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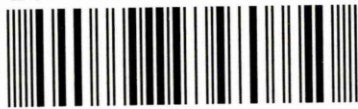
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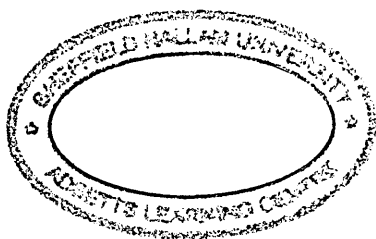
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**5-HT_{1A} receptor expression; studies in
postmortem tissue and
characterisation of a model system**

Lindsey Janice Bunn

A thesis submitted in partial fulfilment of the requirements
of Sheffield Hallam University
for the degree of Doctor of Philosophy

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Abstract

Serotonin (5-HT) neurotransmission is involved in the psychopharmacology of several psychiatric disorders including, depression, anxiety disorders and schizophrenia. The release of 5-HT in neurons is mediated by somatodendritic 5-HT_{1A} autoreceptors. The presence of 5-HT_{1A} receptor is thought to be increased in depressed patients, producing a reduction in the synthesis of 5-HT. A common single nucleotide polymorphism in the promoter region of 5HT_{1A} receptor C-1019G is also associated with depression and suicide. The nuclear DEAF-1 related (NUDR) protein represses the 5-HT_{1A} promoter region hence regulating both 5-HT_{1A} transcription and receptor expression.

The project involved undertaking a post-mortem study to determine any association between the 5-HT_{1A} promoter polymorphism and the expression of 5-HT_{1A} receptor mRNA and receptor density in control human hippocampal brain tissue. This was achieved by genotyping human brain tissue for the 5-HT_{1A} receptor polymorphism C-1019G and 5-HT_{1A} receptor mRNA levels were quantified using real-time PCR. Radioligand binding was used to determine B_{max} and K_d quantifying 5-HT_{1A} receptor density.

The SHSY-5Y neuroblastoma cell line is a well characterised cell line model used in neurotransmitter studies when differentiated. The 5-HT_{1A} receptor couples to G_i proteins inhibiting AC activity and hence mediating a variety of intracellular changes such as, decreasing cAMP leading to decreased Ca²⁺ levels. The SH-SY5Y cell line study investigated whether the 5-HT_{1A} agonist 8-OH-DPAT, inhibited forskolin stimulated Ca²⁺ release in the SHSY-5Y cell line and whether the 5-HT_{1A} antagonist p-MPPI reversed this effect using flow cytometry.

The post-mortem study showed that the G-1019 allele had significantly higher 5-HT_{1A} expression compared to the C allele in control hippocampal tissue. Radioligand binding data demonstrated that control samples with a GG or G/C genotype had a significantly higher 5-HT_{1A} receptor density compared to samples with a CC genotype. SH-SY5Y cells differentiated with RA for 5 days or NGF and aphidicolin for 10 days had significantly increased 5-HT_{1A} receptor mRNA levels compared to undifferentiated cells. Western blots and immunocytochemistry confirmed the presence of the 5-HT_{1A} receptor in this cell line. An increase in NUDR expression was observed at the same time there is an increase in 5-HT_{1A} receptor expression in SH-SY5Y cells treated with RA or NGF and aphidicolin. Flow cytometry showed that 8-OH-DPAT efficiently diminished forskolin-stimulated increase in intracellular Ca²⁺ in RA differentiated cells. 5-HT also a 5-HT_{1A} agonist had a similar effect. SH-SY5Y cells treated with both p-MPPI and 8-OH-DPAT demonstrated that cells treated with p-MPPI at higher concentrations significantly increased forskolin-stimulated intracellular Ca²⁺ levels and therefore effectively reversed the agonistic effect of 8-OH-DPAT.

The findings presented in the post-mortem study are novel and the SH-SY5Y cell line study demonstrates that this cell line when differentiated with either RA or NGF and aphidicolin is a useful cell-line model system for studying the 5-HT_{1A} receptor.

Abbreviations

AC	Adenylyl cyclase
ACTH	Adrenocorticotrophic hormone
APS	Ammonium Persulfate
ARMS	Amplification refractory mutation system
ASO	Allele-specific oligonucleotides
BCA	Bicinchonic acid
B _{max}	Binding site density
BP	Binding potential
BSA	Bovine serum albumin
CA	Cornu ammonis
cAMP	Cyclic AMP
CAMK	Calmodulin-dependent protein kinase
cDNA	Single-stranded complementary DNA synthesised from RNA template
CNS	Central nervous system
CRF	Corticotrophin-releasing factor
CRH	Corticotrophin-releasing hormone
CSF	Cerebrospinal fluid
C _t	Threshold cycle
DAPI	4'6-diamino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DR	Dorsal raphe area
DRE	Dual repressor element
dsDNA	Double stranded DNA
ERK	Extracellular signal related kinases
FCS	Foetal bovine (calf) serum
FRET	Fluorescence resonance energy transfer
GABA	γ-Aminobutyric acid
GDP	Inactive guanine diphosphate
GPCR'S	G-protein coupled receptor superfamily
GR	Glucocorticoid receptor
GTP	Activated guanine triphosphate
HPA	Hypothalamic pituitary adrenal axis
ICC	Immunocytochemistry
Kd	Measure of the concentration of radioactive ligand that is required to occupy 50 percent of receptors
LC	Locus coeruleus
MGB	Minor groove binding
MAOI's	Monoamine oxidase inhibitors
MAP	Mitogen-activated protein kinases
MDD	Major depressive disorder
mRNA	Messenger RNA
MR	Median raphe area
MR	Mineralocorticoid receptor
MTC	Mesiotemporal cortex
NA	Noradrenaline
NGF	Nerve growth factor
NFQ	Non-fluorescent quencher
NRI's	Norepinephrine selective reuptake inhibitors
NUDR	Human nuclear deformed epidermal autoregulatory factor
PBS	Phosphate buffered saline

PBST	PBS and Tween 20
PCR	Polymerase chain reaction
PET	Position emission tomography
PKA	Protein kinase A
PMI	Post-mortem delay
p-MPPI	4(2-methoxy-phenyl)-1-1[2'-(n-2"-pyridinyl)-p-Iodobenzamido]-ethyl- piperazine
PNS	Peripheral nervous system
PRS	Polymorphism ratio sequence
PTSD	Panic disorder
PVDF	Polyvinylidene fluoride
PVN	Paraventricular nucleus of hypothalamus
RA	Retinoic acid
RARE	Retinoic acid response element
Rn	Normalised intensities
RNA	Ribonucleic acid
RT-PCR	Real-Time PCR
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SNP	Single oligonucleotide polymorphism
SSCP	Single stranded conformational polymorphism
ssDNA	Single stranded DNA
SSRI's	Selective serotonin reuptake inhibitors
TEMED	N,N,N',N'-Tetramethylethylenediamine
TCAs	Tricyclic antidepressants
T_m	Melting temperature
TPA	12-O-tetradecanoylphorbol
TPH	Tryptophan hydroxylase
WAY100635	(N-[2-[4-(2-methoxyphenyl)1-piperazinyl]ethyl]n-(2-pyridinyl)cyclohexanecarboxamidetrihydrochloride
5-HT	Serotonin
5-HIAA	5-hydroxyindoleacetic acid
5-HT1A	Serotonin 1A receptor
8-OH-DPAT	8-hydroxy-2-di-n-propylaminotetralin

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Publications

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Bunn LJ et al (2007) Induction of 5-HT_{1A} receptor and NUDR mRNA in differentiated SHSY-5Y neuroblastoma cells. Poster presented at Lifesciences conference, Glasgow, UK

Bunn LJ et al (2006) Induction of 5-HT_{1A}receptor mRNA in differentiated SHSY-5Y neuroblastoma cells. Proceedings of the British Pharmacological Society at <http://www.pA2online.org/abstracts/Vol4Issue2abst038P.pdf>

Bunn LJ et al (2006) A 5-HT_{1A} receptor promoter polymorphism has effects on receptor density and mRNA expression. Journal of Psychopharmacology abstract supplement, 20, 5, A11.

Bunn LJ et al (2005) Expression of 5-HT_{1A} receptor mRNA is influenced by a common promoter region polymorphism. Journal of Psychopharmacology abstract supplement, 19, 5, A35

Conferences attended

British Association for Psychopharmacology (BAP) Harrogate July, 2005

Collegium Internationale Neuro-Psychopharmacologicum (CINP) Belfast April, 2006

British Association for Psychopharmacology (BAP) Oxford July, 2006

British Pharmacological Society (BPS) Winter meeting, 2006

Lifesciences conference Glasgow, 2007

British Association for Psychopharmacology (BAP) Harrogate July, 2007

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Chapter 1

Introduction

1.0- Serotonin (5-HT)

The isolation and characterisation of serotonin (5-HT) and its final identification as serotonin took place between 1940 and 1949. However, back in 1868 it was already widely acknowledged that blood contained a vasoconstrictive substance that was released in serum during platelet breakdown (Green, 2006). This substance proved to be a problem for Irvine Page in his studies on malignant hypertension due to the substance's ability to elicit large pressor responses and, depending on dose administered, this substance could act as either a vasoconstrictor or vasodilator (Rapport, Green and Page, 1948b). The substance was isolated and characterised and in 1949 it was finally identified by Maurice Rapport as serotonin, named after its vasoconstrictor properties (Rapport, Green and Page, 1948a).

One year later in 1950 Gaddum observed that serotonin was present in the brain; he also showed that the action of 5-HT in the gut was antagonised by the hallucinogen, lysergic acid diethylamide (LSD) (Gaddum and Hameed, 1954). Erspamer in the 1950's demonstrated that "enteramine" a substance now known to be serotonin was distributed widely and involved in smooth muscle contraction. Erspamer named serotonin "enteramine" as large amounts of this substance were stored in enterochromaffin cells of the gastrointestinal tract (Erspamer, 1963).

Also in the 1950's, Wooley and Shaw suggested that there was a role for serotonin in mental illness. This was hypothesised due to the knowledge of psychotomimetic activity of serotonin analogues and of LSD. It was not until the 1970's that a role for serotonin in depression was established (Clarke et al, 1975). Underlying this theory was work by a group of Scandinavian scientists who developed selective inhibitors of serotonin uptake and showed these inhibitors to be successful antidepressants (Carlsson et al, 1969).

The discovery and the classification of 5-HT receptors began in 1954 by Gaddum and Hadeem and has since been reviewed to include new developments in the discovery of new receptor subtypes. This has provided a greater understanding of serotonin and its receptors which enabled new drug

development. Presently, there is a substantial amount of information on the neuropharmacology of serotonin (5-HT) which implicates the serotonin system as an important modulator in a variety of central nervous system processes (Green, 2006). These processes include: anxiety, fear, depression and aggression; control of sleep and modulation of ingestive behaviours and the cardiovascular system (Gingrich and Hen, 2001; Hoyer et al, 2002).

1.1- Synthesis and metabolism of serotonin

The precursor amino acid utilised in the biosynthesis of 5-HT is tryptophan. Tryptophan hydroxylase (TPH) catalyzes the hydroxylation of L-tryptophan via the oxidation of tetrahydrobiopterin in the presence of the reductive incorporation of molecular oxygen (Kappock and Caradonna, 1996). This is the first step in the biosynthesis of the indoleamines (serotonin (5-HT) and melatonin) (Martinez, Knappskog and Haavik, 2001), (Figure 1.1).

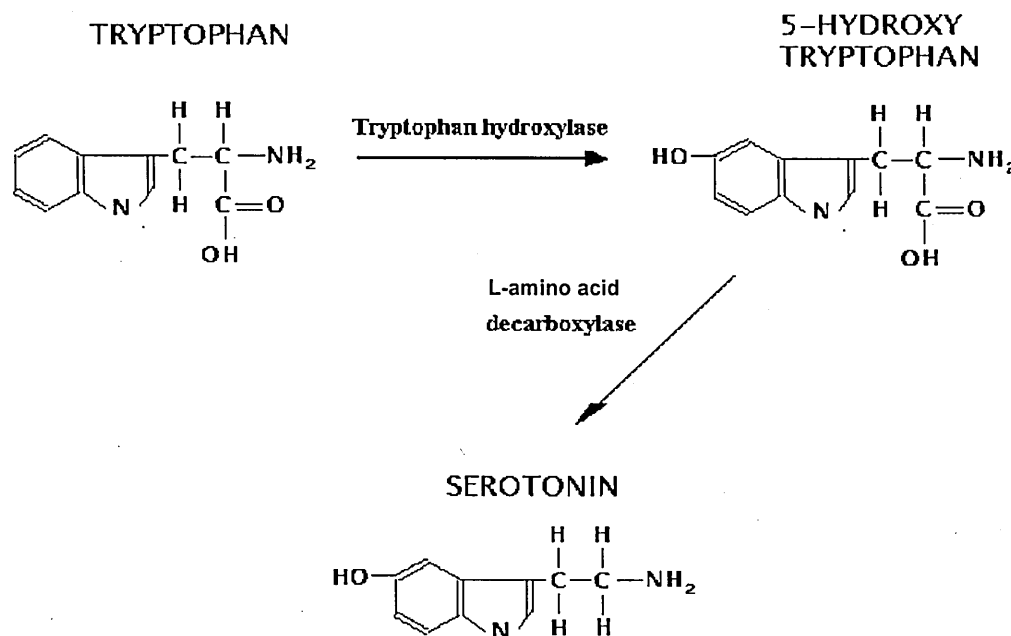


Figure 1.1- Biosynthesis of 5-HT

The conversion of tryptophan to 5-hydroxytryptophan, occurs in the chromaffin cells and neurons. The second step is the decarboxylation of 5- hydroxytryptophan to 5-HT by the aromatic L-amino acid decarboxylase.

In mammalian metabolism of 5-HT, the reaction catalysed by TPH preceding α -decarboxylation is thought to be the rate limiting step in the production of 5-HT (Lovenberg, Jequier and Sjoerdsma, 1967; Jequier, Lovenberg and Sjoerdsma, 1967).

Once 5-HT has been synthesised, 5-HT is stored in secretory vesicles, as the free compound can be rapidly oxidised to 5-hydroxyindoleacetic acid (5-HIAA) through the actions of the enzymes monoamine oxidase and aldehyde dehydrogenase. Stored 5-HT is then released in response to mechanical and neuronal stimuli (Boadle-Biber, 1993).

1.2- Serotonin release and reuptake at a serotonergic synapse

5-HT as previously mentioned is taken up into and stored in storage vesicles, where the neurotransmitter is released into the synaptic cleft only when an action potential occurs at the pre-synaptic neuron. 5-HT then diffuses across the synaptic cleft and can bind with any of the 5-HT receptor classes (1,2,3,4,5,6 and 7) on the post-synaptic neuron.

Levels of synaptic 5-HT are tightly regulated with re-uptake of 5-HT into the presynaptic 5-HT terminals facilitated by the 5-HT transporter (5-HTT). 5-HTT suppresses the action of 5-HT on its receptors by functioning as a sodium-dependent plasma membrane transporter with 5-HT being recycled into the presynaptic terminal (Hranilovic et al, 2004).

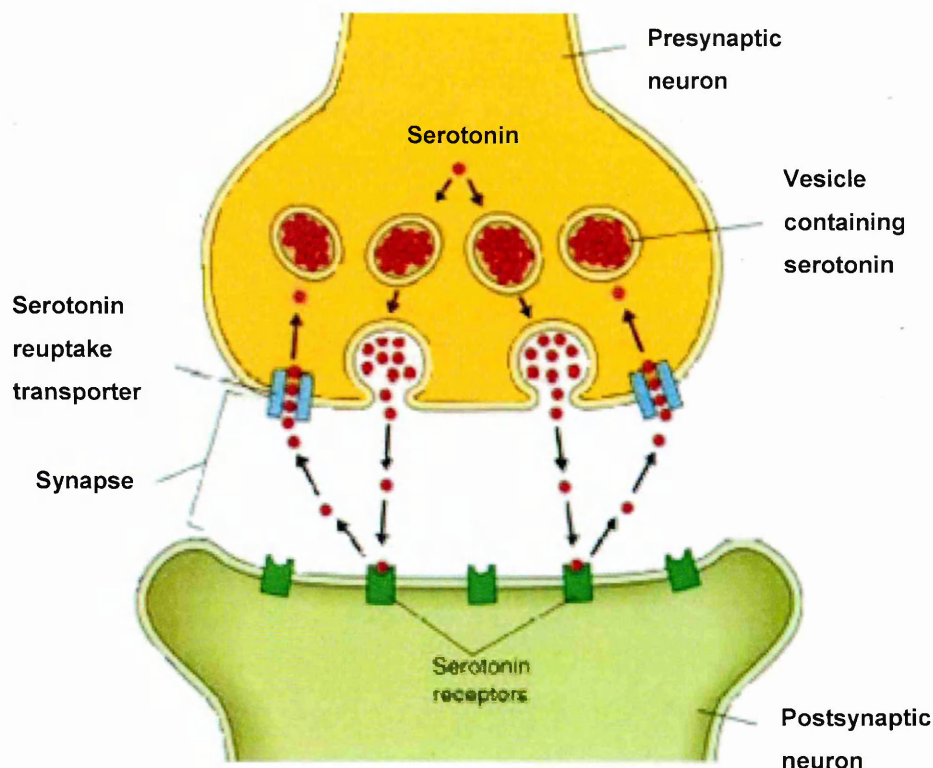


Figure 1.2- 5-HT neurotransmission

An action potential initiates the release of 5-HT from synaptic vesicles into the synaptic cleft. 5-HT binds to 5-HT receptors present on the postsynaptic neuron. Excess 5-HT is reuptaken by 5-HTT or degraded by MAO.

1.3- Receptor subtype identification and classification

The first attempt to categorise 5-HT receptor subtypes was by Gaddum and Picarelli in 1957 although it had been previously published that 5-HT possessed two receptor subtypes by Gaddum and Hadeem in 1954. Gaddum's classification of 5-HT receptors divided them into D and M receptor subtypes. The naming of these receptors was based on the selectivity of the receptor subtypes to dibenzylamine and morphine as blockers. However, Gaddum's proposal was generally accepted to be relevant to peripheral receptors only and not those present in the brain (Gaddum and Picarelli, 1957).

Further research into serotonin led to the discovery of several additional subtypes and classes of 5-HT receptors. The nomenclature system has subsequently been adapted to align it with the human genome (Hoyer and Martin, 1997).

The current classification of 5-HT receptors and their subtypes are based on their molecular structure, signal transduction pathway and operational properties (Kelly, 1995). There are seven classes of 5-HT receptors 5-HT₁-5-HT₇, with some of these receptor classes being further subdivided into subtypes such as 5-HT_{1A}, B, D, E and F (Hoyer and Martin, 1997).

In accordance with the recommendations of NU-IUPHAR (the main IUPHAR nomenclature committee), newly described recombinant receptors are described in lower case i.e. 5-ht_n and described in uppercase only when operational and transductional information are available.

1.3.1- 5-HT₁ receptors

The 5-HT₁ receptor class is subdivided into five receptor subtypes (5-HT_{1A}, 1B, 1D, 1E and 1F), which share 40-63% overall sequence identity in humans and couple preferentially, although not exclusively to, G_{i/o} to inhibit cAMP formation.

The 5-HT_{1A} receptor is widely distributed throughout the CNS in particular in the raphe nuclei and limbic structures including the hippocampus. The 5-HT_{1A} receptor will be discussed in more detail in section 1.4.2. 5-HT_{1B} receptors are expressed in the CNS and thought to be concentrated in the basal ganglia, striatum and frontal cortex. This receptor subtype may function as terminal autoreceptors (Pauwels, 1997). 5-HT_{1D} receptors have been located in the human heart where they modulate 5-HT release (Hoyer et al, 2002) whereas the 5-HT_{1E} receptor was first identified in binding studies in homogenates of human frontal cortex (Bruinvels et al, 1994). Bai et al, (2004) have cloned and characterised the 5-HT_{1E} receptor from guinea pig genomic DNA. The 5-HT_{1E} receptor gene shares 95% sequence homology with the human receptor. Presently, little is known about the distribution and function of the 5-HT_{1F} receptor, mRNA for the human receptor protein has been identified in the brain particularly in the dorsal raphe, hippocampus and cortex (Hoyer et al, 2002).

1.3.2- 5-HT₂, 5-HT₃ and 5-HT₄ receptors

The 5-HT₂ class comprises of the 5-HT_{2A}, 2B and 2C subtypes which exhibit 46-50% overall sequence identity and this receptor class preferentially couple to G_{q/11} to increase the hydrolysis of inositol phosphates and elevate cytosolic calcium. The 5-HT_{2A} receptor is widely distributed in peripheral and central tissues with particularly high expression found in cortical regions (Eison and Mullins, 1996). 5-HT_{2B} receptor expression has been found in organs including vascular smooth muscle (Ullmer et al, 1995) spinal cord (Helton and Colbert, 1994) and the brain (Choi and Maroteaux, 1996). 5-HT_{2C} receptors are found in the choroid plexus (Pazos, Hoyer and Palacios, 1984), the cortex, basal ganglia, hippocampus and hypothalamus (Molineaux et al, 1989).

5-HT₃ receptors can be found on neurones of both central and peripheral origin, the CA1 pyramidal cell layer in the hippocampus and the dorsal motor nucleus (Hoyer and Martin, 1997). The 5-HT₄ receptor is widely distributed within the CNS and peripheral tissues where it is thought to play an important role in the function of several organ responses including the alimentary tract, urinary bladder, heart and adrenal glands (Hedge and Eglen, 1996).

1.3.3- 5-HT₅, 5-HT₆ and 5-HT₇ receptors

The 5-HT₅ and 5-HT₆ receptor classes are both putative. To date there is no evidence to confirm the expression of the 5-HT₅ receptor endogenously, whereas the 5-HT₆ receptor has been shown to be expressed endogenously in neuronal tissue (Hoyer and Martin, 1997). 5-HT₇ receptors share low homology (<50%) with other members of the 5-HT receptor family and are mainly found in CA2 and CA3 pyramidal layers of the hypothalamus and in the human stomach, the descending colon, ileum and coronary artery (Bard et al, 1993).

5-HT1					
Nomenclature	5-HT _{1A}	5-HT _{1B}	5-HT _{1D}	5-HT _{1E}	5-HT _{1F}
Selective agonists	8-OH-DPAT	Sumatriptan	Sumatriptan	-	LY334370
Radioligands	[³ H]WAY100635	[¹²⁵ I]GTI	[¹²⁵ I]GTI	[³ H]5-HT	[¹²⁵ I]LSD
G protein effector	G _{i/o}	G _{i/o}	G _{i/o}	G _{i/o}	G _{i/o}

5-HT2,3 and 4					
Nomenclature	5-HT _{2A}	5-HT _{2B}	5-HT _{2C}	5-HT ₃	5-HT ₄
Selective agonists	Ketanserin	SB200646	Mesulergine	Granisetron	GR113808
Radioligands	[¹²⁵ I] DOI	[³ H]5-HT	[¹²⁵ I]LSD	[³ H](S)zacopride	[¹²⁵ I]SB207710
G protein effector	G _{q/11}	G _{q/11}	G _{q/11}	-	G _s

5-HT5,6 and 7				
Nomenclature	5-HT _{5A}	5-HT _{5B}	5-HT ₆	5-HT ₇
Selective agonists	-	-	-	-
Radioligands	[¹²⁵ I] LSD	[¹²⁵ I]LSD	[¹²⁵ I]SB25885	[¹²⁵ I]LSD
G protein effector	G _{i/o}	None identified	G _s	G _s

Table 1.1- 5-HT receptor nomenclature

Abbreviations: G_{q/11}- activates phospholipase C, G_{i/o}- Inhibits adenylyl cyclase, G_s- Stimulatory G-protein activates adenylyl cyclase. Modified from Hoyer and Martin (1997).

1.4 - Serotonergic system

The serotonergic system has been implicated in a vast array of physiological and behavioural processes in vertebrates by exerting a tonic and modulatory influence on a variety of targets (Jacobs and Azmitia, 1992). The serotonergic system has also been implicated in the pathophysiology of depression and anxiety. The most compelling evidence involves the alleviation of depression observed when selective serotonin reuptake inhibitors (SSRIs) have been administered. SSRIs increase the availability of 5-HT at the synapse (Malagie et al, 2002).

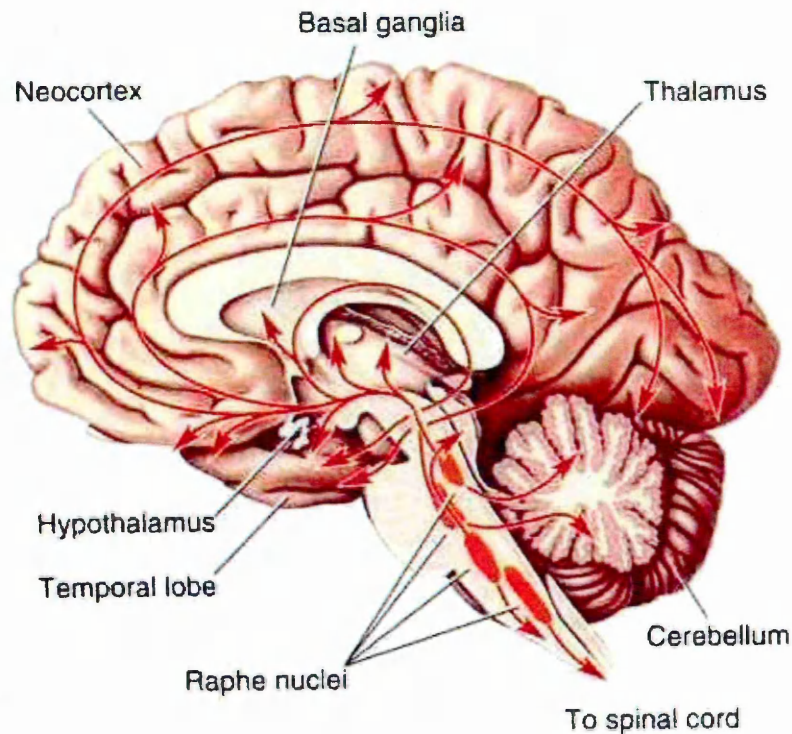


Figure 1.3- The serotonergic system

Ascending projections arise from the dorsal and median raphe nuclei and travel through to target regions including the limbic system (hippocampus, hypothalamus and amygdala), striatum and cerebral cortex. The descending projections arise from the raphe magnus and project towards the spinal cord (Bear, Connors and Paradiso, 2001).

1.4.1- Anatomical localisation

5-HT secreting neurons are distributed and contained throughout the brain and the gastrointestinal tract.

The gut is the main source of 5-HT in the body (Vialli and Erspamer, 1937). Enterochromaffin cells in the mucosa contain >90 percent of the body's 5-HT. It has recently been recognised that 5-HT is also contained in intrinsic neurons of the gastrointestinal tract (De Ponti, 2004). In the gut, 5-HT is an important mucosal signalling molecule that targets enterocytes, smooth muscle cells and enteric neurons (De Ponti, 2004). 5-HT is thought to be involved in the

pathophysiology of a number of clinical entities such as functional gut disorders including irritable bowel syndrome.

5-HT acts as a mucosal signalling molecule for mucosal enterochromaffin cells which act as sensory transducers that respond to mechanical pressure (Bulbring and Crema, 1959) or nutrients (Kim, Cooke and Javed, 2001; Raybould et al, 2003; Fukumoto, Takewaki and Yamada, 2003) to secrete 5-HT into the wall of the bowel and initiate peristaltic (Grider, Kuemmerle and Jin, 1996) and secretory reflexes (Cooke, 2000).

The central nervous system accounts for 5 percent of the 5-HT present in the body with 5-HT being present within several areas of the midbrain including the hippocampus, frontal cortex, limbic system and the hypothalamus (Tork, 1990). Within the central nervous system (CNS) 5-HT acts as a neurotransmitter and high concentrations of 5-HT can be predominantly found in localised nerve projections of the mid-brain particularly in several large clusters of cells referred to as Raphe nuclei (Rang and Ritter, 1999), (Figure 1.3).

1.4.2- Anatomical localisation of 5-HT_{1A} receptors

1.4.2.1 - Raphe nuclei

The raphe nuclei are distributed near the midline of the brainstem along its entire rosto-caudal extension (Meessen and Olszewsky, 1949). The raphe nuclei are distributed near are a group of nuclei at the centre of the reticular formation present in the midbrain, pons and the medulla which are all part of the brain stem. The serotonergic neuron clusters are allocated on the basis of their distribution and main projection, into two groups: the rostral group with major projections to the forebrain, and the caudal group with major projections to the spinal cord (Hornung, 2003). 85 percent of serotonergic neurons in the brain are part of the rostral group. These serotonergic pathways are believed to be distributed capaciously throughout the brainstem, the cerebral cortex and the spinal cord. Presynaptic 5-HT_{1A} receptors are located primarily on cell bodies (soma) and dendrites such as somatodendritic neurons of the dorsal raphe and are thought to act as inhibitory autoreceptors that exert a negative feedback

influence on 5-HT neuronal firing (Nestler et al, 2001; Blier and Ward, 2003; Stahl, 1996; Ou et al, 2000).

In the raphe area the activation of 5-HT_{1A} autoreceptors reduces the release of 5-HT at the level of terminals in the hippocampus (Sharp and Hjorth, 1990; Blier, Serrano and Scatton, 1990). It is therefore assumed that changes in the firing rate of 5-HT neurons which is induced by drugs acting at 5-HT_{1A} receptors can alter the level of activation of post-synaptic 5-HT receptors in the brain (Mongeau, Blier and de Montigny, 1997).

1.4.2.2 - Limbic system

The limbic system is made from several heavily interconnected nuclei and several regions of the cerebral cortex. The limbic system including the hypothalamus, cingulate gyrus and the hippocampus and their interconnections comprise a harmonious mechanism, which involve the function of central emotion as well as participating in emotional expression (Morgane et al, 2005; Papez, 1937).

1.4.2.3 - Hippocampus

The hippocampal formation is one of the most complex and vulnerable brain structures which is recognised as a crucial brain area subserving the human long-term memory (Henke et al, 1999). The hippocampal formation consists of the dentate gyrus and the cornu ammonis (CA) plus the subiculum collectively known as the hippocampus.

The hippocampus has sensory inputs arriving to the entorhinal cortex which are relayed to granule cells of the dentate gyrus. Synapses on CA3 pyramidal neurons are formed from "mossy" fibres composed of axons arising from granule cells. CA1 pyramidal neurons project to the subiculum which provides the main output of the hippocampus (Mongeau, Blier and de Montigny, 1997) (Figure 1.4).

