}H]-IP_x production and [^{3}H]-*m*I uptake without any significant effect on 5-HT_{2A}-R radio-ligand binding. However, imipramine pre-treatment significantly increased receptor function. These results may explain why *m*I was found to be effective exclusively in selective serotonin reuptake inhibitor sensitive disorders. In previous studies from our laboratory conducted on mAChR function it was found that *m*I, fluoxetine and imipramine pre-treatments all reduce mAChR function and that the attenuating effect of *m*I on mAChRs is partially dependent on the phosphoinositide (PI) metabolic pathway. Taken together, the current data suggests that *m*I may exert its antidepressant action via down regulation of 5-HT_{2A}-R signalling and by attenuating cholinergic hypersensitivity. Further detailed studies are, however, necessary to resolve the full mechanism of action of *m*I at subcellular level.

Keywords: *myo*-Inositol, depression, fluoxetine, G-protein, imipramine, signal transduction system, serotonin 2A receptor.

Opsomming

Die rol van *mio*-inositol in G-proteien gekoppelde reseptor-gemedieerde subsellulêre transduksie meganismes, in neuronale sellyne

mio-inositol (*m*I), 'n eenvoudige poliol-isomeer van glukose, is 'n belangrike osmoliet in die brein en 'n voorloper in die metaboliese fosfatidielinositol weg. *m*I fasiliteer ook 'n verskeidenheid gebeure in die sel bv. membraanregulering en dit reguleer ook sel dood en-oorlewing. Tydens die toediening van hoë dosisse *m*I is gevind dat dit effektief is in die behandeling van psigiatriese toestande soos depressie, paniek versteurings en obsessiewe kompulsiewe toestande. *m*I kom natuurlik in die dieët voor bv. in vrugte en volgraan produkte en daar is ook nog geen ernstige newe-effekte gedokumenteer wat verband hou met die inname van hoër dosisse *m*I nie.

Veranderinge in die farmakologiese beheer van depressie is noodsaaklik om geneesmiddels te ontwerp wat minimum newe-effekte openbaar, 'n groter verdraagbaarheid en beter pasiëntmeëwerkendheid te weeg bring. Volgens hierdie kriteria is *m*I 'n potensiële aantreklike geneesmiddel vir die behandeling van hierdie siektetoestande.

Die doel van die studie was om die meganisme van werking van *m*I op molekulêre vlak te ontrafel, spesifiek ten opsigte van die modulerende effekte van *m*I op seratonien 2A reseptor (5-HT_{2A}-R) funksie en reseptorbinding (relatiewe reseptor hoeveelheid) in menslike neuroblastoom (5-HT_{2A}-SH-

SY5Y) selle. Die effek van *m*I word vergelyk met twee prototipe antidepressante nl. fluoksetien ('n selektiewe serotonien heropname remmer) en imipramien ('n trisikliese antidepressant).

Om die modulerende effekte van die voorafbehandelings te ondersoek is die 5-HT_{2A}-SH-SY5Y selle vooraf behandel (24 ure) met verskillende konsentrasies van die afsonderlike geneesmiddels. Daarna is funksionele studies, radio-ligand binding studies en intrasellulêre [^{3}H]-*m*I opname studies uitgevoer. Membrane is voorberei en [^{35}S]-GTPγS binding studies is uitgevoer. Reseptor funksie is gemeet deur tweede boodskapper [^{3}H]-IF_x (inositolmultifosfate) akkumulasie en [^{35}S]-GTPγS binding aan $G_{\alpha q}$ proteien te bepaal. Relatiewe reseptor hoeveelheid is bepaal deur die uitvoering van toepaslike radio-ligand binding eksperimente en intrasellulêre opname van [^{3}H]-*m*I deur die selle is ook bepaal.

Uit die huidige studie blyk dit dat *m*I terapeutiese effekte in depressie en verwante toestande het deur die onderdrukking van 5-HT_{2A}-R funksie en spesifiek deur die verlaging van reseptor sein kapasiteit deur G_q proteiene. *m*I voorafbehandelings veroorsaak 'n verlaging in [^{3}H]-IP_x produksie en ook 'n verlaging in [^{3}H]-*m*I opname sonder enige beduidende verlaging in 5-HT_{2A}-R radio-ligand binding.

Fluoxetine voorafbehandelings het ook [^{3}H]-IP_x produksie en [^{3}H]-*m*I opname verlaag sonder enige beduidende effek op 5-HT_{2A}-R radio-ligand binding. Imipramine voorafbehandelings het egter 'n beduidende verhoging in reseptor funksie tot gevolg gehad.

Hierdie resultate mag dalk gedeeltelik verduidelik hoekom *m*I effektief is in psigiatriese toestande wat suksesvol behandel word met slegs selektiewe serotonien heropname remmers. Tydens vorige studies wat in ons laboratorium uitgevoer is om muskariene reseptor funksie te ondersoek is gevind dat *m*I, fluoksetien en imipramien voorafbehandelings 'n verlaging in muskariene reseptor funksie tot gevolg gehad het. Die modulerende effekte van *m*I op die muskariene reseptore is gedeeltelik afhanklik van die fosfoinositied weg.

Ter afsluiting kan gestel word dat die huidige data daarop dui dat *m*I se antidepressante werking via afregulering van 5-HT_{2A} -R funksie en verlaging van cholinergiese hipersensitiwiteit (in muskariene reseptore) plaasvind. Verdere intensiewe studies moet egter nog uitgevoer word om die werkingsmeganisme van *m*I op subsellulêre vlak volledig op te los.

Sleutelwoorde: *mio*-Inositol, depressie, fluoksetien, G-proteien, imipramien, sein transduksie sisteem, seratonien 2A reseptor.

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Chapter 1 Introduction

1.1 Problem Statement

Depression is one of the most prevalent mental disorders and is potentially severely disabling, also placing an economical burden on society. In fact, the World Health Organisation estimated it to become the second most debilitating disease after cardiovascular disease by the year 2020 (Peveler *et al.*, 2002). The morbidity and mortality associated with alteration of mood, as seen in depression, is one of the major problems facing psychiatry. There is sobering evidence that the most important complication of severe depression is in suicide and the lifetime risk of suicide rises to 15% in individuals with depression (Eisendrath & Lichtmacher, 2000). Due to the high incidence of psychiatric disorders and the severe complications associated with these disorders, it is essential to improve our knowledge of the actions of the drugs used in the treatment of these disorders. Although there are many antidepressants available, drug treatment is still associated with a disturbingly high incidence of unwanted side effects, as well as drug-resistant depression (Anderson, 2000).

Targeted drug design has led to significant advances in the pharmacological management of depression. Drugs have been developed that act on predetermined neurobiological targets recognised to be involved in the pathology

of depression (Harvey, 1997). For the improvement of the efficacy of antidepressant therapy, it is essential to design drugs with less side effects and greater tolerability. This in itself will have a positive therapeutic outcome in patients.

The endogenous substance, *m*I, has demonstrated marked clinical effects in animal models of various psychiatric disorders (Einat & Belmaker, 2001). Moreover, *m*I has demonstrated marked therapeutic effects in humans with various psychiatric disorders such as obsessive compulsive disorder (OCD), panic and depression (i.e. disorders that respond to serotonin-enhancing drugs) (Levine, 1997). It is also noteworthy that behavioural and biochemical studies indicate that this efficacy does not involve simply the replenishing of the membrane PI pool and that *m*I appears to exert distinct pharmacological effects of its own (Harvey *et al.,* 2002). In addition to its well-described precursory role in cell signalling, inositol lipids have been found to alter receptor sensitivity (Harvey *et al.,* 2001), direct diverse membrane trafficking events (Balla *et al.,* 2000), and also modulate an increasing array of signalling proteins (Payrastre *et al.,* 2001). Dietary *m*I and *m*I from *de novo* synthesis is incorporated into neuronal cell membranes as inositol phospholipids where it serves as a key metabolic precursor in G-protein-coupled receptors (Harvey *et al.,* 2002). Several subtypes of adrenergic, cholinergic, serotonergic and metabotropic glutamatergic receptors in the brain (all associated with a role in depression) are coupled with the hydrolysis of phosphoinositides (PI) with *m*I crucial to the re-synthesis of PI and the maintenance and effectiveness of signalling (Harvey *et al.,* 2002). Thus, due to its obligatory role in subcellular neuronal transduction pathways, the ability of *m*I to modulate the interaction between neurotransmitters, drugs, receptors and signalling proteins may represent an important neurobiological target for drug action and drug development.

Therefore, insight into the subcellular mechanism of action of *m*I in the treatment of psychiatric illnesses is of great importance. Specifically, since disorders of

neuronal *m*I metabolism are believed to lead to neurobiological and psychiatric disease (Novak *et al.*, 1999), insight into the possible modulating effects of *m*I on the phosphoinositide metabolic pathway, as regulated by 5-HT_{2A}-Rs, will provide useful answers.

1.2 Study objectives

The primary study objectives will be to investigate and define the possible modulatory effect and associated subcellular mechanism of chronic treatment action with high concentrations of *m*I on:

- 5-HT_{2A} receptor (5-HT_{2A}-R) relative number in a human neuroblastoma (SH-SY5Y) cell line transfected to express the human 5-HT_{2A}-R.
- The function and signal-transduction mechanisms of this G protein-coupled receptor in the transfected SH-SY5Y cell line.

The modulatory effect of *m*I pre-treatment on 5-HT_{2A}-R function and number will be compared to the same possible modulatory effects of two prototype antidepressants from different classes, namely a tricyclic antidepressant imipramine, and a selective serotonin re-uptake inhibitor, fluoxetine.

1.3 Project layout

All experiments were performed in the Laboratory for Applied Molecular Biology at the Potchefstroom University for Christian Higher Education, Potchefstroom, South Africa.

In order to address the project objectives, a human neuroblastoma cell line (SH-SY5Y) was genetically manipulated to express the human 5-HT_{2A}-R. The plasmid to express human 5-HT_{2A} receptors (5-HT_{2A}-Rs), was a kind gift from Dr.

Brian Roth (Case Western Reserve University, Cleveland, OH, USA). This neomycin-resistant construct was characterised, multiplied and purified for transfection purposes in the laboratory of Dr. Francois van der Westhuizen and were used to transfect the SH-SY5Y cells using DOTAP liposomal transfection reagent. Testing for successful transfection was done pharmacologically by performing appropriate radio-ligand binding studies and by showing receptor function through measuring agonist-induced second messenger formation. The transfected cells were then utilised to investigate the effect of 24-hour pre-exposure to high concentrations of *m*I, whereafter receptor function and number was measured by performing appropriate radio-ligand binding studies and by constructing concentration-effect curves of serotonin, measuring whole-cell second messenger formation or [^{35}S]-GTPγS binding to G_q proteins in cell membrane preparations. The modulatory effect of *m*I exposure was compared to that of two classical antidepressants; imipramine and fluoxetine.

Chapter 2 Literature Background

In the current chapter serotonin and its receptors are discussed, as well as the disorders in which serotonin plays a prominent role. In a second section depression and related anxiety disorders are discussed. A third section discusses neurotransmitter and receptor interactions in psychotropic action. Finally background relating to the role of *myo*-inositol (*mI*) in these disorders and the link between classical antidepressant therapy and *mI* treatment in depression is also discussed, more specifically involving the serotonergic systems in the brain.

2.1 Serotonin and its receptor subtypes

2.1.1 Introduction

Psychiatric illnesses have been recognised since the dawn of civilisation. The earliest documentation of the use of substances to alleviate the suffering of the mentally ill date to more than 5000 years ago. Evidence suggests that the Sumarians in the Tigris-Euphrate valley were aware of the mood-elevating effects of the juice of the opium poppy and cultivated these plants for that purpose (Leonard, 1997). Opiates were still being used in the 19th century to relieve depression. A drug of major importance in modern psychopharmacology

arose from the discovery by medicinal chemists of the alkaloids of *Rauwolfia serpentina*. Reserpine, as it is commonly known, was used for alleviating "insanity".

The action of reserpine helped investigators to lay the foundation for psychopharmacology by demonstrating how depletion of central and peripheral stores of biogenic amines correlated with a reduction in blood pressure and sedation. An unexpected discovery also arose during the therapeutic use of reserpine for the treatment of hypertension when it was found that approximately 15% of patients treated with reserpine became clinically depressed. As it had been shown that reserpine depletes both the central and peripheral nervous system of norepinephrine, it was postulated that depression could be a consequence of the defective synthesis of norepinephrine (NE) and possibly serotonin (5-HT). This helped to form the basis of the biogenic amine theory of depression (Leonard, 1997; Jones & Blackburn, 2002). Today it is well-established and generally accepted that 5-HT is involved in the aetiology of depression (Leonard, 1997) and serotonergic neurones appear to play a critical role in the maintenance of mental health (Graeff *et al.*, 1996). 5-HT research is now more than 50 years old and has had a major impact on disease management (Jones & Blackburn, 2002). Importantly, as more effective psychotropic drugs were discovered and as our understanding of the complex functioning, physiology, biochemistry and biomolecular functioning of the brain and its neurons improved, there is increasing evidence that depression (as with many other psychiatric disorders) has a biological basis. In the following section (§ 2.1.2) the normal physiology of 5-HT and its receptor subtypes will be discussed, followed by the disorders in which 5-HT has a primary role.

2.1.2 The normal physiology of serotonin

5-HT has diverse physiological roles in the central nervous system (CNS), the gastrointestinal tract (GIT) (Tamir & Gershon, 1990) and the cardiovascular system. In the CNS a variety of functions are influenced by 5-HT, which include:

- Sleep behaviour
- Cognition
- Sensory perception
- Temperature regulation
- Appetite
- Sexual behaviour
- Hormone secretion

Multiple 5-HT receptor subtypes with similar or contradictory actions are expressed in individual neurones. 5-HT is released at synapses and also at sites of axonal swelling called varicosities, which do not form distinct synaptic contacts. 5-HT released from these sites diffuse to outlying targets where 5-HT not only acts as a neurotransmitter, but also as a neuromodulator (Sanders-Bush & Mayer, 2001). Most of the body's 5-HT is stored in the enterochromaffin cells in the mucosa of the GIT, with the highest density in the duodenum, where 5-HT acts locally to regulate gastrointestinal function. 5-HT can inhibit or enhance motility, depending on which receptor subtype is stimulated. 5-HT and the 5-HT_3 receptors play an important role in emesis (Brunton, 1996; Sanders-Bush & Mayer, 2001). Platelets also express 5-HT receptors and when 5-HT binds to a platelet 5-HT_{2A}-R, it has a vasoconstrictor effect (Sanders-Bush & Mayer, 2001), Since blood is relatively easily obtainable from human subjects, the effect of central drugs on serotonin receptors has been studied in many cases on 5-HT_{2A} -Rs in platelets (Franke *et al.*, 2003).

2.1.2.1 CNS distribution, synthesis and metabolism of serotonin

A high concentration of 5-HT-containing neurones are found as clusters of cells around the midline of the pons and upper brain stem (Leonard, 1997), known as the raphe area of the midbrain, while their neurones extend to other parts of the brain (see Figure 2-1). Furthermore, immunoreactivity studies found serotonin-containing fibres in virtually every part of the brain. In addition, according to studies of the rat brain, cells containing 5-HT are located in the area postrema and in the caudal locus ceruleus, which anatomically connects the serotonergic and noradrenergic systems. In general, it would appear that the noradrenergic and serotonergic systems are co-localised in most limbic areas of the brain, which may provide the anatomical basis for the major involvement of these transmitters in the affective disorders (Leonard, 1997; Blier, 2003).

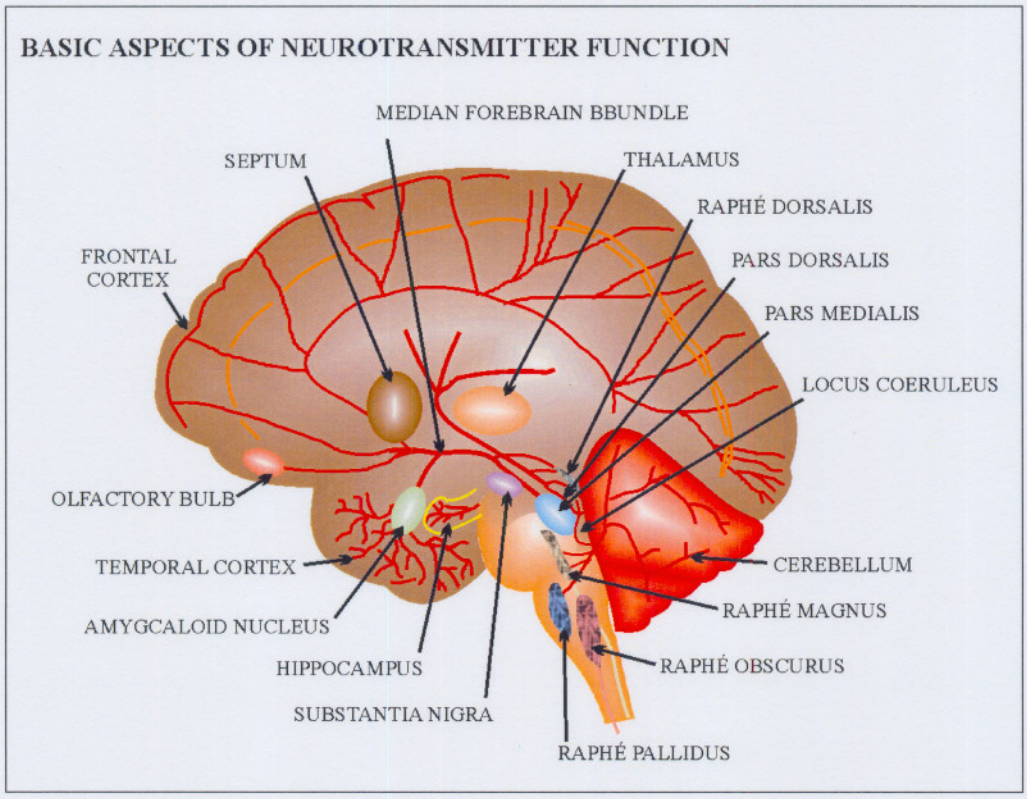

Figure 2-1 Anatomical distribution of the serotonergic pathways in human brain [reconstructed from Leonard (1997)].

5-HT is an indole amine transmitter that is synthesised within the nerve ending from the essential amino acid *l*-tryptophan (see Figure 2-2). *l*-Tryptophan is obtained from dietary sources and endogenous stores and cannot be synthesised *de novo*. The first step in the synthesis of 5-HT is the transportation of free *l*-tryptophan into the brain and nerve terminal by an active transport system (Baskys & Remington, 1996). *l*-Tryptophan is hydroxylated by tryptophan hydroxylase and 5-hydroxytryptophan (5-HTP) is synthesised. 5-HTP is then decarboxylated by the enzyme aromatic L-amino acid decarboxylase to 5-HT (Leonard, 1997).

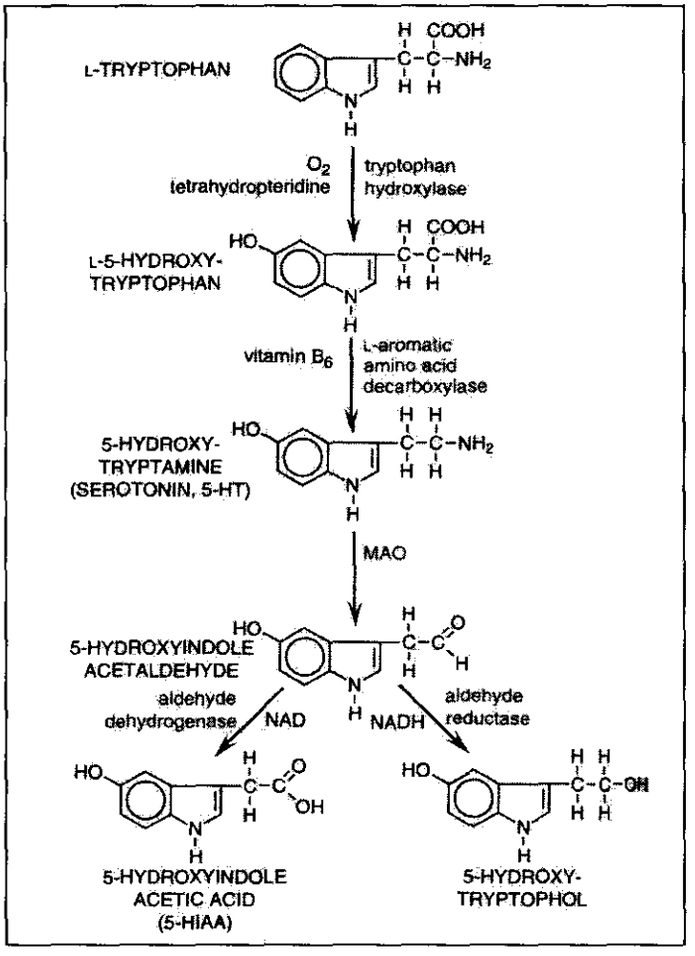

Figure 2-2 Synthesis and inactivation of serotonin (Sanders-Bush & Mayer, 1996).

The principal route of metabolism of serotonin involves the formation of 5-hydroxyindole acetic acid (5-HIAA) by monoamineoxidase (MAO). The aldehyde

formed by the action of MAO is converted to 5-HIAA by the enzyme aldehyde dehydrogenase, which is actively transported from the brain and excreted in the urine (in a normal adult 2 to 10 mg daily).

Two isoforms of MAO have been distinguished, namely MAO-A and MAO-B. MAO-A preferentially metabolises serotonin and norepinephrine, while MAO-B prefers β-phenylethylamine and benzylamine as substrates. Dopamine and tryptamine are metabolised equally well by both isoforms. Neurons contain both isoforms, which is primarily located on the outer membrane of the mitochondria. MAO-B is found primarily in platelets. In addition to metabolism by MAO, a Na^+-dependant, carrier-mediated uptake process (5-HT transporter) is involved in terminating the action of serotonin. The 5-HT transporter is localised in the outer membrane of serotonergic axon terminals where it terminates the action of 5-HT in the synapse by transporting it back into the presynaptic neurone. The 5-HT transporter is also localised in the outer membrane of platelets where it transports serotonin from the blood into platelets, where it plays a role in platelet aggregation (Sanders-Bush, 1996).

2.1.3 The serotonin receptor subtypes

As mentioned in § 2.1.2, 5-HT has many different functions. Early studies of peripheral tissues have advanced the hypothesis that the multiple actions of 5-HT are explained by an interaction with more than one 5-HT receptor subtype. Support for this concept has been provided by research and an accepted classification scheme has been proposed for the subfamilies of 5-HT receptors (Sanders-Bush, 1995). Seven families, 5-HT 1-7, and a total of 14 structurally and pharmacologically distinct mammalian 5-HT receptor subtypes, have been identified, while the functional properties of these different 5-HT receptor subtypes have since been revealed (Barnes & Sharp, 1999).

Table 2-1 Serotonin receptor subtypes and signalling characteristics (Adapted from Sanders-Bush & Mayer, 1996; Raymond *et al.*, 2001).

SUBTYPE	SIGNAL TRANSDUCTION	G PROTEIN COUPLING	FUNCTION
5-HT$_{1A}$	Inhibition of AC Activates K^+ channels Inhibits Ca^{2+} conductances	$G_{i\alpha3} > G_{i\alpha2} \geq G_{i\alpha1} \geq G_{0\alpha} > G_{z\alpha}$	Autoreceptor
5-HT$_{1B}$	Inhibition of AC	$G_{i\alpha3} > G_{i\alpha1} \geq G_{i\alpha2} \geq G_{0\alpha}$	Vasoconstriction
5-HT$_{1D}$	Inhibition of AC	$G_{i\alpha}$ & $G_{o\alpha}$	Regulate K^+ & Ca^{2+} channels
5-HT$_{1E}$	Inhibition of AC	$G_{i\alpha}$ & $G_{o\alpha}$	Unknown
5-HT$_{1F}$	Inhibition of AC	$G_{i\alpha}$ & $G_{o\alpha}$	Platelet aggregation Contraction Neuronal excitation
5-HT$_{2A}$	Activation of PLC	$G_{q\alpha}$ & $G_{11\alpha} \geq G_{i\alpha}$	Contraction
5-HT$_{2B}$	Activation of PLC	$G_{q\alpha}$ & $G_{11\alpha}$	Vasorelaxation
5-HT$_{2C}$	Activation of PLC	$G_{q\alpha}$ & $G_{11\alpha}$	Neuronal excitation
5-HT$_3$	Ligand-operated ion channel		Neuronal excitation
5-HT$_4$	Activation of AC	$G_{s\alpha}$	Aldosterone secretion
5-ht$_{5a}$	Unknown	Unidentified	Unknown
5-ht$_{5B}$	Unknown	Unidentified	Unknown
5-HT$_6$	Activation of AC	$G_{s\alpha}$	Unknown
5-HT$_7$	Activation of AC	$G_{s\alpha}$	Vasorelaxation

The 5-HT receptor subtypes cloned to date are the largest of all known neurotransmitter receptor families. The 5-HT receptor subtypes are expressed in distinct but often overlapping patterns and are coupled with different transmembrane-signalling mechanisms (see Table 2-1) (Sanders-Bush & Mayer, 1995). At molecular level it has been established that all the 5-HT receptors

(with the exception of the 5-HT_3 receptor) are metabotropic G protein-coupled receptors (GPCRs) containing seven hydrophobic (Raymond *et al.,* 2001) transmembrane domains. The domains are connected by three intracellular loops (termed i1- i3) and three extracellular loops (termed e1-e3). The 5-HT_3-R, is a ligand-gated ion channel.

Specific physiological responses have been associated with many 5-HT receptor subtypes (see Table 2-1). According to Barnes and Sharp (1999), responses may include modulation of neuronal activity, transmitter release and behavioural change. The multiplicity of coupling pathways for each of the receptors suggests that each individual 5-HT receptor subtype can regulate a broad array of potential signals. This could be affected by variables such as cell type, receptor number, numbers and types of G proteins expressed in the target cells and the specific agonist through which the receptor is activated (Raymond *et al.*, 2001). The following discussion provides background information on each 5-HT receptor subtype and subsequently reviews in more detail the functional responses attributed to each receptor in the brain. By investigating these receptors and understanding their normal pharmacology, pharmacological manipulation and intervention of the central 5-HT system might have great therapeutic potential in disorders where this neurotransmitter is involved. For the purpose of this dissertation, more extensive discussions will be provided on the 5-HT_{1A} and 5-HT_{2A} receptors, which are believed to be the most important 5-HT receptors in the pathology and drug treatment of depression.

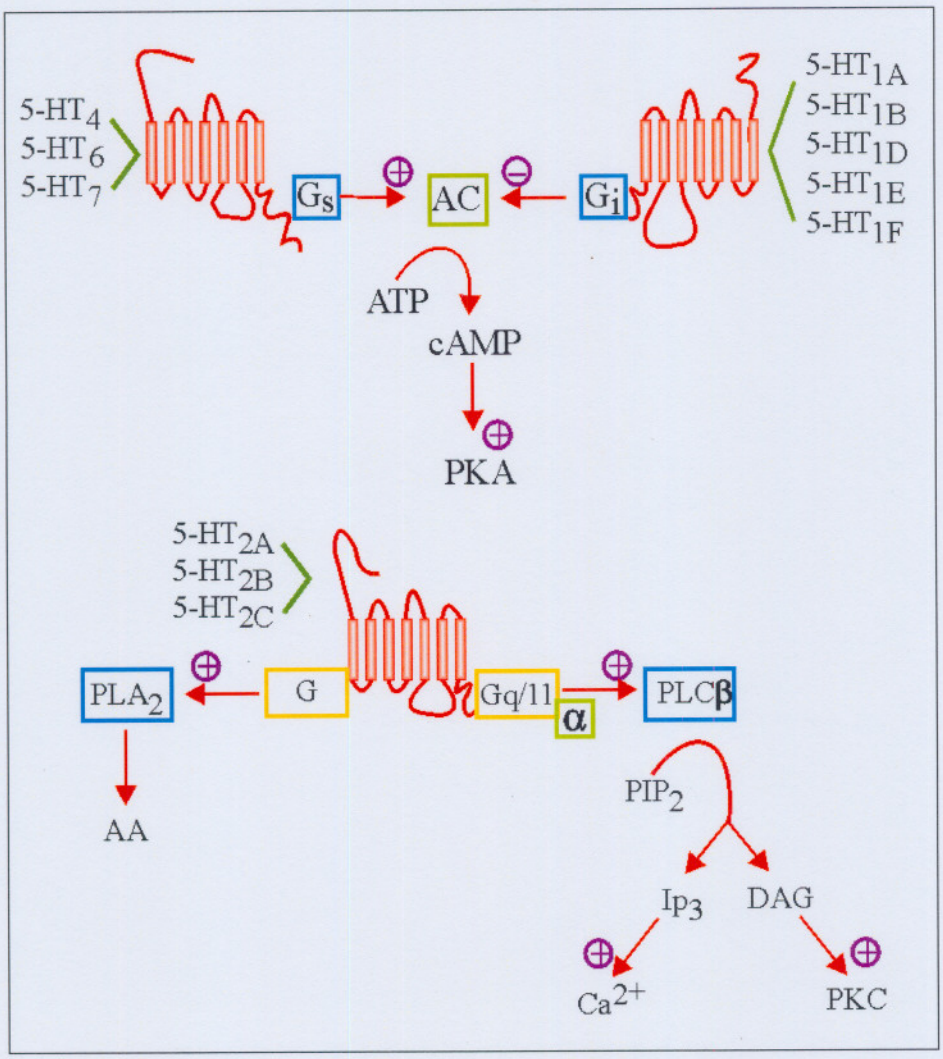

Figure 2-3 Prototypical signalling enzyme linkages of the G protein-coupled 5-HT receptors (Adapted from Raymond *et al.*, 2001).

2.1.3.1 The 5-HT$_1$ receptor family

The 5-HT$_1$ receptor (5-HT$_1$-R)[1] family consists of the 5-HT$_{1A}$-R, 5-HT$_{1B}$-R, 5-HT$_{1D}$-R, 5-HT$_{1E}$-R and the 5-HT$_{1F}$-R. The receptors of the 5-HT$_1$ family have high amino acid sequence homology and are all couple negatively with adenylate cyclase (see Figure 2-3) (Barnes & Sharp, 1999; Sanders-Bush & Mayer, 1995). The 5-HT$_1$-Rs signal primarily through $G_{i/o}$ proteins to inhibit adenylyl cyclase

[1] The former 5-HT$_{1C}$ was reclassified as the 5-HT$_{2C}$ receptor.

(AC) and to modulate a multitude of other signalling pathways and effectors. Other effects of this receptor in transfected cell lines include a decrease in intracellular calcium (Ca^{2+}) and activation of phospholipase Cβ (PLCβ) with the formation of inositol-1, 4,5-triphosphate (IP_3) and the mobilisation of intracellular Ca^{2+}. According to Barnes and Sharp (1999), the diverse effects of these receptors could be attributed to different G protein sub-unit coupling or different isoforms of the effector enzymes being expressed.

2.1.3.1.1 The 5-HT_{1A} receptor

This serotonin receptor subtype is particularly relevant to antidepressant and anxiolyitic responses in human beings (Blier & Ward, 2003). The density of 5-HT_{1A}-R binding sites is high in limbic brain areas, notably the hippocampus, lateral septum, cortical areas (particularly the cingulate and etorhinal cortex), and also the mesencephalic raphe nuclei. It is clear that 5-HT_{1A}-R is located both post-synaptic to 5-HT neurones (in forebrain regions) as well as on the 5-HT neurones themselves at the level of the soma and dendrites in the mesencephalic and medullary raphe nuclei. This receptor couples negatively via G_i proteins to adenylyl cyclase in both rat and guinea pig hippocampal tissue and cell lines (pituitary GH4Cl cells, COS-7 cells, HeLa cells). There are, however, reports of the positive coupling of the 5-HT_{1A}-R with adenylyl cyclase in hippocampal tissue. However, given similarities between the pharmacology of the 5-HT_{1A}-R and newer 5-HT-Rs (5-HT_7 in particular), it is a possibility that these effects have been inadvertently attributed to the wrong receptor or a combination of receptors.

In the rat, administration of 5-HT_{1A}-R agonists causes a wide range of effects like hyperphagia, hypothermia, altered sexual behaviour and a tail-flick response. In addition, 5-HT_{1A}-R agonists display anxiolytic and antidepressant activity in animal models (Barnes & Sharp, 1999). The 5-HT_{1A}-R also plays an important

role in the neuro-endocrine function and thermoregulation, vasoreactive headaches, food intake, memory, immune function and aggression (Raymond *et al.*, 2001). The involvement of the 5-HT_{1A}-Rs remains controversial regarding its involvement in pre- (5-HT_{1A} auto receptors) or post-synaptic mechanisms. The 5-HT behavioural syndrome is clearly mediated via activation of post-synaptic 5-HT_{1A} -Rs.

Studies underlying the anxiolyitic properties of 5-HT_{1A}-R agonists tend to favour pre-synaptic action (Blier & Ward, 2003), although the involvement of post-synaptic mechanisms cannot be ruled out. Neuro-endocrine studies in rats have found that 5-HT_{1A}-R agonists cause an elevation of plasma adrenocorticotropic hormone (ACTH), corticosteroids and prolactin. In humans, the secretion of growth hormone is also increased by stimulation of 5-HT_{1A}-Rs. Animal and human studies show that 5-HT_{1A} antagonists block these neuro-endocrine responses (Barnes & Sharp, 1999).

2.1.3.1.2 The 5-HT_{1B} receptor

In autoradiographic studies, high-density sites of 5-HT_{1B}-Rs are found in the rat basal ganglia, where they are located pre-synaptically and post-synaptically relative to the 5-HT neurones. The anatomical location of the 5-HT_{1B}-R suggests that this receptor has both a 5-HT autoreceptor function (inhibiting 5-HT release) and a heteroceptor function (Arango *et al.*, 2003). The 5-HT_{1B}-R couples negatively with adenylate cyclase via G_i proteins (see Figure 2-3).

2.1.3.1.3 The 5-HT_{1D} receptor

Studies in rat brain suggest that the 5-HT_{1D}-R is present in various regions, especially the basal ganglia, the hippocampus and the cortex. In the human brain the receptor is detected in the basal ganglia (globus pallidus and substantia

nigra) as well as specific regions of the midbrain and spinal cord. In transfected cells, the cloned 5-HT_{1D}-R couples negatively with adenylate cyclase via G_i and G_o proteins (see Table 2-1) (Barnes & Sharp, 1999).

2.1.3.2 The 5-HT_2 receptor family

Three receptor subtypes are currently known in this family, namely the 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C}-Rs (Porter *et al.*, 2001). Although they are similar in terms of their molecular structure, pharmacology and signal transduction pathways, they have differences in their signalling properties (Raymond *et al.* 2001). All three are positively coupled with phospholipase C via G_q proteins and mobilise intracellular calcium (Barnes & Sharp, 1999). This receptor family will be discussed in more detail because of its importance in the current study.

2.1.3.2.1 The 5-HT_{2A} receptor

High levels of 5-HT_{2A}-Rs are found in the forebrain regions, but particularly cortical areas (neocortex, entorhinal and pyriform cortex, claustrum), caudate nucleus, nucleus accumbens, olfactory tubercle and hippocampus. Evidence suggests that in various brain areas the 5-HT_{2A}-Rs are located on local gamma aminobutyric acid (GABA) interneurones. The 5-HT_{2A}-R is also present in cells of smooth muscle, the kidneys and platelets (Raymond *et al.*, 2001). 5-HT_{2A}-Rs are important for many physiological processes, including platelet aggregation, smooth-muscle contraction and the modulation of mood and perception (Gray & Roth, 2001).

The 5-HT_2-R subtypes all signal through G_q proteins to activate PLCβ, leading to increased formation of IP_3 (to mobilise intracellular Ca^{2+}) and diacylglycerol (DAG) from the precursor, phosphatidylinositol 4,5-bisphosphate (PIP_2). These effects result in the activation of L-type Ca^{2+} channels and stimulation of protein

kinase C (PKC) (Raymond *et al.,* 2001). Stimulation of the 5-HT_{2A}-R activates PLCβ in tissues. 5-HT_{2A}-Rs are desensitised after prolonged exposure to serotonin and other agonists, as well as inverse agonists (Gray & Roth, 2001). However, in some *in vitro* and *in vivo* models, 5-HT_{2A}-Rs down-regulate in the face of constant exposure to certain antagonists (mianserin, spiperone). One possible explanation for this phenomenon is that under certain conditions, 5-HT_{2A}-Rs are constitutively active and that some ligands act as inverse agonists. Stimulation of the 5-HT_{2A}-R causes activation of a biochemical cascade, leading to altered expression of a number of genes, including that of the brain-derived neurotrophic factor (BDNF). These changes may be linked at least in part to the increase in expression of BDNF, following repeated treatment with antidepressants. There is evidence that the latter changes lead to altered synaptic connectivity in the brain that may even contribute to the therapeutic effects of antidepressants (Barnes & Sharp, 1999). Behavioural effects in rodents after stimulation with $5HT_{2A}$ -R agonists range from changes in both unconditioned (e.g. increased motor activity and hyperthermia) and conditioned responses (e.g. punished responding, drug discrimination). Other responses induced by the $5HT_{2A}$ -R include neuro-endocrine responses such as increased secretion of cortisol, ACTH, renin and prolactin.

2.1.3.2.2 The 5-HT_{2B} receptor

The human 5-HT_{2B}-R is relatively homologous with the human 5-HT_{2A} and 5-HT_{2C}-Rs. In heterologous expression systems the cloned rat and human 5-HT_{2B}-R stimulate PI hydrolysis, in common with the other members of the 5-HT_2-R family. One interesting putative function of the 5-HT_{2B}-R is to mediate the mitogenic effects of serotonin during neural development. Little data is available on the functional effects of the central 5-HT_{2B}-R, however, it seems that the 5-HT_{2B}-R plays a role in anxiety (Kaiyala *et al.,* 2003).

2.1.3.2.3 The 5-HT_{2C} receptor

There is little evidence for the expression of 5-HT_{2C}-Rs outside the CNS. In addition to the very high distribution of these receptors in the choroid plexus, 5-HT_{2C}-R-binding sites are widely distributed and present in areas of the cortex (olfactory nucleus, pyriform, cingulate and retrosplenial), limbic system (nucleus accumbens, hippocampus, amygdala) and the basal ganglia (caudate nucleus, substantia nigra). The 5-HT_{2C}-Rs are clearly located post-synaptically, but the possibility of a pre-synaptic location needs further study. 5-HT_{2C}-R stimulation increases PLCβ activity via a G_q protein-coupled mechanism (Sanders-Bush *et al.,* 1988). Activation of central 5-HT_{2C}-Rs has been associated with hypolocomotion, hypophagia, anxiety, penile erection and hyperthermia.

2.1.3.3 The 5-HT_3 receptor

The 5-HT_3-R is unique in that it is a ligand-gated ion channel, although it is likely to be comprised of multiple sub-units in common with other members of this superfamily. The highest levels of 5-HT_3-R-binding sites are within the dorsal vagal complex in the brainstem, comprising the nucleus tractus solitarius, area postrema and dorsal motor nucleus of the vagus nerve. These regions are intimately involved in the initiation and co-ordination of the vomiting reflex. 5-HT_3-R expression in the forebrain is low, but is found in the hippocampus, amygdala and superficial layers of the cerebral cortex (Bufton *et al.,* 1993).

2.1.3.4 The 5-HT_4 receptors

Relatively high levels of the 5-HT_4-R are present in the nigrostriatal and mesolimbic systems of the brain in species like the rat, guinea pig, cow and man. This receptor couples positively with adenylate cyclase via G_s proteins and may also regulate the release of dopamine (Barnes & Sharp, 1999).

2.1.3.5 The 5-ht$_5$ receptor

Two subclasses of this receptor have been identified, namely the 5-ht$_{5A}$ and 5-ht$_{5B}$-R subtypes. As functional 5-ht$_5$ receptors have not been identified *in vivo* yet, the lower case designation is used (Raymond *et al.*, 2001). The 5-ht$_5$-R class is not understood very well, but are members of the GPCR superfamily in common with native 5-HT$_4$-Rs. Heterologously expressed receptors couple positively with adenylate cyclase.

2.1.3.6 The 5-HT$_6$ receptor

The 5-HT$_6$-R is expressed in several regions of the brain, most prominently in the caudate nucleus, the olfactory tubercle, the striatum, the hippocampus, and the nucleus accumbens. They appear to regulate cholinergic (rather than dopaminergic) neurotransmission in the brain, implicating it as a target for the treatment of learning and memory disorders. The 5-HT$_6$-R activates AC (see Figure 2-3).

2.1.3.7 The 5-HT$_7$ receptor

The 5-HT$_7$-R is highly expressed in the CNS, especially in the hippocampus, the hypothalamus and the neocortex. It has been speculated that this receptor participates in the control of circadian rhythm, because it is expressed in the suprachiasmatic nucleus. This receptor is also expressed in glial cells, the spleen, vascular smooth muscle and the intestine (Raymond *et al.*, 2001). This receptor subtype couples with G$_S$ proteins and stimulates adenylyl cyclase.