5-HT stimulates 5-HT_{1A} receptors which are localised on both excitatory pyramidal and granule neurons (Gulyas, Acsadi and Freund, 1999) resulting in neuronal hyperpolarisation and inhibition of neuronal activity in the hippocampus (Schmitz et al, 1998). 5-HT acting via its 5-HT_{1A} receptor has been implicated in rhythmic slow activity, which has been associated with learning and memory processes in the hippocampus (Vanderwolf and Barker, 1986; McEntree and Crook, 1992 and Pompeiano et al, 1992).

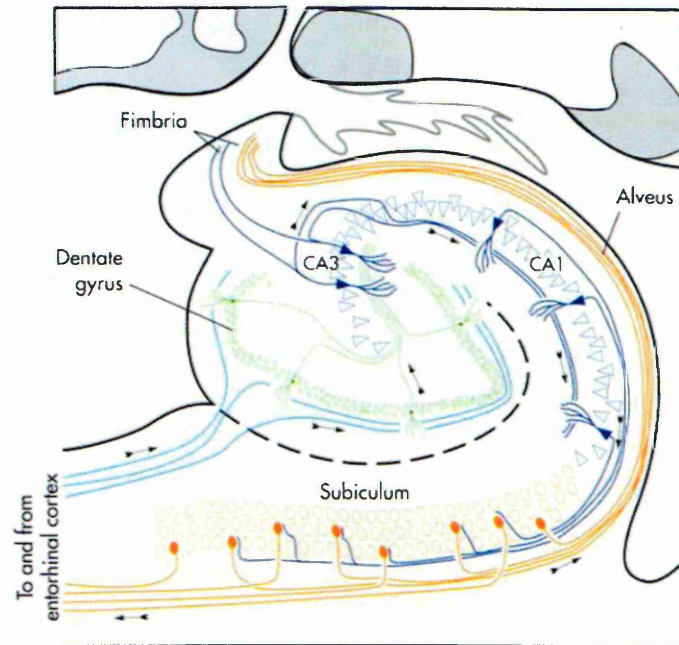


Figure 1.4- Schematic representation of the hippocampus

The hippocampal pyramidal neurons (CA1-3) and the dentate gyrus granule cells (DG) receive a direct cortical input via the perforant pathway from the entorhinal cortex (EC). Hippocampal pyramidal neurons (CA1) and the subiculum are involved in hippocampal cortical output to associated limbic cortices. 5-HT_{1A} receptors are located on both pyramidal CA1-3 neurons and on granule neurones present in the DG (Nolte and Angevine, 2000).

1.4.2.4- Function of the Hippocampus

The hippocampus is believed to be involved in information processing and behaviour (Mongeau, Blier and de Montigny, 1997). The memory formation function of the hippocampus comprises a core structure of the medial temporal

lobe where information about relationships, combinations, and conjunctions among and between stimuli is processed (Riedel and Micheau, 2001). Both the medial temporal lobe and the hippocampus (CA, DG, and subiculum) are the place for either the temporary storage of to-be consolidated information (Squire, 1992) or as the locus of permanent information storage through multiple memory traces (Nadel and Moscovitch, 1997).

Specific encoding of new information requires the dentate gyrus granule cells and processes of memory consolidation, either short-term or long-term, by contrast, should depend on the network activity of the hippocampus proper (CA1-CA3), (Riedel and Micheau, 2001).

1.4.2.5 - Serotonergic transmission in the hippocampus

1.4.2.6- Pre-synaptic mechanisms in the hippocampus

5-HT neuron firing activity is credited to a pacemaker cycle that involves a calcium dependent potassium current. The discharge rate of these neurons is mediated by 5-HT_{1A} autoreceptors localised in the somatodendritic region on the presynaptic neuron (Mongeau, Blier and de Montigny, 1997). The activation of 5-HT_{1A} autoreceptors is regulated by an hyperpolarisation of the membrane occurring by the opening of potassium channels (Aghajanian and Lakoski, 1984; Sprouse and Aghajanian, 1987; Williams, Henderson and North, 1985).

Contrary to somatodendritic autoreceptors of the raphe area, terminal presynaptic 5-HT autoreceptors of the hippocampus are not of the 5-HT_{1A} subtype and control the release of 5-HT without interfering with the propagation of action potentials (Starke, Gothert and Kilbinger, 1989). Terminal 5-HT autoreceptors in mammalian species excluding rodent are of the 5-HT_{1D} subtype (Mongeau, Blier and de Montigny, 1997).

The majority of studies agree that terminal 5-HT autoreceptors reduce the release of 5-HT by reducing the calcium influx via voltage dependent calcium channels (Starke, Gothert and Kilbinger, 1989).

1.4.2.7 – Post-synaptic mechanisms in the hippocampus

Activation of postsynaptic 5-HT_{1A} receptors, results in an inhibition of the activity of neurons of the limbic system (Sprouse and Aghajanian, 1988).

There are two subsets of post-synaptic 5-HT_{1A} receptors in the hippocampus that differentially couple to G-proteins that suppress pyramidal cell firing. Extrasynaptic 5-HT_{1A} receptors are located on the soma of hippocampal pyramidal cells, which can be activated by microiontophoretic application of agonists and are inactivated by pertussis toxin (Blier, de Montigny and Lista, 1993). However, the other subset intrasynaptic 5-HT_{1A} receptors are localised on dendrites of hippocampal pyramidal cells and activated by endogenous 5-HT. Intrasynaptic 5-HT_{1A} receptors are un-affected by pertussis toxin (Blier, de Montigny and Lista, 1993). It has therefore been hypothesised that extrasynaptic 5-HT_{1A} receptors are coupled with G_{i/o} proteins, whereas intrasynaptic 5-HT_{1A} receptors are not.

1.4.2.8 - Other Limbic brain regions

The prefrontal cortex, amygdala-hippocampus complex, thalamus, basal ganglia are all present in the proposed neuroanatomic model of mood regulation (Soares and Mann, 1997). The previously mentioned brain areas are thought to have extensive interconnections, the two major neuroanatomic circuits in the brain believed to be involved in mood regulation are the limbic-thalamic-cortical circuit comprising of the amygdala, mediodorsal nucleus of the thalamus and medial and ventrolateral prefrontal cortex and the second major circuit a limbic-striatal-pallidal-thalamic-cortical circuit, that includes the striatum, ventral pallidum and other regions (Soares and Mann, 1997).

1.4.2.9 - Prefrontal Cortex (PFC)

The prefrontal cortex can be divided into the ventromedial and dorsolateral regions, each of which is associated with posterior and subcortical brain regions (Wood and Grafman, 2003).

The ventral medial PFC has been strongly implicated in the expression of behavioural, neuroendocrine and autonomic responses to emotionally relevant stimuli. Recent imaging studies have indicated that abnormalities in structure and function of this region is present in patients with mood disorders (Drevets et al, 1997; Kennedy et al, 2001). Tracing studies have shown that 5-HT pathways ascend from the midbrain dorsal (DRN) and median raphe nuclei (MRN) which project extensively in to the ventral mPFC (O'Hearn and Molliver, 1984; Steinbusch, 1981). This region has also been shown to contain a high density of 5-HT transporter sites (Battaglia et al, 1991; Herbert et al, 2001) and 5-HT receptors including 5-HT_{1A} and 5-HT_{2A} (Pazos and Palacios, 1985; Pompeiano et al, 1992). The DRN and MRN are thought to receive projections from the ventral mPFC (Hajos et al, 1998; Peyron et al, 1998; Sesack et al, 1989; Varga et al, 2001) activation of which has been shown to mediate 5-HT neuronal activity (Celada et al, 2001; Hajos et al, 1998; Varga et al, 2001).

From anatomical and electrophysiological observations it has been suggested that there is an excitatory mPFC-DRN projection that brings about inhibition of 5-HT neurones in the DRN through the activation of local raphe GABA neurones (Hajos et al, 1998; Varga et al, 2001).

1.4.2.10- Hypothalamus

The hypothalamus's function is to mediate many neuroendocrine functions. The hypothalamus is a highly organised structure. In depression, the hypothalamus has been studied in regard to the hypothalamic-pituitary-adrenal (HPA) axis (Nestler et al, 2002). The HPA axis involves corticotrophin-releasing factor (CRH) and vasopressin (AVP) which are both produced in the parvocellular neurons of the hypothalamic paraventricular nucleus (PVN).

It has been shown that serotonergic mechanisms exert an excitatory influence on the HPA axis (Chaouloff, 1993) and 5-HT has also been shown to elicit the release of ACTH release directly from the pituitary (Spinedi and Negro-Vilar, 1983) by activation of 5-HT_{1A} and 5-HT_{2A} receptors (Calogero et al, 1990; Ritten-House et al, 1994).

The 5-HT system acting through 5-HT_{1A} receptors may be able to mediate the negative feedback control of the HPA axis.

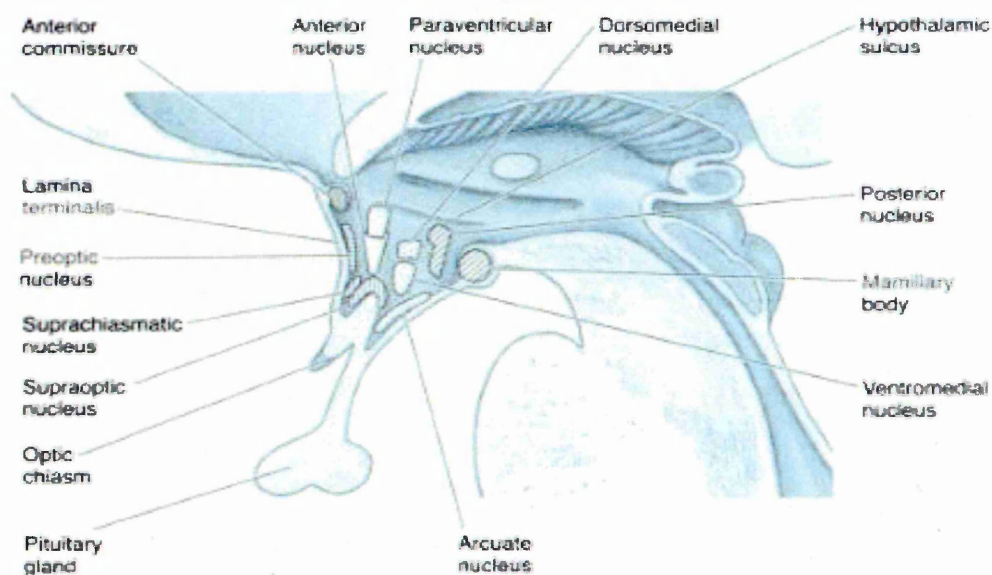


Figure 1.5 - Diagram of the cross section of the hypothalamus

The hypothalamus is divided into a lateral area and a medial area which are separated by the fornix and the mamillothalamic tract. The lateral hypothalamic area includes the preoptic nucleus and the medial hypothalamic area includes the supraoptic region, anterior nucleus, and the supraoptic nucleus (Afifi and Bergman, 2005).

1.4.2.11 Amygdala

Several studies have shown the role of the amygdala to be associated with conditioned fear (Davis, 1998; Catill et al, 1999; Le Doux, 2000). The amygdala is thought to mediate the ability of previously non-threatening stimuli, when associated with naturally frightening stimuli (including the exposure to severe stress) to elude a wide range of stress responses (Nestler et al, 2002).

Postsynaptic 5-HT_{1A} receptors can be found in the amygdala mainly in the central nucleus (Pazos and Palacios, 1985; Ohuoha et al, 1993), (Figure 1.6). Recent data has shown that somatodendritic 5-HT_{1A} autoreceptors in the claudal linear raphe nucleus are capable of modulating extracellular 5-HT levels in this area by reducing 5-HT cell firing by an as yet unknown pathway (Bosker, Klompmakers and Westenberg, 1997).

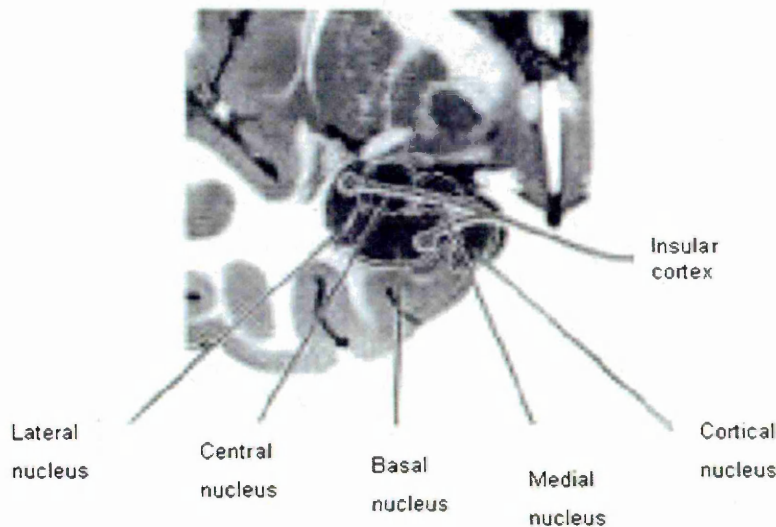


Figure 1.6- Cross- section of the Amygdala

The amygdala is a complex of nuclei which are divided into three main groups the lateral nuclei, the cortical nucleus and the central nucleus (Gilliam et al, 2005).

1.5- Non 5-HT Neurotransmitter systems

Serotonergic neurons in the central nervous system impinge on many other neurons and modulate their neurotransmitter release. Serotonergic neurons interact with GABAergic, noradrenergic, cholinergic and dopaminergic neurons.

1.5.1- GABAergic

γ -Aminobutyric acid (GABA), a neurotransmitter that is inhibitory, is present in the CNS and is distributed across all brain regions (Zachmann, Tocci and Nyhan, 1966).

In the majority of brain regions the release of GABA is controlled by inhibitory presynaptic 5-HT_{1A} receptors that are present at GABAergic nerve terminals. This control of GABA release is thought to be caused by the inhibition of adenylyl cyclase and cAMP signal transduction pathway. This pathway directly acts on the GABA release process in independent K⁺ and Ca²⁺ channels (Koyama et al, 1999). Hence, this pathway has a negative effect on GABA release.

The hypothesised involvement of GABAergic dysfunction in mood disorders came from a study by Enrich et al (1980). The study looked at the mood stabiliser valproate which is used as an effective treatment for bipolar patients. The pathophysiology of mood disorders has been linked with a GABAergic deficiency (Enrich et al, 1980). Preclinical animal studies have also shown that GABA levels may be decreased in animal models of depression and clinical studies have reported low plasma and CSF GABA levels in mood disorder patients (Bambilla et al, 2003).

1.5.2- Noradrenergic system

The Noradrenergic system runs parallel with the serotonergic system, with the noradrenergic system being a valuable target for antidepressants. Throughout

the brain norepinephrine functions as a general regulator of mood responses to stimuli including stress (Wang et al, 1999).

In the hippocampus NA terminals are thought to originate exclusively from the locus coeruleus (LC) (Haring and Davis, 1985; Jones and Moore, 1977). It is generally accepted that an increase in the availability of NA in the biophase of adrenoceptors in this brain region is involved in the mechanism of action of antidepressants. It has also been suggested that 5-HT receptor ligands that enhance NA release may be expected to positively influence mood disorders (Fink and Gothert, 2007).

Inhibitory presynaptic 5-HT_{1B/1D} receptors have been identified on the noradrenergic axon terminals of the cardiovascular system of various species both *in vitro* and *in vivo* (Charlton et al, 1986; Gothert et al, 1986; Medhurst et al, 1997 and Harris et al, 2002). Therefore, it has been hypothesised that noradrenergic nerve terminals in the CNS could also have inhibitory presynaptic 5-HT_{1B/1D} receptors present (Taube et al, 1977; Schlicker et al, 1983).

Microdialysis studies in awake rats in which 5-HT_{1A} ligands were injected subcutaneously provided the first evidence for the involvement of 5-HT_{1A} receptors in the regulation of NA release. An increase in NA release in the hippocampus (Done and Sharp, 1994; Hajos-Korcsok and Sharp, 1996) and the frontal cortex (Suzuki et al, 1995) was observed in the presence of 8-OH-DPAT (a 5-HT_{1A} agonist). The increasing effect observed with 5-HT_{1A} receptor agonists on NA release was suggested to be due to activation of somadendritic 5-HT_{1A} receptors on the serotonergic neurons themselves (Done and Sharp, 1994). GABAergic activity is inhibited by the activation of 5-HT_{1A} receptors thought to be located presynaptically on GABAergic neurons leading to a reduction of GABA and consequently a disinhibition of NA release (Katsurabayashi et al, 2003).

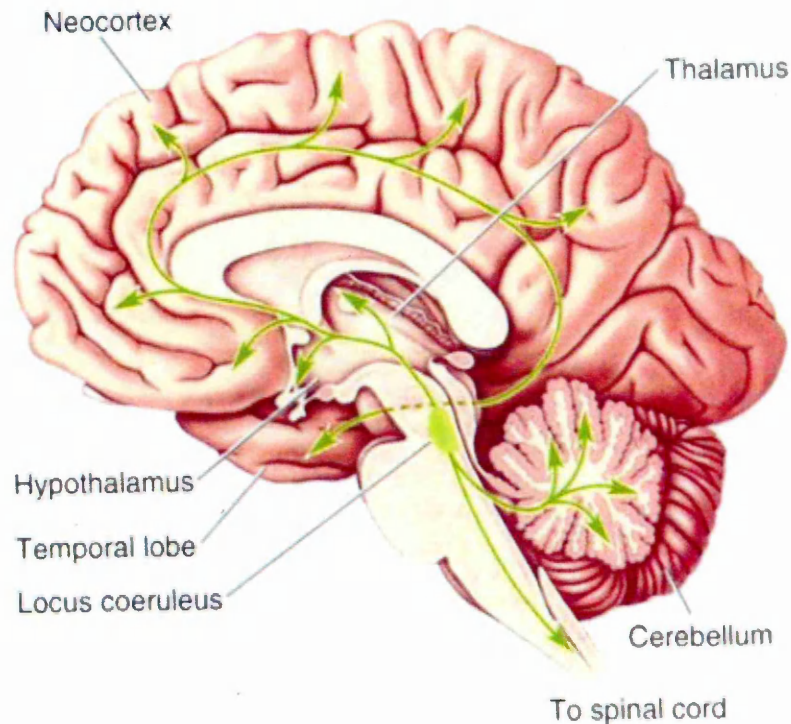


Figure 1.7- The noradrenergic system

The noradrenergic system extends from the locus coeruleus sending projections towards the neocortex, thalamus, hypothalamus and cerebellum (Bear, Connors and Paradiso, 2001).

1.5.3- Cholinergic system

The cholinergic system is important for the role of memory and cognition (Cassel and Jeltsch, 1995; Steckler and Sahgal, 1995; Feuerstein and Seeger, 1997; Ruotsalainen et al, 1998), an observed increase in acetylcholine concentration in the synaptic cleft represents a therapeutic option in dementia which is associated with Alzheimer's disease.

Immunohistochemical double staining has demonstrated that 5-HT_{1A} receptors occur on cholinergic cell bodies in the septum and project to the hippocampus and neocortical areas (Kia et al, 1996). In the presence of 5-HT_{1A} receptor agonists such as 8-OH-DPAT inhibitory somatodendritic autoreceptors on

serotonergic neurons are activated. The activation of these autoreceptors inhibits the activity of serotonergic neurons and therefore, there is an assumed decrease in the stimulation of inhibitory GABAergic interneurons, which, in turn leads to the disinhibition of cholinergic neurons (Fink and Gothert, 2007).

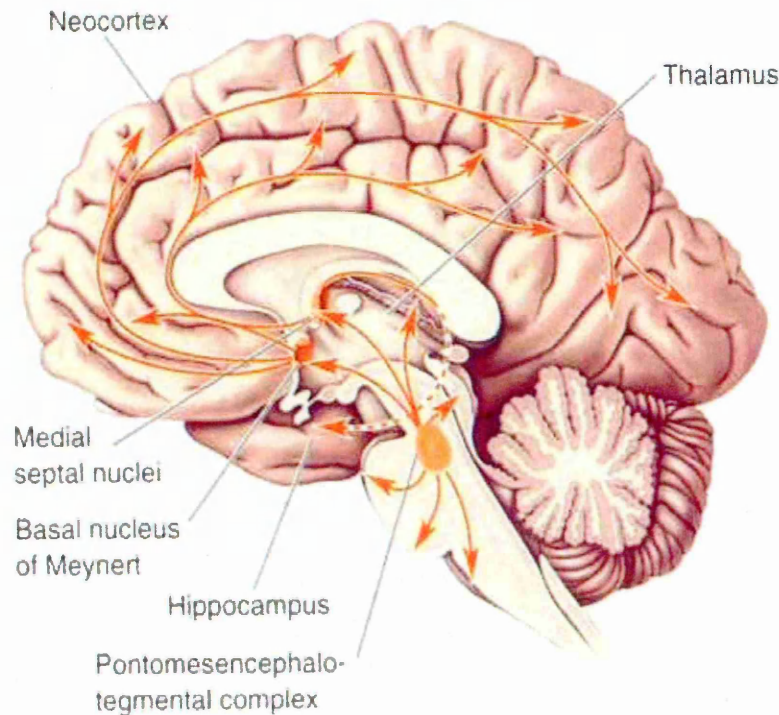


Figure 1.8- Cholinergic system

Ascending projections arise from the pontomesencephalo-tegmental complex towards the hippocampus, neocortex and the thalamus (Bear, Connors and Paradiso, 2001).

1.5.4- Dopaminergic system

The dopaminergic system in the brain arises from the midbrain and in particular from a group of cells in this region and from the hypothalamus (Figure 1.9), (Kapur and Mann, 1992). The rat and mouse brain have been used to investigate the mechanism in which 5-HT receptors regulate dopamine (DA) release.

5-HT_{1A} receptors are thought to increase DA release and it is likely that the 5-HT_{1A} receptors are located as pre-and/or postsynaptic 5-HT_{1A} receptors on inhibitory GABAergic interneurons which exert an inhibitory tone on the activity of the dopaminergic neurons (Fink and Gothert, 2007).

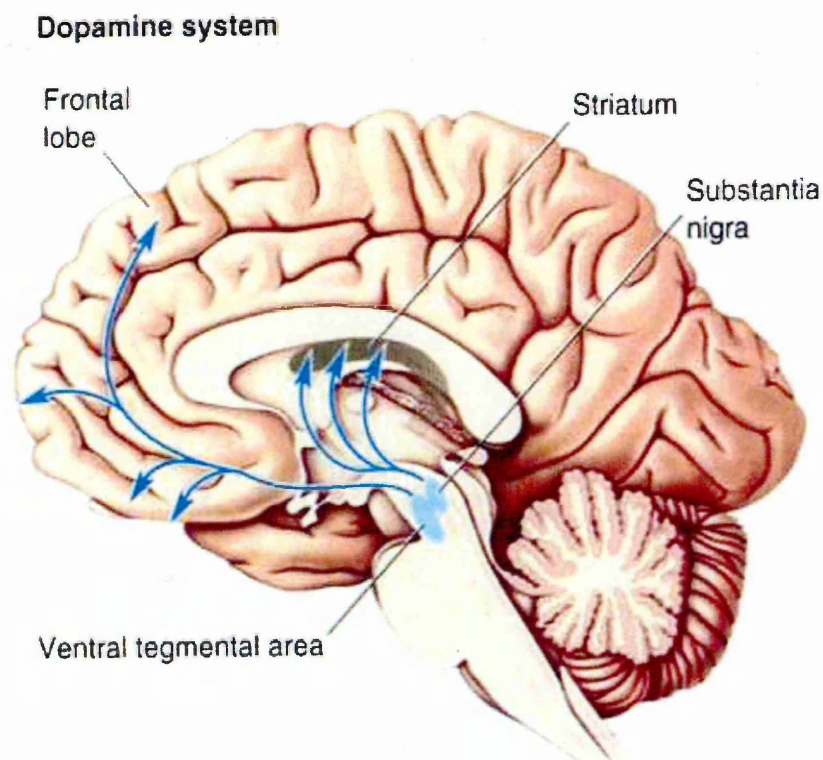


Figure 1.9- Dopaminergic system

Mesolimbic and mesocortical systems arise from the ventral tegmental area. The mesolimbic system projects to elements of the limbic system including the hippocampus and amygdala. The mesocortical system projects to the frontal cortex. (Bear, Connors and Paradiso, 2001).

The serotonergic system as previously mentioned interacts with several non-serotonergic neurons within the CNS and these neurotransmitter systems are present within the limbic region of the brain i.e. the hypothalamus, amygdala and the hippocampus with projections expanding to the dorsal raphe (DR), the locus coeruleus and the pre-frontal cortex (PFC) as summarised in Figure 1.10. The figure shows a simplified summary of a series of neurotransmitter neurons in the brain, which interact with the afore-mentioned brain regions and thus contribute to depressive symptoms.

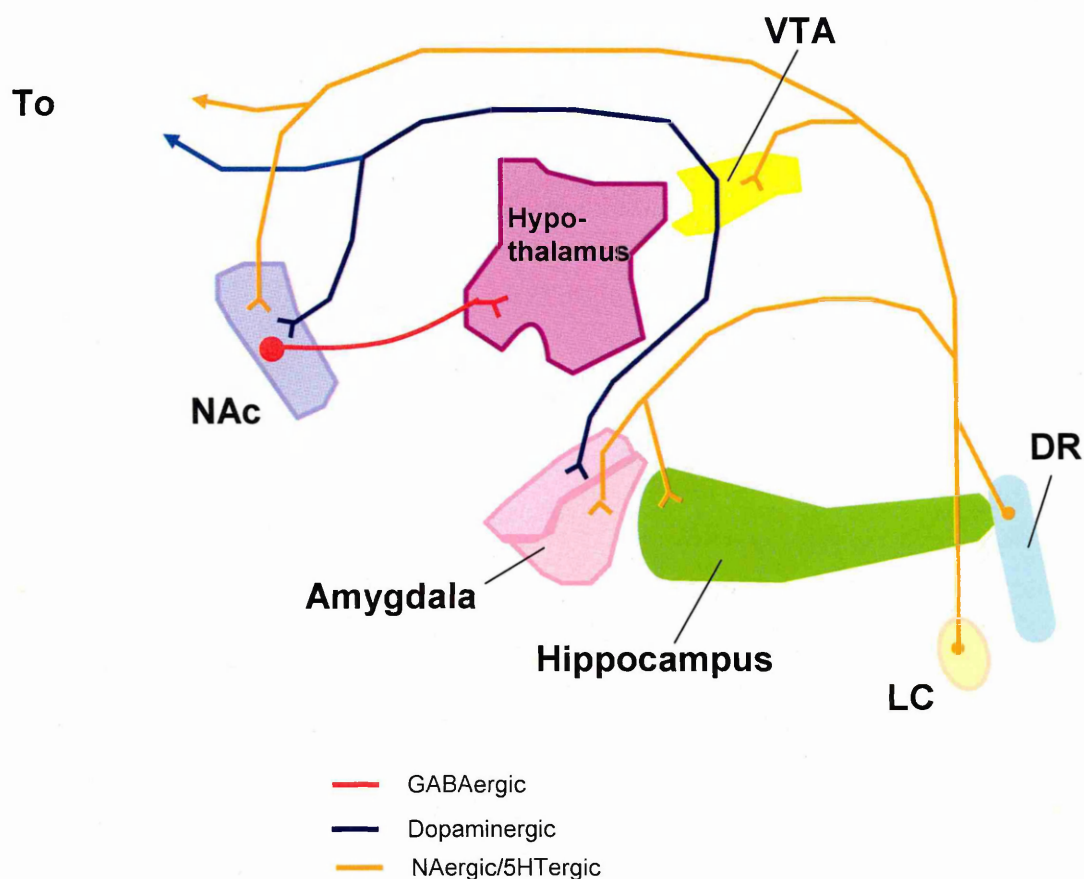


Figure 1.10- Summary of the neural circuitry of the brain

Schematic representation showing the interconnections of neural circuitry in the brain.

Abbreviations: NAc- nucleus accumbens, VTA- ventral tegmental area. Modified from Nestler et al, 2002.

1.6- Depression

Depressive disorders are amongst the most common psychiatric diseases with prevalence estimates ranging from 5 percent to a maximum of 20 percent (Hamet and Tremblay, 2005). Less severe forms of depression may affect an additional 10 percent of the American population. The interaction between genes and the environment have been acknowledged to play a role in the pathophysiology of depression (Hamet and Tremblay, 2005).

Depression is recurrent and tends to have chronic course, and can often be comorbid in nature. It is thought that depression is a clinically heterogeneous disorder thought to result from an interaction of multiple genes cooperating with environmental and developmental epigenetic components (Hamet and Tremblay, 2005).

There are two etiologically different forms of depression, bipolar disorder (manic depression) and unipolar disorder (Lesch, 2004). There are also many different symptoms of depression including disturbance of mood, thinking, sleep, appetite, and motor activity, with suicidal thoughts or attempts that occur to different degrees (American Psychiatric Association, 1994).

1.6.1- Major depression (Unipolar)

Major depression (unipolar) is a serious medical condition. The risk of developing major depression is thought to be approximately one in ten of the population at some time in their lives and twice as great among women than amongst men in almost all cultures studied (Elliot, 1998). Major depression is characterised by sad mood, loss of interest, sleep disturbances and recurrent thoughts of death and suicide (Rajkowska, 2003; Lucki, 1998). In the United States and worldwide, major depression is the leading cause of disability (Costello et al, 2002). 80 percent of people with clinical depression are treated successfully with medication, psychotherapy or a combination of both. However, if clinical depression is left untreated or is inadequately treated it can often lead to suicide (Rajkowska, 2003).

Many individuals suffering from panic disorder (PTSD) or other anxiety related disorders tend to develop major depression. This observation has lead to the thought that there may be overlapping neural circuitry involved in the pathophysiology of depression and that of certain anxiety disorders.

1.6.2- Bipolar depression (Manic Depressive illness)

Bipolar depression is a major public health problem. It is estimated that there is a 0.3-1.5 percent worldwide lifetime prevalence of bipolar depression (Weissman, et al, 1996). Bipolar depression has also been associated with a mortality risk; approximately 25 percent of patients attempt suicide at some point during their lives and 11 percent of patients die by suicide (Prien and Potter, 1990). Bipolar depression is also characterised by familial transmission the incidence of bipolar depression among first-degree relatives of affected individuals is 8-25 percent.

This type of depression is often characterised by episodes of mania, with or without distinct episodes of depression. Mania is characterised by euphoria or irritability, increased energy, and a decreased need for sleep (American Psychiatric Association, 1993).

Both manic and depressive states of this disorder are thought to be due to low serotonergic function through defective dampening of other neurotransmitters such as, norepinephrine and dopamine (Hilty, Brady and Hales, 1999). Lesions in the frontal and temporal lobes are linked with bipolar disorder. Left-sided lesions are related with depression and right-sided lesions are associated with mania (Hilty, Brady and Hales, 1999).

For the treatment of bipolar disorder lithium is effective for the treatment of acute manic and depressive episodes and for the prevention of recurrent manic and depressive episodes (Goodwin and Jamison, 1990).