Although emotional states cannot be attributed to imbalances of just one neurotransmitter and certainly not one receptor type, it is generally acknowledged that 5-HT has a prominent role or participation in disorders such

as depression, anxiety and stress (Graeff *et al.*, 1996). In § 2.2, some of the prominent disorders associated with serotonin imbalances will be discussed.

2.2 Depression and related anxiety disorders

As has already been mentioned, 5-HT has a significant (but certainly not exclusive) role in the core behaviours that are evident in the affective disorders. Among the most persuasive evidence for a role for 5-HT in mood disorders is the lowered brain 5-HT concentration in depressed patients compared to non-depressed controls (Owens & Nemeroff, 1994). Decreasing the availability of tryptophan, the precursor of 5-HT (by restricting tryptophan intake), could induce a mild depression in normals or a more severe depression in recovering depressed patients. Furthermore, when the same TRP-lowering amino acid mixture is given to unmedicated depressed patients, it appears to have a mood-elevating effect (Meltzer, 1990).

Depression is an illness, not a choice. Unfortunately, only approximately one third of individuals with depression are treated (Eisendrath & Lichtmacher, 1999). Not only does society often perceive depression as a type of moral deficiency that is shameful and should be hidden, it is also under-recognised by health-care providers. According to Stahl (2000), up to 15% of severely depressed patients will commit suicide. The reality is that mood disorders are common, debilitating and life-threatening illnesses. In an investigation conducted by the World Heath Organisation it was estimated that depression would likely become the second most important cause of disability world-wide by 2020, after ischaemic heart disease, which is the number one cause of disability (Peveler *et al.*, 2002). Although the affective disorders can be effectively treated, they are often not treated, because of under-recognition by medical practitioners and poor patient compliance. If a drug could be found with little to no adverse effects, it might

help treat this disorder more effectively. Therefore it is essential to investigate new strategies of drug treatment.

Mood disorders are often called affective disorders. Affective disorders are actually syndromes, defined by clusters of symptoms. Depression and mania are often seen as opposite ends of an affective or mood spectrum. Unipolar depression describes patients that only experience the "down" or depressed pole, while in the case of "bipolar" disorder, the patient will at different times experience either the "up" (manic) pole or the "down"(depressed) pole.

Depression is universally experienced by virtually everyone at some time of his or her lives. Accepted, standardised diagnostic criteria are used to separate "normal" depression caused by disappointment or of "having a bad day" from the disorders of mood. Diagnostic criteria for mood disorders are in constant evolution, with current nosologies being set by the Diagnostic and Statistical Manual of Mental Disorders, 4th ed. (DSM-IV) (Stahl, 2000). Some of the criteria for major depression include five or more of the following symptoms during the same two- week period (Peveler *et al.*, 2002):

- Depressed mood
- Substantial weight loss or gain
- Insomnia or hypersomnia
- Feelings of worthlessness or inappropriate guilt
- Recurrent thoughts of death or suicide attempt
- Decreased interest or pleasure
- Psychomotor retardation or agitation
- Fatigue or loss of energy
- Diminished ability to think or concentrate

2.2.1 The biological basis of depression

2.2.1.1 The monoamine hypothesis of depression

The monoamine hypothesis of depression has received the most recognition and research in the past two decades. According to this hypothesis, depression is due to a deficiency of monoamine neurotransmitters, notably norepinephrine (NE) and serotonin (5-HT). Evidence for this is, however, rather simplistic. The known antidepressants (tricyclic and the MAO inhibitors) act to boost synaptic levels of these neurotransmitters. Stahl (2000) has suggested that the normal amount of monoamine neurotransmitters become somewhat depleted and thus precipitate the symptoms of depression. Drugs such as reserpine that cause depletion of these brain monoamines could induce symptoms of depression (Coppen, 1967). It has also been found that some depressed patients have reduced levels of monoamine metabolites in especially cerebrospinal fluid (Barkai *et al.*, 1978). Evidence exists for the participation of both 5-HT and NA neurotransmission as the cause of depression. According to the "permissive hypothesis", a reduction in CNS 5-HT allows an affective state regulated by NA. Depression will therefore arise because of a decrease in 5-HT and NA levels, while mania is associated with decreased 5-HT and increased NA levels (Harvey, 1997).

2.2.1.2 The neurotransmitter receptor hypothesis

The neurotransmitter theory argues that the key monoamine neurotransmitter receptors in depression are dysfunctional. Such a disturbance in neurotransmitter receptors itself may be caused by a depletion of monoamine neurotransmitters and the depletion of the neurotransmitters cause compensatory up-regulation of post-synaptic neurotransmitter receptors. Direct evidence for this is generally lacking, but post-mortem studies show increased numbers of the serotonin

receptor in the frontal cortex of depressed patients who have committed suicide (Stahl, 2000).

2.2.1.3 The monoamine hypothesis of gene expression

There is growing evidence that despite apparently normal levels of monoamines and their receptors, these systems do not respond normally. Such observations have led to the idea that depression may be a pseudo-monoamine deficiency in signal transduction from the monoamine neurotransmitter to its post-synaptic neurone in the presence of normal amounts of neurotransmitter and receptor. If there is a deficiency in the molecular events that cascade from receptor occupancy by neurotransmitter, it could lead to a deficient cellular response and thus a form of pseudo-monoamine deficiency (i.e. the receptor and the neurotransmitter are normal, but the transduction of the signal from the neurotransmitter to its receptor is somehow flawed) (Stahl, 2000). Thus, second messenger systems leading to the formation of intracellular transcription factors that control gene regulation could be the site of deficient functioning of monoamine systems.

2.2.1.4 Cholinergic supersensitivity hypothesis

In depressed people, the cholinergic systems become hyper-responsive and in part might explain the symptoms evident in people suffering from depression. Previous studies have shown that stimulating central cholinergic transmission with cholinomimetics or cholinesterase inhibitors could cause severe depression, dysphoria, behavioural withdrawal, reduced hedonic capacity and psychomotor retardation. These drug-induced syndromes closely emulate the profile of major depressive disorder and have formed the foundation of the "cholinergic hypothesis" (Daws & Overstreet, 1999).

2.2.2 Classification of depression

The term depression describes a spectrum of mood disturbances ranging from mild to severe and from transient to persistent episodes (Peveler *et al.*, 2002). Diagnostic criteria (see section 2.2) for mood disorders are set by the Diagnostic and Statistical Manual of Mental Disorders, 4th ed. (DSM-IV) (Stahl, 2000). According to Eisendrath and Lichtmacher (1999), there are generally four major types of depression, with similar symptoms in each group.

2.2.2.1 Adjustment disorder with depressed mood

Depression may occur in reaction to some identifiable stressor or adverse life situation, usually the loss of a loved one (grief reaction), divorce and financial crisis (Potter & Hollister, 2001). Anger is frequently associated with the loss, and this in turn often produces feelings of guilt. The disorder occurs within months of the stressor and causes significant impairment in social or occupational functioning. The symptoms range from mild sadness, anxiety, irritability, worry, lack of concentration, discouragement and somatic complaints to the more severe symptoms of the next group (see § 2.2.2.2) (Eisendrath & Lichtmacher, 1999).

2.2.2.2 Depressive disorders

This sub-classification includes major depressive episodes and dysthymia. A major depressive episode (endogenous, unipolar disorder, melancholia) is a period of serious mood depression that occurs at any time of life. Many consider a physiological or metabolic aberration to be a cause. Complaints vary widely but most frequently include loss of interest and pleasure (anhedonia), withdrawal from activities and feelings of guilt). Also included are the inability to concentrate, cognitive dysfunction, anxiety, chronic fatigue, feelings of worthlessness, somatic

complaints and loss of sexual drive. Diurnal variation with improvement as the day progresses is common. Vegetative signs that frequently occur are insomnia, anorexia with weight loss and constipation. Occasionally, severe agitation and psychotic ideation (paranoid thinking, somatic delusions) are present. These symptoms are more common in postmenopausal depression (involutional melancholia). Paranoid symptoms may range from general suspiciousness to ideas of reference with delusions. The somatic delusions frequently revolve around feelings of impending annihilation or hypochondriac beliefs (e.g. that the body is rotting away with cancer). Hallucinations are uncommon (Eisendrath & Lichtmacher, 1999). Dysthymia is a chronic depressive disturbance. Sadness, loss of interest and withdrawal from activities over a period of two or more years with a relatively persistent course is necessary for this diagnosis. Generally, the symptoms are milder but longer lasting than those in a major depressive episode.

2.2.2.3 Bipolar disorders

This includes manic and depressive episodes. These episodes usually occur earlier (late teens or early adulthood) than major depressive episodes. A manic episode is a mood change characterised by elation with hyperactivity, over-involvement in life activities, increased irritability and flight of ideas, easy distractibility and little need for sleep. The person then swings into depression and aggressive behaviour. Generally the manic episodes are of shorter duration than the depressive episodes (Eisendrath & Lichtmacher, 1999).

2.2.2.4 Mood disorders secondary to illness and medication

Any illness, severe or mild, could cause significant depression. Conditions such as rheumatoid arthritis, multiple sclerosis, AIDS and chronic heart disease are particularly likely to be associated with depression, as are other chronic illnesses.

Hormonal variations clearly play a role in some depressions. Alcohol dependency frequently coexists with depression. The classical model of drug-induced depression occurs with the use of reserpine. Corticosteroids and oral contraceptives are also commonly associated with mood changes. Antihypertensive medications such as methyldopa, guanethidine and clonidine have been associated with the development of depressive syndromes, as have digitalis and anti-parkinsonism drugs such as levodopa. Prolonged use of stimulants results in a depressive syndrome when the drug is withdrawn. Alcohol, sedatives, opiates and most of the psychedelic drugs are depressants and paradoxically are often used in self-treatment of depression (Eisendrath & Lichtmacher, 1999).

2.2.2.5 Depressive disorder not otherwise specified

This disorder includes several subcategories. **Atypical depression** is characterised by hypersomnia, overeating, lethargy, and rejection sensitivity. These patients should be carefully evaluated for bipolar disorder. **Seasonal affective disorder (SAD)** is a dysfunction of circadian rhythms that occurs more commonly in the winter and is believed to be due to decreased exposure to full-spectrum light. Common symptoms include carbohydrate cravings, lethargy, hyperphagia and hypersomnia. **Premenstrual dysphoric disorder** usually has depressive symptoms during the late luteal phase of the menstrual cycle throughout the year. **Pre-natal and post-partum depressive disorders** usually occur two weeks to six months postpartum. Most women (up to 80%) experience some mild depression in the postpartum period. For 10-15%, the symptoms are more severe and similar to those usually seen in serious depression, with an increased emphasis on concerns related to the baby. Biologic vulnerability with hormonal changes and psychosocial stressors also play a role (Eisendrath & Lichtmacher, 1999).

Depression is a complex syndrome of widely varying severity and the patient loses interest in almost all of his/her usual activities or pastimes (Baldessarini, 1996; Julien, 2001). The longer the depression continues, the more entrenched it becomes with the most important complication being suicide. In individuals with depression, the lifetime risk of suicide is 10-15%. Males tend to be more successful in suicide attempts, particularly in older age groups. Women on the other hand, make more attempts with lower mortality rates (Eisendrath & Lichtmacher, 1999). It is therefore critical that the illness is recognised early and treated correctly. In the next section, treatment strategies will be discussed.

2.2.3 Treatment of depression

The main aims of depression management are to improve the mood and quality of life of the patient and reduce the risk of medical complications (Peveler *et al.*, 2002). The treatment of depression relies on a diverse group of antidepressant therapeutic agents. Antidepressant pharmacotherapy is ineffective in about one third of patients and it takes several weeks to ameliorate the symptoms (Levine *et al.*, 1999). The action of antidepressants has not been completely and adequately explained, but it is known that all effective antidepressants have identifiable, immediate interactions with one or more monoamine neurotransmitter receptors or enzymes. Although theories of depression have focused on the monoamines, it is now suggested that the disorder is not simply related to neurotransmitter release or degradation. It seems as if other mechanisms at receptor level, second messengers and gene activation are involved in the therapeutic action of antidepressants (Einat *et al.*, 1999). In order to act, antidepressants are likely to have one or more primary molecular targets. Those targets may be at or near the membrane, and altered intracellular signalling is often among the initial effects of antidepressant treatment (see Figure 2-4). More specifically, the various mechanisms proposed for

antidepressant action are consistent with an increase in cAMP production (Donati & Rasenick, 2003).

There are at least eight separate pharmacological mechanisms of action and more than two dozen antidepressants (Stahl, 2000). The first agents used successfully were the tricyclic antidepressants. These agents inhibit norepinephrine and serotonin uptake into nerve endings and thus sustain facilitation of noradrenergic and serotonergic function in the brain. Inhibitors of monoamine oxidase, an enzyme that metabolises serotonin, have also been used. Recently, agents that selectively inhibit serotonin re-uptake have been introduced (Baldessarini, 1996). The new selective noradrenergic re-uptake inhibitors, as well as norepinephrine and dopamine re-uptake inhibitors, have recently been introduced to treat depression (Stahl, 2000). There are also a variety of atypical treatments, which cannot be readily classified. Another approach to treat this affective disorder is by means of electroconvulsive therapy (ECT) (Donati & Rasenick, 2003).

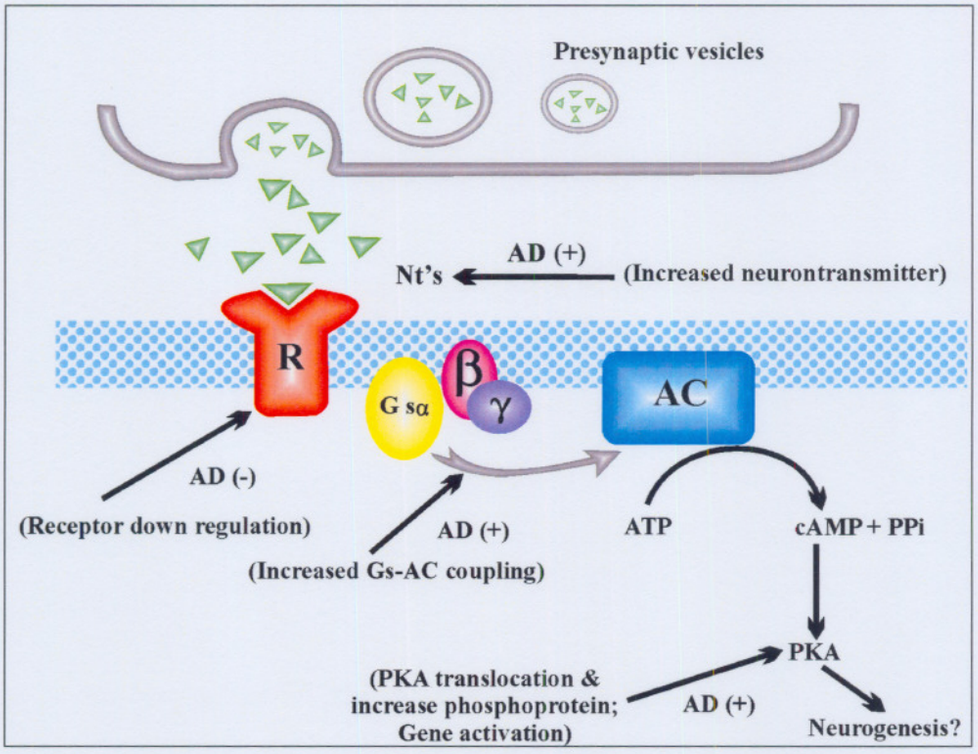

Figure 2-4 Possible targets of chronic antidepressant treatment. 1) Increased duration neurotransmitter in the synaptic cleft. 2) β-adrenergic and serotonin receptor down regulation. 3) Increased Gsα-adenylyl cyclase coupling. 4) Increased PKA translocation and protein phosphorylation (Adapted from Donati & Rasenick, 2003).

2.2.3.1 The tricyclic antidepressants

The tricyclic antidepressants or TCAs have been used for many years to treat depression. These drugs are also effective in the treatment of panic disorders, pain syndromes and anxiety states. Specific TCAs selective for 5-HT has been effective in obsessive-compulsive disorder (clomipramine), while enuresis has been treated successfully with imipramine (Schuessler & Baessler, 2003). A reduction of craving in cocaine withdrawal is also seen in patients treated with desipramine. Most of the TCAs can be given in a single dose at bedtime, starting at fairly low doses and increasing the dose every several days until the therapeutic response is achieved.

The TCAs tend to affect both serotonin and norepinephrine re-uptake, although some of the TCAs act mainly on serotonin, e.g. clomipramine and others principally on norepinephrine re-uptake (Eisendrath & Lichtmacher, 1999). This group of antidepressants is also characterised by potent antagonism of muscarinic, cholinergic, α_1 adrenergic and H_1 receptors (Brunello *et al.,* 1995). These antidepressant drugs are efficacious in treating depression, but are often associated with unpleasant side effects caused by the non-specificity of their pharmacological action (Brunello *et al.*, 1995). The TCAs evoke anticholinergic side effects to varying degrees. The anticholinergic effects also predispose to other medical problems such as heat stroke or dental problems from xerostomia. Orthostatic hypotension is fairly common, may not remit with time and is a major problem in elderly women with osteoporosis. Cardiac effects include anticholinergic effects, direct myocardial depression (quinidine-like effect), and interference with adrenergic neurones. These factors may cause cardiac abnormalities such as altered rate, rhythm and contractility, particularly in patients with pre-existing cardiac disease. TCAs lower the seizure threshold that is of particular concern in patients with a propensity for seizures. Other side effects include loss of libido and erectile, ejaculatory and orgasmic dysfunction. These side effects often compromise patient compliance. Sudden discontinuation of some of these drugs could also produce cholinergic rebound, manifested by headaches and nausea with abdominal cramps. Overdoses of the TCAs are often serious and potentially life-threatening, because of their narrow therapeutic index (Potter & Hollister, 2001).

2.2.3.2 Monoamine oxidase inhibitors

MAOI (monoamine oxidase inhibitors) are now generally used as third-line drugs for depression (after the failure of the tricyclics or the newer agents) because of the dietary and other restrictions they have. They should be considered as drugs of first choice in atypical depression (with rejection sensitivity) or as useful agents

for panic disorder or refractory depression. The MAOIs commonly cause symptoms of orthostatic hypotension and sympathomimetic effects such as tachycardia, sweating and tremor. Nausea, insomnia (often associated with intense afternoon drowsiness) and sexual dysfunction are common. Central nervous system effects include agitation and toxic psychoses. Some drugs containing phenylephrine, dextromethorphan, pseudoephidrine, if administered with a MAOI, can precipitate a hypertensive crisis. Dietary limitations and abstinence from foods containing tyramine are mandatory for treatment with MAOI. Cheeses (not cream cheese and cottage cheese), fermented or aged meats, liver, meat and yeast extracts, red wine, sherry, beer are some of the foods that should rather be avoided during treatment and one month after cessation of therapy with a MAO inhibitor (Eisendrath & Lichtmacher, 1999).

2.2.3.3 The selective serotonin re-uptake inhibitors: e.g. Fluoxetine

The selective serotonin re-uptake inhibitors (SSRIs) have emerged as a major therapeutic advance in psychopharmacology (Vaswani, 2003). Examples of SSRIs include citalopram, fluoxetine, sertraline, paroxetine and fluvoxamine. They have undoubtedly established the pathophysiological role of 5-HT in affective disorders and the spectrum of anxiety disorders. The indications of SSRIs are many, but the focus of this study is mainly on the role of the SSRIs on depression. The SSRIs are also the first to confirm the inhibition of neurotransmitter re-uptake as an important therapeutic principle. The mechanism of action of SSRIs is not totally clear but its acute action involves perturbation of the serotonergic system, specifically 5-HT re-uptake (Einat *et al.*, 1999). At very high concentrations the SSRIs also inhibit the re-uptake of norepinephrine and, to a lesser extent, the dopamine neurotransmitter. The discovery of these drugs marks a milestone in neuropsychopharmacology. Prior to the SSRIs, all psychotropic medications were the result of chance observation. The SSRIs were a rationally designed class of psychotropic medications and are

currently among the most frequently prescribed therapeutic agents in medicine (Vaswani, 2003). These drugs do not cause significant cardiovascular or anticholinergic side effects or significant weight gain, as do the TCAs (Brunello *et al.*, 1995). However, like the TCAs, the SSRIs have a delayed onset of action of two to six weeks before the therapeutic effect becomes established (Jones & Blackburn, 2002). The SSRI used in this study, namely fluoxetine, was the first SSRI found to be effective in the treatment of depression.

2.2.3.4 Atypical agents

The atypical antidepressant bupropion appears to exert its effect primarily through the dopaminergic system. Venlafaxine inhibits the re-uptake of both serotonin and norepinephrine, while nefazodone blocks the re-uptake of serotonin but also inhibits 5-HT_{2A} post-synaptic receptors. Mirtazapine selectively blocks presynaptic α_2-adrenergic receptors and in this manner enhances both noradrenergic and serotonergic transmission. All of the above antidepressants are effective in the treatment of depression, but have also proved to be effective in the treatment of panic attacks, bulimia and OCD. Bupropion may also have some efficacy in the treatment of rapid-cycling bipolar disorder, while venlafaxine may have efficacy in treatment of neuropathic pain (Eisendrath & Lichtmacher, 1999). Most of these drugs tend to be activating and are therefore given in the morning, although some patients might experience sedation, requiring that the drug be given at bedtime. This latter reaction occurs most commonly with mirtazepine.

2.2.3.5 General considerations

The SSRIs can be given as a once-daily dosage. There is usually some delay in response and fluoxetine requires a treatment of two to six weeks to act in depression, 4-8 weeks to be effective in panic disorder and 6-12 weeks to be

effective in obsessive compulsive disorder. Side effects common to all of these drugs are headache, nausea, tinnitus, insomnia and nervousness. Athisia has been common with these drugs. Sexual side effects of impotence, retrograde ejaculation and dysorgasmia are very common with the use of the SSRIs.

The SSRIs could in high doses or in combination therapy with MAO inhibitors cause a serotonin syndrome. Rigidity, hyperthermia, autonomic instability, myoclonus, confusion, delirium and coma manifest in this syndrome. This syndrome could be a troublesome problem in the elderly. Several cases of angina have been reported in association with SSRIs. Research indicates that fluoxetine does not cause an increased risk of major fetal malformation when used during pregnancy. Both the TCAs and fluoxetine are associated with a higher risk of miscarriage, but this may be related to underlying depressive disorder (Eisendrath & Lichtmacher, 1999).

In this study fluoxetine (FLX) was used and therefore the focus will be on this particular SSRI. FLX is almost completely absorbed after oral administration. Due to hepatic first pass metabolism, the oral bio-availability is < 90%. The volume of distribution of FLX is by far the highest among all the SSRIs. FLX undergoes extensive metabolic conversion leading to the active metabolite norfluoxetine. FLX has a half-life of 1-4 days, whereas the half-life of norfluoxetine ranges between 7 and 15 days. At least 4 weeks of constant medication are necessary to reach steady state levels of FLX. Moreover, in the case of FLX non-response, long washout periods are necessary before switching the patient to a TCA or a MAO inhibitor to avoid drug interactions or the development of a 5-HT syndrome. After oral administration, FLX is mainly excreted in urine with 10% excreted unchanged or as fluoxetine *N*-glucoronide. FLX is a potent inhibitor of the CYP iso-enzyme, CYP2D6 activity. FLX has low affinity for neurotransmitter receptors, e.g., the 5-HT_{2A} receptors, muscarinic acetylcholine receptors, dopamine D_2-receptor or β-adreno-receptor (Vaswani *et al.*, 2003).

2.2.3.6 Maintenance and tapering

When clinical relief of symptoms is obtained, medication is continued for 12 months at the effective maintenance dosage, which is usually the dosage required in the acute stage. Current research suggests that when an individual is over the age of 40 with two episodes or over 50 with one episode, the full dosage should be continued indefinitely. This research suggests that major depression should often be considered a chronic disease. If the medication is tapered, it should be done gradually over several months and the patient monitored closely for relapse (Eisendrath & Lichtmacher, 1999).

2.2.4 Obsessive compulsive disorder

OCD is characterised by obsessions and or compulsions, which together last at least an hour per day and are sufficiently bothersome that they interfere with normal social or occupational functioning (Stahl, 2000). Obsessions are experienced internally and subjectively by the patient as thoughts, impulses or images (Eisendrath & Lichtmacher, 1999). These obsessions drive the compulsions, which are repetitive behaviours, or purposeful mental acts that are geared to diminish the obsession. OCD was originally thought to be a rare disease, but recent epidemiological studies suggests that OCD exists in about 1 out of 50 adults and in about 1 out of every 200 children (Stahl, 2000). This finding has lead to intensive research into finding effective treatments for OCD. It is known that all the SSRIs are effective treatments for OCD. Treatment ameliorates but does not eliminate OCD symptoms in many patients. Another problem is that relapses are very common after discontinuing treatment.

The biological basis for OCD remains unknown. However, some evidence implicates abnormal neurone activity as well as alterations in neurotransmitters in OCD. The serotonin hypothesis stems largely from pharmacological treatment studies. Clomipramine, a TCA, has been used effectively to alleviate the symptoms of OCD. The hypothesis that SSRIs work in OCD by a serotonergic mechanism is also supported by studies showing a strong positive correlation between improvement in OCD symptoms during clomipramine treatment and drug-induced decreases in cerebrospinal fluid levels of the serotonin metabolite 5-HIAA and platelet serotonin concentrations. Thus, peripheral markers of 5-HT function link the symptomatic improvement in OCD symptoms produced by SSRIs to changes in 5-HT function (Baldessarini, 1996). Dopamine (DA) is implicated in the mediation of some symptoms of OCD. Other neurotransmitters may also be involved in OCD. In some studies that have been conducted, high doses of dopaminergic agents, such as bromocriptine and apomorphine, induce

stereotyped movements in animals, which resemble compulsive behaviour in OCD patients.

Based on the studies of both 5-HT and DA in OCD, it seems possible that at least in some forms of OCD (OCD with a history of Tourette syndrome), both 5-HT and DA transmitter systems may be involved in the pathophysiology of symptoms (Stahl, 2000). It is not clear whether the primary abnormality is in 5-HT function, DA function or serotonin-dopaminergic balance.

Various treatments can be given in combination for OCD. The basis of all combination treatments is an SSRI (Jones & Blackburn, 2002) or clomipramine (Potter & Hollister, 2001; Baldessarini, 1996). These should be augmented by using a 5-HT_{1A} partial agonist, a 5-HT_{2A} antagonist, lithium, a benzodiazepine or a sedative-hypnotic, a conventional antipsychotic or an antipsychotic or behavioural psychotherapy (Stahl, 2000).

2.2.5 Panic attacks and panic disorder

Anxiety disorders include disorders in which anxiety and associated symptoms are irrational and impairs functioning. Anxiety disorders are classified as generalized anxiety disorder (GAD), panic disorder, social anxiety disorder (SAD), specific phobia and posttraumatic stress disorder (PTSD).

A panic attack is a discrete episode of unexpected terror accompanied by a variety of physical symptoms. Associated symptoms include fear and anxiety as well as catastrophic thoughts with a sense of impending doom or the belief that loss of control, death or insanity is imminent. Physical symptoms could be neurological, gastrointestinal, cardiac or pulmonary and therefore may mimic different types of medical illnesses. A panic attack usually lasts from 5 to 30 minutes, with the symptoms peaking at about 10 minutes. However, attacks

have been reported to last for hours. When an attack occurs during sleep, it is called a nocturnal panic attack (Potter & Hollister, 2001). Panic attacks should not be confused with panic disorder. Panic disorder is the presence of recurrent unexpected panic attacks followed by at least a one-month period of persistent anxiety or behavioural changes related to the attack (Stahl, 2000). Panic disorders affect up to 2% of the population, but less than one percent receives treatment. This disorder is more prevalent in women than in men (Wells *et al.*, 2003). Studies have shown that patients with panic disorder have a suicide rate comparable with that of patients with major depression. The high rate of suicide attempts does not appear to be caused by the presence of depression in panic disorder patients. PTSD is characterised by anxiety, startle, painful recollection of the traumatic events and usually disturbed sleep (Baldessarini, 1996).

The neurological basis of panic disorder includes a neurotransmitter dysregulation. Neurotransmitters include norepinephrine, GABA (gamma aminobutyric acid) and cholecystokinin (CCK). One theory suggests that there is an initial excess of norepiniphrine. This theory is supported by evidence that panic disorder patients are hypersensitive to alpha-2 antagonists and hyposensitive to alpha-2 agonists (Stahl, 2000).

Many medications originally developed or used in the treatment of depression have been found to be effective in treating posttraumatic stress disorder (PTSD), especially the SSRIs that are used as first-line treatment in this disorder. (Wells *et al.*, 2003). Since many patients have co-existing depression and panic disorder, SSRIs could treat both conditions in the same patient at the same time. Imipramine and clomipramine, both tricyclic antidepressants, have been the most extensively studied in treating panic disorder. Both have shown efficacy in treating panic disorder (Stahl, 2000; Baldessarini, 1996). Various treatments can be given in combination for panic disorder. The basis of all treatments is an SSRI. Other antidepressants such as venlafaxine, nefazodone, mirtazapine, TCAs and MAO inhibitors could all have anti-panic actions, but they are second-

line treatments, like benzodiazepines. On the other hand, benzodiazepines are often added to SSRIs, particularly at the initiation of a SSRI and intermittently when there is break-through panic. Cognitive and behavioural psychotherapies could also be added to any of these drug treatments (Wells *et al.*, 2003).

2.3 Neurotransmitter and receptor interactions in psychotropic action

Serotonergic pathways make synaptic connections via heteroreceptors, with dopaminergic, cholinergic and noradrenergic pathways. This leads to a modulation of their function. The release of both NA and 5-HT is regulated by α_2 receptors on noradrenergic neurones (autoreceptors) and 5-HT heteroreceptors. This results in attenuation of release. If these receptors were inhibited, it would result in the enhanced release of both neurotransmitters (Harvey, 1997). Although serotonergic systems are most often implicated in mood, dopaminergic systems are traditionally associated with reward and appetitive motivation. Dopaminergic effects may be involved in the antidepressant effects of SSRIs. Such a putative effect would likely involve serotonin-dopamine interactions. Although less commonly prescribed, drugs that enhance dopaminergic function also are effective antidepressants (Sasaki-Adams & Kelley, 2001).

Functional cross-talk describes a sub-cellular interaction between different receptors within single cells or neurones. This is mediated by second messenger release acting as mutual co-regulators of cell function. Various sub-cellular pathways utilise second messengers such as cyclic adenosine monophosphate (cAMP), calcium ions (Ca^{2+}), cyclic guanosine monophosphate (cGMP) and nitric oxide (NO) to modulate events mediated by extra-cellular receptor interactions. Various animal studies have demonstrated the interactions and mutual interdependency of DA-ergic (dopaminergic), NA-ergic (noradrenergic) and 5-HT-

ergic (serotonergic) pathways on their respective neuronal effects (Harvey, 1997).

2.3.1 Subcellular mechanisms and onset of antidepressant action

Extracellular and intracellular dynamics determine the onset of therapeutic activity of antidepressants. There is an acute decrease in synaptic levels of 5-HT after administration of an antidepressant, because of stimulation of somatodendritic and synaptosomal 5-HT_{1A} autoreceptors that inhibit 5-HT release. This effectively acts as a brake on the onset of antidepressant action. Once these receptors have been down-regulated via changes in sub-cellular elements, the full antidepressant action can be realised. If a 5-HT_{1A} receptor antagonist, such as pindolol is given, it might hasten the onset of SSRI action (Goodwin, 1996). Once 5-HT has activated its specific receptor, a cascade of events is initiated that conveys the extra-cellular signal through the cell membrane into the cytoplasm and the nucleus where cell function is controlled (see § 2.1.3).

2.3.2 The 5-HT receptors as G protein-coupled receptors

In § 2.1.3 the G protein coupling of these receptors was mentioned. The 5-HT receptor subtypes are expressed in distinct but often overlapping patterns and are coupled with different transmembrane signalling mechanisms (see section 2.1.3 and Table 2-1) (Sanders-Bush & Mayer, 1995). Many types and subtypes of GPCRs are known, as well as different types of G proteins. Most of the mACHRs (cholinergic muscarinic receptor), 5-HT (serotonin) receptors, dopaminergic and adrenergic receptors are GPCRs. G proteins include $G_{q/11}$, which activates phospholipase C, G_s that stimulate adenylyl cyclase and $G_{i/o}$,

which inhibits adenylyl cyclase. G protein-regulated effectors include enzymes such as adenylyl cyclase and phospholipase C and plasma membrane ion channels selective for Ca^{2+} and K^+ (Ross & Kenakin, 2001).

G proteins are involved in at least 1000 different receptors for a great variety of ligands, such as hormones, neurotransmitters, lipids, alkaloids, peptides and biogenic amines, and even for direct sensory stimuli such as odorants, pheromones and light (Gether & Kobilka, 1998). Thus, G proteins play a crucial role in perception and neurotransmission.

All GPCRs share a common feature of structure and consist of a single polypeptide chain that forms seven transmembrane helices, connected by three intracellular loops (see Figure 2-3).

Their signal is transduced and amplified by G proteins, which act as molecular switches, activated by GTP binding and switched off by hydrolysation of bound GTP (Sóvágó *et al.*, 2001). G proteins are membrane-anchored hetrotrimeric protein molecules composed by α, β and γ sub-units. The α sub-unit can bind guanine nucleotides, therefore the name G protein. Four main classes of G proteins are distinguished, namely the α sub-unit: $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$ and $G\alpha_{12/13}$. The sub-unit composition determines the signal transduction pathway activated by agonist binding. The best characterised actions of these G protein sub-units are activation ($G\alpha_s$) or inhibition ($G\alpha_{i/o}$) of adenylyl cyclase, activation of phospholipase C ($G\alpha_{q/11}$), but they could also modulate other enzymes and ion-channels (Sóvágó *et al.*, 2001).

In the inactive state, the α sub-unit of the heterotremer binds GDP. Binding of an agonist induces conformational change of the receptor, which in turn interacts with and activates a G protein molecule. When the G protein is activated, the α sub-unit releases its bound GDP and binds GTP instead. Subsequently, it

dissociates into two active components, namely Gα-GTP and Gβγ. It has been verified that not only the α sub-unit, but also the βγ sub-unit, could activate downstream signalling elements. The activation is terminated by the GTPase activity of G_{α}. This process is called the guanine-nucleotide exchange cycle (Sóvágó *et al.*, 2001). Important features of G protein actions are signal amplification, convergence and divergence. Each receptor could activate several G proteins and each G protein could activate more second messenger molecules by its α and βγ sub-units. Moreover, a single receptor could activate several different G protein subtypes (divergence), whereas a discrete G protein could be coupled to numerous distinct receptors (convergence) (Sóvágó *et al.*, 2001). Hur and Kim (2001) also found that propagation of GPCR signalling involves cross-regulation of many specific pathways, including cross-talk between different GPRCs as well as with other signalling pathways.

Antidepressants may alter the neurotransmitter function indirectly through the regulation of intracellular signalling. Antidepressant agents may be effective because they modulate converging post-synaptic signals generated in response to multiple endogenous neurotransmitters, including norepinephrine and serotonin. In this context, the signal-transducing G proteins, which play a major role in the amplification and integration of signals in the CNS, are in a unique position to affect the functional balance between neurotransmitter systems.

four times that of the whole brain. With the exception of the kidneys, the testis and lens of the eye, the mammalian CNS contains significantly more inositol than the non-neural tissues do (Fisher *et al.*, 2002).

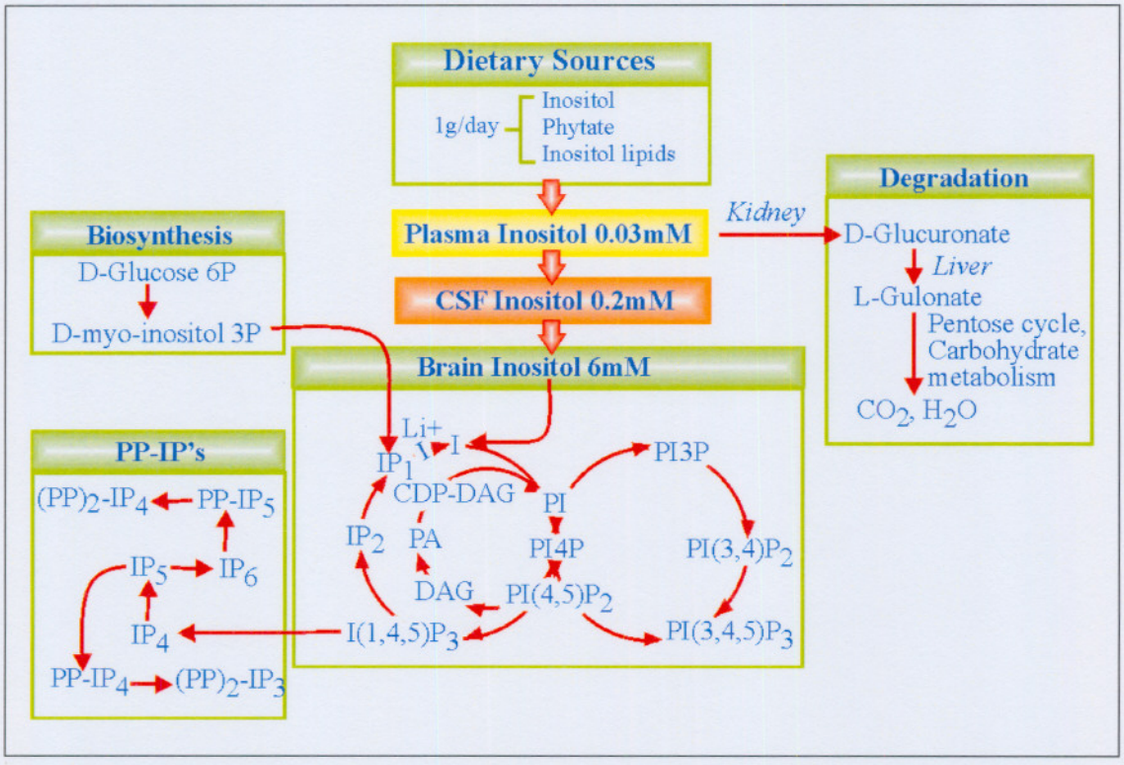

Figure 2-6 The synthesis, sources and metabolism of *myo*-inositol (reconstructed from Fisher *et al.*, 2002).

2.4.2.2 Functions of myo-inositol

2.4.2.2.1 Inositol as a physiologically important osmolyte

Electrolytes, or osmotically-active particles inside the cell, are in a constant state of flux. The osmolyte concentration is constantly regulated, because bulk flow of water across the membrane may result in damaging changes in cell volume.

termed inosit, from *inos,* the Greek root for muscle. It was then translated into English as inositol. It was found to be naturally abundant and a compound of many foods, especially grains (Fisher *et al.,* 2002). To distinguish the name of the prevalent isomer in nature from the eight other isomeric inositols, it was renamed *myo*-inositol. When the term inositol is used, it refers to the *myo*-inositol isomer (Fisher *et al.,* 2002). Inositol is one of a number of 'bios' growth factors required by some micro-organisms (Fisher *et al.,* 2002). In 1957 it was found that this substance was essential for the growth of all human and other animal cells in tissue culture (Marcus & Coulston, 1996). A special function for *myo*-inositol (*m*I) in the CNS was first suggested by the discovery of a relatively high inositol content in the brains of many species. More recently, alterations in brain and cerebro-spinal fluid (CSF) inositol concentrations have been reported in a number of pathological conditions (Fisher *et al.,* 2002). Moreover, lithium salts, which are used in the treatment of mania and manic-depressive disorders, inhibits the inositol phosphatases, thus leading to a reduction in inositol and its metabolites. This reduction in the inositol content impairs the capability of brain cells to react to transmitters like serotonin, noradrenaline and acetylcholine. It has since been found that treatment of depressed patients with high doses of inositol has distinct therapeutic efficacy (Wolfson *et al.,* 1998). Consequently, the role of *m*I in affective disorders has been more intensely investigated in recent years.

The CNS possesses relatively high concentrations of *m*I as well as the means to synthesise it. *m*I serves not only as a precursor molecule for inositol lipid synthesis, but also as a physiologically important osmolyte (Fisher *et al.,* 2002). It is now well known that inositol phospholipids play a prominent role in signal transduction events within the CNS (Berridge & Irvine, 1984). Inositol's role in membrane trafficking, maintenance of the actin cytoskeleton, regulation of cell death and survival and anchors for plasma membrane proteins, has also been documented (Fisher *et al.,* 2002). One of the inositol lipids, phosphatidylinositol 4,5-bisphosphate (PIP_2), is hydrolysed to diacylglycerol (DAG) and inositol

trisphosphate (IP_3). DAG operates within the plane of the membrane to activate protein kinase C (PKC), whereas IP_3 is released into the cytoplasm to function as a second messenger for mobilising intracellular calcium (Berridge & Irvine, 1984).