1.6.3- Stress-induced depression

Stress and the HPA axis have both been implicated as a factor involved in the onset of depression (Taylor et al, 2004).

The release of stress hormones, such as cortisol and also corticotrophin-releasing hormone (CRH), which are secreted from the hypothalamus; this occurs in many individuals diagnosed with mood disorders and may result from hyperfunctioning of the amygdala (which is known to activate the paraventricular nucleus of the hypothalamus (PVN)), or by the hypofunctioning of the hippocampus (which exerts a potent inhibitory influence on the PVN), (Young, Lopez and Murphy-Weinberg, 2003; Muller et al, 2002). The release of stress hormones from the hypothalamus in turn stimulates the release of glucocorticoids from the adrenal cortex (Liberzon, Krstov and Young, 1997). Excessive amounts of glucocorticoids can be damaging and therefore the HPA axis is under tight regulation by a negative feedback system (Liberzon, Krstov and Young, 1997) which occurs mainly through mineralocorticoid and glucocorticoid receptors (Young, Lopez and Murphy-Weinberg, 2003).

Cortisol in humans is the main glucocorticoid that modulates metabolism, induces catabolism, suppresses the immune system and is thought to have temporary elevating effects on mood and emotions, especially fear and anxiety (Muller et al, 2002). Short-term administration of glucocorticoids often generates euphoria and increased energy in patients with depression. However, the long-term increased levels of endogenous glucocorticoids produced during depression can be toxic to hippocampal neurons in both animals and humans. The hippocampus is required for the feedback inhibition of CRF neurons. Episodes of depression marked by severe hypercortisolemia may produce further impairment in the feedback regulation of the HPA axis or an impaired cortisol negative feedback mechanism and therefore, predispose affected individuals to chronic depression or future recurrences (Nemeroff, 1996; Young et al, 1995).

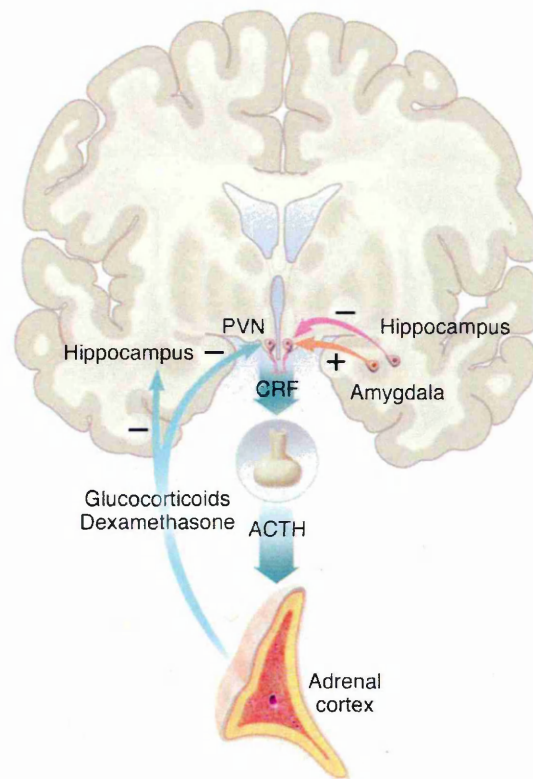


Figure 1.11- HPA axis

The HPA axis receives prominent neural inputs that include excitatory afferents from the amygdala. The HPA axis may contribute to depression not only via the amygdala and inhibitory (postsynaptic) afferents from the hippocampus but also through enhanced CRF transfer (Nestler et al, 2002).

1.6.3.1- Glucocorticoid receptor and Mineralocorticoid receptors

Glucocorticoids produce their biologic effects by binding to one of two cytosolic receptors: the glucocorticoid receptor (GR) or the mineralocorticoid receptor (MR). The MR has a much higher affinity for glucocorticoids than the GR. The GR is expressed in essentially every tissue in the body but is mainly concentrated in the hippocampus. MR expression is more restricted; in addition to being expressed in the brain it is expressed in the kidney, gut and heart. Antidepressants have been shown to upregulate levels of MR and GR (Pariante and Miller, 2001). It has been reported that MR in hippocampal region may be dysfunctional in human depression (Heuser et al, 2000; Rubin et al, 1995).

1.6.4- 5-HT receptor(s) involvement with depression

Several 5-HT receptors have been linked with being involved in depression although exact roles for many of the 5-HT receptors have not yet been fully identified. The main 5-HT receptors of interest are the 5-HT_{2A}, 5-HTT and particularly the 5-HT_{1A} receptor.

Increases in 5-HT_{2A} receptors are frequently mentioned in support of the hypothesis of alterations in serotonergic neurotransmission in suicide and affective disorders (Stockmeier, 2003). Various studies have investigated 5-HT_{2A} binding in platelets of depressed patients, these studies reported an increase in binding sites (B_{max}) in both depressed and suicidal patients (Arora and Meltzer, 1989). Studies investigating 5-HT_{2A} binding in post-mortem tissue have shown 5-HT_{2A} receptors to be found in the frontal cortex suggesting a role in cognitive aspects of depression. In the hippocampus of suicide victims a significant decrease in the number of 5-HT_{2A} receptors has been reported (Cheetham et al, 1988).

Two polymorphisms of the 5-HTT gene have been identified; a variable number of tandem repeats in the second intron (VNTR) (Lesch et al, 1994) and a 44bp insertion/deletion in the 5-HTT linked polymorphic region (long and short alleles) (Heils et al, 1996). An association between the short allele of the 5-HT transporter has been reported with both unipolar and bipolar patients (Collier et al, 1996). This functional polymorphism in the promoter region of the 5-HTT has also been found to moderate the influence of stressful life events on depression. It has been hypothesised that individuals possessing one or two copies of the short allele may exhibit more depressive symptoms and are more suicidal in relation to stressful life events than individuals who are homozygous for the long allele (Caspi et al, 2003).

Evidence for an association between the short allele and depression is however, inconclusive as Kunugi et al (1997), reported to find no association between the short allele with either unipolar or bipolar depression. Therefore, the 5-HTT gene may not be directly associated with depression but could be involved in moderating the serotonergic response to stress (Caspi et al, 2003).

5-HT_{1A} receptor has a heterogenous localisation, presynaptically as an autoreceptor on the soma and dendrites found mainly in the median and dorsal raphe nuclei and postsynaptically in the limbic regions of the brain (Jacobs and Azmitia, 1992). Somatodendritic receptors have an autoinhibitory function. It has been shown that on stimulation a decrease in firing in serotonergic neurons, 5-HT synthesis and 5-HT release was observed (Blier, de-Montigny, Chaput 1987; Sprouse and Aghajanian, 1987; Hjorth and Magnusson, 1988; Hutsen et al, 1989; Martin-Ruiz and Ugedo, 2001).

Activation of postsynaptic 5-HT_{1A} receptors, located in limbic areas (Pazos and Palacios, 1985; Li, Battagila and Van de Ker, 1997) results in an inhibition of the activity of neurons of the limbic system (Sprouse and Aghajanian, 1988; Blier, de montigny and Lista, 1993). When 5-HT_{1A} receptors are activated by excess amounts of 5-HT they hyperpolarise the neuron, causing it to slow down its firing activity (Nestler et al, 2001; Blier and Ward, 2003; Stahl, 1996; Ou et al, 2000).

There is great interest in this receptor due to its involvement in the pathogenesis and treatment of anxiety and depression (Veenstra-VanderWeele; 2000). In a study performed by Lemonde et al (2003), results indicated that depressed patients were twice as likely as controls to have the homozygous G-1019 genotype, and suicide victims were four times as likely to carry the same genotype. Also as a part of this study nuclear proteins from raphe cells were analysed to see whether they bound to the 26-bp palindrome sequence surrounding the polymorphism. A nuclear protein complex was identified only in serotonergic neuron derived cells that bind to the C-allele of the polymorphism but not the G-allele. Several transcription factors were found to specifically bind to and activate the C(-1019) allele, these included NUDR/Deaf-1 and Hes5 (Figure 1.12).

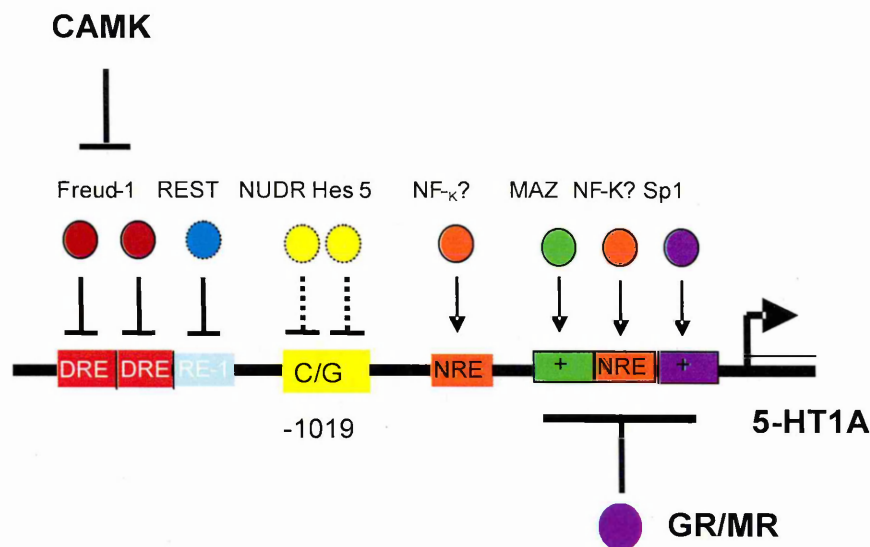
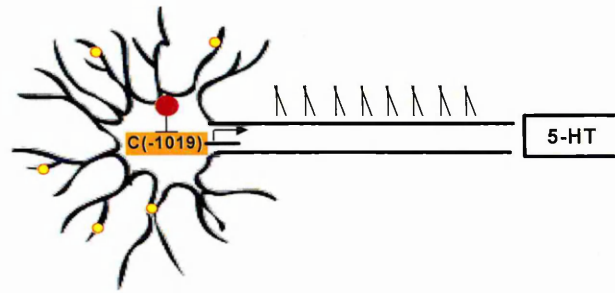


Figure 1.12- Transcriptional regulatory elements of the human 5-HT_{1A} gene.

Highlighted in bold are the key repressor elements and proteins, activation is indicated by the arrows and blocked lines indicate repression; The C(-1019) allele dependence of NUDR/Hes5 binding is indicated by the dashed lines. There are two tandem copies of the dual repressor element (DRE), and the RE-1 (which binds REST/NRSF). Adapted from Albert and Lemonde (2003).

It has been suggested from data obtained from transcriptional reporter assays, that Human nuclear deformed epidermal autoregulatory factor (NUDR) and Hes5 repress the transcription activity of the C (-1019) allele of the 5-HT_{1A} promoter. With the G (-1019) allele, this transcriptional repression is significantly decreased (Lemonde et al, 2003). The G allele, unlike the C allele fails to bind and mediate NUDR repression generating an overexpression of the 5-HT_{1A} autoreceptor and hence decreasing action potential firing rates and therefore reducing the release of 5-HT (Albert and Lemonde, 2004), (Figure 1.13).

Normal



Depression/Suicide

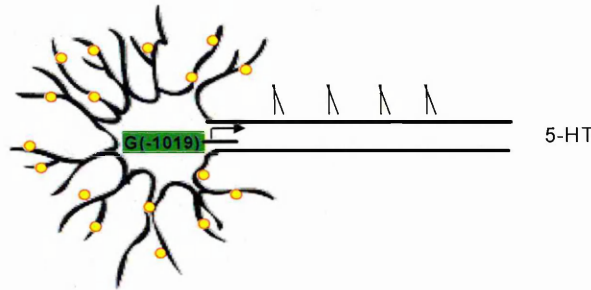


Figure 1.13- Actions of the C-1019G 5-HT_{1A} polymorphism in 5-HT neurons

In non-depressed subjects those with a C allele, NUDR (red dot) binds and 5-HT_{1A} receptor (yellow dots) expression is repressed leading to an increase in 5-HT firing rate (\wedge). Whereas, the G-allele fails to bind to NUDR leading to an overexpression of 5-HT_{1A} receptors and hence a decrease in 5-HT firing rate (Adapted from Albert and Lemonde, 2004).

In addition, under chronic stress conditions 5-HT_{1A} receptor mRNA and binding in the hippocampus is downregulated (Lopez et al, 1998). However, it is yet to be fully identified whether the G (-1019) 5-HT_{1A} allele is associated with a rise in 5-HT_{1A} receptors in the midbrain of depressed suicide post-mortem tissue samples (Stockmeier et al, 1998). It is believed that the C-1019G polymorphism may be linked with depression and this could provide a transcriptional model looking at the transcription factors NUDR, Hes5 and others for its aetiology.

Studies of post-mortem brains from depressed suicide victims have provided the main evidence for increased levels of 5-HT_{1A} autoreceptors in human depression and suicide (Stockmeier et al, 1998). From studies on nonsuicidal subjects, a specific upregulation of 5-HT_{1A} autoreceptors in the raphe region

has been shown with no change in postsynaptic 5-HT_{1A} receptors sites (Drevets et al, 1999).

The raphe and the prefrontal cortex are not the only regions of the brain studied, in the hippocampus a decrease in postsynaptic 5-HT_{1A} RNA has been observed in post mortem studies of major depression (Lopez-Figueroa et al, 2004). The reduction in 5-HT_{1A} receptors may reflect a decrease in cell number in these regions in depression.

A study by Huang et al (2004), found an association of the 5-HT_{1A} C-1019G locus with schizophrenia, substance abuse and panic attacks. However, binding of the 5-HT_{1A} receptor in the prefrontal cortex of post-mortem brain tissue samples showed no relationship with suicide and genotype, therefore the study concluded that the relationship can not be explained by binding differences, but receptor affinity and transduction can not be ruled out completely.

Position emission tomography (PET) imaging studies of human patients with bipolar depression, major depression, and panic disorder have shown a decrease in 5-HT_{1A} receptor density, mainly in the dorsolateral prefrontal cortex (Albert and Lemonde, 2004). Further studies need to be performed to determine whether these changes are due to alterations in 5-HT_{1A} expression or changes in neuronal number.

5-HT_{1A} receptors have been examined in the hippocampus of suicide victims. An increase in 5-HT_{1A} receptor sites in the stratum pyramidale (CA1) of the hippocampus of suicide victims probably suffering from an affective disorder was reported by Joyce et al, (1993). Other studies, including those where subjects were psychiatrically characterised, however, did not observe any significant changes in agonist binding to the 5-HT_{1A} receptor in the hippocampus of suicide victims (Dillon et al, 1991; Cowther et al, 1997; Stockmeier et al, 1997). One study reported that in the cortex there were no differences in binding between suicide and control patients, however, it did report a significant decrease in 5-HT_{1A} receptors in the hippocampus of suicide patients (Cheetham et al, 1990). Studies of 5-HT_{1A} receptors in the prefrontal cortex have generated various results. One study of the prefrontal cortex

detected an increase in 5-HT_{1A} receptors in suicide victims (Matsubara et al, 1991), whereas, Arango et al (1995), observed an increase in radioligand binding to 5-HT_{1A} receptors in the ventrolateral, but no other areas. Several other studies of the prefrontal cortex report no significant changes in 5-HT_{1A} receptor in classified suicide victims (Dillion et al, 1991; Arranz et al, 1994; Lowther et al, 1997; Stockmeier et al, 1997).

The above examples highlight the fact that it is not simply one mechanism that can reduce 5-HT neurotransmission but multiple mechanisms that can contribute to predisposition to depression or suicide (Albert and Lemonde, 2004).

Several studies have established interactions between the serotonergic system and the HPA axis and glucocorticoid secretion (Dinan, 1994). Glucocorticoid hormones have major effects on behaviour, hippocampal morphology, 5-HT neurotransmission (Dickinson, Kennett and Curzon, 1985; Joels, Heslen and Kloet, 1991; Mendelson and McEwen, 1992; Nausjeda, Carve and Weiner, 1982; Woolley, Gould and McEwen, 1990) and down regulating mRNA 5-HT_{1A} receptor expression (Chalmers et al, 1993). In depressed patients, elevated cortisol levels may lower L-tryptophan availability and therefore decreasing 5-HT turnover thus downregulating presynaptic 5-HT_{1A} receptors and upregulating 5-HT₂ receptors. Contrarily, 5-HT is known to stimulate the release of CRH and ACTH and could possibly modulate the negative feedback of the HPA axis by glucocorticoids (Maes et al, 1994). Elevation of corticosterone levels by stress or from direct stimulation from dexamethasone on glucocorticoid receptors decrease 5-HT_{1A} receptor binding levels in discrete subfields of the hippocampus (Mendelson and McKewen, 1992; Chalmers et al, 1994). Glucocorticoid receptor mRNA levels were shown to be decreased in the selective neurotoxic lesion of 5-HT neurons of the hippocampus (Seckl et al, 1990). However, antidepressant treatments that inhibit 5-HT uptake increase glucocorticoid receptor mRNA levels in the hippocampus (Seckl and Fink, 1992). The expression of 5-HT_{1A} receptors is thought to be negatively mediated by corticosterone in the hippocampus but not in the raphe nucleus, and adrenalectomy (suppresses endogenous corticosterone) increased the concentration of both 5-HT_{1A} receptor mRNA and binding sites in the

hippocampus (Burnet et al, 1992). Hence, under stressful conditions when endogenous corticosterone is secreted it is only somatodendritic 5-HT_{1A} autoreceptors that are desensitised and not postsynaptic 5-HT_{1A} receptors (Laaris et al, 1999).

The ability of corticosteroids to modulate postsynaptic 5-HT_{1A} receptor function has been well documented. There is less evidence available regarding the effects of corticosteroids on somatodendritic 5-HT_{1A} receptor autoreceptor function (Fairchild, Leitch and Ingram, 2003). An electrophysiological study demonstrated that corticosterone attenuated dorsal raphe 5-HT_{1A} receptor functions when applied acutely to rat brain slices (Laaris et al, 1995). Subsequently, Laaris et al (1999) also showed that exposure to unpredictable stress, in combination with social isolation desensitised 5-HT_{1A} autoreceptors in the DRN (Laaris et al, 1999).

1.6.5- Medical management of depression

The desensitization of 5-HT_{1A} autoreceptors is assumed to be one of the main adaptive changes that allow antidepressant actions. Studies have demonstrated that after 2 to 3 weeks of selective serotonin reuptake inhibitor (SSRI) treatment, there is internalization and a reduction of 5-HT_{1A} autoreceptors (Hervas et al, 2001). With the desensitization of 5-HT_{1A} neuronal firing of 5-HT is un-inhibited producing an increase in 5-HT production that correlates with a seen improvement in depressed symptoms (Parsons, Kerr and Tecott, 2001).

Antidepressant drugs are a heterogeneous group of compounds that are effective in the treatment of major depression. Antidepressant drugs are often subdivided into groups according to their structure and neurochemical properties, these groups include tricyclic, SSRIs, norepinephrine selective reuptake inhibitors (NRIs), and MAOIs. MAOIs were one of the first groups to be administered as antidepressants, followed by TCAs which led to the more recent developments of SSRIs and NRIs antidepressants.

1.6.5.1- Monoamine oxidase inhibitors (MAOI's)

The monoamine theory of depression heavily focused on noradrenaline rather than dopamine or 5-HT (Jones and Blackburn, 2002). This theory was proposed from observations that reserpine depleted monoamines and caused depression, whereas the monoamine oxidase inhibitors enhanced monoamine function and thereby relieved depression (Jones and Blackburn, 2002). A definite decrease in the firing activity of locus coeruleus nucleus (LC) NA neurons was observed after a 2-day treatment with MAOIs including clorgyline and phenelzine (Blier and de Montigny, 1985; Blier, de Montigny and Azzaro, 1986). Dorsal raphe nucleus (DR) 5-HT neurons were shown to reduce their firing rate in response to a two day treatment with MAOIs (Blier and de Montigny, 1985; Blier, de Montigny and Azzaro, 1986). In contrast to NA neurons 5-HT neurons firing activity displays a partial recovery after one week and a complete recovery after three weeks of treatment. The steady revival of 5-HT neuronal discharge was accounted for by the finding that 5-HT_{1A} autoreceptors desensitise after long-term MAOI treatments (Blier and de Montigny, 1985; Blier, de Montigny and Azzaro, 1986).

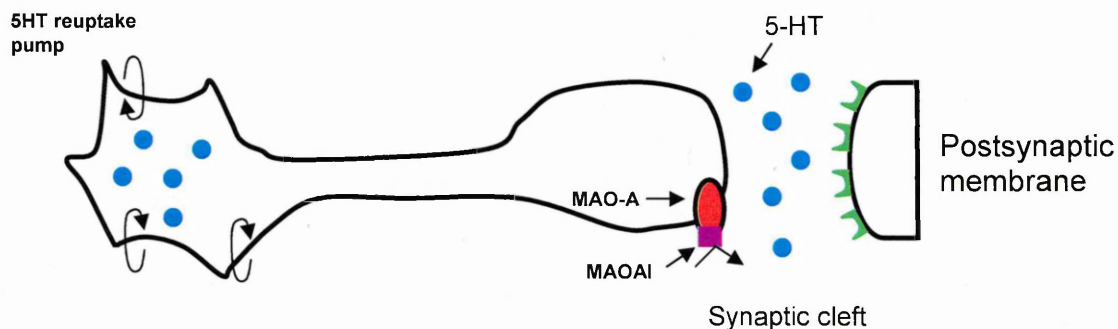


Figure 1.14- Mechanism of action of Monoamine oxidase inhibitors antidepressants

Monoamine oxidase A (MAOA) is an enzyme involved in the metabolism of 5-HT. MAO-A regulates the free intraneuronal concentration and releasable stores of 5-HT. MAO-A inhibitors (MAOAI) bind to and inhibit MAO-A preventing 5-HT degradation. This leads to an increase in 5-HT in the synaptic cleft.

1.6.5.2- Tricyclic antidepressants (TCAs)

TCAs have been shown to have a similar effect of action of blocking the re-uptake of noradrenaline (NA) and 5-HT like MAOIs antidepressants. However, TCA antidepressants block the re-uptake with different potencies. The therapeutic efficacy of TCA antidepressants was thus considered to be related to the prolongation of the synaptic action of these neurotransmitters (Willner, 1985).

TCA antidepressants decrease the firing rate of dorsal raphe nucleus 5-HT neurons when administered acutely. Of all the TCAs chlorimipramine is the most potent of all the TCAs currently available (Scuvee-Moreau and Svensson, 1982; VanderMaelen and Braselton, 1992). Imipramine and amitriptyline are known to suppress 5-HT neurons' discharges at high doses (Scuvee-Moreau and Svensson, 1982).

TCAs also preferentially block NA uptake for example, nortriptyline and desipramine, however, these antidepressants are very weak or inactive inhibitors of 5-HT reuptake (Quineaux, Scuvee-Moreau and Dresse, 1982).

Long term treatment of TCAs has been claimed to desensitise the α -adrenergic autoreceptors which mediate the inhibitory feedback on the firing activity of NA neurons, which in turn leads to a increase NA neurotransmission (Mongeau et al, 1997).

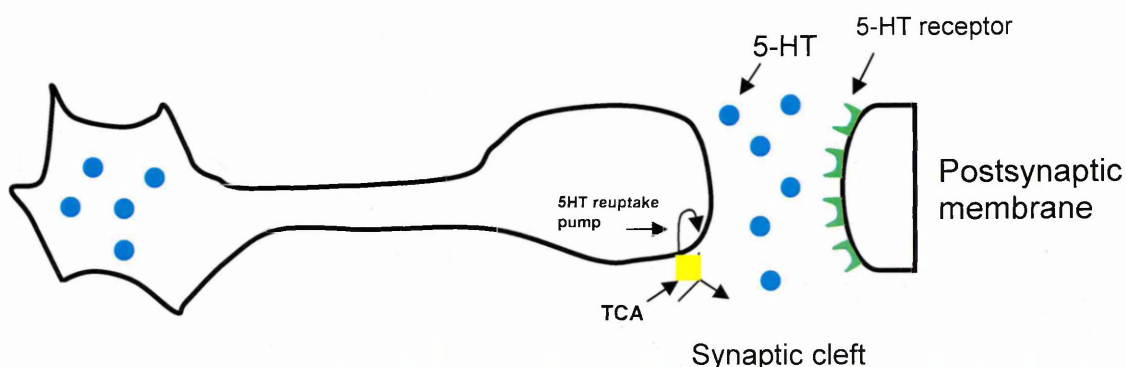


Figure 1.15- Tricyclic antidepressant (TCA) mode of action on 5-HT

TCAs bind to the 5-HT re-uptake transporter preventing the re-uptake of 5-HT from the synaptic cleft. This leads to the accumulation of 5-HT in the synaptic cleft and concentration of 5-HT returns to normal levels.

1.6.5.3- Selective reuptake inhibitors

1.6.5.4- Selective serotonin reuptake inhibitors (SSRIs)

SSRIs are the most commonly prescribed antidepressant drugs due to their tolerability and absence of severe side effects (Celada et al, 2003). Common selective serotonin reuptake inhibitors e.g. fluoxetine, paroxetine and citalopram used in the treatment of depression have a selective effect on the serotonin reuptake pump. An initial increase in 5-HT at the cell body and dendrites is observed. The immediate effect is to inhibit the rate of firing of 5-HT neurons and hence the release of 5-HT by the action of 5-HT_{1A} autoreceptors (Albert and Lemonde, 2004; Celada et al, 2003), (Figure 1.16).

The activation of 5-HT_{1A} receptors increases potassium conductance and hence hyperpolarizing the neuronal membrane and leading to a reduction in serotonergic neuron firing rate in the cortex and hippocampus (Sprouse and Aghajanian, 1987; Araneda and Andrade, 1991; Tanaka and North, 1993; Ashby, Edwards and Wang, 1994). The 5-HT_{1A} autoreceptor mediates the inhibition of cell firing but also generates a reduction of terminal 5-HT release which augments the rise in extracellular 5-HT produced by re-uptake blockade (Adell and Artigas, 1991; Artigas et al, 1996; Invernizzi, Belli and Samanian, 1992).

However, the efficacy of this negative feedback resulting in attenuation of cell firing and terminal 5-HT release is less noticeable after long-term treatment with SSRIs. Long-term treatment outcome shows a recovery of the 5-HT firing in the dorsal raphe nucleus cells and an increase in extracellular 5-HT greater than after single administration (Bel and Artigas, 1993; Blier and Ward, 2003). Both effects are thought to result from the 5-HT-induced desensitisation of raphe 5-HT_{1A} autoreceptors (Blier and Ward, 2003).

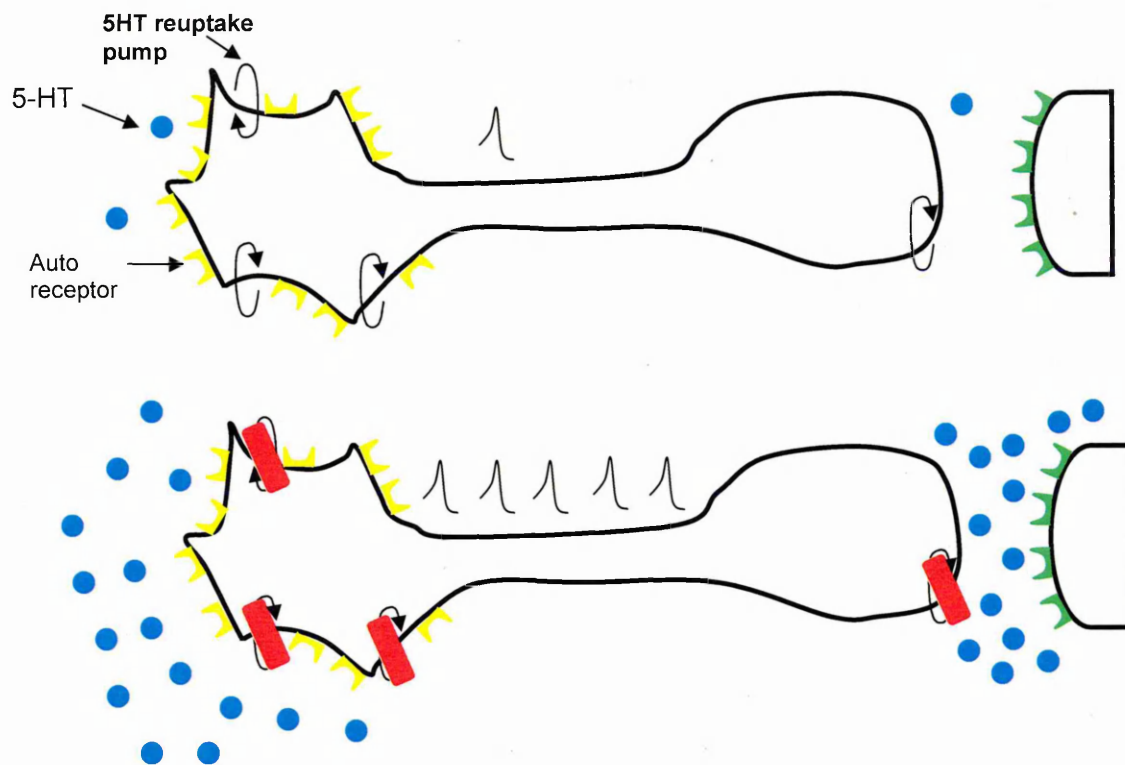


Figure 1.16- Mechanism of action of SSRIs

SSRIs (red rectangles) are thought to restore the levels of 5-HT (blue dots) in the synaptic cleft by binding at the 5-HT re-uptake transporter preventing the re-uptake and subsequent degradation of 5-HT. This re-uptake blockade leads to the accumulation of 5-HT (blue dots) in the synaptic cleft and 5-HT firing rate is increased thus returning 5-HT concentration to within the normal range. This action of SSRIs is thought to contribute to the alleviation of the symptoms of depression (Stahl and Grady, 2003).

To confirm the hypothesis that 5-HT_{1A} autoreceptor desensitization plays a substantial role in the antidepressant effect of 5-HT_{1A} agonists, the agonist buspirone was given with the preferential 5-HT_{1A} autoreceptor antagonist pindolol (Blier et al, 1997). The study revealed that depressed patients improved quickly with the buspirone-pindolol combination compared to patients receiving tricyclics (tricyclics do not block 5-HT reuptake). This study, along with others, suggests that when the desensitization of the 5-HT_{1A} autoreceptor is bypassed, by blocking it, the antidepressant response can be accelerated (Albert and Lemonde, 2004).