Chronic inositol treatment has been found to be effective in a select group of psychiatric illnesses, including obsessive compulsive disorder (OCD), panic and depression. It seems as if inositol efficacy is specific for illnesses that respond positively to SSRIs (Harvey *et al.*, 2002). Inositol seems to be ineffective in disorders like Alzheimer's disease, autism and schizophrenia and appears to worsen the symptoms of attention-deficit hyperactivity disorder (ADHD) (Levine, 1997). It also seems that there is an increased activity of the *m*I monophosphatase enzyme in Alzheimer's disease, so that *m*I might play a role in the pathology of the disease (Shimohama *et al.*, 1998). The mechanism whereby *m*I exerts these selective therapeutic responses remains unknown, but research suggests that *m*I may target specific interactions between G protein-coupled serotonergic receptors and their ligands (Harvey *et al.*, 2002). It seems as if *m*I influences pathologically altered receptor activation states and signals transduction pathways. Disorders responsive to *m*I are at best 60-70% responsive to current drug treatment, therefore understanding the neuropharmacology of *m*I in these disorders might hold great promise in understanding the neuropathology of disorders like OCD and depression. Viljoen (2002) studied the effect of *m*I treatment on SH-SY5Y cells, establishing that the modulating effects of *m*I involves the phosphatidylinositol (PI) metabolic pathway, presumably by desensitisation of the muscarinic receptor signal transduction pathway. In this study a closer examination of the pharmacology of *m*I with respect to depression within the framework of a dysfunction of the PI transduction system and more specifically from a serotonergic functional approach, will be undertaken.

2.4.2 Myo-inositol physiology

2.4.2.1 Synthesis, sources & metabolism of myo-inositol

Some tissues in the body, including brain endothelium, the kidneys (4g/day) and the testis, can convert D-glucose-6-phosphate to D-inositol-3-phosphate, which could then be dephosphorylated to inositol (see Figure 2-6).

The human diet provides approximately 1 g inositol a day. It is obtained from fruits, whole-grain cereals and other plant sources (Marcus & Coulston, 1996). In addition, dietary phytate (phytic acid or *myo*-inositol hexakisdihydrogenphosphate, IP_6) (Barrientos & Murthy, 1996) can in some instances give rise to free inositol, either by the action of ingested dietary phytases or from endogenous (gut bacteria) phytase activity in the intestinal mucosa (Fisher *et al.*, 2002; Marcus & Coulston, 1996).

Inositol is easily absorbed from the gut and then metabolised to glucose. The normal human plasma concentration of inositol is 5 mg/l (28 μM). The concentration of inositol is particularly high in heart muscle, skeletal muscle and brain tissue. The inositol content in urine is small, but markedly increased in diabetics (Marcus & Coulston, 1996).

The kidneys are the major organs involved in the catabolism and excretion of inositol (see Figure 2-6). Inositol degradation occurs exclusively in the kidneys via an oxygenase pathway that cleaves the polyol ring of the inositol, with the production of D-glucuronic acid. The D-glucuronic acid is then metabolised in the liver to D-xylulose 5-phosphate and enters the pentose phosphate pathway.

Cerebrospinal fluid is seven times more enriched in inositol relative to blood (plasma). Inositol levels in neuronal and glial cells could be between three and

four times that of the whole brain. With the exception of the kidneys, the testis and lens of the eye, the mammalian CNS contains significantly more inositol than the non-neural tissues do (Fisher *et al.*, 2002).

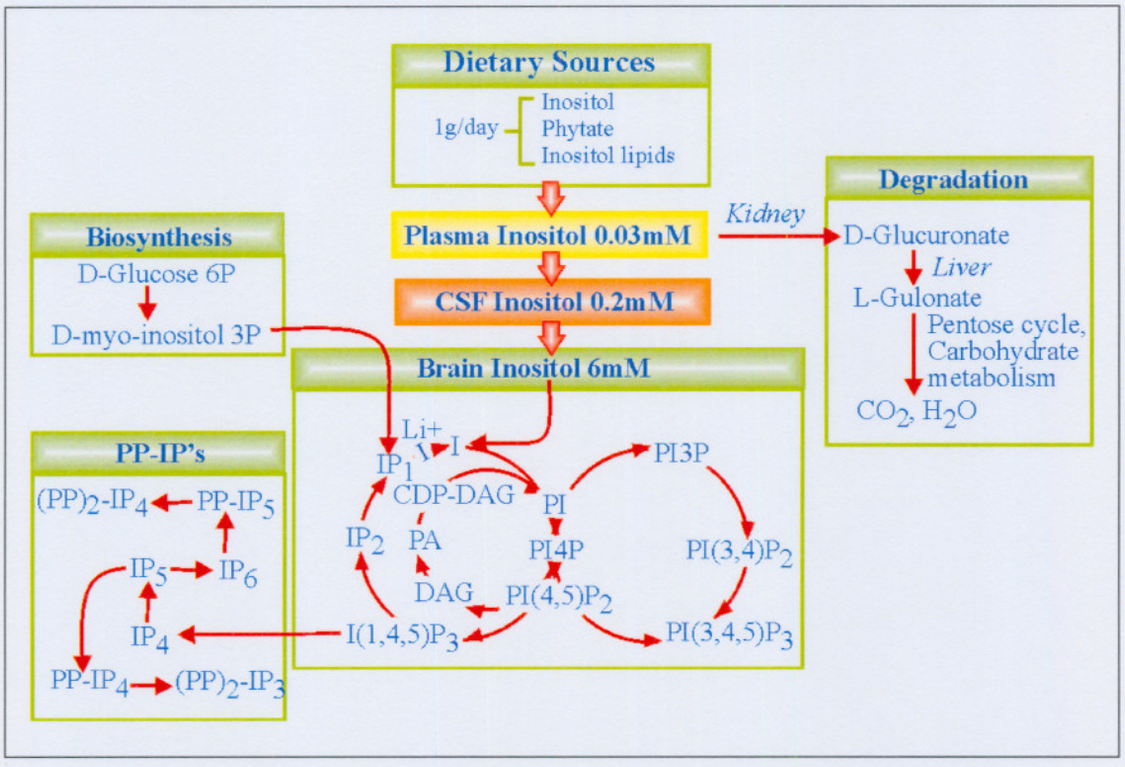

Figure 2-6 The synthesis, sources and metabolism of *myo*-inositol (reconstructed from Fisher *et al.*, 2002).

2.4.2.2 Functions of myo-inositol

2.4.2.2.1 Inositol as a physiologically important osmolyte

Electrolytes, or osmotically-active particles inside the cell, are in a constant state of flux. The osmolyte concentration is constantly regulated, because bulk flow of water across the membrane may result in damaging changes in cell volume.

Acute changes in tonicity are regulated by the transport of Na^+, K^+, H^+ or Cl^- across the plasma membrane. Chronic changes in tonicity are offset by the transport of non-perturbing organic osmolytes, primarily inositol. Research has shown that imbalances related to the function of inositol as an osmolyte in the CNS might be at least part of the pathogenesis of Down's syndrome (Fisher *et al.*, 2002).

2.4.2.2.2 The role of inositol in the synthesis of phosphoinositides

*m*I serves as a metabolic precursor molecule for inositol lipid synthesis. Dietary inositol is incorporated into neuronal cell membranes as phospholipids. One of the most important phospholipids is phosphatidyl-inositol- (4,5) bisphosphate (PIP_2). PIP_2 has a structural role but also serves as the precursor of receptor-activated PIP_2 hydrolysis by PLC enzymes. The hydrolysis of PIP_2 produces the second messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 mediates the release of intracellular calcium ions (Ca^{2+}) via IP_3 receptors, while DAG activates protein kinase C. IP_3 can be phosphorylated or sequentially dephosphorylated to *m*I. The signals generated by the PI kinases and PLC are terminated by inositol polyphosphate 5-phosphatase. The biosynthesis of PI, involving the DAG pathway and *m*I, is catalysed by PI-synthase. PI undergoes two phosphorylations to yield PI-4-phosphate and PIP_2. The PLC pathway has to mediate Ca^{2+} release within the cell and/or Ca^{2+} entry through the cell membrane. This action is mediated by the action of IP_3 at three IP_3 receptor subtypes. The Type-1 receptor mediates Ca^{2+} mobilisation from the sarcoplasmic reticulum, while the Type-3 receptor regulates Ca^{2+} influx across the plasma membrane. The turnover of membrane inositol phospholipids constitutes a signal transduction mechanism that mediates the cellular responses to hormones, growth factors and neurotransmitters. Several adrenergic, cholinergic, serotonergic and metabotropic glutamatergic receptors are coupled with PIP_2 hydrolyses in the brain. *m*I is crucial for the resynthesis of the PIs and the maintenance and efficacy of signalling (Harvey *et al.*, 2002). *m*I has an

important role in cellular effects, including the ability to regulate the activity of PLC and to increase membrane fluidity. It can also control the expression of inositol monophosphatase. Monophosphatase is a key enzyme in the PI-PLC pathway. These characteristics of *m*I make it a potential therapeutic agent to modulate neuronal function or activity when given exogenously (Harvey *et al.*, 2002).

2.4.2.2.3 Phosphoinositides and membrane trafficking

The first hint that phosphoinositides were important in membrane trafficking came from studies showing that a bacterial phospholipase C inhibited regulated exocytosis. Membrane trafficking can be controlled by phosphoinositides via their structural role as membrane components and by their ability to engage specific protein domains, which bind with high affinity and specificity to the different phosphorylated species of phosphoinositides. Thus, they recruit molecular machinery driving specific membrane trafficking events, regulating actin assembly (Cockroft & Matteis, 2001).

2.4.3 Inositol's involvement in disease

Administration of inositol has been found to reduce the incidence of spina bifida in mice foetuses. Research has also demonstrated that the administration of inositol can ameliorate the symptoms associated with diabetic peripheral neuropathy. The inhibition of inositol uptake in peripheral nerves has been implicated in the aetiology of the disease (Fisher *et al.*, 2002). Inositol concentrations are 30-50% higher in the brain and CSF of Down syndrome patients than in controls. Inositol transport is also altered in stroke, while physical trauma to the brain can trigger an up-regulation of cellular inositol uptake (Fisher *et al.*, 2002). Hepatic encephalopathy also leads to alterations in inositol transport, which results in the loss of cerebral inositol (Fisher *et al.*,

2002). Inositol supplementation has been found to be beneficial in pathologies such as fatty liver syndrome, cirrhosis of the liver (due to inositol deficiency) and respiratory distress syndrome in premature infants (Kofman *et al.*, 2000).

2.4.4 Inositol and the treatment of human neuropsychiatric disorders

Significantly lower concentrations of inositol were found to be present in the CSF of both unipolar and bipolar depressed patients than in healthy controls (Fisher *et al.*, 2002). Oral administration of inositol (6 g/day) has been found to increase the concentration of inositol in the CSF with as much as 70%. These studies suggest that *m*I may be decreased in subjects with affective disorders, leading to a decrease in the efficiency of the PI second messenger system and subsequent mood changes (Moore *et al.*, 1999).

Many of the psychiatric disorders form a continuum of overlapping symptoms, pathology and treatment strategies. The disorders responsive to *m*I, namely OCD, panic and depression, are either specific anxiety disorders (panic and OCD) or disorders that present with significant comorbid anxiety symptoms. The exact underlying pathology of depression remains illusive, but 5-HT, NA, DA, glutamate (Glu), γ-amino butyric acid (GABA), and various neuropeptides seem to play a vital role in its pathology. The 5-HT_2-R has been strongly implicated as a therapeutic target in anxiety and depression (Harvey *et al.*, 2002). Therefore, this study has focused on the 5-HT_{2A}-R (also see § 2.1.3.2). During the last few years the efficacy of *m*I in the treatment of depression has been demonstrated (Einat *et al.*, 2000) and also in other affective disorders (Pettegrew *et al.*, 2001). In double-blind control studies where inositol was chronically administered to patients, inositol induced a significant decrease in the Hamilton depression scale scores compared to placebo (Einat *et al.*, 2000). Unlike other antidepressants, inositol does not seem to exert a direct effect on the synapse and no changes

have been evident in monoamine levels in the brain (Einat *et al.,* 2000). Behavioural studies in rodents have suggested that inositol's effects are mediated by the serotonergic and not the adrenergic system (Einat *et al.,* 2000)

The emerging clinical profile of inositol suggests that, like specific serotonin re-uptake inhibitors, it is effective both as an antidepressant and in disorders like OCD and panic disorder. The mechanism of action of inositol remains however illusive and further investigation is needed to shed more light into this phenomenon. Thus far, inositol has been shown to affect serotonergic, and cholinergic (Viljoen, 2002) neurotransmission. The role of *m*I and the second messengers in the PI cycle have great therapeutic potential when given exogenously (Kofman *et al.,* 1998).

The observations of the clinical efficacy of *m*I in depression and the important role of the 5-HT_{2A}-Rs in this disorder have prompted the idea of using inositol as a treatment for depression (Fisher *et al.,* 2002).

Chapter 3 Experimental Procedures

3.1 Study design

3.1.1 Introduction

In the following chapter the materials, the cell line, and the experimental layout and assays for the functional studies, binding studies and [^{3}H]-*myo*-inositol ([^{3}H]-*m*I) cellular uptake studies are discussed.

High dose *m*I has been shown to be clinically effective in the treatment of depression, obsessive compulsive disorder and panic, although its mechanism of action remains illusive (see § 2.4.1). In the current study the primary aim was to investigate the possible modulating role of *m*I, as compared with the antidepressant prototypes fluoxetine (FLX) and imipramine (IMI), on serotonergic 5-HT$_{2A}$ receptor (5-HT$_{2A}$-R) function and concentration.

A human neuroblastoma cell line, transfected to express the human 5-HT$_{2A}$-R, was employed. Cells were pre-treated with different concentrations respectively of *m*I, FLX or IMI for a period of 24 hours, whereafter 5-HT$_{2A}$-R function and binding was determined. 5-HT$_{2A}$-R function was investigated by constructing concentration-effect curves with the endogenous agonist serotonin, measuring whole cell tritiated inositol phosphates ([^{3}H]-IP$_x$) accumulation or [^{35}S]-GTPγS binding in cell membranes. 5-HT$_{2A}$-R binding (indicative of relative receptor number) was determined by appropriate

radioligand binding assays. In addition, the influence of the pre-treatments on the total cellular uptake of [^{3}H]-*m*I was determined, as this may effect the interpretation of results of functional studies measuring [^{3}H]-IP$_x$ accumulation. The study can be divided into four phases (see Figure 3-1), namely:

- **Phase 1**: Transfection of neuroblastoma cells with the cDNA for the human 5-HT$_{2A}$ receptor.
- **Phase 2**: Confirmation of successful transfection and characterisation of transfected neuroblastoma cells.
- **Phase 3**: Investigation of the possible modulating role of *m*I, fluoxetine and imipramine pre-treatments on 5-HT$_{2A}$-R function and concentration.
- **Phase 4**: Investigation of the level in the 5-HT$_{2A}$-R signal-transduction system at which *m*I exerts its modulating effect on 5-HT$_{2A}$-R function.

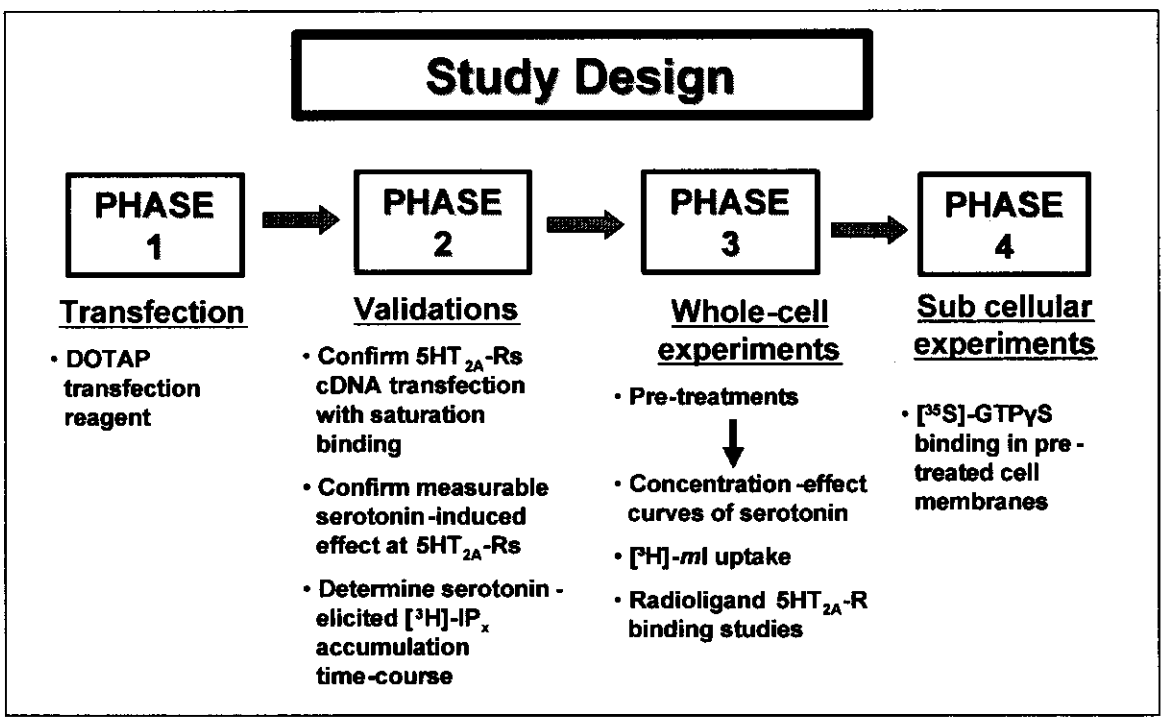

Figure 3-1 A schematic layout of the experiments conducted

3.1.2 Phase 1

Firstly neuroblastoma cells (SH-SY5Y cell line) were genetically manipulated to express the 5-HT_{2A}-R. This was done with the help and assistance of Dr. F.H. van der Westhuizen (Ph.D.) who had sufficient experience in genetic manipulation of cells. The receptors were kind gifts from Dr. Brian Roth (Case Western Reserve University, 2109 Adelbert Road, SOM W 438, Cleveland OH 44106 USA). These neomycin-resistant constructs had already been characterised, multiplied and purified for transfection purposes in the laboratory of Dr. van der Westhuizen. DOTAP liposomal transfection reagent (Roche Diagnostics) was used to transfect the SH-SY5Y cells with the 5-HT_{2A}-R.

3.1.3 Phase 2

During the next phase we had to establish whether the transfection was successful. To establish this we had to construct dose-response curves of the 5-HT_{2A}-R agonist serotonin on the function of the transfected cells. Assays were performed according to the principles of Godfrey (1992) and Cassarosa *et al.* (2001).

3.1.4 Phase 3

In phase 3 dose-response curves were constructed of the 5-HT_{2A}-R agonist, serotonin, on the transfected receptor, after the appropriate pre-treatments as described in § 3.5.2. Any alterations in receptor number were determined by standard saturation-binding and other appropriate radio-ligand binding studies.

3.1.5 Phase 4

During phase 4 receptor function and G protein coupling were investigated by utilising appropriate cell membranes and performing [^{35}S]-GTPγS binding experiments.

3.2 Cell line employed

The SH-SY5Y cultured cell line (from American Type Culture Collection (ATCC), catalogue number CRL-2266) was used in this study. This human neuroblastoma cultured cell line was established in 1970 from a metastatic bone tumour of a 4-year-old patient. Being a neuronal cell line, SH-SY5Y cells provided a simplistic *in vitro* biological model of neuronal cells in the central nervous system, where antidepressants exert their primary therapeutic effect. Cell cultures are grown *in vitro* under artificial conditions, so that they sometimes respond slightly differently to drugs than cells *in vivo*. SH-SY5Y cells are cancerous with a duplication time of about 48 hours under optimal conditions *in vitro*. For this reason a 48-hour incubation time may be sufficient to investigate modulating effects of antidepressant drugs. Furthermore, the 5-HT_{2A}-R is central to the theme of the current study, because of evidence suggesting its involvement in psychiatric illnesses, such as depression (see § 2.3). SH-SY5Y cells do not endogenously express the 5-HT_{2A}-R at significant numbers, which was evident from the absence of any measurable serotonin-induced accumulation of [^{3}H]-IP_x in these cells (see Chapter 4). The SH-SY5Y cells were transfected to express the human 5-HT_{2A}-R, using a standard transfection technique as described below.