1.6.5.5- Norepinephrine selective reuptake inhibitors (NRIs)

The evidence for a role of noradrenaline in depression is well established (Leonard et al, 1997), beginning with the discovery that drugs which either caused or alleviated depression acted to alter noradrenaline metabolism (Brunello et al, 2002). Common NRIs include reboxetine and maprofiline, which selectively inhibit noradrenaline reuptake in the synapse via the noradrenaline transporter (Waller, Renwick and Hillier, 2001; Buschmann et al, 2007). The selective inhibition of noradrenaline reuptake is thought to enhance serotonergic transmission maybe by augmenting 5-HT_{1A} postsynaptic neurotransmission (Waller, Renwick and Hillier, 2001).

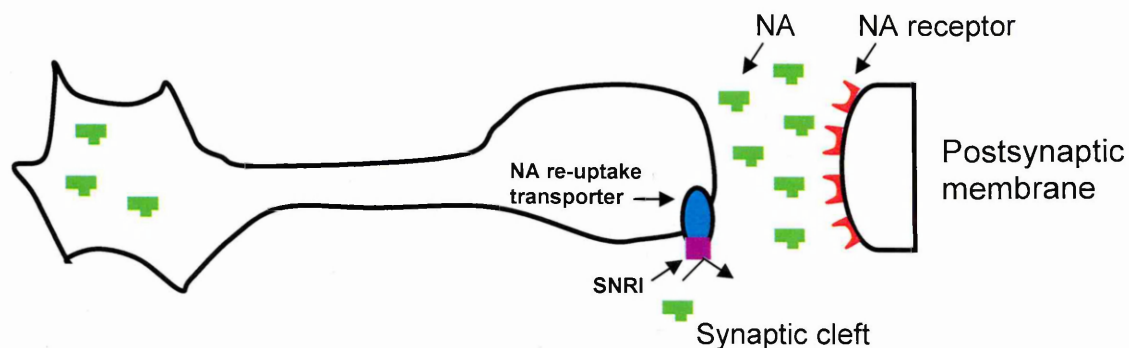


Figure 1.17- Norepinephrine selective reuptake inhibitors (NRI's) mode of action on NA

NRI's restore the levels of NA in the synaptic cleft by binding to the NA re-uptake transporter preventing the re-uptake of NA providing an increase in NA in the synaptic cleft returning NA back to normal levels.

1.7- 5-HT_{1A} structure and pharmacology

1.7.1- 5-HT_{1A} structure

One of the first G-protein coupled receptors to have its gene identified by molecular cloning methods was the 5-HT_{1A} receptor. The development of cell transfection methods has enabled large amounts of information regarding potential signal transduction pathways linked to the receptor, correlations of receptor structure to its various functions and pharmacological properties of the receptor to be obtained (Raymond et al, 1999).

5-HT_{1A} is a member of the G-protein-coupled receptor superfamily (GPCRs) (Iismaa et al, 1995) and therefore the basic structure of the 5-HT_{1A} receptor comprises of the characteristic seven transmembrane domains that span the cell membrane, with an N-terminus and C-terminus present extracellularly and intracellularly respectively (Adham et al, 1994; Wurch, Colpaert and Pauwels, 1998). The 5-HT_{1A} receptor is composed of 422 amino acids, with a core molecular weight of \approx 46KDa and an isoelectric point of 8.8. From hydrophobicity analysis studies it has been shown that the seven transmembrane domains form α -helices and that the receptor is orientated in the plasma membrane facing the extracellular domain. Hydrophilic sequences which form three intracellular and three extracellular loops connect the 7 hydrophobic transmembrane regions (Raymond et al, 1991). The second extracellular domain contains a cysteine residue (Cys¹⁸⁶) which could form a disulphide bond with Cys¹⁰⁹ (Refer to Figure 1.18). This disulphide bond may help to stabilise the receptor conformation (Emerit et al, 1991; Gozlan et al, 1988).

As 5-HT_{1A} is a member of the G-protein family it can interact with G_{ai} (inhibitory)/G_o proteins to inhibit adenylyl cyclase and modulate ionic effectors, e.g. potassium and /or calcium channels (Lanfumeij and Hamon, 2004)

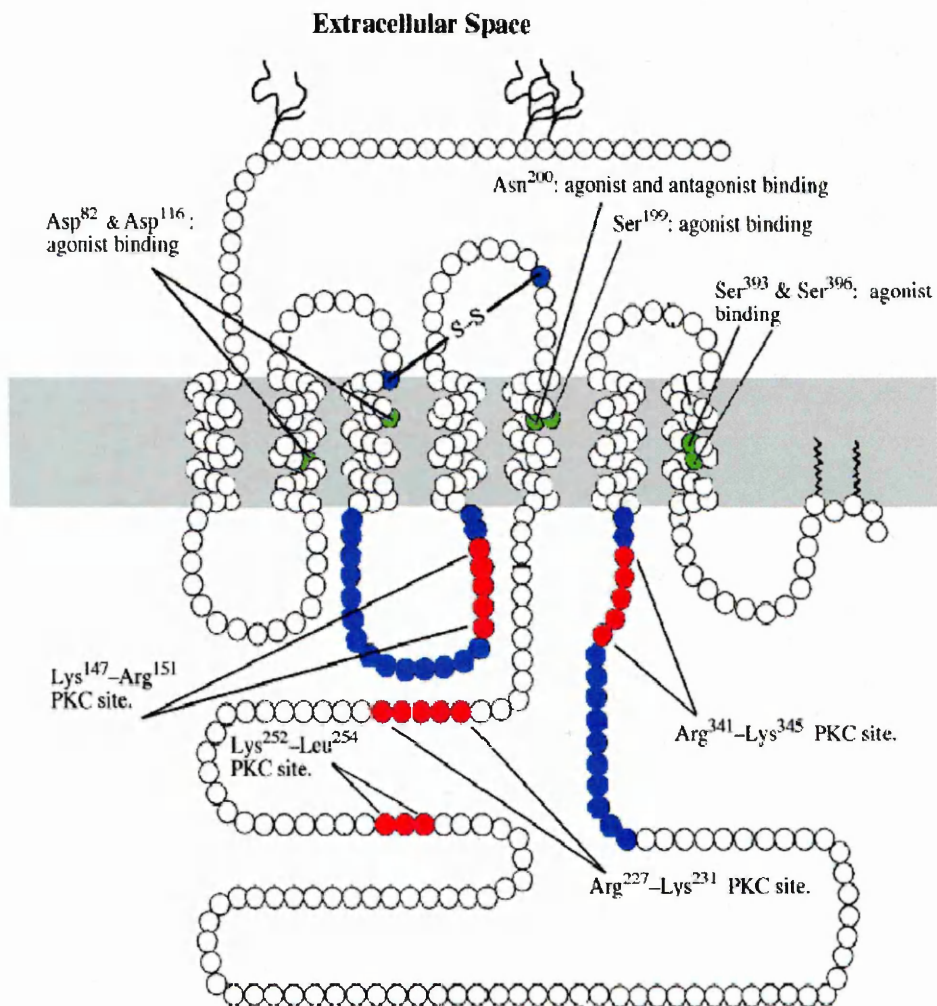


Figure 1.18- Diagrammatic representation of the structure of the 5-HT_{1A} receptor

5-HT_{1A} has a seven transmembrane domain that spans the cell membrane, an N-terminal is present extracellular and an intracellular C-terminal. The active site for ligands (agonists and antagonists; shown in green) in this family of proteins is located in the transmembrane regions, known as α -helices confirmed by site directed mutagenesis and chimeric receptors (Adapted from Raymond et al, 1999).

1.7.2- 5-HT_{1A} receptor polymorphisms

The possibility that the 5-HT_{1A} receptor could possess allelic variants was supported by two studies that had cloned the 5-HT_{1A} receptor in the rat (Albert et al, 1990; Fujiwara et al, 1990). Subsequently, further research into allelic variants has been published in regards to the human 5-HT_{1A} receptor gene. Variants published include; Pro¹⁶-Leu, Ile²⁸-Val, Gly²²-Ser, Arg²¹⁹-Leu, Gly²⁷²-

Asp, Asn⁴¹⁷-Lys, and C-1019G (Erdmann et al, 1995; Harada et al, 1996; Kawanishi et al, 1998; Nakhai et al, 1995; Lemonde et al, 2003). Through the discovery of these allelic variants, the interesting possibility that there may be functional differences in the 5-HT_{1A} receptor has been raised and it has been suggested this may lead to disease manifestations (Raymond et al, 1999).

Recent molecular studies have demonstrated that the 5-HT_{1A} gene variants Pro¹⁶-Leu, (a substitution of a single-base pair (47C → T) on codon 16 produces an amino-acid exchange from Proline to Leucine) and Gly²⁷²Asp (A single base-pair substitution (815G → A) on codon 272 resulting in an amino-acid exchange from Glycine to Aspartic acid), have been associated with schizophrenia (Kawanishi et al, 1998). The Gly²⁷²Asp polymorphism can be exactly located in the intermediate portion of the third intracellular loop and as this area is involved in the activation and binding of G-proteins, it is thought that this mutation could alter signal transduction function through G-protein coupling (Kawanishi et al, 1998).

The rare Ile²⁸-Val polymorphism of the 5-HT_{1A} receptor is located in the coding region of the gene. The coding mutation (A → G) is present at nucleotide position 82 leading to an amino-acid exchange from Isoleucine to Valine. Brüss et al (1995), demonstrated that this receptor variant had ligand binding properties that were practically identical to those of the wild type receptor in transfected COS-7 cells.

Studies investigating this allelic variant suggest that this polymorphism is unlikely to play a major role in the genetic predisposition to bipolar disorder or schizophrenia (Erdmann et al, 1995). Another coding mutation Gly²²-Ser is present at position 22 and involves an amino acid exchange from Glycine to Serine. When expressed this polymorphism can alter the extracellular amino terminal domain of the 5-HT_{1A} receptor (Nakhai et al, 1995).

The C-1019G polymorphism of the 5-HT_{1A} receptor gene is the most commonly studied polymorphism as it is associated with depression. As previously discussed the C-1019G polymorphism is located in a transcriptional regulatory region. The sequence of the polymorphism is situated within a 26-bp

palindrome, a site believed to be important for protein-DNA interactions (Lemondé et al, 2003; Huang et al, 2004). The variation in this region could affect efficacy of binding of regulatory proteins and thus impairment of the repression of 5-HT_{1A} receptor, which correlates with depression or suicidal behaviour or both.

1.7.3- 5-HT_{1A} receptor ligand pharmacology

Research into ligand pharmacology of 5-HT_{1A} has been studied in much detail. Cell lines transfected with 5-HT_{1A} have been used to characterise radioligands (Sundaram et al, 1992; 1995), in particular to identify and characterise partial (Arthur et al, 1993; Assie et al, 1997; Pauwels et al, 1993) and inverse agonists (Barr and Manning, 1997; Newman-Tancredi et al, 1998). However, the usefulness of the information gained requires some measure of validation (Deupree and Bylund, 2002). It is important that the appropriate binding conditions are used. Data analysed is based on equilibrium conditions being present when unbound drug is separated from bound and the unbound drug concentration remains constant. Receptor subtype conformations can be altered by metal ions and GTP, therefore, it is necessary to ensure that similar assay conditions are used when comparing one study to another (Deupree and Bylund, 2002).

1.7.3.1- 5-HT_{1A} receptor agonists

There is evidence that some 5-HT_{1A} agonists that are anxiolytic agents could also demonstrate antidepressant activity (Schatzberg and Cole, 1978; Rickels et al, 1982; Feighner et al, 1983; Schweizer et al, 1986). Several 5-HT_{1A} partial agonists have been found to possess antidepressant activity in some depressed patients (Schweizer et al, 1986; Amsterdam et al, 1987; Jenkins et al, 1990; Robinson et al, 1990; Rickels et al, 1990).

Buspirone was one of the first discovered 5-HT_{1A} receptor agonists in 1972 by Wu and Rayburn. However, buspirone is not considered to be an antipsychotic but an anxiolytic drug instead. The exact mechanism by which buspirone is

thought to act still remains uncertain (Goa and Ward, 1986; Robinson et al, 1990). Although it has been suggested that buspirone exerts the majority of its clinical effects by modulating the serotonergic system (Pecknold, 1994; Tunnieliff, 1991). Buspirone acts at both pre- and post-synaptic 5-HT receptors. Presynaptically in the raphe nuclei it acts as a full agonist where it inhibits the synthesis and the firing rate of 5-HT neurons (Pecknold, 1994; Tunnieliff, 1991). Buspirone acts as a partial agonist on postsynaptic 5-HT_{1A} receptors resulting in a reduction in 5-HT_{1A} functioning (Peroutka, 1988). The selectivity of buspirone has been debated as it has been shown to have a high affinity for dopamine receptors (Yocca et al, 1986) and therefore its use as a 5-HT_{1A} receptor agonist has yet to be determined.

Gepirone (4-4-dimethyl-1-1-[4[4-(2-pyrimidinyl)-1-piperazinyl]butyl]-2,6-pipendinedione hydrochloride) is a selective partial agonist used in the treatment of generalised anxiety disorder (GAD) and has been suggested to have antidepressant action similar to that of buspirone (Jenkins et al, 1990). Gepirone demonstrates differential activity at 5-HT_{1A} pre- and postsynaptic receptors resulting in an increase in 5-HT activity (Blier and De Montigny, 1990). Furthermore, gepirone has been demonstrated to down-regulate 5-HT₂ receptors similar to more conventional antidepressant compounds (Eison and Yocca, 1985). In several animal models of depression gepirone has been shown to demonstrate antidepressant activity (Kennett et al, 1987; Giral et al, 1988). Gepirone has been proven to be selective for 5-HT_{1A} as it does not react with noradrenergic, GABA or dopaminergic receptor sites (Riblet et al, 1982; Jenkins et al, 1990).

Tandospirone (3an, 413, 76, 7act-hexahydro-2-(4-(4-(2-pyrimidinyl)-1-piperazinyl)-butyl)-4,7-methano-1H-isoindole-1,3(2H)-dione dihydrogen citrate) a novel anxiolytic piperazine derivative like buspirone, gepirone and ispsapirone all have pharmacological (Schimizu et al, 1987) and clinical (Tsutsui et al, 1992) efficacy. Tandospirone binds the 5-HT_{1A} receptor with high affinity and selectivity (Schimizu et al, 1988), from behavioural and physiological studies tandospirone has demonstrated it self to be a highly efficient 5-HT_{1A} receptor agonist (Schimizu et al, 1992; Tsuji et al, 1990). Tatsuno et al (1989), reported that a decrease in 5-HT metabolite level and an increase in levels of NA and DA

in the rat brain were reported with the addition of tandospirone. Furthermore, tandospirone has also been shown to suppress hippocampal adenylate cyclase activity via 5-HT_{1A} receptors (Tanaka et al, 1995). Tanaka et al (1995), have suggested that tandospirone is almost a full agonist for 5-HT_{1A} receptors and more potent than other receptor related anxiolytics.

The most commonly used 5-HT_{1A} agonist is 8-OH-DPAT (8-hydroxy-2-(di-n-propylamino)tetralin) which is known to be a powerful and selective 5-HT_{1A} receptor agonist. This notion was derived from a series of behavioural and biochemical investigations (Arvidsson et al, 1981; Hjorth et al, 1982) and has subsequently been further substantiated by a wealth of *in vivo* and *in vitro* studies (Middlemiss and Fozard, 1983; Hamon et al, 1984; Tricklebank et al, 1984; Hall et al, 1985; Dourish et al, 1985).

In the rat CNS *in vivo* 8-OH-DPAT can inhibit a number of aspects of 5-HT neuronal activity. Hence, 8-OH-DPAT decreases transmitter synthesis, utilisation, release and turnover (Hjorth et al, 1982; 1987) and 5-HT cell firing (Fallon et al, 1983; Sprouse and Aghajanian, 1986) in 5-HT neurons.

1.7.3.2- 5-HT_{1A} receptor antagonists

Antagonist ligands that have previously been used to define the 5-HT_{1A} receptor are either non-selective or have agonist activity at the presynaptic 5-HT_{1A} receptor. It is only in more recent years that selective and silent 5-HT_{1A} receptor antagonists have emerged (Routledge, 1996).

(S)-UH-301, was the first ligand to be described to be a 5-HT_{1A} receptor antagonist at both pre-and postsynaptic sites. (S)-UH-301 is a single enantiomer of the racemic 5-fluoro-analogue of 8-OH-DPAT (Hilliver et al, 1990). This compound is known to have a high affinity for the 5-HT_{1A} receptor and is thought to antagonise the effects of 8-OH-DPAT in a number of functional assays (Routledge, 1996). (S)-UH-301 has also been shown to increase the dorsal raphe neuron firing rate (Arborelius and Svensson, 1992). However, there is one major disadvantage to this compound which limits its use as an *in*

vivo tool in that it is also a potent dopamine 2 receptor (D_2) agonist and therefore has the ability to decrease both dopamine synthesis and the firing of dopamine neurones in the ventral tegmental area (Arborelius and Svensson, 1992).

SD2 216,525 is also a 5-HT_{1A} receptor antagonist that shows selectivity over other 5-HT and monoamine receptors (Schoeffer et al, 1993). The effect of 8-OH-DPAT on forskolin stimulated hippocampal adenylyl cyclase hypothermia was antagonised by SD2 216,525. It was thus concluded that SD2 216,525 is an antagonist based on its functional activity at postsynaptic 5-HT_{1A} receptors (Schoeffer et al, 1993). However, there is some evidence that suggests that this compound acts as a 5-HT_{1A} receptor partial agonist presynaptically and as an antagonist postsynaptically. Hence, SD2 216,525 is not an optimal choice as a research tool *in vivo* to study the 5-HT_{1A} receptor function (Routledge, 1996).

The first 5-HT_{1A} receptor ligand to display unequivocal antagonist properties both pre and postsynaptically was (RS)-WAY100135 (Fletcher et al, 1993; Cliffe et al, 1993). (RS)-WAY100135 blocked the effects of 8-OH-DPAT on dorsal raphe neuronal firing and hippocampal 5-HT release in a presynaptic 5-HT_{1A} receptor model (Routledge et al, 1993). However, like the afore-mentioned antagonists there are some disadvantages to this compound as a *vivo* tool, at high doses (>400µg/kg i.v.), (RS)-WAY100135 induced a small but dose dependent decrease in raphe neuronal cell firing, consistent with a 5-HT_{1A} partial agonist profile (Fletcher et al, 1993).

Recent research performed has given evidence of silent antagonists of the 5-HT_{1A} receptor, such as WAY100635 (N-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl)-n-(2-pyridinyl)cyclohexanecarboxamidetrihydrochloride), which is described as a silent antagonist due to its lack of intrinsic activity in both presynaptic and postsynaptic 5-HT_{1A} receptor function models (Routledge, 1996). The common antagonist WAY100635 has been portrayed to be a true antagonist that is selective at both somatodendritic and postsynaptic 5-HT_{1A} receptors (Cliffe, 1993) and also in a study of transfected Chinese hamster ovary (CHO) cell system using GTP γ S binding (Newman-Tancredi et al, 1998). WAY100635 has no effect on 5-HT neuronal activity, signifying that this

compound does not have the use-limiting properties like other 5-HT_{1A} antagonist ligands. Hence, it is the first selective and silent 5-HT_{1A} receptor antagonist and a very useful ligand suitable for assessing 5-HT_{1A} receptor function *in vivo* (Routledge, 1996).

Finally, another commonly used 5-HT_{1A} receptor antagonist is 4(2'methoxyphenyl)-1-[2'[N-(2''pyridinyl)-p-iodobenzamido]ethyl] piperazine (p-MPPI) (Zhuang et al, 1994). p-MPPI inhibits the agonist activity induced by 8-OH-DPAT in inhibiting forskolin induced adenylyl cyclase activity in rat hippocampal tissue (Kung et al, 1994). This 5-HT_{1A} antagonist has an advantage in that it can be readily iodinated with [¹²³I] or [¹²⁵I] to enable its use in radioligand binding studies both *in vivo* and *in vitro* to study 5-HT_{1A} receptor function (Zhuang, Kung and Kung, 1994; Kung et al, 1994; Kung et al, 1995).

1.7.4- 5-HT receptors and second messenger signalling pathways

1.7.4.1- 5-HT_{1A} receptor signalling

Martin Rodbell in 1971 hypothesised that a guanine-nucleotide regulatory protein could functionally connect receptors with their effectors in the context of the hormonal stimulation of adenylyl cyclase (AC) system, which generates second messenger cyclic AMP (cAMP), (Rodbell et al, 1971). The guanine-nucleotide regulatory protein was later discovered to be Gs, once purified Gs protein was shown to be heterotrimeric comprising of α , β , and γ -subunits. It is known that the α -subunit is responsible for GTP and GDP binding and also for GTP hydrolysis, whereas the β , and γ - subunits have been associated in a tightly linked $\beta\gamma$ complex (Gilman, 1987).

5-HT receptors are involved and regulate multiple signalling pathways and effector molecules. It has been shown that all the 5-HT₁ receptor subtypes couple to the G_{i/o} effector. The 5-HT_{1A} receptor subtype is the most characterised receptor in its receptor class.

As previously mentioned the 5-HT_{1A} receptor is a member of the G-protein coupled superfamily consisting of α , β , and γ -subunits. The 5-HT_{1A} receptor can

couple to the broadest range of second messengers compared to any of the other 5-HT receptors. Raymond et al (1999), have demonstrated that the recombinant 5-HT_{1A} receptor is coupled to the inhibition of AC through pertussis toxin-sensitive G-proteins (De Vivo and Maayani, 1986; Weiss et al, 1986). AC inhibition coupling is universally expressed (Banerjee et al, 1993; Fargin et al, 1989; Lui and Albert, 1991; Varrault et al, 1992) and that this coupling is extremely efficient in that the efficacy of coupling is maximal at low physiologically relevant levels of receptor expression. In some systems the coupling of the 5-HT_{1A} receptor to AC is manifested by an ability to inhibit forskolin-stimulated AC activity.

5-HT_{1A} receptors that have been transfected into polarised epithelial LLC-PK1 cells and were shown to be expressed on both basolateral and apical membranes. It was determined that the 5-HT_{1A} receptors present on both surfaces had the ability to inhibit cAMP accumulation and therefore, the 5-HT_{1A} receptor has been shown to consistently inhibit AC in multiple cells (Langlois et al, 1996) including cultured neuronal cells (the NCB-20, P-11 and HNZ lines), (Hensler et al, 1996; Singh et al, 1996).

The 5-HT_{1A} receptor has also been reported to activate phospholipase (PL)C, src kinase, and mitogen-activated protein (MAP) kinases (Raymond et al, 1999). Several groups have reported that the 5-HT_{1A} receptor activates ERK MAP kinases in CHO cells (Cowen et al, 1996; Garnovskaya et al, 1996; 1998; Della Rocca et al, 1999; Mendez et al, 1999; Mulifun et al, 2000). Similarly, to other GPCRs the 5-HT_{1A} receptor activates ERK through an intricate signalling pathway that consists of an assembly of signalling complexes that require many of the same molecules used by growth factor receptor tyrosine kinases (Marshall, 1995; Luttrell et al, 1997). The activation of ERK by the 5-HT_{1A} receptor is initiated by the $\beta\gamma$ -subunits that are released from pertussis toxin-sensitive G proteins (Raymond et al, 1999).

5-HT_{1A} receptor activation through the activation or inhibition of numerous effectors leads to both the α -subunit and $\beta\gamma$ -dimer signalling to occur. Activation of the receptor by agonists generates conformational changes which are still poorly understood (Ballesteros et al, 2001; Farahbakhsh et al, 1995).

Once the 5-HT_{1A} receptor is activated it can interact with the heterotrimeric G protein and can act as a guanine nucleotide exchange factor (GEF) to promote GDP dissociation and GTP binding and activation. In the current models of 5-HT_{1A} receptor signalling the activated receptor dissociates into a α -subunit and a $\beta\gamma$ -dimer both subunits have the capability to regulate separate effectors (Gilman, 1987). The $\beta\gamma$ -dimer is thought to induce the opening of potassium channels and the closing of calcium channels as shown to occur mainly in neuroendocrine cells (Albert et al, 2001). Depending on cell type the model of 5-HT_{1A} signalling can be modified. In pituitary GH4 cells transfected with the 5-HT_{1A} receptor, agonist induced receptor activation decreases calcium concentration by the closing of calcium channels and the opening of potassium channels which leads to a reduction in intracellular levels of cAMP through the inhibition of both the basal and G_s-stimulated activity of AC (Albert et al, 2001). However, in transfected LIK⁻ fibroblast cells the activated 5-HT_{1A} receptor increases calcium concentration by augmenting PI turnover through phospholipase activation C (PKC). Activation of PKC leads to the generation of two key second messengers. The first is inositol trisphosphate (IP₃) (Berridge et al, 1998), a rise in IP₃ is observed in LIK⁻ fibroblast cells which, in turn releases intracellular calcium stores. The second key messenger is diacylglycerol (DAG) that binds to and activates PKC. In LIK⁻ cells 5-HT_{1A} receptor mediated inhibition of basal cAMP is not observed, but forskolin-induced cAMP accumulation has been shown to be reduced (Albert et al, 2001).

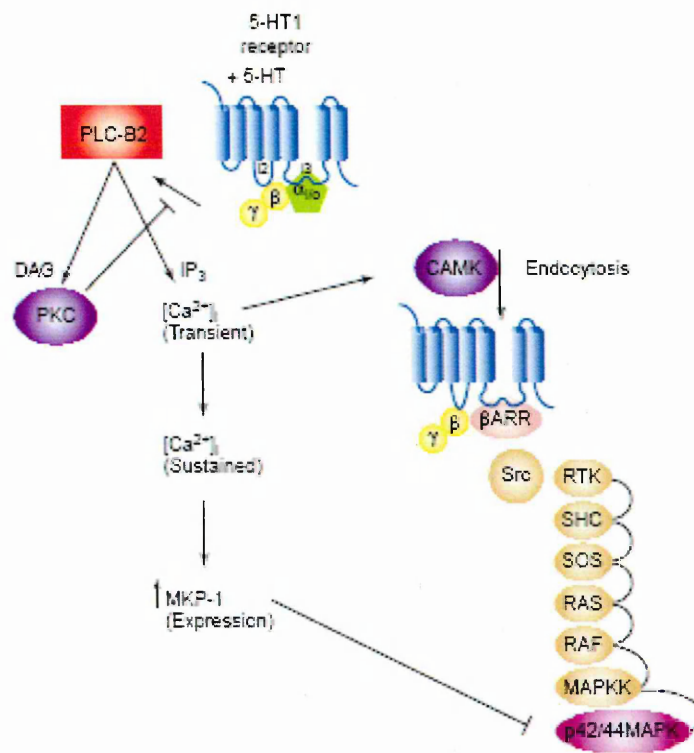


Figure 1.19- Regulation of MAPK by 5-HT_{1A} receptors

On activation of the 5-HT_{1A} receptor it becomes phosphorylated by GRK. The acute activation of MAPK has been associated with a Ca²⁺-CAMK dependent pathway. This leads to the recruitment of SRC-RTK and the inhibition of the MAPK cascade (Adapted from Albert and Tiben, 2001).

1.7.4.2- Other 5-HT receptor signalling pathways

All three of the 5-HT₂ receptor subtypes couple positively to phospholipase C and therefore, produce an accumulation of inositol phosphates and intracellular calcium. When the 5-HT_{2A} receptor is stimulated it activates phospholipase C in both transfected cell lines (Alberts, et al, 1999) and brain tissue (Conn and Saunders-Bush, 1986) via G_{q/11} coupling (Figure 1.20). Activation of the 5-HT_{2C} receptor in the choroid plexus of various species has been shown to increase phospholipase C activity via a G-protein coupled mechanism (Saunders-Bush et al, 1988). There is broad range of evidence suggesting that both 5-HT_{2A} and 5-HT_{2C} receptors can couple to other effector pathways including the mitogen activated protein kinase pathway (Aghajanian and Saunders-Bush, 2005).

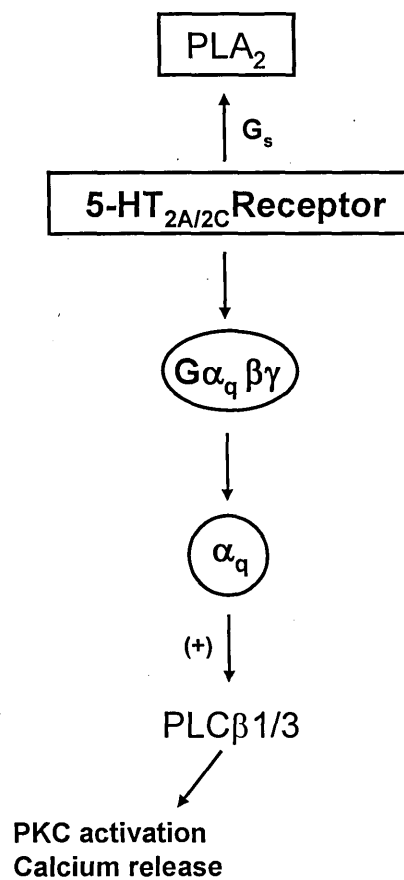


Figure 1.20- Schematic of 5-HT₂ receptor second messenger signalling

5-HT₂ receptor subtypes couple with Gα_q βγ leading to the dissociation of α_q initiating PKC and calcium release (Adapted from Agahajanian and Saunders-Bush, 2005).

5-HT₄, 5-HT₆ and 5-HT₇ receptors have the ability to activate adenylate cyclase via G_{as} (Figure 1.21). 5-HT₄ receptors in several studies have demonstrated that this receptor mediates an increase in cAMP levels producing the phosphorylation of a range of target proteins such as cAMP-dependent protein kinase. The 5-HT₇ receptor has been shown to increase intracellular calcium which activates calmodulin-stimulated adenylate cyclase. Both 5-HT₄ and 5-HT₇ receptors have been hypothesised to be involved in cAMP formation in the rat hippocampus (Aghajanian and Saunders-Bush, 2005).

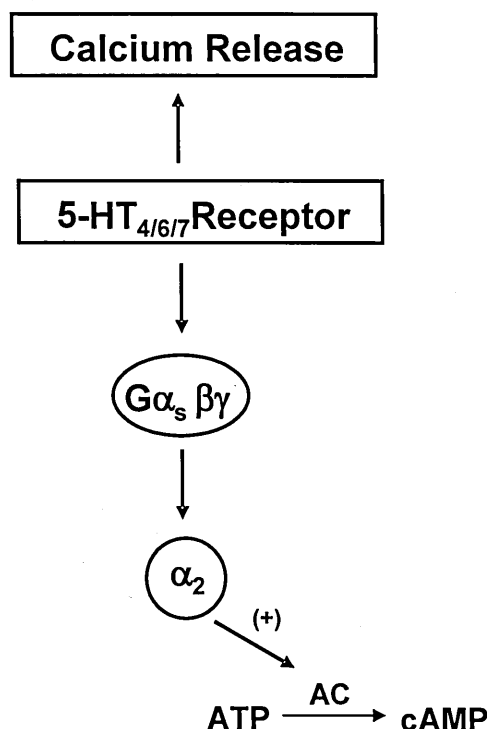


Figure 1.21- Schematic of 5-HT₄, 5-HT₆ and 5-HT₇ receptor second messenger signalling

When activated these 5-HT receptor classes couple to $G\alpha_s\beta\gamma$ and initiating the conversion of ATP to cAMP via the activation of adenylyl cyclase leading to an overall increase in calcium release (Adapted from Agahajanian and Saunders-Bush, 2005).