3.3 Materials

3.3.1 Chemicals

24-well plates and 150 cm^2 culture flasks was obtained from Corning (New York, U.S.A.). DOTAP liposomal transfection reagent was from Roche molecular biochemicals, Ethanol Absolute 99.9% from Bio-zone, Geneticin disulphate from Sigma Aldrich, Wortmannin was obtained from Sigma Aldrich, *myo*-Inositol (*mI*), imipramine (IMI), trichloroacetic acid (TCA), HEPES (N-(-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)), lithium chloride (LiCl), Dowex 1x8-400, 200-400 mesh (1-chloride form) was obtained from Sigma Aldrich (Johannesburg, South Africa). Fluoxetine hydrochloride (FLX-HCl) was a gift from Eli Lilly (Johannesburg, South Africa). Dulbecco's Modified

Eagles Medium (DMEM):Ham's F-12 (1:1 ratio mixture), DMEM Powder foetal calf serum (FCS), Minimum Essential Media with Earle's Base (EMEM), Trypsin-versene (0.05% trypsin 1:250 + 0.02% EDTA) and bovine serum albumin was obtained from Highveld Biologicals (Johannesburg, South Africa). 2-Amino-2-hydroxymethyl-propan-1,3,-diol (Tris) was obtained from Acros (Geel, Belgium). Ascorbic acid, Bradford reagent, ethylenediaminetetraacetic acid (EDTA), NaCl, KCl, Na_2HPO_4, KH_2PO_4, formic acid, acetone and cacodylate was obtained from Merck (Johannesburg, South Africa). Penicillin, Streptomycin and fungizone were obtained from Bio-Whittaker (Walkersville, MD, U.S.A.). Formic acid was from Saarchem-Holpro Analytic (Krugersdorp, Gauteng, SouthAfrica). 5-hydroxytryptamine creatinine sulphate complex (serotonin creatinin sulphate), acetyl-β-methylcholine chloride (methacholine chloride), guanine diphosphate (GDP) and ritanserine were obtained from Sigma Chemical (St Louis, MO, U.S.A.). Liquid N_2 was obtained from Afrox (Johannesburg, South Africa). Ultima Gold XR scintillation fluid was obtained from Packard BioScience (Meriden, CT, U.S.A.). Nonidet P-40 (10%) from Roche Diagnostics.

3.3.2 Antibodies

Anti-$G_{q/11}$ from Whitehead Scientific, Santa Cruz (SC-392), Anti-rabbit SPA beads (Amersham cat# RPNQ 0016).

3.3.3 Radio chemicals

[^{35}S]-guanosine 5'-3-o-(thio)triphosphate ([^{35}S]-GTPγS) (1146 Ci/mmol) and *myo*-[2-^{3}H]-Inositol ([^{3}H]-MI) (17 Ci/mmol) were obtained from Amersham Pharmacia Biotech (U.K.). [Benzene ring-^{3}H]-spiperone ([^{3}H]-spiperone) (24.9 Ci/mmol) were obtained from NEN Life Science Products (Boston, MA, U.S.A.) from Separation Scientific (Johannesburg, South Africa), [^{35}S]-GTPγS from Amersham.

3.4 Instruments

Tri-carb 2100 TR liquid scintillation analyser (Packard, A.D.P. South Africa), haemocytometer (0.1 mm depth, 0.0025 cm^2). Beckman ultracentrifuge, 96-

well plate reader and a 560 nm filter (Labsystems Multiskan RC) Teflon® homogenizer.

3.5 Experimental design

3.5.1 Seeding of cells in 24-well plates

For experiments, cells were grown and cultured in 150 cm^2 culture flasks with normal culture medium (DMEM : Ham's F12 + 10% FBS) until 95% confluency. Cells were detached from the flask bottom with lyses solution and seeded with normal culture medium in 24-well plates at a density of 8 x 10^6 cells per well. Cells were incubated for 6-7 hours to allow cells to attach to the well bottoms, whereafter the medium was aspirated and the cells were rinsed twice with PBS. Thereafter the 24-hour pre-treatments were initiated as described in § 3.5.2 below.

3.5.2 Pre-treatments

The pre-treatments of the cells involved a 24-hour incubation of the cells with *m*I, FLX or IMI in serum-free minimum essential medium (Earle's base) (EMEM) at 37 °C in 5% CO_2 and water-saturated atmosphere. The following concentrations were used for the pre-treatments.

- *m*I: 10 mM, 1 mM and 0.1 mM (control, normal *m*I concentration in medium);
- FLX: 10 µM, 97 nM and a control (0.1 mM *m*I)
- IMI: 10 µM, 71 nM and a control (0.1 mM *m*I)
- Ritanserin: 10µM.

Any drug effects and mechanisms provided by these *in vitro* studies, should be confirmed by observations from *in vivo* studies or preferably clinical studies.

3.5.2.1 Concentrations used in pre-treatments

- **Myo-inositol concentrations**

  Ham's F12 medium contains 18 mg/ml *m*I (0.1 mM) and Ham's F12 : DMEM mixture contains 12.51 mg/l *m*I (0.07 mM). A concentration of 0.1 mM *m*I can therefore be regarded as a normal or standard concentration. If compared to *in vivo* brain concentrations in humans, concentration, 0.1 mM represents relatively low physiological concentrations. Normal brain concentrations of *m*I ranges from 2 to 15 mM and in plasma it has been about 5mg/l (28µM) (Marcus & Coulston, 2001). Therefore 0.1 mM *m*I was used as the standard concentration in control experiments for all the pre-treatments and all pre-treatment drugs were added to EMEM with 0.1 mM *m*I. For investigating the modulating effects of *m*I on $5HT_{2A}$-R function and concentration, a medium concentration of 1 mM *m*I (10x) and a high concentration of 10 mM (100x), all within physiological range, were chosen.

- **Fluoxetine & Imipramine concentrations**

  FLX and IMI have been used in concentrations of 10 µM in *in vitro* studies (Viljoen, 2002; Willets *et al.,* 1996). Their respective maximal effective plasma concentrations were obtained from Tummel & Shen (2001). The free plasma concentration was calculated from the maximal effective plasma concentration and the fraction bound to plasma protein:

  - FLX is 94% bound to plasma proteins and the maximal effective plasma concentration is 500 ng/ml. The free plasma concentration was calculated as 6% of 500ng/ml = 30 ng/ml = 97 nM. Therefore FLX pre-treatments were used at concentrastions of 0 M (control), 97 nM (medium concentration) and 10 µM (high concentration).

  - IMI is 90.1% protein bound and has a maximal effective plasma concentration of 200 ng/ml. The free plasma concentration was calculated as 10% of 200 ng/ml = 20 ng/ml = 71 nM. Therefore

IMI pre-treatments were used at concentrations of 0 M (control), 71 nM (medium concentration) and 10 µM (high concentration).

In all cases the drugs were added to EMEM with 0.1 mM *m*I.

Table 3-1 Drug pre-treatment series

Drug series	Drug concentration 1	Drug concentration 2	Drug concentration 3
Drug series 1: *m*I in EMEM	0.1 mM *m*I (control)	1 mM *m*I (10x)	10 mM *m*I (100x)
Drug series 2: FLX in EMEM	0.1 mM *m*I + 0 M FLX (control)	0.1 mM *m*I + 97 nM FLX	0.1 mM *m*I + 10 µM FLX
Drug series 3: IMI in EMEM	0.1 mM *m*I + 0 M IMI (control)	0.1 mM *m*I + 71 nM IMI	0.1 mM *m*I + 10 µM IMI

3.5.2.2 Pre-treatment procedure

Cells were grown and maintained in 150 cm^2 flasks. A suspension of cells was prepared and the cells were seeded (see § 3.5.1). On the day of the pre-treatment, the cells were washed in the wells with 2x EMEM medium (500 µl), and the pre-treatment medium was added (1 ml). The drugs were diluted in EMEM without FCS, because FLX and IMI is highly protein bound. The cells were then incubated for a period of 24 hours. Previous studies done by Viljoen (2002) indicated that FCS did not have a modulating effect on intracellular *m*I uptake, whether DMEM:Ham's F12 + 10 % FCS or EMEM (serum free) medium was used in the *m*I pre-treatments.

3.5.3 Assays

3.5.3.1 Transfection of adherent cells

Neuroblastoma cells (SH-SY5Y cell line) were genetically manipulated to express the human 5-HT_{2A}-R. The human $5HT_{2A}$-R plasmid cDNA in the pIRES (Neo^r) mammalian expression vector was kindly provided by Dr. B. Roth (Department of Biochemistry, Case Western Reserve University,

Cleveland, OH, U.S.A.). pIRES is a mammalian expression vector, which expresses the human 5-HT_{2A} receptor. pIRES uses the neomycin resistance gene (Neo) to permit selection of transformed cells.

The transfection, using the transfection agent DOTAP, was performed with the kind assistance of Dr. Francois van der Westhuizen (Division of Biochemistry at the Potchefstroom University for Christian Higher Education). DOTAP is a monocationic transfection reagent based on liposome technology. Liposomes are formed by cationic lipid N-[1-(2,3-Dioleoyloxy)]-N,N,N-trimethylammonium propane methylsulfate ($C_{43}H_{83}NO_8S$, MW: 774,21), incorporating the cDNA, and thereby it facilitates the internalisation of the genetic material via the cell membrane into the intracellular domain, where it is incorporated into nuclear chromosomes to express the protein it encodes for. Other methods available to transfer DNA into eukaryotic cells include the use of calcium phosphate or other divalent cations, polycations, liposomes, retroviruses, microinjection and electroporation. However all of these methods suffer from problems related to cellular toxicity, poor reproducibility, inconvenience or inefficiency of DNA delivery. The transfection reagent DOTAP has been proven to be highly effective for the transfection of DNA, RNA, oligonucleotides, ribonucleoprotein particles and proteins (Roche Diagnostics). After transfection, attempts to clone cells from a single colony that express a particular number of 5-HT_{2A}-Rs, using the dilution cloning technique, failed and the transfection mix (designated 5-HT_{2A}-SH-SY5Y cells) was used.

5-HT_{2A}-SH-SY5Y cells were cultured in 150 cm^2 culture flasks and maintained in 1:1 DMEM:Ham's F12, foetal calf serum (FCS), 100 units penicillin/ml, 100 µg streptomycin/ml and 0.25 µg fungizone /ml in an incubator at a constant temperature of 37 ºC in 5% CO_2. Under these optimal conditions the cells had a duplication time of approximately 48 hours. Cells were grown to 95 % confluency, whereafter they were detached from the flask bottoms by means of trypsination and reseeded at a density of ≥ 20% confluency.

3.5.3.2 [^{3}H]-IP_x accumulation assay

The aim of this study was to construct concentration effect curves of the serotonin agonist on the function of the transfected 5-HT_{2A}-R in the SH-SY5Y cell line, after the pre-treatments. The assay protocol was followed as described by Godfrey (1992) with minor modifications. IP_x includes all the inositolphosphates, that is inositolphosphate (IP), inositolbiphosphate (IP_2) and inositoltriphosphate (IP_3). In this study we are more interested in the measurement of the IP_3.

- After the pre-treatments as described in § 3.5.2, the medium was aspirated.
- Each well was then washed twice with 500 µl of EMEM (37°C) to remove any serum.
- The cells were labelled by adding 300 µl EMEM + 1% BSA + 1 µCi/ml [^{3}H]-*m*I and by incubating the cells for 18-20 hours at 37°C and 5% CO_2.
- After the labelling, the cells were rinsed twice with DMEM and then incubated for 10 min with 500 µl/well assay medium (DMEM + 0.5 M Hepes + 0.4 M LiCl[1]).
- The assay medium was then aspirated and 500 µl assay medium plus the appropriate concentration of the serotonin agonist were added to each well.
- The cells were incubated for 180 min at 37°C and 5% CO_2.
- After the labelling, the cells were rinsed twice with DMEM and then incubated for 10 min with 500 µl/well assay medium (DMEM + 0.5 M Hepes + 0.4 M LiCl).
- The assay medium was then aspirated and 500 µl assay medium plus the appropriate concentration of the serotonin agonist were added to each well.
- The cells were incubated for 180 min at 37°C and 5% CO_2.
- The reaction of the agonist stimulation was terminated after the incubation period with 1 ml lyses solution. The medium was aspirated

[1] Li+ is known to inhibit the enzyme which brakes down IPx

and 1 ml ice-cold 10 mM formic acid was added and let to stand for 90 min to allow the cells to lyse.

- One hundred Bio-Rad poly-prep columns were fitted into double layer racks[2].
- The Dowex columns were prepared by using Dowex 1x8 – 400, 200 – 400 mesh, 1-chloride form.
- The columns were washed with the following solutions:
  - 5 ml double distilled water (ddH_2O)
  - 2.5 ml Solution **1** (see Table 3-2 for composition)
  - 10 ml ddH_2O
  - 2 times with Solution **2** (see Table 3-2 for composition)
- After the lyses period, the 1 ml cell supernatant from each well was transferred to a Dowex column.
- Each well was rinsed with 1 ml lyses solution (4°C), which was also added to the column.
- After the samples were transferred to the columns, the columns were rinsed with 2 x 5 ml Solution **3**.
- Columns were placed over scintillation vials and the IP_xs in each Dowex column were eluded with 3 ml Solution **4**.
- 7 ml Ultima Gold XR scintillation fluid was added to each scintillation vial.
- Vials were mixed thoroughly and then the [^{3}H]-IP_x was counted in a Tri-carb 2100 TR liquid scintillation analyser (Packard, A.D.P. South Africa).

[2]These racks were designed to fit over collecting buckets (for radioactive material) and scintillation vial racks

Table 3-2 Chromatographic solutions (All the solutions were made up in ddH_2O)

Solution	Composition	Function
1	5 M Ammonium formate & 3 M Formic acid	Dowex column regeneration
2	0.1 M *Myo*-inositol & 3 M Formic acid	Dowex column regeneration
3	1 M Sodium formate & 0.1 M Borax decahydrate	Washing through unwanted [^{3}H]-biochemicals
4	5 M Ammonium formate & 3 M Formic Acid	Washing through [^{3}H]-IP_x

3.5.3.3 Cell counting assay

Cell counting was done for each experiment to ensure a uniform cell number per well between experiments. Cells were seeded homogenously at 8 million cells/well.

- After loosening cells by trypsinisation, cell suspensions were diluted as necessary and homogenous suspensions ensured by pipetting cells up and down.
- 20 µl of the cell suspension was injected into a haemocytometer for counting purposes under an inverted microscope, as is standard for this technique.

3.5.3.4 Whole-cell radioligand binding assay

In this study we wanted to determine the specific binding of 10 nM of the 5-HT_{2A}-R ligand [^{3}H] spiperone to 5-HT_{2A}Rs. The non-specific binding was defined by 10 nM (> 1000 x K_D) of the reversible antagonist ritanserin (10

µM). The specific binding could be calculated by subtracting non-specific binding from total binding.

- Cells were seeded in 24-well plates, pre-treated and dummy labelled (as labelling, without [^{3}H]-*m*I) as before (see § 3.6.2).
- The cells were then rinsed twice with DMEM and then incubated for 60 minutes at 37°C and 5% CO_2 with 300 µl/well EMEM + radioligand ± cold ligand (see Table 3-3).
- The medium was aspirated and each well was rinsed with 2 × 1 ml PBS.
- 5% trichloroacetic acid was added to each well to terminate the reaction and allowed to stand for 60 min to allow cells to lyse.
- The lyses solution from each well was then transferred directly to scintillation vials; 7 ml Ultima Gold XR scintillation fluid was added to each vial. The [^{3}H]-spiperone was then counted in the scintillation counter.

Table 3-3 Ligand/EMEM solutions for whole cell radioligand binding

HOT ligand **= Specific & Non-specific binding**	**HOT + COLD ligand** **= Non-specific binding**
EMEM 10 nM [^{3}H]spiperone	EMEM 10 nM [^{3}H]spiperone 10 µM ritanserin

3.5.4 Myo-[2-^{3}H]-inositol uptake assay

The aim of this study was to investigate the possible effect of *m*I, FLX and IMI pre-treatments on [^{3}H]-*m*I uptake into the SH-SY5Y cells.

- Cells were seeded in 24-well plates, pre-treated, labelled and lysed as before (see § 3.6.2), whereafter the 1 ml lyses solution supernatant was transferred directly to 20 ml scintillation vials.

- 7 ml Ultima Gold XR scintillation liquid was added to each vial and the total radioactivity (total intracellular radioactivity) was counted in the scintillation counter.

3.5.5 [^{35}S]-GTPγS experiments

The [^{35}S]-GTPγS assay is a method to study the distribution and function of neurotransmitter receptors in tissue sections. It provides anatomical and functional information at the same time (Sóvágó *et al.*, 2001). This assay is based an on increase in guanine nucleotide exchange at G-proteins upon agonist stimulation.

3.5.5.1 Preparing cell purified membranes from cultured 5HT$_{2A}$-SH-SY5Y cells

Cells were seeded in 24-well plates as described in § 3.5.1 and pre-treated as described in § 3.5.2. Thereafter the cells were dummy-labelled and membranes were prepared as described below, in preparation for [^{35}S]-GTPγS binding assays.

- The cells were then "dummy labelled"[3] for 18 hours. This step was necessary to ensure a uniform incubation period for all the assays performed. After pre-treatments and "dummy labelling" the cells were rinsed twice with 2 ml sterile PBS, whereafter the cells were loosened by trypsinisation and by using a cell scraper.
- Thereafter 6 ml PBS was added and cells pipetted up and down to ensure a homogenous suspension.
- After pooling cells from all flasks with corresponding pre-treatments, the respective cell suspensions were added to conical centrifuge tubes and centrifuged in a bench top centrifuge for 10 min at 4°C at 5000 rpm.

[3] Incubated in EMEM, but without [^{3}H]-*m*I, at 37°C, 5% CO_2 and humidified atmosphere.

- After centrifugation the pellet was collected and the supernatant decanted and discarded. The pellet was then re-suspended in PBS and centrifuged twice to remove all trypsin.
- The pellet was then re-suspended in 25 ml Tris buffer (1 mM, pH 7.4 & 4°C. see Table 3.3). The suspension was then rotated in the cold room (4°C) for 15 min.
- After the rotation in the cold room the pellet nuclei and undisrupted cells were homogenised with a Teflon homogeniser until the suspension had no visible particles (± 7 times up and down).
- Now the homogenate was centrifuged for 10 min at 4°C at 3000 rpm in benchtop centrifuge.
- After centrifugation the supernatant was collected. The supernatant contained the membrane – the unwanted cell nuclei and remnant membrane, which are in the pellet.
- The pellet was re-suspended in 15 ml of 1 mM tris Buffer (pH 7.4 and 4°C). Then the pellet had to repeat rotating in cold room, being homogenised and centrifuged as before to make sure all the remnant membranes were collected in the pellet.
- The supernatant were collected (now containing the membranes) and centrifuged at 18 196 rpm in type 50 rotor for 60 min at 4°C in the ultracentrifuge.
- Only after the 60 min the pellet would contain the wanted membranes. This pellet was re-suspended in 1 ml HNM buffer (see Table 3.3) and homogenised with the Teflon homogeniser.
- The homogenate was divided into 50 µl and 100 µl aliquots in eppendorf tubes.

Table 3-4 Composition of buffers

BUFFER	COMPOSITION
Tris-buffer	121,14 mg tris acid and add ddH_2O to 1000 ml (pH 7.4)
HNM-buffer	20 mM Hepes 5 mM $MgCl_2$ 100 mM NaCl made in ddH_2O.

3.5.5.2 [^{35}S]-GTPγS assay

The assay protocol for [^{35}S]-GTPγS binding (Amersham cat# SJ1308) to $G\alpha_{q/11}$ was done using anti-$G_{q/11}$ (Santa Cruz SC-392) antibody and anti-rabbit scintillation proximity assay (SPA) beads (Amersham cat# RPNQ 0016), according to the method of DeLapp *et al.* (1999), but in microcentrifuge tubes (Eppendorf, 1.5 ml, clear).

- The frozen membranes were quick-thawed to 25°C. The membrane aliquots with same pre-treatments were added together to yield a homogenous suspension.
- The membrane suspension was then diluted with HNM-buffer solution (see Table 3-4 for composition) at 25°C to yield a 0.25 mg protein per ml suspension. 100 µl of the buffer or sample were added to appropriate eppendorf tubes
- Then 50 µl of the appropriate serotonin concentration were added to each test tube and incubated for 30 min at 25°C.
- 50 µl of the 0.8 nM [^{35}S]-GTPγS in buffer solution were added to each tube to yield a 0.2 nM final concentration. At this stage the membrane suspension were again incubated for 30 min at 25 °C.
- After the incubation time 5,4 µl of the 10% Nonidet (protein emulsifier) was added to the tubes and incubated for 30 min, with gentle agitation in a shaking water bath. After the time has expired, anti-$G_{q/11}$ antibody was added to the appropriate tubes and another incubation period of 60 min followed (at 25°C).

- Then 50 µl of anti-IgG-coated SPA beads were added to each tube and the tubes were incubated for 3 hours at 25°C.
- The suspensions were then centrifuged in the benchtop centrifuge for 10 min at 4°C.
- After centrifugation, the radioactivity was counted in a scintillation counter.