1.7.4.3- 5-HT_{1A} signalling in depression

Studies to investigate the extent to which 5-HT_{1A} receptors are responsive to the stimulation from agonists that initiate the activation of second messengers could provide a more meaningful index of serotonergic system function (Hsiung et al, 2003).

As previously mentioned the activation of the 5-HT_{1A} receptor requires the activation and the dissociation of a heterodimeric G protein to G_α and $G_{\beta\gamma}$ subunits, the 2 subunits can activate different transduction pathways. $G_{\beta\gamma}$ subunits pathway was first discovered for adrenergic receptors (Touhara et al,

1995) and includes activation of PI3-K and its downstream effector, protein kinase B.

In suicide victims cAMP and PKA activity which couple to $G_{\alpha i/o}$, are thought to be downstream effectors of AC (Dwivedi et al, 2002; Hsiung, 2003). Hsiung et al (2003) have found $G_{\alpha i/o}$ subunit expression to be reduced in suicide victims their data also suggests that both the total activity (induced by forskolin) and the inhibition of this activity (caused by stimulation of the 5-HT_{1A} receptor by 8-OH-DPAT) are attenuated in suicide victims. In brains of patients with major depressive disorder (MDD) and suicide victims little is known about the 5-HT_{1A} receptor activation potential changes in second messenger activities (Hsiung, 2003).

However, studies by Cowburn et al (1994), have begun to functionally assess serotonergic neurotransmission by measuring signal transduction molecules in post-mortem tissues. Cowburn et al studied the post-mortem frontal cortex of suicide victims and showed that G proteins as well as the forskolin-induced adenylyl cyclase activity changes were decreased compared with matched controls even though expression levels of $G_{\alpha s}$ and $G_{\alpha i}$ proteins were unaltered. These observations were confirmed by Reisch et al (1999); they demonstrated reduced adenylyl cyclase immunolabeling and activity in the temporal cortex of depressed suicide victims.

One second messenger pathway activated by 5-HT_{1A} receptors (Cowen, 1996; Della Rocca et al, 1999) and regulated by cAMP (Kim et al, 2001; Lou et al, 2002) involves ERK's. ERK's are members of the MAP kinase family which is involved in pathways that activate transcription factors (Gutkind, 1998). Dwivedi et al, 2001 found that brain regions from suicide victims when compared with matched controls have lower ERK1/2 enzymatic activity.

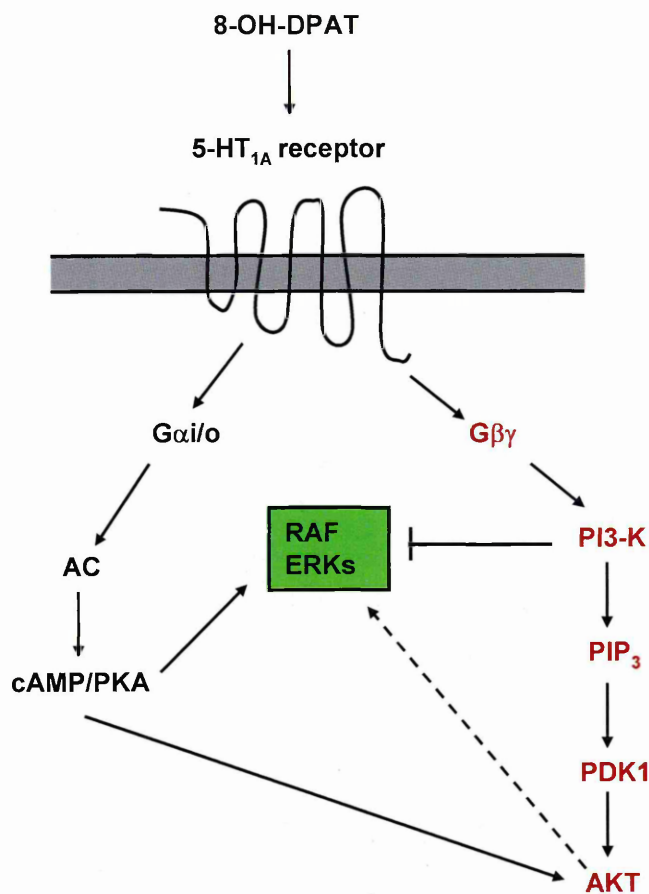


Figure 1.22- 5-HT_{1A} receptor activated transduction pathways

The 5-HT_{1A} receptor, a G-protein is activated by 5-HT_{1A} receptor agonists such as 8-OH-DPAT. This leads to the dissociation of the G_{αi/o} and G_{βγ} subunits. The dissociation of these subunits activates different transduction pathways. The G_{βγ} subunit activates PI3-K and protein kinase B (AKT). The G_{αi/o} subunit decreases the concentration of AC and its downstream effectors cAMP and PKA. 5-HT_{1A} receptors in depressed subjects initiates the ERK's pathway which has been shown to have lower activity in suicide victims (Adapted from Hsiung et al, 2003).

1.8-Summary

5-HT is synthesised from tryptophan and stored in pre-synaptic vesicles. An action potential activates serotonergic neurons causing 5-HT to be released from storage vesicles into the synaptic cleft where it can bind to any of the 5-HT receptor classes present on the post-synaptic neuron. Depending on which post-synaptic 5-HT receptor 5-HT has bound to different signalling transduction pathways are activated. All 5-HT₁ receptor subtypes have been shown to couple to the $G_{i/o}$ effector leading to the inhibition of adenylyl cyclase and a decrease in cAMP accumulation and calcium levels (discussed in section 1.6.4.2).

Synaptic 5-HT levels are mediated by the 5-HT transporter which leads to the re-uptake of 5-HT into the pre-synaptic neuron where it is recycled (Hranilovic et al, 2004). Alternatively, 5-HT is degraded by oxidative deamination catalysed by the enzyme monoamine oxidase (MAO) and converted by oxidation into the metabolite 5-HIAA.

The 5-HT transporter is also the site where antidepressants such as SSRIs bind to prevent the re-uptake of 5-HT from the synaptic cleft and therefore act to alleviate the symptoms of depression by elevating levels of 5-HT in the synaptic cleft leading to an increase in 5-HT firing rate thus returning 5-HT concentration to within the normal range.

In addition, 5-HT activates pre-synaptic somatodendritic 5-HT_{1A} autoreceptors which can act as a negative feedback mechanism on 5-HT neuronal firing. These autoreceptors can be blocked by selective antagonists such as p-MPPI and WAY100635 (discussed in section 1.6.3.2).

The processes and factors involved in 5-HT neurotransmission and its regulation are summarised in Figure 1.23. It has been shown that the 5-HT_{1A} receptor possess allelic variants in coding and regulatory regions. The C-1019G 5-HT_{1A} receptor promoter polymorphism is of particular interest as it affects the expression of the 5-HT_{1A} receptor and hence 5-HT neuronal firing rate. This polymorphism is also involved in the pathogenesis and treatment of anxiety and

depression (Veenstra-VanderWeele, Anderson and Cook, 2000). The nuclear protein complex NUDR has been previously demonstrated to bind to the C-allele of the C-1019G promoter polymorphism and not the G-allele, and therefore, repress the transcriptional activity of the C-1019 allele whereas NUDR fails to bind to the G-1019 allele generating an overexpression of the 5-HT_{1A} receptor this leads to a decrease in action potential firing rates reducing the release of 5-HT. Allelic variants of the 5-HT_{1A} and other 5-HT receptors could influence antidepressant efficacy therefore the study of the 5-HT_{1A} receptor promoter polymorphism C-1019G is important to further elucidate the mechanisms of 5-HT neuronal firing and the effect this polymorphism has in the response to antidepressant treatment for pharmacogenomics.

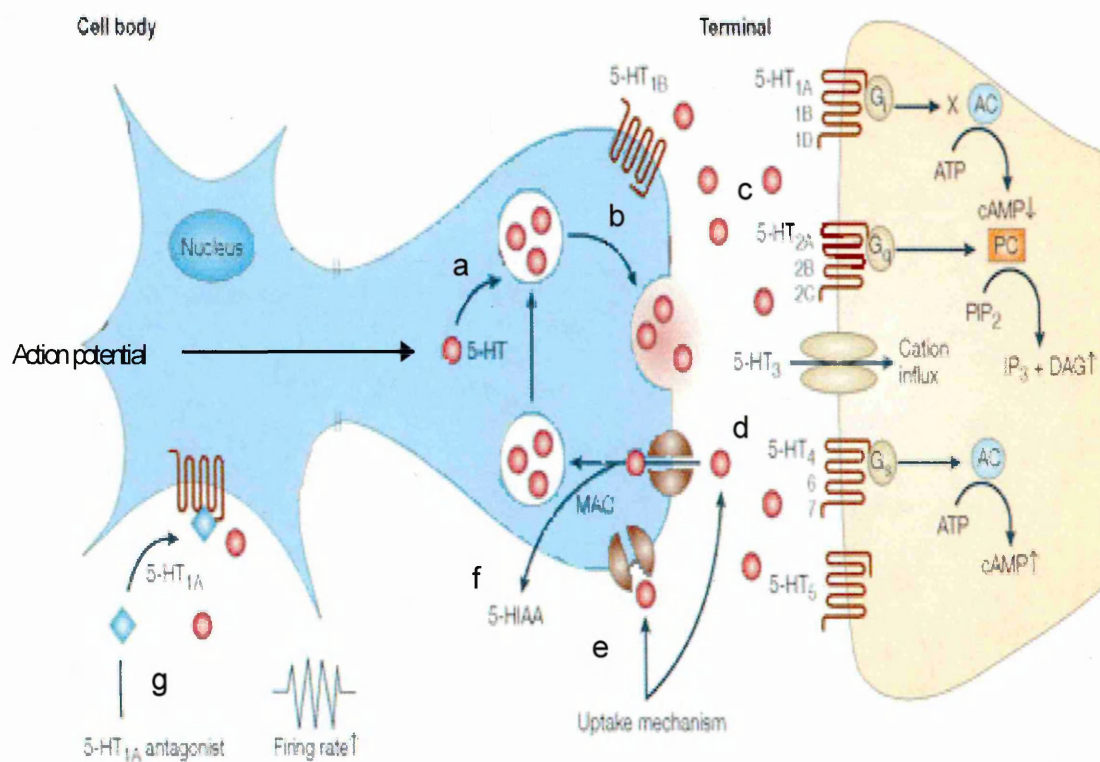


Figure 1.23- Overview of 5-HT neurotransmission

a- 5-HT is taken up into storage vesicles, **b-** 5-HT is released at the pre-synaptic neuron by exocytosis, **c-** 5-HT is released in the synaptic cleft, **d-** 5-HT is degraded by MAO, **e-** 5-HTT re-uptakes 5-HT into the presynaptic neuron, **f-** 5-HT is oxidised into 5-HIAA, **g-** 5-HT can activate 5-HT_{1A} autoreceptors which can be blocked by 5-HT_{1A} antagonists (Wong, Perry and Bymaster, 2005).

1.9- Aims of thesis

The work presented in this thesis was divided into two parts. The first part of this thesis aims to investigate the 5-HT_{1A} receptor promoter polymorphism C-1019G in control hippocampal human post-mortem tissue and to quantify 5-HT_{1A} receptor mRNA expression using real-time PCR and 5-HT_{1A} receptor density by radioligand binding using WAY100635 a 5-HT_{1A} receptor antagonist **(Chapter 2)**.

In the second part of the work presented in this thesis, the SH-SY5Y cell-line was studied to assess whether the retinoic acid (RA) or nerve growth factor (NGF) and aphidicolin differentiated cell line are suitable models to study the 5-HT_{1A} receptor. Real-time PCR was used to investigate the presence of 5-HT_{1A} receptor and NUDR mRNA in this cell line. To determine the presence of 5-HT_{1A} receptor protein western blots and immunocytochemistry were used **(Chapter 3)**. To investigate whether the 5-HT_{1A} receptor is a functional receptor in the SH-SY5Y cell line by studying calcium signalling using fura-2AM assays on a flow cytometer **(Chapter 4)**.

Chapter 2

A study of the 5-HT_{1A} receptor in post-mortem tissue

2.0- Aims

- To determine whether the genotype of the C-1019G 5-HT_{1A} receptor polymorphism affects expression using real-time PCR to quantify 5-HT_{1A} receptor mRNA transcript levels in control human hippocampal post-mortem brain tissue samples.
- To determine 5-HT_{1A} receptor density (fmol/mg) in post-mortem tissue using radioligand binding correlates with C-1019G genotype.

2.1- Introduction

In the past 15 years, studies involving the use of human post-mortem brain tissue have become an essential part of the effort to understand the neurobiology of psychiatric disorders (Lewis, 2002).

Animal models have been used to study the pathogenesis of a wide variety of these disorders but it is thought that these animal models do not always or exactly reflect the human condition (Cummings et al, 2001). Therefore, studying human post-mortem brain tissue directly can provide several essential advantages in the study of psychiatric disorders that are not currently available through other approaches (Lewis, 2002).

In many psychiatric disorders, susceptibility genes are being identified. It is believed that post-mortem tissue studies may provide a crucial means for determining how those genetic liabilities are converted into altered expression of gene products (Lewis, 2002).

2.1.1- Post-mortem tissue

When using post-mortem tissue there are some factors to take into consideration including post-mortem delay (PMI), quality of RNA, age, pH, sex and pre-mortem illnesses. These factors may affect the biological status of post-mortem human brain tissue; for example, the tissue concentrations of individual members of a family of proteins may also change in different ways as a result of one or more of these factors (Gilbert et al, 1981; Bowen et al, 1976; Perry and Perry, 1983).

Post-mortem delay is classified as the time elapsed between death and the freezing or immersion of brain tissue in a fixative (Lewis, 2002). It is reasonable to assume that the shorter the PMI the better the quality of the tissue, however, several other factors need to be considered as well including the fixative used and storage of samples (Barton, 1993; Harrison, 1995; Trotter, 2002).

More recently, the addition of tissue pH and specific markers of RNA quality such as the RNA integrity number (RIN) have been introduced (Hynd, 2003; Imbeaud, 2005; Johnston, 1997) as other quality markers to consider when using post-mortem tissue.

2.1.2- 5-HT_{1A} polymorphism C-1019G

The C-1019G polymorphism of the 5-HT_{1A} receptor gene is the most commonly studied polymorphism due to its association with depression (Lemondé et al, 2003). The polymorphism is located in a transcriptional regulatory region within the 26bp palindromic site believed to be important for protein-DNA interactions (Lemondé et al, 2003; Huang et al, 2004).

It is thought that variation in this region could initiate impairment in the repression of the 5-HT_{1A} receptor, which could correlate with depression or suicidal behaviour or both.

An association between the functional C-1019G promoter polymorphism of the 5-HT_{1A} gene and major depressive disorder (MDD) has been reported; MDD subjects are three times more likely to be homozygous for the GG genotype compared to control subjects (Parsey et al, 2006).

2.1.3- Genotyping methods

A Single Nucleotide Polymorphism (SNP) is the variation of a single base pair in the DNA sequence between either the members of a species or between the paired chromosomes of an individual.

SNP detection and genotyping can be used to explain and diagnose many diseases, to study the variation in drug responses, to establish the origin of biological material and to study the relatedness between individuals. SNPs are highly abundant, and are estimated to occur at 1 out of every 1,000 bases in the human genome (Sachidanandam et al, 2001).

Presently, there are several different methods readily available for the detection of SNP-genotypes ranging from hybridisation of allele-specific oligonucleotides to TaqMan probes.

2.1.3.1- Real-time PCR

The polymerase chain reaction (PCR) is a technique that can amplify a specific DNA segment *invitro* by using two specific primers that hybridise to opposite DNA strands (Sharma, Singh and Sharma, 2002).

The PCR method generates large quantities of specific DNA from a complex DNA template in a single enzymatic reaction within a matter of hours (Sharma, Singh and Sharma, 2002). The traditional form of PCR involves the detection of the PCR target at the end-point after the last PCR cycle, whereas, in more advanced techniques such as, Real-time PCR (RT-PCR) an accurate measure of the amount of PCR product is taken throughout every cycle.

This was achieved using a selection of different fluorescent chemistries that enable to correlate PCR product concentration with fluorescent intensity (Wong and Medrano, 2005).

Real-time PCR has now become the technique of choice for quantifying mRNA, as real-time PCR allows the rapid analysis of gene expression from low quantities of starting template (Peirson, Butler and Foster, 2003). This technique has enabled swift and reproducible high-throughput quantification combined with high sensitivity (Peirson, Butler and Foster, 2003).

More traditional approaches, such as northern blots and RNase protection assays are in several cases unsuitable as they have low sensitivity which requires high concentrations of starting template to achieve detection (Peirson, Butler and Foster, 2003).

2.1.3.2- PCR amplification phase

The PCR amplification can be divided into 3 phases the linear ground phase, exponential phase and the plateau phase (Tichopad et al, 2003). During the first 10-15 PCR cycles, fluorescence emission at each cycle has not yet risen above that of background this is known as the linear phase. During this phase baseline fluorescence is calculated. The second phase is known as the exponential phase at this stage the fluorescence has reached a threshold and is significantly higher than background levels (Threshold line in Icyler, Biorad literature). At this point the PCR reaction has reached its optimal amplification period with PCR product doubling every cycle under ideal reaction conditions. The final phase is the plateau phase where all the reaction components become limited (Bustin, 2000).

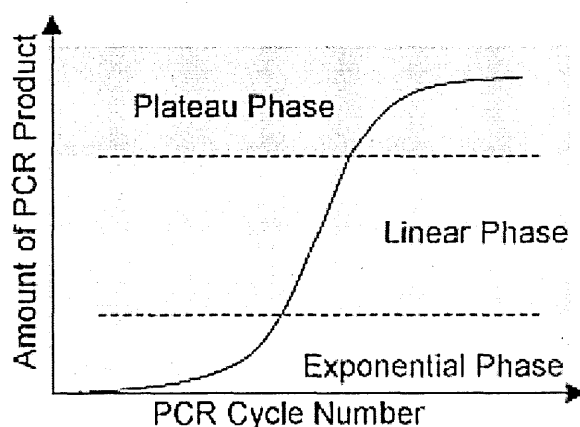


Figure 2.1- Diagrammatic representation of the PCR phases

In the exponential phase the level of fluorescence is significantly above that of background. The linear phase involves the doubling of PCR product every cycle. The final phase is the plateau phase this occurs when the reaction components become limited.

2.1.3.3- Melt curve analysis

A melt curve illustrates the variation in the fluorescence signal observed as double-stranded DNA (dsDNA) separates or "melts" into single-stranded (ssDNA) when amplicon temperature is increased. Verification of Real-time PCR product can be accomplished by plotting fluorescence as a function of temperature, hence the melting curve of the amplicon. Software such as iCycler plots change in fluorescence ($-dF$)/ change in temperature (dT) plotted against

temperature. The melting temperature (T_m) of an amplicon depends entirely on its nucleoside composition, G and C content and the presence of base mismatches. It is therefore, possible to distinguish the fluorescent signals obtained from the correct product from amplification artefacts that melt at lower temperatures and have broader peaks (Bustin, 2000). The specificity of the PCR reaction is given by the primers and reaction conditions used. Even well designed primers can form primer dimers or amplify non-specific product, therefore using a melt curve analysis you can clearly see if you have non-specific product amplified.

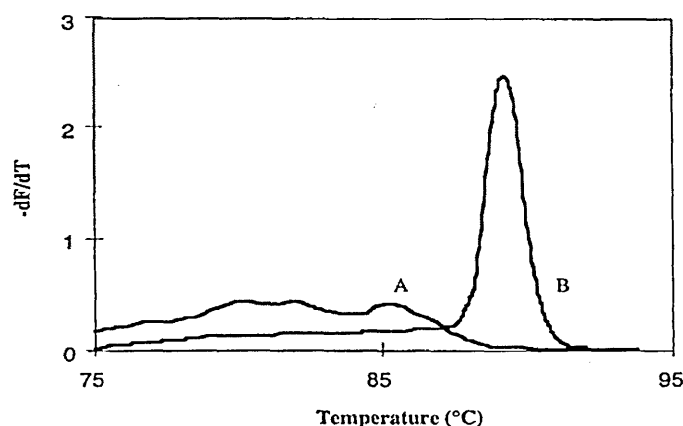


Figure 2.2- Melt curve analysis

The peak labelled with B shows specific product the peak displaced to the left (A) shows non-specific product. An ideal melt curve should have non-specific product separated from specific product by at least 2°C.

2.1.3.4- Housekeeping genes

Real-time PCR gene expression data is normalised to correct for sample to sample variation. Starting material obtained from different individuals can vary in tissue mass, cell number, RNA integrity or quality. It can be difficult to standardise whole tissue samples for mRNA levels whereas, when using a cell line cell number can be standardised (Vandesompele et al,2002). Therefore, real-time PCR results are often normalised against a control gene (Housekeeping gene). The ideal housekeeping gene should be expressed constantly regardless of experimental conditions, including cell type. As it is quite difficult to find one gene that meets all the above criteria for every experimental conditions it is therefore necessary to validate the expression and

stability of a control gene to run with every new real-time PCR experiment (Schmittgen and Zakrajsek, 2000).

2.1.3.5- Optimisation of PCR reaction

The primer length and sequence are important parameters to take into account when designing PCR primers to obtain successful amplification. Optimum primer length should have the ideal length ranging from 17 to 28 base pairs (Rybicki, 2001) and should have a Guanine (G) and Cytosine (C) base composition of 50-60 percent. It is necessary to check that 3' prime ends of primers are not complementary as this may produce primer dimer. Primer self-complementarity, the ability for primers to form hair-pin structures, should also be avoided (Innis and Gelfand, 1991).

2.1.3.6- Amplification efficiency of PCR primers

Amplification efficiency is an important consideration when performing relative quantification (changes in sample gene expression are measured based on a reference gene). Many PCRs do not have ideal amplification efficiencies and, therefore, calculations of relative gene expression without an appropriate correction factor for primer efficiency may lead to an over estimation of gene expression (Lui and Saint, 2002).

As PCR results are based on C_t values which are determined in early exponential phase of the reaction these differences in amplification efficiency only generate minor differences in C_t value (Giulietti et al, 2001). However, after 36 cycles of amplification a 5 percent difference in amplification efficiency can generate a 2-fold difference in PCR product concentration (Freeman, Walker and Vrana, 1999). The delta-delta C_t method ($\Delta\Delta C_t$) and the Pfaffl method are used to calculate gene expression. In the $\Delta\Delta C_t$ method the efficiency of reaction primers is assumed as 2, which can cause variability in calculated gene expression levels. Whereas, the Pfaffl method takes into account the individual primer efficiencies allowing the accurate calculation of relative expression levels.

2.1.3.7- Allele Specific Oligonucleotides (ASO)

Early SNP-genotyping assays were based on ASO hybridisation in a “dot” blot format. Amplified products encompassing the SNP are immobilised onto a solid surface and then hybridised to a radiolabelled oligonucleotide or a reverse dot blot format which involves the binding of biotin or fluorescent labelled PCR products (Saiki et al, 1989). This technique was developed further by several groups to permit allele-specific amplification and genotyping of SNPs. Two allele-specific oligonucleotides are designed with the nucleotide complementary to the allelic variant of the single nucleotide polymorphism positioned at the end of the probe (Syvänen et al, 2001). Hybridisation conditions are chosen such that only those probes with a perfect match will hybridise to the sample. Therefore, it is possible to distinguish by hybridisation between alleles differing by only one nucleotide in sequence.

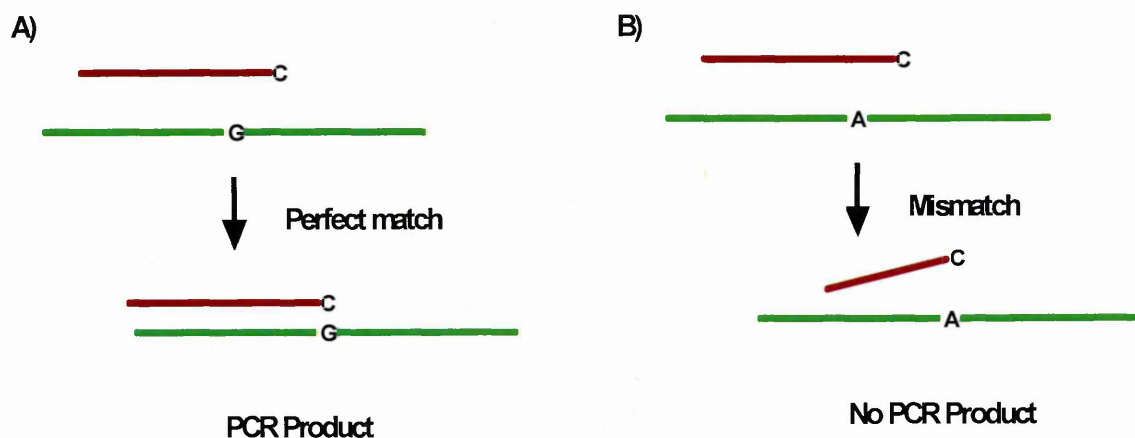


Figure 2.3- Schematic of ASO genotyping method

Allele specific oligonucleotides will only bind to the complementary allelic variant SNP base as shown in A) to generate PCR product which is detected on an Agarose gel. B) shows that when a mismatch of bases occur no PCR product is generated.

2.1.3.8- SNP Real-time PCR genotyping

Several new SNP genotyping technologies have been developed in the past few years. Several of these technologies are based on various methods of allele discrimination and target amplification (Wang et al, 2005). An inexpensive homogenous melting temperature (T_m) - shift genotyping method has been

reported by Wang (2005). The method relies on allele-specific PCR without labelled oligonucleotides. This method uses two allele-specific primers, each containing a 3'-terminal base that corresponds to one of the two SNP allelic variants. The method also includes a reverse primer that amplifies both alleles and the fluorescent dye SYBR Green (Abgene, UK) is used for detection purposes (Wang et al, 2005). GC-rich tails of unequal length are attached to the allele-specific primers, this provides the PCR product with a distinct T_m that depends on which of the two primers is responsible for the amplification and hence genotype can be determined by examining the melt curve on the real-time PCR instrument (Biorad, iCycler).

2.1.3.9- TaqMan probe genotyping

TaqMan probes provide sequence-specific detection that relies on one method of signal generation, the separation of a fluorophore from a quencher (Gibson, 2006). Other technologies involve the association of two fluorophores to generate fluorescent signal by energy transfer between a donor and an acceptor fluor, this is most commonly used in FRET technologies.

Sequence-specific detection permits the unambiguous detection of target sequences without the production of non-specific signals arising from primer dimers and other PCR events.

TaqMan was one of the initial methods introduced for homogeneous or real-time sequence detection (Holland et al, 1991) and has since been widely adopted for both the quantification of mRNA's and for detecting variation.

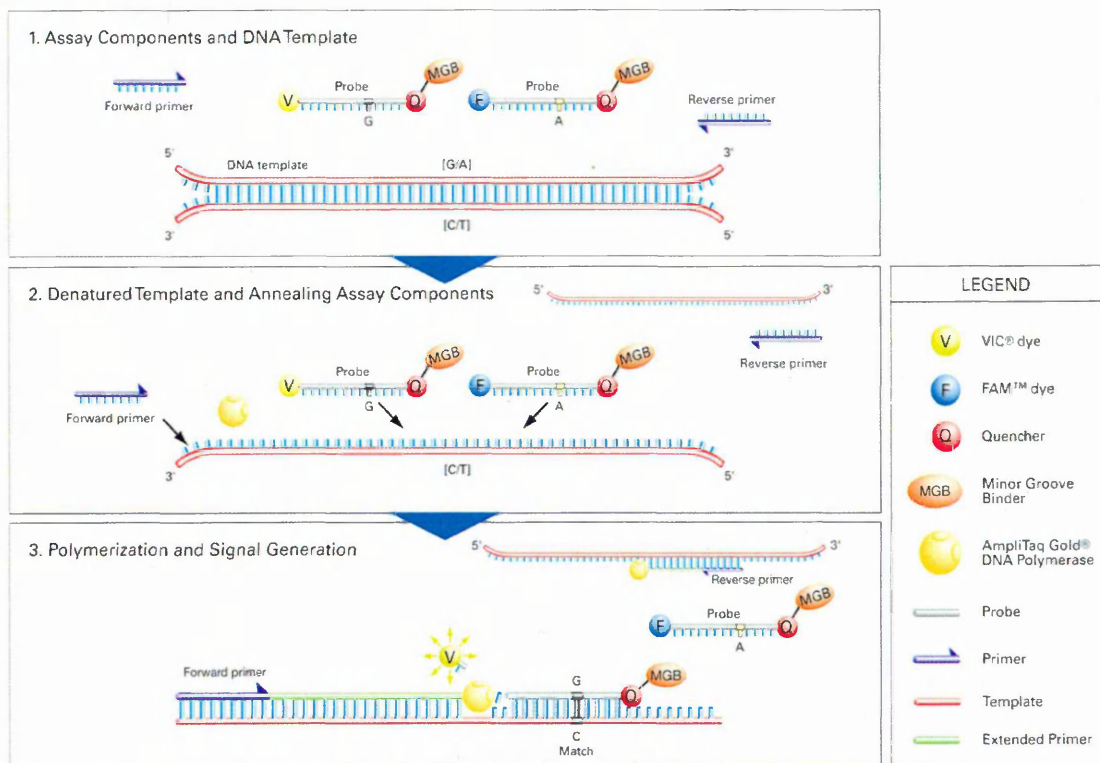


Figure 2.4 - Schematic of TaqMan probe genotyping method

One of the specifically designed TaqMan probes for the target SNP bind to single stranded DNA template where there is a match leading to the separation of the VIC dye or FAM dye from the quencher which generates a fluorescence signal that is detected by the instrument (Applied Biosystems literature).

2.1.4- Radioligand binding

Radioligand binding is a highly regarded tool throughout a wide range of disciplines, including pharmacology, physiology, biochemistry, immunology and cell biology (Toews, 1993).

To enable the analysis of interactions of hormones, neurotransmitters, growth factors, and related drugs with their receptors, studies of receptor interactions with second messenger systems, the characterization of regulatory changes in receptor number and physiological function are required (Bylund and Towes, 1993).

Radioligand binding as a technique is comparably simple, only requiring that the binding should be saturable, due to there being a finite number of receptors.