3.6 Data analysis

Data from assays were obtained as triplicate measurements from at least three separate experiments, and expressed as mean ± S.E.M., unless stated otherwise. Semi-logarithmic concentration-effect curves were constructed as non-linear least square fits, by utilising the computer software Graph Pad Prism® (version 4.01 for Windows®, GraphPad Software, San Diego, CA, U.S.A., www.graphpad.com). The Hill-Slope factor was set at 1. The Dunnet's post-hoc t-test (two-way with 95% and 99% confidence intervals, as performed with The SAS System for Windows Release 8.02 TS Level; 02M0) was implemented to compare all relevant values, where all values were expressed relative to one control value (i.e. after control pre-treatment, without agonist). For all reported statistical probability values $p < 0.05$ was taken as statistically significant.

Chapter 4 Results & Discussion

4.1 Introduction

In the current chapter all the results of the experiments conducted are presented and discussed. All the experiments were performed in the Laboratory for Applied Molecular Biology at the Potchefstroom University for Christian Higher Education.

4.2 Results of control experiments

4.2.1 Confirmation of successful transfection of SH-SY5Y cells with human $5HT_{2A}$ receptor cDNA

Successful transfection of SH-SY5Y cells with the plasmid for the expression of the human $5HT_{2A}$ receptor ($5HT_{2A}$-R) was confirmed by firstly showing serotonin-induced IP_x (second messenger) formation (absent in untransfected cells), as well as by showing specific binding of the $5HT_2$-R ligand [^{3}H]-spiperone to $5HT_2$-Rs with an appropriate K_D value.

4.2.1.1 Dose response curves of serotonin in transfected SH-SY5Y cells

In the current study one human neuroblastoma cell line was employed, namely a SH-SY5Y cell line (from American Type Culture Collection (ATCC), catalogue number CRL-2266) and transfected to express the human 5-HT_{2A}

receptor ($5HT_{2A}$-R). The transfected cell line was designated $5HT_{2A}$- SH-SY5Y.

The SH-SY5Y cell line is known to endogenously express muscarinic acetylcholine receptors, predominantly of the M_3 type (Slowiejko *et al.*, 1996). The current study, however, focuses on the modulating effect of *myo*-inositol (*m*I) on $5HT_{2A}$-R function and binding. Therefore, the SH-SY5Y cells were genetically manipulated to express the 5-HT_{2A}-R. Figure 4-1 shows the concentration-effect curves of serotonin, measuring triturated inositol phosphates ([^{3}H]-IP_x) accumulation, in untransfected SH-SY5Y cells versus in transfected cells (designated $5HT_{2A}$-SH-SY5Y cells).

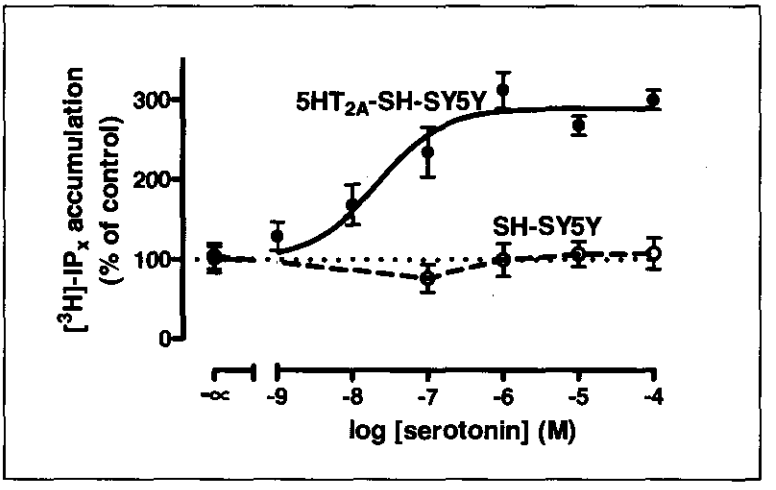

Figure 4-1: Concentration effect curves of 5-HT in SH-SY5Y cells and 5-HT_{2A}-SH-SY5Y cells. IP_x (inositol phosphates) accumulation was measured (% of control). Data are averages of three independent experiments, each with triplicate observations, and are expressed as mean ± standard error of the mean (S.E.M.).

It can be seen in Figure 4-1 that serotonin-induced IP_x accumulation was not found in the untransfected SH-SY5Y cells, while a concentration effect curve was obtained with the transfected $5HT_{2A}$-SH-SY5Y cells. These data therefore suggest that native SH-SY5Y cells do not express $5HT_2$-Rs in sufficient numbers to produce measurable agonist-induced IP_x production, whereas the transfected SH-SY5Y ($5HT_{2A}$-SH-SY5Y) cells do express sufficient $5HT_2$-Rs.

4.2.1.2 Saturation binding study with [^{3}H]spiperone ± 10 µM ritanserin

Figure 4-2 depicts saturation binding of [^{3}H]-spiperone in 5-HT$_{2A}$-SH-SY5Y cells.

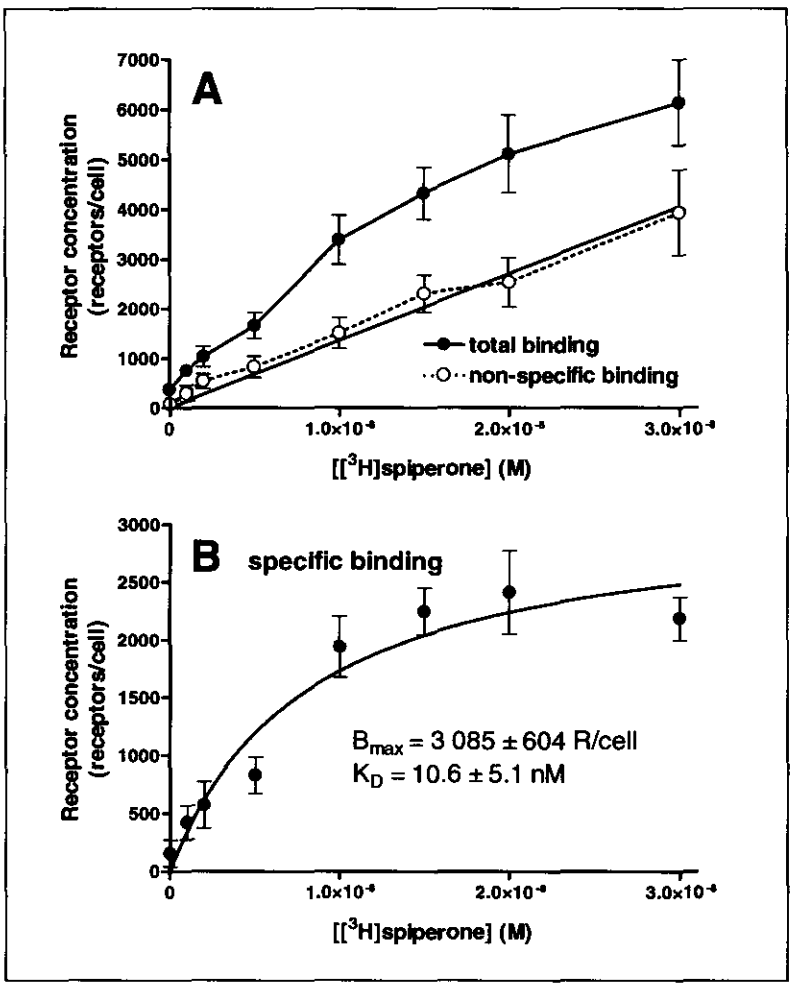

Figure 4-2 Saturation binding of [^{3}H]-spiperone 5-HT$_{2A}$-SH-SY5Y cells. (A) Total and non-specific binding of [^{3}H]-spiperone, with non-specific binding defined by 10 µM ritanserin (> 1000× K_D). (B) Specific binding of [^{3}H]-spiperone. Data are averages of three independent experiments, each with triplicate observations, and are expressed as mean ± standard error of the mean (S.E.M.).

In Figure 4-2 A it can be seen that non-specific binding (as defined by 10 µM ritanserin (> 1000× K_D)) was a significant proportion of the total binding. The use of different media to determine total and non-specific binding (including phosphate buffered saline, DMEM and UltraMEM) were also investigated, but all of these gave larger non-specific to total binding ratios (data not shown).

In this context it should be kept in mind that the 5-HT_{2A}-R expression in 5-HT_{2A}-SH-SY5Y cells was relatively low. In From the specific saturation binding curve in Figure 4-2 B, the K_D value of [^{3}H]-spiperone at 5-HT_{2A}-Rs was calculated as 10.6 ± 5.1 nM and the B_{max} value was calculated as 3.085 ± 604 receptors/cell. This K_D value corresponds with reported K_i values for spiperone at 5-HT_{2A}-Rs, ranging between 0.12 nM and 50.11 nM (Boess & Martin, 1994).

Taken together, the results from Figure 4-1 and Figure 4-2 suggest that the transfection of SH-SY5Y cells to express the human 5-HT_{2A}-R was successful and that the transfection mix of 5-HT_{2A}-SH-SY5Y cells express the 5-HT_{2A}-R at an average of 3.085 ± 604 receptors/cell.

4.2.2 Time course experiments

Two time-course experiments were conducted to validate the experimental procedures. Firstly, it was important to establish whether serotonin-induced IP_x accumulation is linear with time over a period of 180 minutes. It was found that, since 5-HT_{2A}-SH-SY5Y cells express the 5-HT_{2A}-Rs at a relatively low number, 180 minutes incubation with serotonin was necessary to observe sufficient IP_x accumulation above baseline. Secondly, we had to determine the time-dependency of the indicated drug pre-treatments to modulate 5-HT_{2A}-R function in 5-HT_{2A}-SH-SY5Y cells.

4.2.2.1 Validation of the linear relationship between serotonin-induced IP_x accumulation and time in 5-HT_{2A}-SH-SY5Y cells.

Trial experiments indicated that sufficient serotonin-induced IPx accumulation above baseline in 5-HT_{2A}-SH-SY5Y cells is obtained only after 180 minutes (data not shown) and at shorter incubation times resolution of data was less clear. An experiment therefore was conducted to determine whether the relationship between serotonin-induced IP_x accumulation and time was linear in 5-HT_{2A}-SH-SY5Y cells for up to 180 minutes.

Figure 4-3 shows the relationship between serotonin-induced IP_x accumulation and time in 5-HT_{2A}-SH-SY5Y cells, where these cells were incubated with or without serotonin for 0, 30, 60, 90, 120, 150 or 180 minutes, respectively

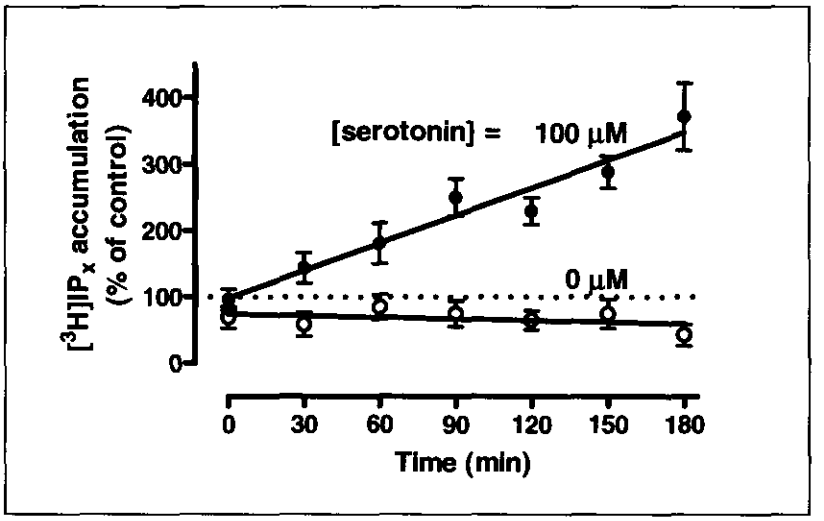

Figure 4-3 The relationship between agonist-induced [^{3}H]-IP_x production and time in 5-HT_{2A}-SH-SY5Y cells. The 5-HT_{2A}-Rs were stimulated with 0 and 100 µM 5-HT, at different time periods and the resulting inositol phosphates accumulation production was measured. Data are averages of three independent experiments, each with triplicate observations, and are expressed as mean ± standard error of the mean (S.E.M.).

In Figure 4-3 it can be seen that, in the absence of serotonin, intracellular IP_x concentration did not change significantly over a period of 180 minutes. However, in the presence of 100 µM serotonin, intracellular IP_x concentration increased with a linear relationship with time. These results suggest that an incubation time of 180 minutes with serotonin would be appropriate and would therefore yield interpretable results.

4.2.2.2 Time course experiment of 10 mM myo-inositol pre-treatment in 5-HT_{2A} SH-SY5Y cells.

To establish an optimum pre-treatment time period for the *in vitro* cell culture system, it was important to investigate the incubation time needed for *m*I to modulate 5-HT_{2A}-R function. Figure 4-4 depicts the influence of different pre-treatment incubation times on the modulating effect of *m*I on the 5-HT_{2A}-R

function, as measured by the ability of 100 µM serotonin to stimulate phospholipase C activity (i.e. IP_x accumulation).

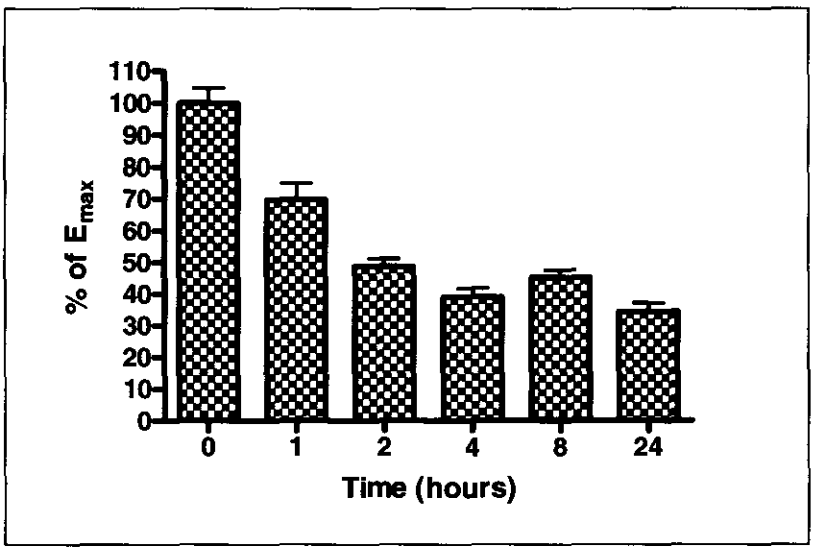

Figure 4-4 IP_x accumulation as induced by 100 µM serotonin in 5-HT_{2A}-SH-SY5Y cells, after different pre-treatment times with 100 µM *m*l. Data are averages of three independent experiments, each with triplicate observations, and are expressed as mean ± standard error of the mean (S.E.M.).

In Figure 4-4 it can be seen that a rapid reduction in responsiveness of the 5-HT_{2A}-SH-SY5Y function occurs within about two to four hours, where after the responsiveness remains reduced but constant up to 24 hours. These results suggest that a pre-treatment period of between 4 and 24 hours will be appropriate to investigate the modulatory effect of *m*l on 5-HT_{2A}-R function.

In a comparable study conducted by Viljoen (2002), measuring muscarinic acetylcholine receptor function in SH-SY5Y cells, maximal supression of receptor function was obtained only after 24 hours. In addition, comparison of a 24-hour, five day and five week pre-treatment period with *ml* showed no differences in the modulation of muscarinic acetylcholine receptor function. In this study a 24 hour pre-treatment time was therefore used. In order to maintain uniform procedures and comparable results, a 24 hour pre-treatment period was also implemented for the current study.

4.2 Results of study objective experiments

The study objective experiments were performed to investigate the possible modulating effects of the different indicated drug pre-treatments on the 5-HT_{2A}-R function and relative 5-HT_{2A}-R number in 5-HT_{2A}-SH-SY5Y cells.

4.2.1 Modulating effect of myo-Inositol pre-treatments

Figure 4-5 depicts the modulating effects of different *m*I pre-treatment concentrations on 5-HT_{2A}-R mediated [^{3}H]-IP_x production, GTPγS binding to $G_{\alpha q/11}$ proteins, total cellular [^{3}H]-*m*I uptake during the radio-labelling of 5-HT_{2A}-SH-SY5Y cells and the specific binding of [^{3}H]-spiperone to 5-HT_{2A}-Rs.

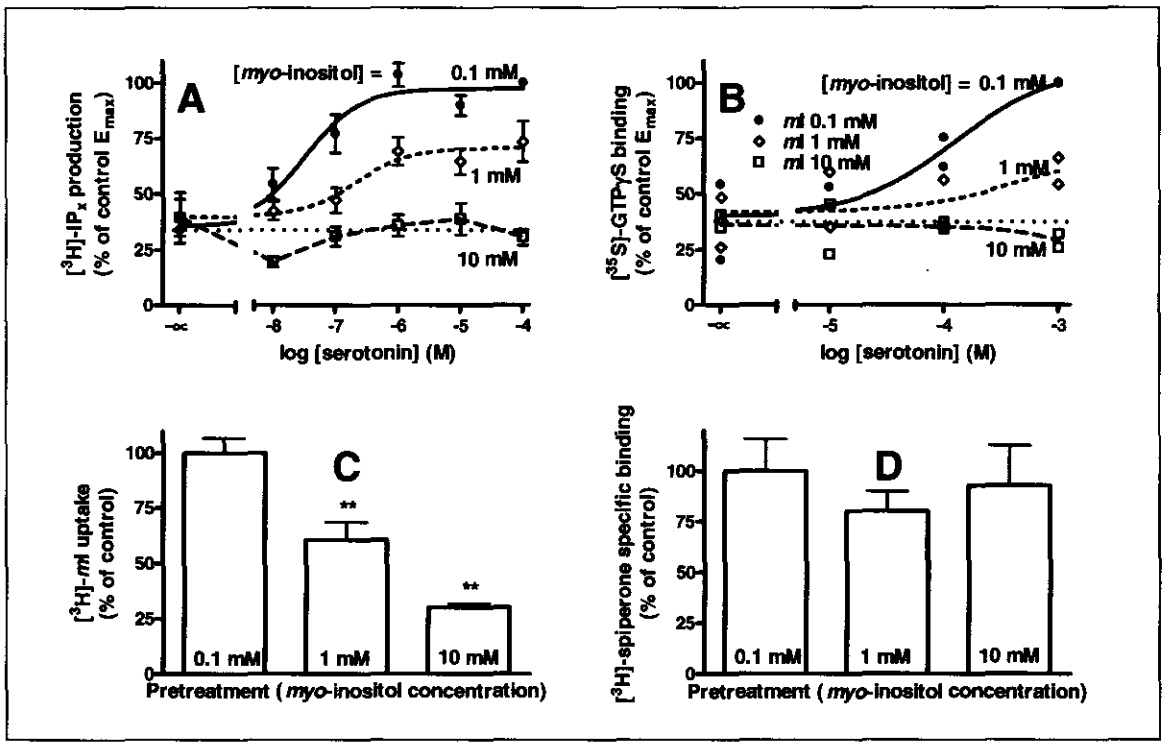

Figure 4-5 The modulating effects of 24-hour pre-treatment with 0, 1 or 10 mM *m*I on 5-HT_{2A}-R function and relative number. (A) Concentration-effect curve of serotonin in 5-HT_{2A}-SH-SY5Y cells, measuring 5-HT_{2A}-R mediated [^{3}H]-IP_x production. (B) Concentration-effect curve of serotonin in 5-HT_{2A}-SH-SY5Y membranes, measuring [^{35}S]-GTPγS binding to $G_{\alpha q/11}$ proteins, (C) Total cellular [^{3}H]-*m*I uptake during the radio-labelling of 5-HT_{2A}-SH-SY5Y cells. (D) Specific binding of 10 nM [^{3}H]-spiperone at 5-HT_{2A}-Rs (non-specific binding defined by 10 μM ritanserin). Data from A, C & D are averages from three independent and comparable experiments, each with triplicate measurements, and are expressed as mean ± standard error of the mean (S.E.M). Data from B is one experiment with duplicate observations.

[^{3}H]-IP_x production was measured from the liquid scintillation counting, expressed as counts per minute (cpm). The cpm data were then processed as the percentage of the maximum effect (E_{max}) of the control experiment (0,1 mM *m*I pre-treatment).

From Figure 4-5 A, it can be seen that the increasing concentrations of *m*I decrease the agonist-induced accumulation of [^{3}H]-IP_x production. A 40% reduction in E_{max} is seen after treatment with 1 mM *m*I. After pre-treatment of the cells with 10 mM *m*I an elimination of the effect is seen ($p < .0.001$). The baseline accumulation of [^{3}H]-IP_x (i.e. in absence of serotonin) was not altered by *m*I pre-treatments.