Binding should maintain a selectivity in competing for radioligand that parallels the selectivity of those competing agents in modifying a response and that the kinetics of binding should parallel the kinetic response (Limbird, 2004).

Studies using radioligand binding are often performed to establish the affinity of different drugs for a receptor and in addition the binding site density (B_{\max}) of receptor subtypes of a particular family can be determined for individual tissues or samples.

K_d is the measure of the concentration of a radioactive ligand that is required to occupy 50% of the receptors, whereas B_{\max} is a measure of density of the receptor in a tissue and is equal to the amount 'Bound' when all the receptors are occupied by a radioactive ligand.

These techniques of genotyping, real-time PCR and radioligand binding described above have been used extensively in the characterisation of serotonin receptors in the brain providing an insight into the functional role of serotonin and its receptors and their involvement in the pathophysiology of many psychiatric diseases.

2.2.1- Ethical aspects

Human post-mortem tissue samples used in this research were provided by Professor Gavin Reynolds, Queens University Belfast. Approval for this study was obtained from the appropriate committees.

2.2.2- Brain tissue samples

All tissue samples used in this study were obtained from control subjects that had no previous history of mental health. The age range, post-mortem delay and sex of subjects were 49-93 years, 4-79 hours and 14 subjects were male and 10 were female.

Human post-mortem tissue samples from the hippocampal brain region were stored in a freezer at -70°C . All tissue samples were weighed and then thawed on ice before use.

2.2.3- Human post-mortem Brain tissue genotyping

2.2.3.1- DNA extraction

150mg of human post-mortem brain tissue was homogenised in kit lysis buffer using a micro pestle (Fisher Scientific UK) and genomic DNA extracted using the tissue DNA purification kit (Nucleon ST, Tepecik Life Sciences, UK). Lysed tissue was pelleted and 0.5 ml of reagent B was added to resuspend pellet. To deproteinise 150 μl of sodium perchlorate was added and sample inverted 7 times, 0.5ml of chloroform was then added and inverted 7 times to emulsify the phases. Nucleon resin (150 μl) was added and centrifuged at 350g for 1 minute. To precipitate DNA, the upper aqueous layer (clear) was removed to a fresh 1.5ml eppendorf and two volumes of cold absolute ethanol were added. The sample was inverted several times until DNA was precipitated. Once DNA was precipitated the sample was centrifuged at 4000g to pellet the DNA. The pellet was then washed with 1.0ml of 70 percent ethanol. The sample was re-centrifuged and supernatant discarded. The pellet was then air dried for 10 minutes to remove all traces of ethanol. DNA was re-dissolved in 100 μl of ddH₂O.

A further ethanol purification step was carried out. 2.5-3 volumes of 95 percent ethanol/0.12M sodium acetate were added to the DNA sample. The sample was

incubated on ice for 10 minutes. After incubation samples were centrifuged at 10,000g in a microcentrifuge for 15 minutes at 4°C, the supernatant was decanted and sample drained by inverting onto a paper towel. 80 percent ethanol (2 volumes of original sample) were added. Samples were incubated at room temperature for 5-10 minutes, then centrifuged for 5 minutes. Samples were decanted and drained as above. Samples were then left to air dry for 10-15 minutes, pellets were then re-suspended in deionised water.

2.2.3.2- Allele specific oligonucleotide (ASO) PCR for 5-HT_{1A} genotyping

ASOs allow specific amplification of a region that includes the variant nucleotide site. The ideal ASO length is 19-21 base pairs, which is short enough to allow differential hybridisation based on a single base change and long enough to provide high probability of locus specificity.

Allele specific primers for the C-1019G polymorphism of the 5-HT_{1A} receptor were designed from the sequence (Z11168) using primer 3 software. Allele-specific primers were synthesised by MWG Biotech. This ASO method was adapted from Prof. Gavin Reynolds (Reynolds et al, 2006).

Primer	Sequence (5'-3')	T _m	Product size
Forward	CTG AGG GAG TAA GGC TGG AC	61.4	174bp
Reverse (C allele)	GAA GAA GAC CGA GTG TGT CTT CG	62.4	
Reverse (G allele)	GAA GAA GAC CGA CTG TGT CTT CC	62.4	

Table 2.1: Oligonucleotide primers used for ASO genotyping

2.2.3.3- PCR reaction

Each 25µl PCR reaction contained PCR master mix (3mM MgCl₂) (ABgene, UK), 5µl of purified DNA and final concentration of 0.5µM of forward and reverse primers.

2.2.3.4- PCR cycle conditions

95°C for 5 minutes

95°C for 30 seconds

58°C for 40 seconds

72°C for 40 seconds

72°C for 10 minutes

hold at 4°C

32 cycles

Two PCR reactions (Forward primer was run with different reverse primers) were run for each sample genotyped. PCR product was visualised on a 3.5 percent agarose gel and genotype determined by either a band being present with both primers (G/C genotype) or only one band present with either the G primer or the C primer.

2.2.4- SNP real-time PCR genotyping

To each of the two allele-specific primers, GC tails of different lengths were added. The long 14-bp GC tail had the sequence 5'- GCGGGCAGGGCGGC-3' and the short 6-bp GC tail had the sequence 5'- GCGGGC-3'. The longer GC rich tail is usually added to an allele-specific primer that has a higher T_m base (G or C) at its 3'end, and a short tail to the other allele-specific primer with the lower T_m base (A or T).

If one allele-specific primer is thought to amplify more efficiently than the other resulting in uneven height of melt curves then the more efficient allele-specific primer is added at half the original concentration (0.25 μ M). Primers were synthesised by MWG Biotech UK. This method was adapted from Wang et al, 2005.

2.2.4.1- Primers

Primer	Sequence (5'-3')	T_m
Forward	CTG AGG GAG TAA GGC TGG AC 3'	61.4
Reverse (C allele)	GCG GGC GAA GAA GAC CGA GTG TGT CTT CG	72.3
Reverse (G allele)	GCG GGC AGG GCG GCG AAG AAG ACC GAC TGT GTC TTC C	>75

Table 2.2: Oligonucleotide primers for Real-time PCR SNP genotyping

2.2.4.2- PCR reaction

Primer stock solution was 100pM, 12.5µl of this stock was added to ddH₂O to make a 12.5µM solution. 1µl of 12.5µM solution in 25µl PCR reaction gave a final concentration of 0.5µM.

Each 25µl PCR reaction contained 12.5µl of SYBR green PCR master mix (ABgene, UK), 5µl of purified DNA and final concentration of 0.5µM of each forward and reverse C primer and 0.25µM of reverse G primer were added.

2.2.4.3- PCR cycle conditions

95°C for 15 minutes to activate SYBR green mix

95°C for 15 seconds
58°C for 15 seconds
72°C for 30 seconds

} 40 cycles

Melt curve

95°C 30 seconds
50°C for 30 seconds increasing in 1°C increments

} 45 cycles

2.2.5- TaqMan custom Genotyping

Applied Biosystems custom designed TaqMan probes for G and C alleles designed from NCBI accession number Z11168. The assay contains two allele specific TaqMan probes labelled with VIC or 6-FAM dye and a primer pair to detect the specific SNP target. Each TaqMan probe incorporates the minor groove binder (MGB) group on the 3' end. Each 25µl PCR reaction consisted of 30ng DNA, 11.857µl Applied Biosystems PCR master mix and 0.657µl of TaqMan probe mix.

2.2.5.1- PCR cycles

The following cycles were performed on ABI Step one Real-time PCR instrument.

90°C 10 minutes
90°C 30 seconds
92°C 15 seconds
72°C 15 seconds

} 40 cycles

2.2.6- RNA extraction

RNA was extracted from human post-mortem tissue samples from the hippocampal region (50-60mg) using a SV total RNA isolation kit (Promega, UK). Tissue was homogenised with a micro pestle (Fisher Scientific, UK) in kit lysis buffer until a liquid homogenate was formed. Buffer N3 was added and the samples were centrifuged at 10,000g for 10 minutes. The clear top layer was removed into a fresh 1.5ml eppendorf tube, 200µl of 70 per cent ethanol was added before loading sample on to a spin column. RNA was eluted in 100µl of elution buffer. Extracted RNA was kept in the freezer at -70°C.

2.2.6.1- Experion analysis of RNA

For quantitative and qualitative analysis of RNA the Bio-Rad Experion system was used. Extracted RNA samples were thawed on ice. Once thawed, 3µl of RNA sample and 3µl of experion RNA ladder were heated in a 1.5ml eppendorf tube at 70°C for 3 minutes. After 3 minutes samples were stored on ice. The Experion StdSens RNA chip was primed with 9µl of filtered gel mixture and was pipetted into the yellow G well. The chip was placed onto the priming station and primed for 30 seconds on pressure setting B. Once primed, 7µl of sample buffer was added to wells labelled 1-12. RNA ladder (1µl) and 7µl of sample buffer were added to L well. 9µl of gel and gel stain mixture were pipetted in to GS well. 1µl of each sample was pipetted into each well 1-12. Any blank wells had water added to them instead of sample. Samples were electrophoresed for 15 minutes.

2.2.6.2- cDNA synthesis

The iScript cDNA synthesis kit (Bio-Rad, UK) was used to transcribe single-stranded cDNA. In each cDNA synthesis reaction RNA, 10µl ddH₂O, 4µl of (5X) iScript buffer and 1µl of reverse transcriptase (1U) was added. Reactions were heated at 25°C in a heat block for 5 minutes, then moved to a water bath and heated at 42°C for 30 minutes. Reactions were finally heated at 85°C for 5 minutes in a heat block for 5 minutes. All samples had 20µl of ddH₂O added to them before being stored at -20°C.

2.2.7- Real-time PCR

Real-time PCR is a common method used for quantifying gene expression. To normalise any PCR reaction housekeeping genes are used. Housekeeping gene expression should remain constant in the tissue or cells of the target gene. The accurate normalisation of levels of gene expression is vitally important to achieve reliable data, especially when the biological significance of a subtle difference in gene expression is investigated (Vandesompele et al, 2002). Therefore, the two most stable housekeeping genes to run with every real-time PCR reaction should be determined using GeNorm software (Vandesompele et al, 2002).

2.2.7.1- Housekeeping gene validation

8 housekeeping genes (B actin, β_2 M, GAPDH, HPRT, RPL13A, SDHA, UBC, and YWHAZ) were tested on 10 human post-mortem brain tissue samples. Threshold (C_t) values were collected and the expression ratio was determined by the comparative C_t method. These values were imported into the GeNorm software (Vandesompele et al, 2002) which determines the most stable housekeeping genes. A gene expression stability measure (M value) was calculated for each housekeeping gene. The housekeeping gene with an M value greater than 1.5 was identified as the least stable and was removed. Housekeeping genes are eliminated until the two most stable housekeeping genes remain.

2.2.7.2- Primer design

The housekeeping gene primers were taken from Vandesompele et al, (2002). The 5-HT_{1A} primers used were designed from accession number Z11168 using Primer 3 software (www.primer3.com). All primers were obtained from MWG Biotech. Primers are shown in the following table.

Primer	Forward primer (5'-3')	T _m	Reverse primer (5'-3')	T _m	Product size
β – ACTIN	CTGGAACGGTGAAGGTGA CA	57.3	AAGGGACTTCCTGTAACA ATGCA	57.3	141bp
β ₂ M	TGCTGTCTCCATGTTTGAT GTATCT	57.9	TCTCTGCTCCCCACCTCTA AGT	55.9	86bp
GAPDH	TGCACCACCAACTGCTTA GC	59.4	GGCATGGACTGTGGTCAT GAG	59.4	125bp
HPRT1	TGACACTGGCAAAACAAT GCA	55.9	GGTCCTTTTCACCAGCAA GCT	59.8	98bp
RPL13A	CCTGGAGGAGAAGAGGAA AGAGA	62.4	TTGAGGACCTCTGTGTATT TGCAA	59.7	126bp
SDHA	TGGGAACAAGAGGGCATC TG	59.4	CCACCACTGCATCAAATTC ATG	58.4	86bp
UBC	ATTTGGGTGCGGTTCTT G	56.7	TGCCTTGACATTCTCGATG GT	57.9	90bp
YWHAZ	ACTTTTGGTACATTGTGGC TTCAA	57.6	CCGCCAGGACAAACCAGT AT	59.4	134bp

Table 2.3: Oligonucleotide primer sequences for housekeeping genes used for Real-time PCR

Primer	Sequence (5'-3')	T _m	Product size
5-ht1a_for	GCG AGA ACG GAG GTA GCT TT	59.4	148bp
5-ht1a_rev	CCC AGA GTG GCA ATA GGA GA	59.4	

Table 2.4: Oligonucleotide primer sequences for 5-HT_{1A} gene

2.2.7.3- PCR reaction

The PCR reaction was optimised by titrating the magnesium chloride (MgCl₂) concentration (3mM, 4mM and 5mM), template concentration (2µl and 4µl) and primer concentration (0.2µM, 0.5µM, and 1µM).

Once optimised each 25µl PCR reaction contained 12.5µl of (2X) SYBR green mix (Abgene, UK), 1µl of MgCl₂ (4mM), 5.5µl of ddH₂O and 1µl of each forward and reverse primer (0.5µM). 4µl of cDNA was added separately to the 96 well plate sample wells.

Two housekeeping genes were run on every plate and used as a reference to normalise data.

2.2.7.4- PCR Cycles

95°C for 15 minutes to activate SYBR green mix

95°C for 15 seconds
60°C for 15 seconds
72°C for 30 seconds } 40 cycles

Melt curve

95°C 30 seconds
50°C for 30 seconds increasing in 1°C increments } 45 cycles

2.2.7.5- Primer efficiency

To calculate primer efficiency template cDNA was diluted in series (neat, 1:5, 1:10, 1:50 and 1:100), each PCR reaction was carried out in duplicate. The C_t values obtained were exported in to the graphical and statistical software programme GraphPad Prism 4. Using the Prism software the slope and 1 over slope were determined.

A good reaction should have efficiency between 90 percent and 110 per cent. In this study, the slope of each primer was calculated using the Prism software.

A 100 per cent efficiency corresponds to a slope of -3.32. The slope of a log-linear phase demonstrates the efficiency of the amplification reaction.

2.2.8- Radioligand Binding of 5-HT_{1A}

Radioligand binding is performed to establish the affinity of different drugs for a receptor. In addition the binding site density (B_{\max}) of receptor subtypes of a particular family can be determined for individual tissues or samples.

K_d is a measure of concentration of radioactive ligand that is required to occupy 50 percent of the receptors, whereas B_{\max} is a measure of density of the receptor in a tissue.

2.2.8.1- Sample preparation

The radioligand used was the 5-HT_{1A} antagonist ³[H] WAY100635. An exact amount in excess of 60mg of control hippocampal human brain tissue was weighed out and homogenised in Tris-HCL buffer (50mM, pH 7.4). The homogenate was centrifuged and the pellet resuspended in buffer to a concentration of 3.125mg/ml.

The radioligand was diluted to produce eight concentrations (8nM, 6nM, 4nM, 2nM, 1nM, 0.5nM, 0.25nM and 0.12nM).

The amount of radioligand added to buffer to make a 8nM solution was calculated using the following equation:

$$\frac{\text{volume of solution required (X) x 81 (specific activity)}}{200}$$

The binding reaction was carried out in a 96 well plate, in each well 400µl of tissue suspension, 50µl of radioligand, 50µl displacer (5-HT (1mM)) or buffer (Control) was added.

The plate was incubated at 37°C for 50 minutes. The Skatron cell harvester was used to transfer the contents of the wells to filter paper. Filter paper was placed in scintillation fluid and counted in a scintillation counter.

2.2.9- Data analysis

2.2.9.1- Comparative C_t method

Housekeeping gene mRNA expression was calculated using the comparative C_t method using the formula below (Livak and Schmittgen, 2001).

$$\text{Ratio} = 2^{-[\Delta C_t \text{ sample} - \Delta C_t \text{ control}]}$$

$$\text{Ratio} = 2^{-\Delta\Delta C_t}$$

2.2.9.2- Pfaffl method

The relative expression ratio of a target gene was calculated based on its efficiency (E) and the difference in C_t value (delta C_t) of an unknown sample against that of a control and expressed in comparison to a reference gene:

$$\text{RATIO} = \frac{(E_{\text{target}})^{\Delta C_t_{\text{target}}(\text{control-sample})}}{(E_{\text{ref}})^{\Delta C_t_{\text{ref}}(\text{control-sample})}}$$

Where, E_{target} is the real-time PCR efficiency of the target gene transcript;

E_{ref} is the real-time PCR efficiency of a reference gene transcript; $\Delta C_t_{\text{target}}$ is the C_t deviation of control minus the sample of the target gene transcript; ΔC_t_{ref} is equal to the C_t deviation of control minus the sample of the reference gene transcript (Pfaffl, 2001).

2.2.9.3-Primer efficiency calculation

$$E = 10^{(-1/\text{slope})} - 1$$

2.2.9.4- B_{\max} and K_d

A common method of determining K_d and B_{\max} values from saturation experiments is to use nonlinear regression analyses provided by software such as GraphPad Prism. A hyperbola curve is fitted to the data, as concentration increases the amount of bound also continues to increase until a saturation point is reached. To determine B_{\max} and K_d using a saturation plot a rectangular hyperbola can be fitted to the data utilising the following equation:

$$\text{Specific Binding} = \text{Fractional Occupancy} \cdot B_{\max} = \frac{B_{\max} \times [L]}{K_d + [L]}$$

Where specific binding is the total binding of receptor and ligand, $[L]$ is the concentration of the free radioligand.

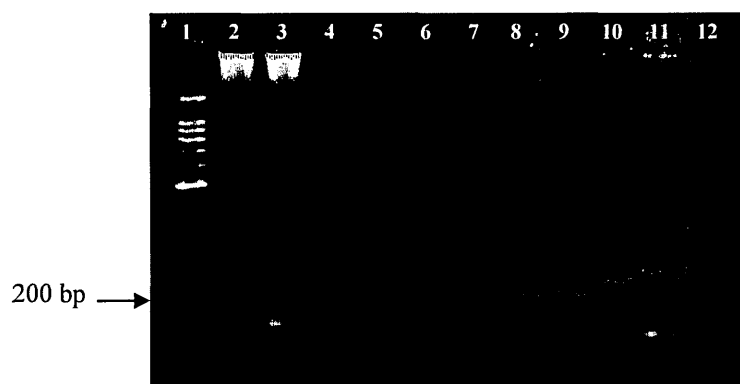
2.3- Results

2.3.1- ASO 5-HT_{1A} genotyping

Specifically designed primers with a single base-pair substitution were used for genotyping samples.

In total 23 human post-mortem brain tissue samples from various brain regions were genotyped for the C-1019G promoter polymorphism.

Bands were present at PCR product size 174bp. The following samples, positive control (genomic DNA, Sigma) and sample 344 both have a band present with both C and G reverse primers therefore these samples have a G/C genotype. One band is present with the G allele with samples 177 and 187 and therefore both samples have a GG genotype. Sample 200 has one band present with the C allele and therefore has a CC genotype (Figure 2.5).



Key

- 1- DNA ladder
- 2- Genomic DNA (Sigma) reverse C
- 3- Genomic DNA (Sigma) I reverse G
- 4- 344 rev C
- 5- 344 rev G
- 6- 177 rev C
- 7- 177 rev G
- 8- 200 rev C
- 9- 200 rev G
- 10- 187 rev C
- 11- 187 rev G

Figure 2.5- A typical agarose gel of 5-HT_{1A} receptor genotypes

An example agarose gel showing the resultant PCR product(s) generated by the ASO genotyping technique.

Sample	Genotype
009	G/C
062	GG
069	GG
131	G/C
177	GG
187	GG
199	-
200	CC
204	-
244	G/C
254	-
255	CC
257	GG
266	G/C
267	GG
283	CC
300	CC
323	G/C
324	G/C
325	GG
334	G/C
343	G/C
344	CC

Table 2.5- Genotype of human hippocampal postmortem brain tissue samples using the ASO method

Each genotyping reaction was repeated twice. Three samples (199, 204 and 254) did not yield any PCR products and therefore, their genotype could not be determined. The percentage genotypes for the ASO method were 35% GG, 40% G/C and 25% CC genotype.

2.3.2- Real-time PCR SNP genotyping

A homogenous melting temperature (T_m) - shift genotyping method has been reported by Wang et al (2005). This method was adapted to enable its use with the Biorad iCycler real-time PCR instrument. This method uses two allele-specific primers, each containing a 3'-terminal base that corresponds to one of the two SNP allelic variants.

GC-rich tails of unequal length are attached to the allele-specific primers, this provides the PCR product with a distinct T_m and hence genotype can be determined by examining the melt curve (Figure 2.6).

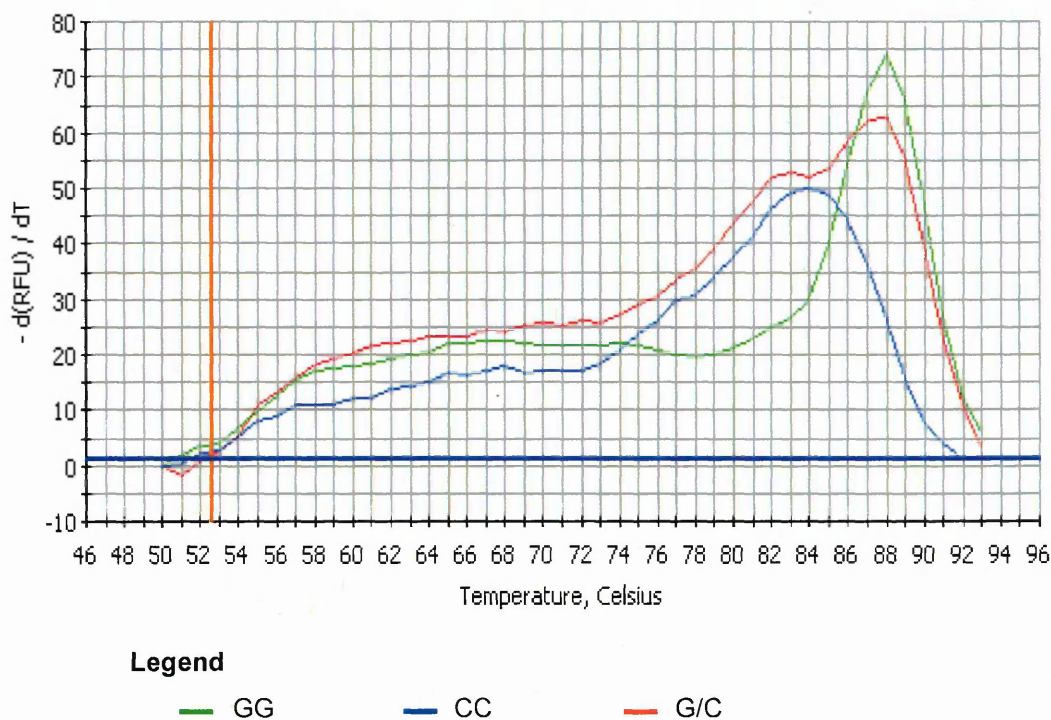


Figure 2.6- Example data of real-time PCR SNP genotyping

The green peak represents the homozygous GG genotype which has a melting temperature T_m of 89°C, homozygous CC genotype is represented by the blue peak which has a T_m of 84°C and the red peak represents the heterozygote G/C genotype with T_m 's of 84°C and 89°C.

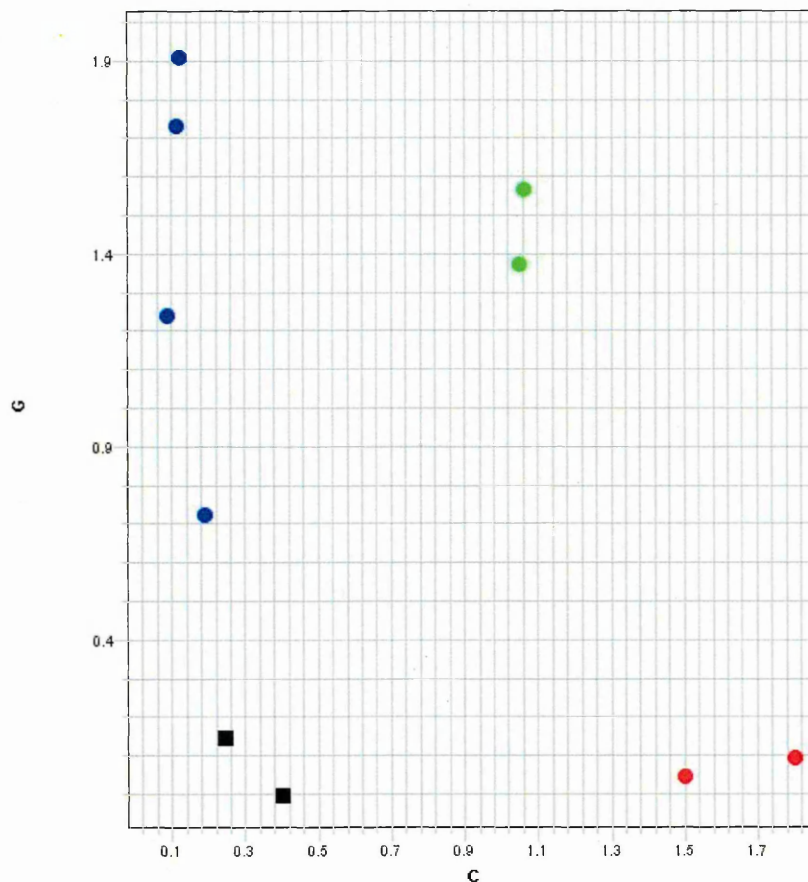
Sample	Genotype
009	CC
062	CC
069	-
131	GG
177	GG
187	-
199	-
200	CC
204	CC
244	GG
254	CC
255	GG
257	CC
266	GG
267	GG
283	GG
300	-
323	CC
324	-
325	-
334	GG
343	CC
344	GG

Table 2.6- Genotype of human hippocampal postmortem brain tissue samples using real-time PCR SNP genotyping method

PCR reactions were performed in triplicate. Genotypes in six samples were undetermined due to inconsistencies in melt curve.

2.3.3- TaqMan genotyping

Applied Biosystems TaqMan custom assays are designed, synthesized and delivered in a single-tube format. Custom assays use TaqMan® minor groove binding (MGB) probe-based assays that provide superior allelic discrimination and assay design flexibility. This custom SNP assay was specifically designed for the C-1019G promoter polymorphism.



Legend

- Homozygous CC
- Homozygous GG
- Heterozygous G/C
- Negative control

Figure 2.7- Example of a TaqMan allelic discrimination plot

The allelic discrimination plot is generated by normalising the fluorescence of reporter dyes to the fluorescence of the passive reference dye in each well. The ABI prism software plots the normalised intensities (R_n) of the reporter dyes in each sample well on an allelic discrimination plot. The software then algorithmically clusters the sample data and assigns a genotype according to the samples position on the plot.

Samples were repeated in triplicate.

Sample	Genotype			Overall genotype
009	G/C	G/C	GG	G/C
062	CC	CC		CC
069	G/C	G/C		G/C
131	CC	G/C	G/C	G/C
177	CC	CC		CC
187	CC	CC		CC
199	GG	GG		GG
200	G/C	G/C		G/C
204	GG	GG		GG
244	G/C	G/C		G/C
254	G/C			G/C
255	CC	GG	CC	CC
257	CC	CC		CC
266	G/C	G/C		G/C
267	GG	GG		GG
283	G/C	G/C	G/C	G/C
300	G/C	G/C		G/C
323	GG	G/C		-
324	GG	GG		GG
325	GG	GG	GG	GG
334	G/C	G/C	G/C	G/C
343	CC	CC		CC
344	G/C			G/C

Table 2.7- Genotypes of human hippocampal postmortem brain tissue using custom TaqMan probes

The genotype of one sample (323) could not be determined.

Each genotyping technique used in this study produced varying genotypes for each sample. It was therefore, decided to combine genotyping results from each method predominantly using the TaqMan genotyping technique and any samples with poor quality and quantity of DNA the ASO method was used. In this study extracted DNA was assessed for quality and quantity and it was observed that the genotype determined using the ASO method was found to be less effected by DNA quantity and quality. Whereas, the genotype determined by the TaqMan SNP genotype method is effected by the quality and quantity of DNA. Six samples (187, 200, 257, 300, 324 and 344) showed poor quality and quantity of DNA and low amplification with the TaqMan genotyping method, which lead to an inconsistent genotyping result. Therefore, in these cases the ASO method was used. A positive correlation between quality and quantity of DNA extracted from human post-mortem tissue samples was observed (Figure 2.8). The SNP real-time PCR method was not utilised due to the lack of identification of any heterozygotes in these samples which contradicts current

literature. Therefore the genotypes from this method were not included when determining the final genotype for each sample. Final genotypes for each sample are shown in Table 2.8.

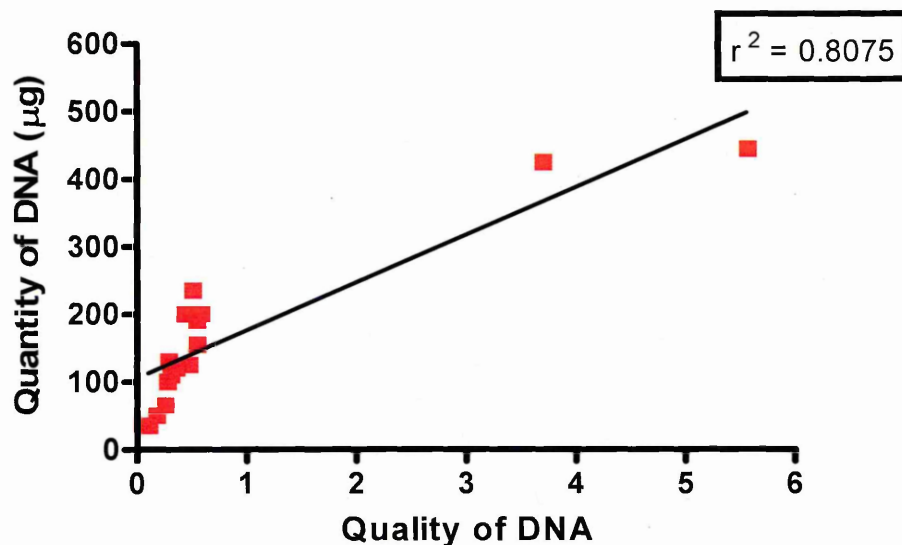


Figure 2.8- Correlation between quality and quantity of DNA

Quality of DNA was determined from the ratio of absorbance readings taken at 260 and 280nm. The quantity of DNA was determined by absorbance reading taken at 260nm. A positive correlation was observed between quality and quantity of DNA.