In Figure 4-5 B the same trend was seen as in Figure 4-5 A. A concentration-dependent reduction in E_{max} was seen, after *m*I pre-treatments when measuring [^{35}S]-GTPγS binding to $G_{\alpha q/11}$ proteins in 5-HT_{2A}-SH-SY5Y membranes. These data suggest that *m*I may reduce 5-HT_{2A}-R function by reducing the signalling capacity through G_q proteins.

In Figure 4-5 C it can be seen that *m*I pre-treatments dose-dependently decrease also the cellular uptake of [^{3}H]-*m*I after radio-labelling of the cells. A decrease of 39% ($p < 0.01$) at 1 mM *m*I and 70% ($p < 0.001$) at 10 mM *m*I. The relative E_{max} values of the concentration – effect curves in Figure 4-5 B correlates well with the corresponding E_{max} values in Figure 4-5 A, and the data therefore suggest that the decrease in maximal response of serotonin obtained after the pre-treatment of the cells with *m*I, cannot be fully explained by the decrease in [^{3}H]-*m*I uptake as presented in Figure 4-5 C. It is possible, however, that high *m*I pre-treatment concentrations may have saturated intracellular *m*I pools and thereby have contributed to the observed decrease in [^{3}H]-*m*I uptake. It can therefore not be excluded that the reduction in whole-cell [^{3}H]-*m*I uptake may have contributed to the observed reduction in whole-cell [^{3}H]-IP_x accumulation, as presented in Figure 4-5 A, in addition to the contribution of the reduced 5-HT_{2A}-R signalling capacity through G_q proteins.

Figure 4-5 D shows specific binding of 10 nM [^{3}H]-spiperone to 5-HT_{2A}-Rs after the respective pre-treatments with *m*I. From the results it can be concluded that *m*I pre-treatment does not alter the 5-HT_{2A}-R binding in the 5-HT_{2A}-SH-SY5Y cells. A reduction in receptor binding does not explain the reduced E_{max} values after the *m*I pre-treatments, as observed in Figure 4-5 A and Figure 4-5 B. These data therefore suggest that *m*I reduces the signalling capacity of 5-HT_{2A}-Rs at the receptor-G-protein level.

It was noted that the EC_{50} values of the concentration-effect curves of serotonin in Figure 4-5 A and Figure 4-5 B differ. This observation is not fully explained and it may be due to differences in the experimental conditions or even suboptimal experimental conditions for the [^{35}S]-GTPγS binding assay.

4.2.2 Modulating effects of fluoxetine and imipramine pre-treatments

In Figure 4-6 the dose -dependent modulating effects of FLX and IMI pre-treatments on 5-HT_{2A}-R function and binding in 5-HT_{2A}-SH-SY5Y cells are depicted.

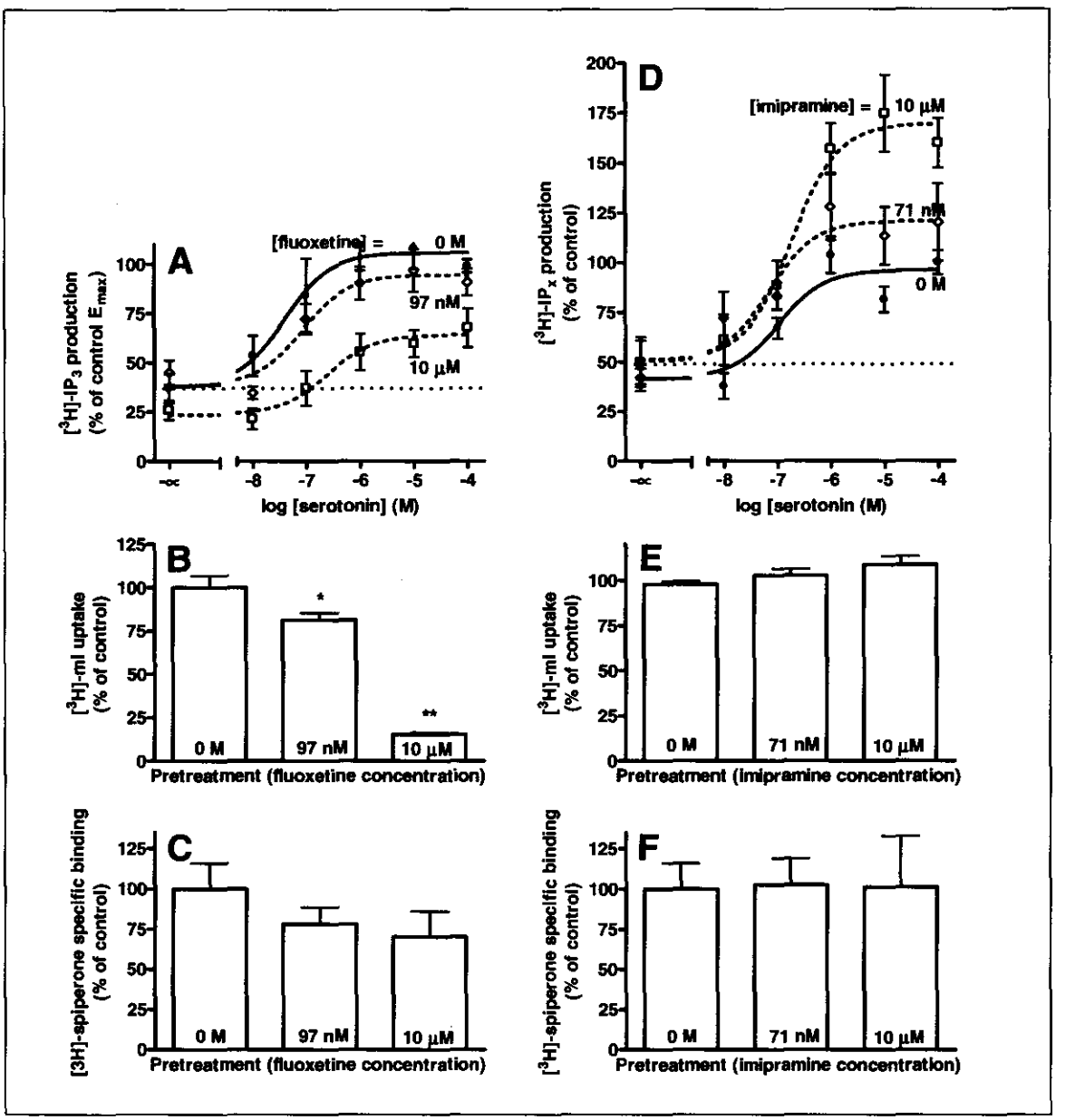

Figure 4-6 The modulating effects of 24-hour pre-treatment with different concentrations of fluoxetine or imipramine on 5-HT_{2A}-R function and relative number. (A) Concentration-effect curve of serotonin in 5-HT_{2A}-SH-SY5Y cells, measuring 5-HT_{2A}-R mediated [^{3}H]-IP_x production, after pre-treatments with 0 M, 97 nM or 10 µM fluoxetine. (B) Total cellular [^{3}H]-*ml* uptake during the radio-labelling of 5-HT_{2A}-SH-SY5Y cells, after pre-treatments with 0 M, 97 nM or 10 µM fluoxetine. (C) Specific binding

of 10 nM [^{3}H]-spiperone at 5-HT_{2A}-Rs (non-specific binding defined by 10 μM ritanserin), after pre-treatments with 0 M, 97 nM or 10 μM fluoxetine. (D) Concentration-effect curve of serotonin in 5-HT_{2A}-SH-SY5Y cells, measuring 5-HT_{2A}-R mediated [^{3}H]-IP_x production, after pre-treatments with 0 M, 71 nM or 10 μM imipramine. (E) Total cellular [^{3}H]-*m*I uptake during the radio-labelling of 5-HT_{2A}-SH-SY5Y cells, after pre-treatments with 0 M, 71 nM or 10 μM imipramine. (F) Specific binding of 10 nM [^{3}H]-spiperone at 5-HT_{2A}-Rs (non-specific binding defined by 10 μM ritanserin), after pre-treatments with 0 M, 71 nM or 10 μM imipramine. Data are averages from three independent and comparable experiments, each with triplicate measurements, except in (D) where data are averages of triplicate measurements from two independent and comparable experiments. Data are expressed as mean ± standard error of the mean (S.E.M). Data from B is one experiment with duplicate observations.

In Figure 4-6 A it can be seen that increasing concentrations of FLX decreases the agonist-induced accumulation of [^{3}H]-IP_x, with a 13% reduction in E_{max} after pre-treatment of the cells with 97 nM FLX (not statistically significant) and 50% after pre-treatment of the cells with 10 μM FLX ($p < 0.01$). These decreases in E_{max} values are less than those observed with *m*I pre-treatments (see Figure 4-5). It is of particular interest that this modulating effect of reduced 5-HT_{2A}-R function is shared by FLX, as seen in Figure 4-6 A. *m*I seems to have a greater capacity to modulate 5-HT_{2A}-R function, at least *in vitro* under the experimental conditions used.

Figure 4-6 B depicts the total cellular uptake of [^{3}H]-*m*I uptake during the radio-labelling of the cells, after the respective pre-treatments with FLX. With increasing concentrations of FLX pre-treatments, the total [^{3}H]-*m*I uptake into the 5-HT_{2A}-SH-SY5Y cells decreases. If Figure 4-6 B is compared to Figure 4-5 C it can be seen that both pre-treatments result in a decrease in the total [^{3}H]-*m*I uptake into the 5-HT_{2A}-SH-SY5Ycells.

Figure 4-6 C depicts specific binding of 10 nM [^{3}H]-spiperone to 5-HT_{2A}-Rs after the appropriate pre-treatments with FLX. Small reductions in the average specific binding of [^{3}H]-spiperone after the pre-treatments were not statistically significant. As seen with the *m*I pre-treatment, the FLX pre-treatment did not alter the receptor binding. Therefore a reduction in [^{3}H]-*m*I production can not be attributed to a reduction in relative receptor number.

Figure 4-6 D depicts the agonist-induced accumulation of [^{3}H]-IP$_x$ in the 5-HT$_{2A}$-SH-SY5Y cells after the IMI pre-treatments. After pre-treatment with 71 nM IMI an increase of 39% in E_{max} was seen (not statistically significant), a 117% increase was seen after pre-treatment with 10 µM IMI ($p<0.01$). These increased values of E_{max} in the IMI pre-treatments differ from the values seen when cells are stimulated with *m*I or FLX.

Figure 4-6 E depicts the total cellular uptake into the 5-HT$_{2A}$-SH-SY5Y cells, after the respective pre-treatments with IMI. It can be seen that IMI pre-treatments do not alter the total [^{3}H]-*m*I uptake into the 5-HT$_{2A}$-SH-SY5Y cells during the radio-labelling of the cells. Figure 4-6 F depicts the specific binding of 10 nM [^{3}H]-spiperone to 5-HT$_{2A}$-Rs after the appropriate pre-treatments with IMI. As was seen with fluoxetine pre-treatments, imipramine pre-treatments did not alter the 5-HT$_{2A}$-R binding.

4.2.3 Synopsis

The study objective experiments showed that:

Phosphoinositides have been implicated in the regulation of various intracellular signalling pathways, such as receptor trafficking, particularly the internalisation of agonist-activated G protein coupled receptors and also increasing the activity of PLCβ. Their role in maintaining the effectiveness of signalling of receptors linked to PI hydrolysis has hinted of the potential value of *m*I in the neurobiology and treatment of various psychiatric disorders such as depression, OCD and panic disorders. The experiments in § 4.3.1 investigate the possible modulatory role of different *m*I pre-treatments on 5-HT$_{2A}$-R function.

The relevance of the similarities between *m*I and FLX pre-treatments might explain why *m*I is effective in disorders that respond to SSRI therapy and it may in part unravel the mechanism of action of *m*I. IMI on the other hand, has less serotonergic properties and is not that effective in the treatment of certain anxiety disorders.

Chapter 5 Summary & Conclusions

5.1 Summary

The current study aimed to investigate the possible modulatory role of *myo*-inositol (*mI*) versus fluoxetine or imipramine pre-treatments on 5-HT_{2A}-R function and binding (relative number), utilising a human neuroblastoma cell line (SH-SY5Y) that was transfected to express the human 5-HT_{2A}-R and designated 5-HT_{2A}-SH-SY5Y.

The following control experiments were performed and results obtained:

- Successful transfection of SH-SY5Y cells with the human 5-HT_{2A}-R plasmid was verified with appropriate concentration-effect curves of serotonin, as well as with saturation binding with an appropriate radio-ligand. Results indicated that 5-HT_{2A}-SH-SY5Y cells express the 5-HT_{2A}-R at sufficient numbers to yield a measurable increase in serotonin-induced IP_x accumulation. Results from saturation binding experiments also suggest specific binding of the 5-HT_2-R radioligand [^{3}H]spiperone, with a K_D value of 10.6 ± 5.1 nM (correlating well with reported K_i values of spiperone for the 5-HT_2-R) and a relatively low receptor number (B_{max} = 3085 ± 604 receptors/cell).

- A linear relationship between incubation time with serotonin and IP_x accumulation for up to 180 minutes was verified where the increase in serotonin-induced IP_x accumulation was sufficient to overcome the barrier of assay sensitivity.

- The time-dependency of the modulating effect of *m*I pre-treatment on 5-HT_{2A}-R function was determined for up to 24 hours of pre-treatment. It was clear from the results that the maximal modulating effect of *m*I on 5-HT_2-R function is obtained already within 4 hours of pre-treatment and maintained for up to 24 hours.

After the pre-treatment of 5-HT_{2A}-SH-SY5Y cells with different concentrations of *m*I, fluoxetine or imipramine, receptor function was determined by measuring whole-cell [^{3}H]-IP_x accumulation or [^{35}S]-GTPγS binding to $G_{\alpha q}$ protein in cell membrane preparations. In addition total [^{3}H]-*m*I uptake into cells was measured, as well as specific receptor binding to determine relative receptor number before and after pre-treatments. Results showed that *m*I pre-treatment caused a concentration-dependent decrease in [^{3}H]-IP_x production and a decrease in [^{3}H]-*m*I uptake without any significant effect on 5-HT_{2A}-R binding. A concentration-dependent reduction in E_{max} was seen, after *m*I pre-treatments when measuring [^{35}S]-GTPγS binding to $G_{\alpha q/11}$ proteins in 5-HT_{2A}-SH-SY5Y membranes. These data suggest that *m*I may reduce 5-HT_{2A}-R function by reducing the signalling capacity through G_q proteins.

- The fluoxetine pre-treatment causes a concentration-dependent decrease in [^{3}H]-IP_x production and a decrease in [^{3}H]-*m*I uptake (to a lesser degree than *m*I) without any significant effect on 5-HT_{2A}-R binding. The imipramine pre-treatments caused however an increase in [^{3}H]-IP_x production and no significant change in [^{3}H]-*m*I uptake. The 5-HT_{2A}-R binding was also not altered, as seen in both the other pre-treatments. In Table 5-1 a summary of the effects of the various pre-treatments are shown.

Table 5-1 Modulating effects of *m*I, FLX & IMI pre-treatments on 5-HT_{2A}-R function and expression

	Pre-treatments		
	10 mM *m*I	10 µM FLX	10 µM IMI
[^{3}H]-IP_x production	Decrease	Decrease	Increase
[^{3}H]-*m*I uptake	Decrease	Decrease	No change
[^{35}S]-GTPγS binding	Decrease	Not applicable	Not applicable
5-HT_{2A}-R binding	No change	No change	No change

5.2 Conclusions

Harvey *et al.* (2002) suggested that *m*I acts selectively on serotonin-specific disorders such as depression, panic disorder, OCD etc. Therefore the modulating effects of *m*I were compared to the effects of a SSRI (fluoxetine) and a TCA (imipramine).

In the current study it was shown that the property of a time-dependent decrease in 5-HT_{2A}-R function in transfected human neuroblastoma cells is shared by *m*I and fluoxetine pre-treatments, but not by the imipramine pre-treatment that revealed a time-dependent increase in 5-HT_{2A}-R function. Would this finding apply also to the *in vivo* situation and in particular to the human brain, this difference may explain why *m*I is effective exclusively in SSRI sensitive disorders.

Viljoen (2002) investigated the possible modulating effect of similar *m*I, fluoxetine or imipramine pre-treatments in SH-SY5Y cells on the function of muscarinic acetylcholine receptors (mAChRs). It was concluded that *m*I

decreased mAChR function.as measured by maximal methacholine-induced IP_x production and this anticholinergic property was more prominent than that observed with fluoxetine and imipramine. This property of mAChR function inhibition may in part explain the therapeutic effect in the treatment of depression. Furthermore, *m*I seems to mimic the modulating effects of fluoxetine on these receptor systems better than of imipramine, which may explain why *m*I are effective selectively in serotonin-reuptake inhibitor-responding disorders.

It is clear that the modulating effects of *m*I pre-treatment involve the PI metabolic pathway-dependent mechanisms that eventually lead to, presumably, desensitisation of the metabolic pathway. It was, however, established that a decrease in 5-HT_{2A}-R signalling capacity was already evident at $G_{\alpha q}$ protein level. The current study has provided useful information on understanding the mechanism of action of *m*I on 5-HT_{2A}-R function, in disorders like depression.

Further detailed *in vivo* and clinical studies are however needed to investigate the possible role of *m*I in the treatment of anxiety related disorders in humans. It is also important to investigate the role of multiple neurotransmitters and the interaction between them in relation to the aetiology and treatment of depression (Janowsky *et al.*, 1972). The current study has provided information on understanding the mechanism of action of *m*I in depression and anxiety disorders relevant to 5-HT_{2A}-R function modulation. This study, however, was done in an in vitro cell culture model and does not necessarily represent in vivo effects. It is therefore important to confirm the results with ex vivo experiments after in vivo treatments in appropriate animal models.

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Appendix A: Abbreviations

Abbreviation	Description
AA	Arachidonic acid
AC	Adenylyl cyclase
ACTH	Adrenocorticotropic hormone
ADHD	Attention-deficit hyperactivity disorder
AIDS	Acquired immune deficiency syndrome
BDNF	Brain-derived neurotrophic factor
Ca^{2+}	Calcium ion
CCK	Cholecystokinin
cGMP	Cyclic GMP
Cl^-	Chloride ion
CEC	Concentration effect curve
CNS	Central nervous system
CO_2	Carbon dioxide
CSF	Cerebrospinal fluid
DA	Dopamine
DAG	Diacylglycerol
DMEM	Dulbecco's Modified Eagles Medium
ECT	Electroconvulsive therapy

EMEM	Minimum Essential Media with Earle's Base
ERK	Extracellular signal-regulated kinase
FCS	Foetal calf serum
FLX	Fluoxetine
GABA	Gamma aminobutyric acid
GDP	Guanosine diphosphate
GPCR	G protein-coupled receptor
GTP	Guanosine triphosphate
5-HIAA	5-hydroxyindole acetic acid
5-HTP	5-Hydroxytryptophan
5-HT	Serotonin, 5-Hydroxytryptamine
H^+	Hydrogen ion
IMI	Imipramine
IP	Inositolphosphate
IP_x	Inositol-multiphosphates
IP_2	Inositol-4,5-biphosphate
IP_3	Inositol-1,4,5-triphosphate
IP_4	Inositol-1,2,3,4-tetrakisphosphate
IP_5	Inositol-1,2,3,4,6-pentakisphosphate
IP_6	Inositol-1,2,3,4,5,6-hexakisphosphate
K^+	Potassium ion
K_D	Dissociation constant of the ligand-receptor complex
l-N_2	Liquid nitrogen
mACHRs	Cholinergic muscarinic receptor
MAO	Mono amine oxidase
*m*I	*myo*-Inositol
Na^+	Sodium ion
OCD	Obsessive compulsive disorder
PBS	Phosphate buffered saline

PI	Phosphatidylinositol
PI-3,4-P_2	Phosphatidylinositol-3,4-biphosphate
PI-3,5-P_2	Phosphatidylinositol-3,5-biphosphate
PI-4-K	Phosphatidylinositol-4-kinase
PI-3-P	Phosphatidylinositol-3-phosphate
PI-4-P	Phosphatidylinositol-4-phosphate
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PLA_2	Phospholipase A_2
PTSD	Posttraumatic stress disorder
R	Receptor
SAD	Seasonal affective disorders
S.E.M.	Standard error of the mean
[^{35}S]GTP	[^{35}S]Guanosine-5'-*O*-(3-thio)triphosphate
SSRI	Selective serotonin re-uptake inhibitor
TCA	Tricyclic antidepressants
TRP	Tryptophan
Wrt	Wortmannin

Appendix B: Publications

BRINK, C.B., VILJOEN, S.L., DE KOCK, S.E., STEIN, D.J. & HARVEY, B.H. 2003. Effects of *myo*-inositol versus fluoxetine and imipramine pre-treatments on serotonin 5-HT_{2A} and muscarinic acetylcholine receptors in human neuroblastoma cells. *In Press*.