Sample	ASO Genotype	TaqMan Genotype	Final Genotype
009	G/C	G/C	G/C
062	GG	CC	CC
069	GG	G/C	GG
131	G/C	G/C	G/C
177	GG	CC	CC
187	GG	CC	GG
199	-	GG	GG
200	CC	G/C	CC
204	-	GG	GG
244	G/C	G/C	G/C
254	-	G/C	G/C
255	CC	CC	CC
257	GG	CC	GG
266	G/C	G/C	G/C
267	GG	GG	GG
283	CC	G/C	G/C
300	CC	G/C	CC
323	G/C	-	G/C
324	G/C	GG	G/C
325	GG	GG	GG
334	G/C	G/C	G/C
343	G/C	CC	CC
344	CC	G/C	CC

Table 2.8- Final genotype assigned to samples

2.3.4- Real-time PCR gene expression results

2.3.4.1- RNA analysis using the Experion system

RNA was quantified using the Experion system. Good quality RNA is represented by two bands at 2000 and 3000 corresponding to the two ribosomal bands characteristic of eukaryotic RNA. All samples contained RNA with samples showing some degradation of RNA, samples 9 and 10 particularly showed poor quality RNA (Figure 2.9).

Bands present between the two ribosomal bands suggest degradation of RNA (Figure 2.10).

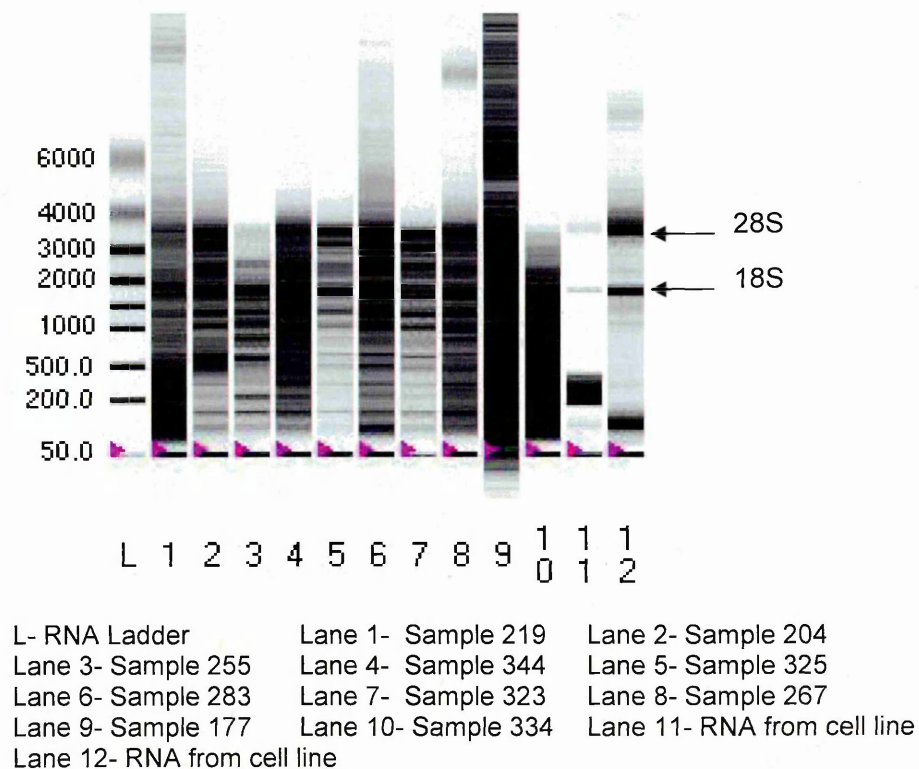


Figure 2.9 - Experion gel data RNA from Human post-mortem brain tissue samples

For good quality RNA two bands, one about 2000 and other about 3000 are expected corresponding to the two ribosomal bands 18S and 28S respectively. Bands present between these two ribosomal bands indicate degradation of the sample. All samples contained RNA Lanes 9 and 10 showed poor quality RNA.

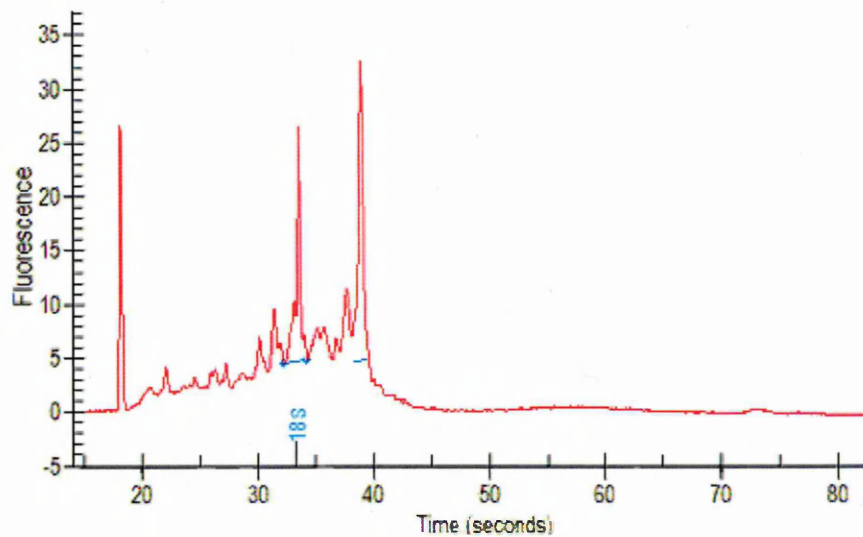


Figure 2.10- Example of a RNA sample electropherogram from the Experion system

Peaks between the two ribosomal (18S and 28S) peaks indicate degradation of the sample.

The human post-mortem tissue used in this study did show some degradation when analysed using the experion system. However, using a highly sensitive technique such as real-time PCR for the detection of mRNA expression, even human post-mortem brain tissue samples with slight RNA degradation due to post-mortem intervals beyond 12hours can still be detected (Gutala and Reddy, 2004).

2.3.4.2- Housekeeping gene validation

Real-time PCR was performed on 10 Human post-mortem brain tissue samples using 8 housekeeping genes (β -actin, β_2 M, GADPH, HPRT, RPL13A, SDHA, UBC, and YWHAZ). 3 housekeeping genes were eliminated from validation due to poor amplification and melt curve analysis. The remaining housekeeping genes data were used to calculate expression ratios by the comparative C_t method. This data was imported into the geNorm software and analysed.

The geNorm software calculates M-values for each housekeeping gene. Housekeeping genes with an M-value above 1.5 are eliminated until the two most stable housekeeping results remained.

Results showed that the two most stable housekeeping genes were SDHA and UBC both with M-values of 0.905.


					
Change Data	B ACTIN	RPL13A	HPRT	SDHA	UBC
177	1.40E-01	1.00E-02	1.20E-01	1.40E-01	1.00E-01
323	1.00E+00	1.00E+00	1.00E+00	1.00E+00	1.00E+00
219	1.00E-02	1.00E-01	3.00E-02	3.00E-02	3.00E-02
204	2.90E-01	2.70E-01	6.00E-02	6.00E-02	7.00E-02
267	1.90E-01	3.70E-01	2.30E-01	4.00E-02	5.00E-02
255	6.00E-02	5.20E-01	1.00E-02	1.00E-02	1.00E-02
344	1.30E-01	1.74E+00	1.74E+00	2.00E-02	6.00E-02
334	4.00E-02	2.46E+00	9.30E-01	1.30E-01	1.70E-01
283	6.40E-01	1.93E+00	1.23E+00	9.00E-02	1.30E-01
325	1.50E-01	1.68E+00	2.14E+00	1.00E-02	6.00E-02
M < 1.5	2.198	2.772	2.455	2.283	1.829

Figure 2.11 - geNorm data of 5 housekeeping genes

The relative expression ratios of each housekeeping gene were imported into the geNorm software. The M value for each gene was calculated and the gene with the highest M value (Shaded in red) is deemed the least stable and is removed. The most stable housekeeping gene is shaded in green. Housekeeping genes are removed until the two most stable genes remain.

geNorm			
Change Data	SDHA	UBC	Normalisation Factor
177	1.40E-01	1.00E-01	1.8127
323	1.00E+00	1.00E+00	15.3205
219	3.00E-02	3.00E-02	0.4596
204	6.00E-02	7.00E-02	0.9929
267	4.00E-02	5.00E-02	0.6852
255	1.00E-02	1.00E-02	0.1532
344	2.00E-02	6.00E-02	0.5307
334	1.30E-01	1.70E-01	2.2776
283	9.00E-02	1.30E-01	1.6572
325	1.00E-02	6.00E-02	0.3753
M < 1.5	0.905	0.905	

Figure 2.12- geNorm data of the 2 most stable housekeeping genes

Through a process of elimination according to the M values of the housekeeping genes the two remaining stable housekeeping genes are SHDA and UBC both have an M value of 0.905.

2.3.4.3- Efficiency of Primers

A dilution series of cDNA was tested with both housekeeping gene primers and the 5-HT_{1A} primer.

The C_t values for each dilution were imported in to GraphPad Prism, the slope of the line was calculated using average C_t values.

A slope of -3.295 represents a 100 percent efficient primer. SDHA, UBC and 5-HT_{1A} efficiencies are shown in Figure 2.13.

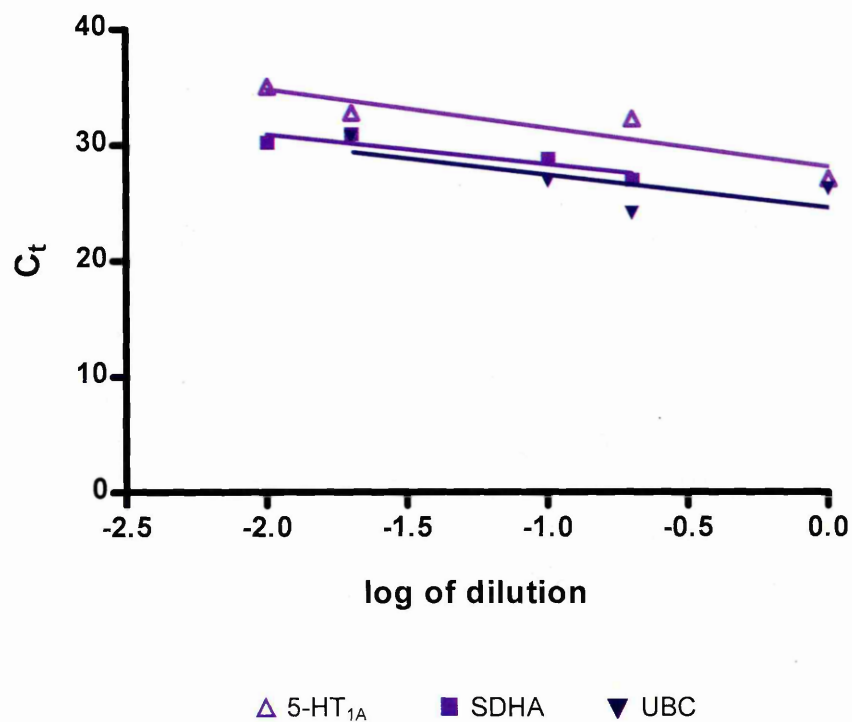


Figure 2.13- Efficiency of the housekeeping primers SDHA, UBC and 5-HT_{1A}

A dilution series of cDNA was tested with the primers SDHA, UBC and 5-HT_{1A}. The C_t values for each dilution were used to calculate slope of the line. Percentage of efficiency is calculated using $E = 10^{(-1/\text{slope})} - 1$. SDHA, UBC and 5-HT_{1A} have a slope of -2.570 ± 0.881 , -2.821 ± 1.881 and -3.344 ± 1.040 , and percentage efficiencies of 144.9, 117.2 and 99.1 percent respectively.

2.3.4.4- Post-mortem factors

The key factor in conducting post-mortem research is the quality of the tissue. Several factors including pre and post-mortem conditions can influence the quality of tissue and its ability to yield accurate results (Stan, 2006). However, the housekeeping genes used in real-time PCR controls for this providing that confounding factors are checked.

Commonly recognised confounds include PMI, age and sex of patient. These tissue quality parameters were assessed in the human brain tissue samples used in this study.

Increasing age at death has been associated with reductions in certain mRNAs (Nichols et al, 1993; Harrison, 1995; Castensson et al, 2002). Figure 2.14 shows that there is no significant correlation between Age of subject and relative expression ratio of the 5-HT_{1A} receptor.

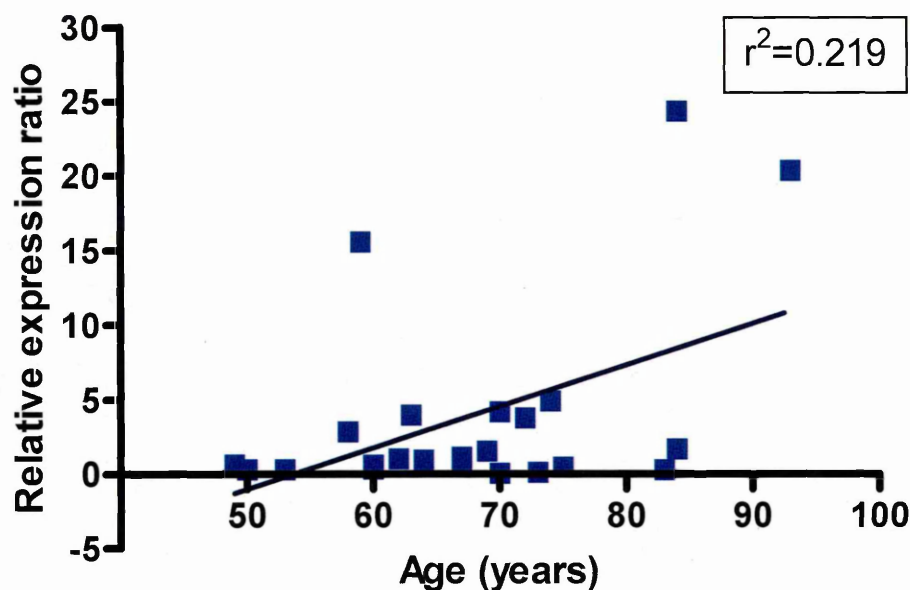


Figure 2.14- Correlation between age and relative expression ratio

No significant correlation is observed between an increase years in age of subject and relative expression ratio of 5-HT_{1A} receptor mRNA ($r^2 = 0.219$).

Gender is one of the main confounds of interest in autopsy studies, presently there is little data on sex differences in human subjects (Hynd, 2003). In this study 13 male and 10 female subjects were correlated to 5-HT_{1A} receptor mRNA levels (Figure 2.15).

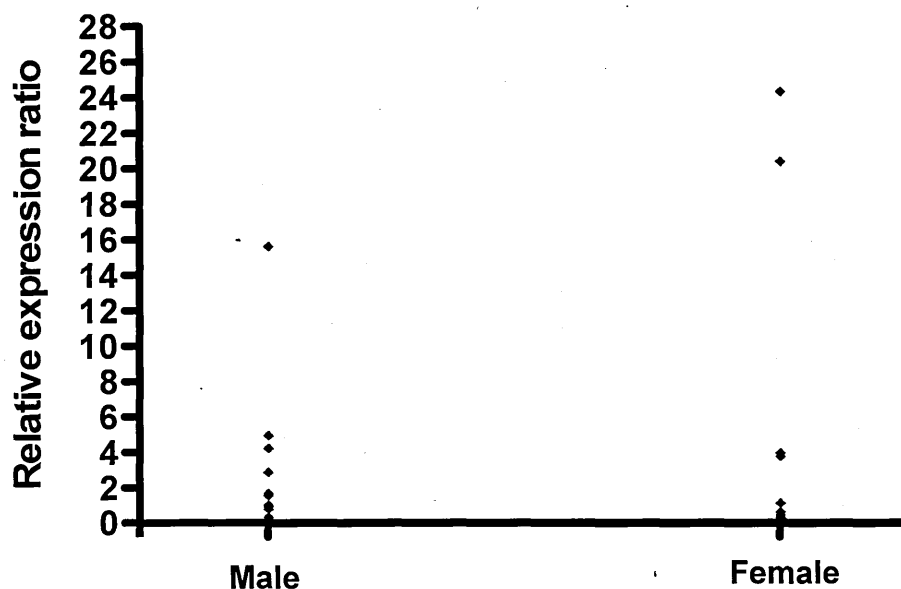


Figure 2.15- correlation between gender of subject and relative expression ratio

No significant correlation was observed with gender and 5-HT_{1A} receptor mRNA expression ratios. 1.0= Male, 2.0= Female.

To assess the quality of post-mortem tissue the main confound is the PMI. Typical intervals of post-mortem delay range from 4 to 36 hours (Whitehouse, 1984; Barton, 1993). Studies of gene expression in the brain often target specific mRNAs therefore it is essential to be able to control for the effect of PMI on that mRNA (Hynd, 2003). Figure 2.16 shows that PMI had no effect on the mRNA expression of the 5-HT_{1A} receptor in the tissue used in this study.

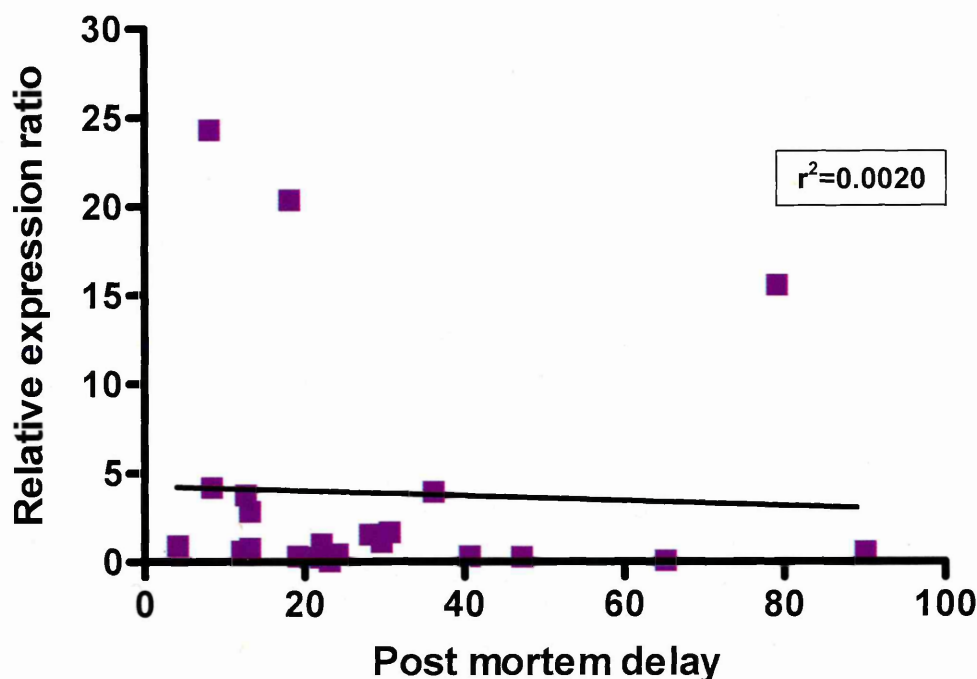


Figure 2.16- Correlation between post-mortem delay and relative expression ratio

There was no correlation observed between PMI and relative expression ratio of 5-HT_{1A} receptor in human brain tissue samples used in this study ($r^2=0.0020$).

2.3.4.5- Real-time PCR data correlated to genotype

The mean C_t values of each sample were imported into Excel and the Pfaffl method was used to calculate the relative gene expression ratios (Pfaffl, 2001). Samples with a GG genotype had higher expression compared to those with a G/C or CC genotype. Data was analysed using one-way ANOVA ($p>0.05$), (Figure 2.17). The log of the relative expression was also compared to that of the C-allele. The results show that samples with a G allele had a 1-fold increase of average expression compared to samples with C allele

Data was analysed with an un-paired Student's t-test. Samples with a G allele had a significantly higher 5-HT_{1A} receptor expression compared to samples with a C allele ($P<0.05$), (Figure 2.18).

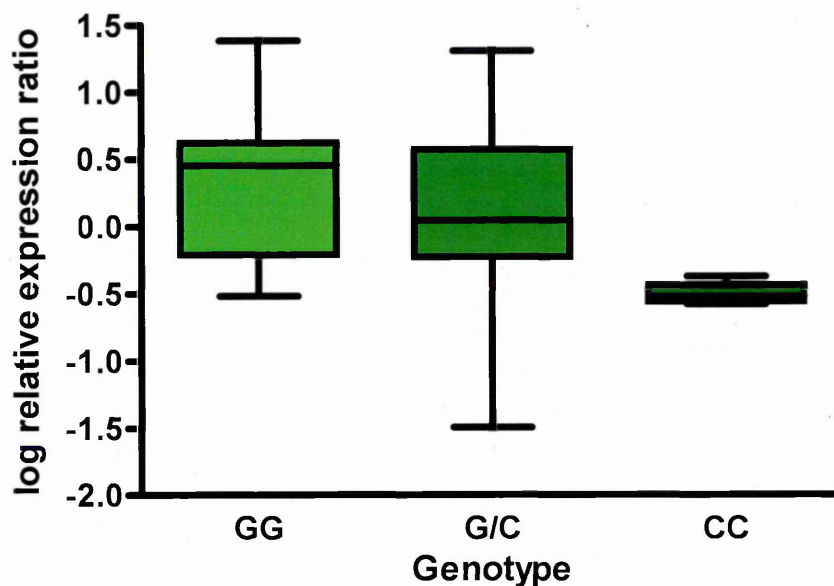


Figure 2.17- Log of relative gene expression correlated with genotype

Post-mortem brain tissue samples with a GG genotype had higher expression of the 5-HT_{1A} receptor than samples with a CC genotype ($p>0.05$). 22 samples were genotyped 7 samples had a GG genotype, 9 samples had a G/C genotype and 6 had a CC genotype. One sample was excluded as it produced an ambiguous result. The boxes displaces the differences between populations and the spacings between the different parts of the box help indicate the degree of dispersion. The line represents the median.

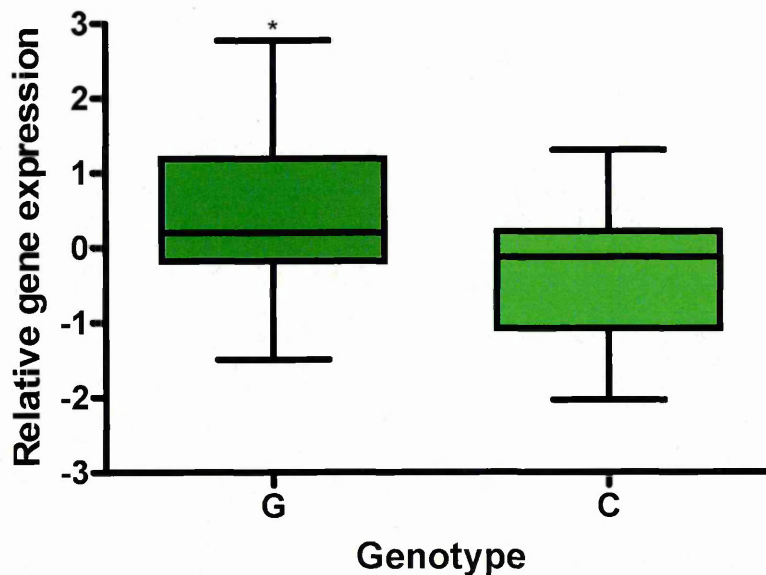


Figure 2.18- Log of relative gene expression of G-allele versus the C-allele

Post-mortem brain tissue samples with a G allele had higher expression of the 5-HT_{1A} receptor than samples with a C allele ($*p<0.05$). 22 samples were genotyped 16 samples had a G allele present, 15 samples had a C allele present. One sample was excluded as it produced an ambiguous result.

The calculated relative expression ratios and genotype for each post-mortem brain tissue sample is shown in Table 2.9.

Sample	Relative Expression Ratio	Genotype
177	0.3127	CC
200	0.2904	CC
255	0.4253	CC
267	0.6191	GG
283	0.5963	G/C
323	0.7541	G/C
325	0.9065	GG
343	0.2636	CC
344	1.1112	CC
199	0.3047	GG
204	3.9292	GG
334	3.7549	G/C
324	1	G/C
187	2.8407	GG
254	1.5250	G/C
257	4.1861	GG
069	24.324	GG
062	4.9038	CC
131	15.5841	G/C
009	20.371	G/C
244	1.6501	G/C
266	0.0320	G/C
300	0.0963	CC

Table 2.9- The relative expression ratios of and genotype for each post-mortem brain tissue sample

Sample 324 was chosen as the control sample to which all other samples were compared when calculating the relative expression ratios using the PffafI method and therefore has a relative expression ratio of 1.

2.3.5- Radioligand binding results

The best and most common method of determining K_d and B_{max} values from saturation experiments is to use nonlinear regression analyses provided by software such as Prism (Graphpad). A hyperbola curve is fitted to the data, as concentration increases the amount of bound also continues to increase until a saturation point is reached. B_{max} and K_d for this sample were 1383 ± 32.6 dpm and 1.15 ± 0.09 nM respectively (Figure 2.19).

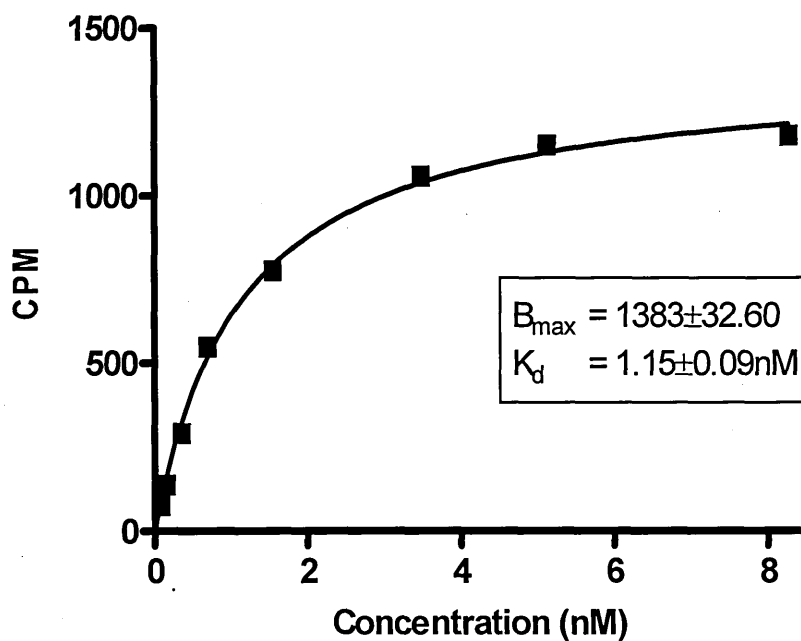


Figure 2.19- Radioligand binding of a human post-mortem tissue sample with the 5-HT_{1A} silent antagonist WAY100635

A saturation plot with Bound on the Y-axis and Free on the X-axis can be used to determine B_{\max} and K_d values by non-linear regression. As the concentration of radioligand increases the amount bound continues to increase until saturation point is reached. The resultant graph is a hyperbola.

2.3.5.1- Binding data correlated with genotype data

B_{\max} values and genotype for each post-mortem tissue sample were imported into Microsoft Excel and from B_{\max} values receptor density in fmol was calculated. Results indicate that samples with a GG or G/C genotype have an increased 5-HT_{1A} receptor density compared to samples with a CC genotype ($p < 0.05$) (Figure 2.20).

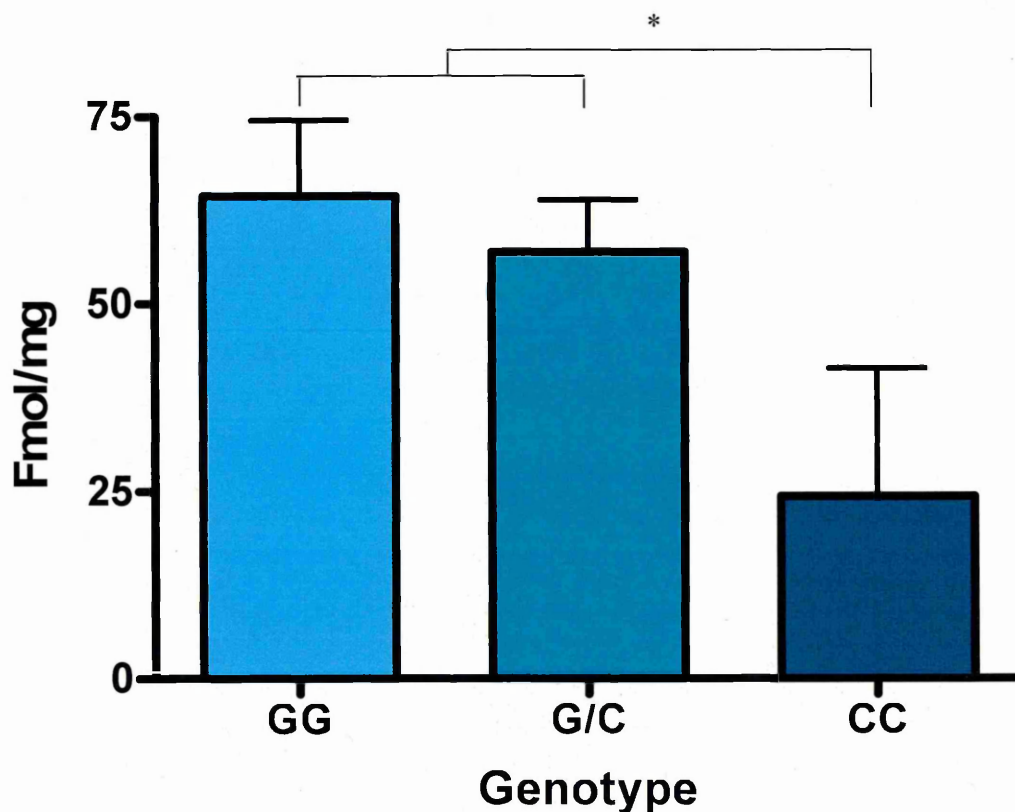


Figure 2.20- 5-HT_{1A} radioligand binding data correlated with genotype

Samples with a GG ($n = 4$) or G/C ($n = 7$) genotype have a significantly higher density of 5-HT_{1A} receptor compared to samples with a CC ($n = 2$) genotype. Data presented as means \pm SEM, Student's un-paired t-test, $*p < 0.05$.

The calculated B_{\max} , K_d , receptor density in fmol and genotype of each post-mortem brain tissue sample is shown in Table 2.10.

Sample	B _{MAX} (DPM)	K _D (nM)	Receptor density (fmol)	Genotype
199	997.5	0.221	55.97	GG
200	737.5	0.08586	41.38	CC
204	255.4	0.3325	14.34	GG
244	1113	0.1477	62.45	G/C
266	695.4	0.4	39.02	G/C
267	941.3	0.06	52.82	GG
283	1117	0.08287	62.68	G/C
300	1432	0.1506	80.35	G/C
323	630	0.2896	35.35	G/C
324	1383	0.1155	77.60	G/C
325	1506	0.1383	84.51	GG
334	254	0.4567	14.25	G/C
343	131	0.3655	7.35	CC
344	723.3	0.1136	40.58	G/C

Table 2.10- The calculated B_{max}, K_d and receptor density (fmol) values with concurrent genotype of each post-mortem brain tissue sample

Genotype	Sample	Receptor density (fmol)
GG	199	55.97
GG	204	14.34
GG	267	52.82
GG	325	84.51
G/C	244	62.45
G/C	266	39.02
G/C	300	80.35
G/C	323	35.35
G/C	324	77.60
G/C	334	14.25
G/C	344	40.58
CC	200	41.38
CC	343	7.35

Table 2.11- Summary table of 5-HT_{1A} receptor density and genotype

2.4- Discussion

The results show a clear association between genotype of the common promoter region polymorphism C-1019G, mRNA expression levels and radioligand binding of 5-HT_{1A} receptor in human brain post-mortem tissue samples.

2.4.1- 5-HT_{1A} genotyping of human post-mortem brain tissue samples

Many new SNP genotyping technologies have been developed in the past few years. Several of these technologies are based on various methods of allelic discrimination and target amplification (Wang et al, 2005).

In this study three different genotyping methods were used, ASO genotyping, real-time PCR SNP genotyping and finally custom designed TaqMan probes (Applied Biosystems). Each method incorporated the use of allele specific oligonucleotides, but the way in which genotype was determined differed greatly. For the ASO genotyping method genotype is determined by the presence of a band with each reverse primer producing a GG or CC genotype and two bands of equal intensity are present with the G/C genotype on an agarose gel, whereas, the real-time PCR SNP genotyping method genotype is determined from the melt curve analysis and genotype is determined by an allelic discrimination plot with the TaqMan probe genotyping method.

In this study 23 samples were genotyped for the common C-1019G 5-HT_{1A} promoter polymorphism. The resultant genotypes for each sample genotyped by the ASO method are shown in Table 2.5. Table 2.6 shows the genotypes determined by the real-time PCR SNP genotyping method and the genotypes determined by the TaqMan probe method are shown in Table 2.7.

The resultant genotypes demonstrate that 25 percent have CC genotype, 35 percent have the GG genotype and 40 percent have G/C genotype with the ASO method. Similarly, the TaqMan probe genotyping method results generated a 26 percent CC genotype, 22 percent GG and 52 percent G/C

genotype split. In contrast, the resultant genotypes from the real-time PCR SNP method produced a 47 percent CC genotype and 53 percent GG genotype split with no heterozygotes present.

Clearly, the real-time PCR SNP genotyping method proved to be unreliable as no heterozygote genotypes were identified. Therefore results using this method were disregarded.

The overall genotype was determined by using genotypes obtained by the ASO method, a previously established method. Any ambiguous genotypes were verified using the TaqMan genotyping method. The results demonstrate control samples had a 30 percent CC genotype, 30 percent GG, 39 percent G/C genotype across 23 samples. Genotype distribution was in Hardy Weinberg equilibrium.

With each genotyping method used in this study there are limitations. The ASO method of genotyping needs to have carefully controlled PCR conditions for accuracy. Genotype is determined on the basis of the presence or absence of PCR products when using oligonucleotides specific for either genotype. The limitation with this technique is the fact that the absence of a product may also be due to sub-optimal PCR conditions or due to low DNA quantity or quality (Mamotte, 2006). Similarly, genotyping using the TaqMan method the PCR reaction is also affected by low DNA quality or quantity of samples.

Genotyping using the real-time PCR SNP method has one main disadvantage which is the use of DNA dyes as they are not sequence specific and are often prone to generating non-specific products, such as, primer dimers (Gibson, 2006). In this study this method was disregarded due to the lack of identification of any heterozygotes in this sample group.

The genotyping results obtained in this study are in agreement with Lemonde et al, (2003), Arias et al (2002), Parsey et al (2006) and Huang et al (2004). Lemonde et al, 2003 genotyped 134 control samples and the results showed that 37% had the CC genotype, 12% the GG genotype and 51% G/C genotype. Another study by Arias (2002) gave a similar trend of percentage of genotype

as seen in the Lemonde et al (2003) study, in which 170 control samples were genotyped and 19%, 26% and 55% had CC, GG, and G/C genotypes respectively. Parsey et al, (2006) genotyped 42 control samples and showed that 28% had a CC genotype, 12% had a GG genotype and 60% had a G/C genotype. A similar study showed comparable results with healthy volunteer samples genotyped for C-1019G polymorphism had a 31 % CC, 22% GG and 47% G/C split across 102 samples (Huang et al, 2004).

2.4.2- Real-time gene expression PCR

2.4.2.1- RNA analysis

It is known that high quality RNA sample is important to obtain successful results with many routine molecular biology applications, especially real-time PCR. However, it has also been proven that degraded RNA can produce accurate and valid results when used in carefully validated PCR reactions (Schoor et al, 1995; Lee et al, 2005; Auer et al, 2003; Wittiver et al, 1997). The Experion automated electrophoresis system (Biorad, UK) can be used in the assessment of RNA quality as it provides a sensitive and accurate analysis.

RNA quality of post-mortem brain tissue used in this study was shown to be degraded when analysed on the experion system (Figure 2.9 and Figure 2.10). It is very well known that RNA is sensitive to degradation especially by post-mortem processes (Perez-Novo et al, 2005). Schoor et al (1995), have shown that gene expression profiles obtained from partially degraded RNA samples that still have visible ribosomal bands present exhibit a high degree of similarity compared to that of intact samples and that of RNA samples that have sub-optimal quality. Thus, gene expression profiles obtained from degraded RNA may still lead to meaningful results if used carefully. Similarly, Lee et al (2005) results indicated that high quality expression data can be generated even when the RNA exhibits significant degradation. Auer et al (2003), were also in agreement and concluded that RNA degradation does not preclude microarray analysis if comparison is done using samples of comparable RNA integrity.

It is also acknowledged that normalisation by an internal reference gene can reduce or diminish tissue derived effects on quantitative real-time PCR (Wittiver et al, 1997).

In addition a study of real-time PCR analysis of mRNA expression in 19 brains with 3 endogenous reference genes (B-actin, 18s RNA and GADPH) suggested that the post-mortem intervals and age at death do not significantly influence mRNA expression (Gutala and Reddy, 2004).

2.4.2.2- Housekeeping gene validation

It is essential to control for error when measuring RNA expression and quality of RNA especially for errors between samples. These errors can be introduced at a number of stages throughout the experimental protocol including input sample, RNA extraction and reverse transcription. One way of controlling these errors is to use housekeeping genes which are used to normalise any PCR reaction and should not vary in anyway in the tissue or cells of the target gene (Karge et al, 1998; Vandesompele and De Preter, 2002). However, from recent research many studies are performed using these housekeeping genes without making sure of their proper validation of their stability of expression (Vandesompele and De Preter, 2002).

The geNorm software (Vandesompele et al, 2002) is used in the accurate validation of housekeeping genes to be run with every real-time PCR reaction performed. The appropriate validation of internal references is, therefore, crucial to avoid misinterpretations of gene expression (Dheda et al, 2004). In this study the housekeeping genes deemed the most stable according to their M-value where SDHA and UBC (Figure 2.12).

2.4.2.3- Efficiency of primers

The efficiency of each primer set used in this study was determined to enable the quantitative analysis of relative gene expression by the Pfaffl method (Pfaffl, 2001). The slope of a log-linear phase demonstrates the efficiency of the amplification reaction.

To obtain primer efficiency close to 100 per cent, the slope should be around -3.32. Under ideal conditions the efficiency of a PCR reaction should be 100 percent; hence the template doubles after each cycle during exponential

amplification. However, this does not always occur and can be due to a number of factors, such as length of primers, the G/C content of amplicon and pipetting. An ideal reaction should have efficiency between 90 percent and 110 per cent.

SDHA, UBC and 5-HT_{1A} primer sets had efficiencies of 144, 117 and 99.8 percent respectively (Figure 2.13). It is acceptable to use primer sets with efficiencies above 110 percent when determining the relative expression ratio using the Pfaffl method as this takes into consideration the efficiency of each primer pair providing accurate relative expression ratios.

2.4.2.4- Effect of Age, Post-mortem interval (PMI) and sex on 5-HT_{1A} receptor mRNA

Age, PMI (time from death to collection of brain tissue) or sex had no effect on the quantification of 5-HT_{1A} mRNA in the human post-mortem brain tissues used in this study. There was no correlation between PMI (ranging from 4 to 79hrs) with mRNA expression of 5-HT_{1A} (Figure 2.16). Also, no correlation was observed between age (ranging from 49 to 93 years) (Figure 2.14) and sex (males 13 and 10 females), (Figure 2.15) with 5-HT_{1A} receptor mRNA expression.

PET studies of several classes of neuroreceptors such as, dopamine D1 (Wang et al, 1998) and 5-HT_{2A} (Adams et al, 2004) demonstrate age-dependent decline of the availability of receptors by 10 percent per decade. However, this decline has not been confirmed with 5-HT_{1A} receptors.

A study by Gray et al found a significant decrease in 5-HT_{1A} receptor density in females compared with males (Gray, 2005). These finding are in contrast with previous studies on human brain samples which found no variation with gender in 11 men and 10 women (Palego et al, 1997). Another study using 8-OH-DPAT has reported decreased levels of 5-HT_{1A} receptor levels in frontal cortex from females compared with males (Arango, 1995).

Many mRNAs are thought to be highly susceptible to post-mortem interval (Barton, 1993; Harrison, 1995). The results in this study are confirmed by Burnet et al (1996) that found there was no significant effect of post-mortem interval on mRNA's extracted from the hippocampus brain region (Burnet et al, 1996). However, in

previous studies post-mortem interval did affect 5-HT_{1A} and 5-HT_{2A} receptor mRNAs in other brain regions (Burnet et al, 1996).

2.4.2.5- Real-time PCR data correlated with 5-HT_{1A} genotype data

In this study the relative expression ratio for the 5-HT_{1A} receptor indicates that human post-mortem brain tissue samples with a G allele had a 1-fold increase of average mRNA expression compared to that of the C allele. Statistical analysis using un-paired Student's t-test, samples with a G allele had significantly higher gene expression than that of samples with a C allele ($p < 0.035$), (Figure 2.18).

Lemondé et al, (2003) study suggested that their results indicated that depressed patients were twice as likely as controls to have the homozygous G-1019 genotype, and suicide victims were four times as likely to carry the same genotype. Similarly, Huang et al found that the G allele is associated with increased expression in cell-lines and has a higher frequency in schizophrenia (Huang et al, 2004). The GG allele has also been associated with the endophenotypes of depression and anxiety on the NEO rating scale for neuroticism in 284 normal subjects (Strobel et al, 2003). These results suggest that the G-1019 allele is associated with a predisposition to a depressed phenotype in normal subjects (Albert and Lemondé, 2004) which is in agreement with the results presented in this study, which show that subjects with a GG genotype have increased expression of the 5-HT_{1A} receptor mRNA levels and therefore a predisposition to anxiety and depression.

2.4.3- Radioligand binding of 5-HT_{1A}

Studies using radioligand binding are often performed to establish the affinity of different drugs for a receptor and in addition the binding site density (B_{max}) of receptor subtypes of a particular family can be determined for individual tissues or samples (Bylund and Toews, 1993).

In this study the radioligand used was the 5-HT_{1A} antagonist ³[H] WAY100635. The ideal saturation plot is one where the hyperbola curve fitted to the data goes through all the data points as seen in Figure 2.19. As concentration increases the amount of bound ligand also continues to increase until a saturation point is reached.

The results in this study indicate a significant ($p > 0.05$, un-paired Students T-test) increase in 5-HT_{1A} receptor binding when correlated with genotype in control human post-mortem tissue samples. The results show that control subjects with a GG or G/C genotype for the C-1019G 5-HT_{1A} receptor promoter polymorphism have higher post-synaptic 5-HT_{1A} receptor binding in human post-mortem hippocampal tissue. Thus, these subjects may have a predisposition to anxiety and depression. These results are in contrast to other studies that have shown that in post-mortem studies an increase in pre-synaptic 5-HT_{1A} binding with the C-1019G promoter polymorphism (Stockmeier et al, 1998). These results differ from Huang et al that find 5-HT_{1A} receptor density is unrelated to genotype of the C-1019G polymorphism (Huang et al, 2004).

Studies using the 5-HT_{1A} radioligand 8-OH-N, N-dipropyl-2-aminotetralin (8-OH-DPAT) have shown that there are no differences in binding between suicide and control samples in the cortex region of the brain. However, a significant decrease in these receptors in the hippocampus brain region of suicide samples has been indicated (Gross-Isseroff et al, 1998). Lopez et al (2004), demonstrated that there was a decrease in postsynaptic 5-HT_{1A} RNA in the hippocampus and dorsolateral prefrontal cortex regions of post-mortem tissue from major depression subjects (Lopez et al, 2004). This was consistent with a reduction of post-synaptic 5-HT_{1A} signalling observed in depressed suicide tissue (Hsiung, 2003). In contrast, Studies of human post-mortem brains from depressed suicide victims have revealed the presence of increased levels of 5-HT_{1A} autoreceptor in depression and suicide compared to non-suicide tissue. This up-regulation of 5-HT_{1A} receptors was seen in the raphe area and no change was observed in post-synaptic 5-HT_{1A} receptor sites (Stockmeier, 1998).

In a positron emission tomography (PET) study using the 5-HT_{1A} the partial antagonist WAY100635 has shown reduced 5-HT_{1A} receptor binding potential (BP) in the mesiotemporal cortex (MTC) and raphe in depressives compared to controls (Drevets et al, 1999) and a 43 percent decrease in 5-HT_{1A} BP in the raphe (Drevets, 2007). The level of the reductions in 5-HT_{1A} receptor binding observed in the Drevet study were similar to those found in 5-HT_{1A} receptor mRNA concentrations in post-mortem hippocampal samples of MDD subjects (Lopez, 1998) and in the 5-HT_{1A} receptor binding capacity in the raphe of depressed suicide victims (Arango et al, 2001).

Presently, there does exist disagreement within the literature regarding the presence and the direction of 5-HT_{1A} receptor binding abnormalities in depression, which may be in some cases explained by differences in anatomical location, for example the study by Stockmeier, where the binding of 8-OH-DPAT to the 5-HT_{1A} receptor was significantly increased in the midbrain dorsal raphe nucleus of suicide victims with major depression compared to normal control patients (Stockmeier, 1998). In other cases these differences in the literature can be accounted for by pathophysiological heterogeneity within MDD subjects. NUDR a repressor associated with the C-1019G promoter polymorphism of the 5-HT_{1A} receptor may exert opposite effects on hippocampal and cortical post-synaptic 5-HT_{1A} receptors as a reduction in NUDR function leads to a decrease in post-synaptic 5-HT_{1A} receptor expression in-vivo (Lemondé et al, 2003). A PET study of 5-HT_{1A} receptor BP reported that the G-allele of the C-1019G 5-HT_{1A} receptor polymorphism was linked with higher 5-HT_{1A} receptor binding in depressed subjects (Parsey et al, 2006). Some depressed subjects hypersecrete cortisol in response to stress, which is thought to down regulate 5-HT_{1A} receptor expression (Lopez, 1998) by lowering the availability of L-tryptophan leading to a reduction in 5-HT turnover and hence downregulating pre-synaptic 5-HT_{1A} receptors (Chalmers et al, 1993). Elevated levels of cortisol may also possibly induce a relatively widespread reduction in 5-HT_{1A} receptor expression (Drevets et al, 2007).

Chapter 3

The SH-SY5Y cell line; a model system

3.0- Aims

- To differentiate SH-SY5Y cells with retinoic acid (RA) or nerve growth factor (NGF) and aphidicolin over a selected time period (0-14 days) to produce a more neuronal cell subtype.
- To assess whether the SH-SY5Y cell line when differentiated is a suitable cell model to study the 5-HT_{1A} receptor, using real-time PCR to investigate the presence of 5-HT_{1A} receptor and NUDR mRNA.
- To use western blots and immunocytochemistry to determine the presence of 5-HT_{1A} receptor protein.

3.1- Introduction

3.1.1- SH-SY5Y cell line

The SH-SY5Y neuroblastoma cell line is a third generation neuroblastoma, cloned from SH-SY5. The original cell line was isolated from a woman's metastatic bone tumour in 1970.

The human SH-SY5Y neuroblastoma cell-line is a well established system for studying neuronal function (Pahlman et al, 1995, Körner et al, 1994). SH-SY5Y cells can be morphologically differentiated into neuronal like cells by several different inducing agents producing two distinct SH-SY5Y cell phenotypes (Feng and Porter, 1999, Pahlman et al, 1995); one by 12-O-tetradecanoylphorbol (TPA) in combination with serum or growth factors and another by retinoic acid (RA). Cell differentiation can be a complex process which is regulated by an interplay among intrinsic cellular programs such as, cell-cell and cell-substrate interactions, and a plethora of soluble extracellular signalling molecules including hormones, growth factors, cytokines, trophic factors and morphogens (Iopez-Garballo et al, 2002).

TPA differentiation of SH-SY5Y cells can be induced by using nanomolar concentrations of TPA initiating an arrest in proliferation of cells but a continuation of cells to differentiate morphologically by releasing growth cone terminated neurites, allowing cells to acquire a neuronal phenotype (Pahlman et al, 1995).

The synchronized regulation of cell differentiation and survival by RA may play an important role in the context of neuronal cell generation, in which an excess of precursor cells is produced to ensure that all of the required nervous connections take place. With this neurotrophile strategy those cells that establish contact with their target cells will receive from them neurotrophic survival factors, whereas those cells that do not succeed in finding their targets will die through apoptosis (Pettmann and Henderson, 1998). RA induced differentiation produces growth inhibited adherent cells which process long neuritic cell processes (Pahlman et al, 1995).

SH-SY5Y cells are commonly differentiated with RA rather than TPA to study 5-HT receptors. This is perhaps due to the inability of the TPA agent to induce a change in G protein expression, even though TPA can produce a 200-fold increase in noradrenaline and generates changes in protein kinase C, whereas RA induces only a fourfold increase in noradrenaline and does not affect protein kinase C but specifically alters the basal levels of several G protein subunits in SH-SY5Y neuroblastoma cells (Pahlman et al, 1984; Ammer, 1994).

SHSY-5Y cells can also be differentiated with Nerve growth factor (NGF), a member of the neurotrophin family which is thought to play a role in the survival and differentiation of neurons within the peripheral and central nervous system (Oe et al, 2005) and aphidicolin (a DNA polymerase α and δ inhibitor). A dramatic increase of morphological neuronal cells is observed (LoPresti et al, 1992) when cells are differentiated with NGF and aphidicolin. Cells become dependent on NGF for survival and therefore continued treatment of cells with NGF maintains the neuronal phenotype for several weeks (Jensen, Zhong and Shooter, 1992). The withdrawal of NGF from differentiated cells results in a loss of cell viability and cellular adhesion.

SH-SY5Y cells are more commonly differentiated with retinoic acid or nerve growth factor and aphidicolin as they induce morphological change in cell phenotype to become more neuronal (Ammer, 1994; LoPresti et al, 1992).

The morphology of differentiated cells is one way of assessing the level of differentiation of neuroblastoma cells *invitro*. Another method used to confirm differentiation is testing for biochemical and functional markers. Therefore, with the combination of the change of morphology of differentiated neuroblastoma cells and the presence of functional markers, the neuroblastoma cell-line could be considered to be a useful model system to study the initial phases of neuronal differentiation (Sidell, 1982).

3.1.2- Human nuclear deformed epidermal autoregulatory factor (NUDR)

NUDR is a 59kDa protein which shows sequence similarity to the *Drosophila* deformed epidermal autoregulatory factor-1 (DEAF-1). NUDR has been shown to have similarities to other proteins providing evidence for functional domains in NUDR including an alanine-rich region prevalent in developmental transcription factors, a domain found in the promyelocytic leukaemia-associated SP100 proteins, and a zinc finger homology domain associated with the AML/MTG8 oncoprotein (Huggenivik et al, 1998).

NUDR is also a transcription factor that can function as a repressor (Lemondé et al, 2003). The transcription factor NUDR has been associated with major depression and completed suicide (Lemondé et al, 2003). A molecular mechanism by which the 5-HT_{1A} promoter C-1019G polymorphism may regulate 5-HT_{1A} gene expression *in vivo* has been suggested. The polymorphism is thought to regulate 5-HT_{1A} by derepression of the 5-HT_{1A} promoter in presynaptic raphe neurons leading to an overall decrease in serotonergic neurotransmission (Lemondé et al, 2003).

3.1.3- Real-time PCR analysis of gene expression

Real-time PCR was used in this study to determine the relative quantification of target gene expression. This involves determining the change in expression level relative to another set of experimental samples such as a reference sample (Peirson, Butler and Foster, 2003, Wong and Medrano, 2005).

This technique has many advantages as it allows rapid analysis of gene expression from low quantities of starting template, it is reproducible, and high-throughput quantification can be achieved along with high sensitivity (Peirson, Butler and Foster, 2003).

3.1.4- Immunocytochemistry

Immunocytochemistry is based on the binding of antibodies to a specific antigen in cells. Antibody specificity is used to detect a cellular antigen of interest within a cell. Cells are grown in culture media before being fixed to a surface using a fixative. This conserves the antigen and maintains the attachment of the cells to prevent multiple washing steps affecting the sample.

The fixation of cells and tissue is necessary to adequately preserve cellular components, including soluble and structural proteins, to prevent autolysis and displacement of cell constituents such as, antigens and enzymes and finally to stabilise cellular materials against deleterious effects of subsequent procedures. Fixing of cells or tissue can facilitate conventional staining and immunostaining (Hayat, 2002).

Detection systems are classified as direct or indirect methods. Direct method is the simplest of the immunocytochemical methods as it involves a one-step process with a primary antibody conjugated with a reporter label (Coons and Kapan, 1950). Several labels have been used including, fluorochromes, enzymes and biotin (Polack and Van Noorden, 2003).

The indirect method provides a more sensitive antigen detection method, with detection taking place over two-steps. The primary antibody is un-labelled, but the secondary antibody is labelled (Polack and Van Noorden, 2003). The sensitivity of this method is higher than a direct method because the primary antibody is not labelled this retains the activity of the antibody generating a stronger signal, the number of labels per molecule of primary antibody are higher and, therefore, increasing the intensity of reaction (Ramos-Vera, 2005).

3.1.5- Western blots

The procedure of western blotting and subsequent immunodetection has become a powerful tool to detect and characterise a whole host of proteins, in particular, proteins which are in low abundance (Kurien and Scofield, 2003).

The western blot technique involves the transfer of proteins that have been previously separated on a sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) to a solid support (Laemmli, 1970).

Once the proteins from the SDS-PAGE gel have been blotted, the blotted proteins provide an exact replica of the gel producing a useful starting step for a variety of experiments. The following employment of antibody probes directed against a membrane such as, nitrocellulose bound proteins has revolutionised the field of immunology (Kurien and Scofield, 2003).

The transfer of proteins from native or SDS-PAGE gels to a nitrocellulose or PVDF membranes has been achieved in 3 different ways, the first way is by simple diffusion (Renart, reiser and Stark, 1979; Kurien and Scofield, 1997); the second method is by a vacuum-assisted solvent flow (Peferoen, Huybrechts and Deloof, 1982); and finally the third method by "Western" blotting or electrophoretic elution (Towbin, Stacheln and Gordon, 1979).

Detection with enzyme-linked reagents involves the use of chemiluminescence, which has become an important technique due to its sensitivity and selectivity. The majority of chemiluminescence methods involves the use of a few chemical components to generate the required light (Nieman, 1989).

To increase the sensitivity of detection the avidin-streptavidin system has been developed. This method exploits the specificity of the interaction between the low-molecular weight vitamin biotin and the protein avidin (Dunn, 1994). Antibodies can be easily conjugated with biotin and used as a secondary detection reagent for probing blots.

Real-time PCR, immunocytochemistry and western blots described above are techniques, which have been used in the characterisation of 5-HT receptors in cell lines providing an insight into whether cell lines are suitable model systems for studying 5-HT receptors.

3.2- Methods

3.2.1- Cell culture

The SH-SY5Y cells were grown in DMEM containing 10% FCS, penicillin (100 Uml⁻¹) and streptomycin (100µg/ml⁻¹).

3.2.2- Differentiation with Retinoic acid (RA), Nerve growth factor (NGF) and aphidicolin

SH-SY5Y cells were grown in DMEM for different lengths of time (0, 3, 5, 7, 10 and 14 days) in the presence of either retinoic acid (10⁻⁵M) or nerve growth factor (100ng/ml) and aphidicolin (0.3µM).

3.2.3- SH-SY5Y cell line genotyping

1X10⁶ SH-SY5Y cells were centrifuged at 600g for 5 minutes. The pellet was resuspended in 1.0ml buffer A of kit for 5 minutes before being centrifuged at 1300g for 5 minutes. The DNA from the cell line was then extracted as previously described in section 2.2.3.1.

The ASO method of genotyping was used as previously mentioned in section 2.2.3.2.

3.2.4- Gene expression studies by real-time PCR

3.2.4.1- Cell preparation before RNA extraction

Confluent cells were washed with PBS then trypsinised for 2 minutes in a 37°C incubator. Trypsinised cells were resuspended in DMEM media before being centrifuged at 1,000 rpm for 5 minutes. The supernatant was discarded.

3.2.4.2- RNA extraction

RNA was extracted from SH-SY5Y cell pellet (10^7 cells) using a GenElute mammalian total RNA miniprep kit (Sigma-aldrich, UK). The cell pellet was vortexed with Lysis buffer (500 μ l of lysis buffer and 5 μ l β -mercaptoethanol) until all clumps disappeared. The homogenate was transferred to a GenElute filtration column and centrifuged at 12,000 g for 2 minutes. The filtered lysate had an equal volume of 70 percent ethanol added to it before loading the sample on to a binding column. After binding the RNA to the column, the column was washed with wash solution one from the kit and wash solution two from the kit. Finally, RNA was eluted in 50 μ l of elution buffer solution.

Extracted RNA was treated with DNase 1 (Sigma-aldrich, UK) to prevent contamination of the sample with genomic DNA. 50 μ l of extracted RNA was treated with 5 μ l of 10X reaction buffer and 5 μ l of amplification grade DNase 1. The sample was incubated at room temperature for 15 minutes and then DNase 1 was inactivated by adding 5 μ l of stop solution. The sample was incubated at 70°C for 10 minutes. Extracted RNA was kept in the freezer at -70°C.

3.2.4.3- cDNA synthesis

The iScript cDNA synthesis kit (Bio-Rad, UK) was used to transcribe single-stranded cDNA. In each cDNA synthesis reaction RNA, 10 μ l ddH₂O, 4 μ l of iScript buffer and 1 μ l of reverse transcriptase (1U) was added. Reaction tubes were heated at 25°C in a heat block for 5 minutes, reaction tubes were then moved to a water bath and heated at 42°C for 30 minutes. Reaction tubes were finally heated at 85°C for 5 minutes in a heat block. All samples had 20 μ l of ddH₂O added to them before being stored at -20°C

3.2.4.4- Housekeeping validation

8 housekeeping genes (B actin, β_2 M, GAPDH, HPRT, RPL13A, SDHA, UBC, and YWHAZ) were tested on 11 SH-SY5Y cell line time-point samples differentiated with both RA and NGF. Primer sequences for housekeeping genes and the 5-HT_{1A} receptor are shown in Table 2.3 and 2.4 respectively.

The primer sequences for NUDR were designed from NCBI accession number NM_021008 using Primer 3 software.

Primer	Sequence (5'-3')	T _m	Product size
NUDR_F	AGC CAG TAA GGA CTG GA	59.4	201bp
NUDR_R	GGA GTT GTG GGC AGT TCA TT	57.3	

Table 3.1-Oligonucleotide primer sequences for 5-HT_{1A} and NUDR

3.2.4.5- PCR reaction

Each 25µl PCR reaction contained 12.5µl of (2X) SYBR green mix (Abgene, UK), 1µl of MgCl₂ (4mM), 7.5µl of ddH₂O and 1µl of each forward and reverse primer (0.5µM). 2µl of cDNA was added separately to the 96 well plate.

Two housekeeping genes were run on every plate and used as a reference to normalise data.

3.2.4.6- PCR Cycles

95°C for 15 minutes to activate SYBR green mix

95°C for 15 seconds	}	40 cycles
60°C for 15 seconds		
72°C for 30 seconds		

Melt curve

95°C 30 seconds	}	45 cycles
50°C for 30 seconds increasing in 1°C increments		

3.2.4.7- Primer efficiency

To calculate primer efficiency template cDNA was diluted in a dilution series (neat, 1:5, 1:10, 1:50 and 1:100) each PCR reaction was carried out in duplicate. The C_t values obtained are exported into the graphical software programme GraphPad Prism 4. Using the Prism software the slope and 1 over slope were determined.

3.2.5- Immunocytochemistry

There are many methods of immunocytochemistry the more commonly used are the direct and indirect method as mentioned previously. The indirect method was used in this case.

SHSY-5Y cells were treated with retinoic acid (RA, 10^{-5} M) for 5 days and were grown in 8 well chamber slides (1×10^5 cells per well). Cells were fixed with ice cold methanol for 10 minutes at -20°C . SH-SY5Y cells were then blocked with 1 percent BSA in PBST (PBS and Tween 20) for 30 minutes. After blocking cells were washed 3 times with PBST over 15 minutes.

SH-SY5Y cells were incubated in a humidified chamber for 1 hour with a 1/50 dilution of SR-1A (Santa-Cruz, USA) primary antibody directed at the 5-HT_{1A} receptor. Negative control wells (no primary antibody) had PBS added to them. After incubation cells were washed with PBST 3 times over 15 minutes.

Secondary antibody alexa-flura goat anti-rabbit (Invitrogen, UK) was used at a 1/1000 dilution. Cells were incubated at 37°C in the dark for 1 hour in a humidified chamber. Before mounting the slides they were washed 3 times with PBST. Slides were mounted with DAPI (Vector shield, UK). Slides were stored in the dark at $4-8^{\circ}\text{C}$ until visualised under the fluorescent microscope.

3.2.6- SH-SY5Y cell line western blot

3.2.6.1- Sample preparation

SH-SY5Y cells (1×10^6 in total) were RA treated (10^{-5}M) for 5 days then pelleted, the pellet was resuspended in lysis buffer (5mM Tris-HCL, 2mM EDTA and protease inhibitor cocktail).

3.2.6.2- Protein concentration using Amicon Centricon centrifugal filter devices

Protein extracted from cells at each time-point was concentrated using an Amicon Centricon. A maximum of 2mls of sample was added to the sample reservoir, the sample was then spun at 3000g until the desired concentration of sample was reached. The sample was stored in the retentate vial.

3.2.6.3- Bicinchoninic acid (BCA) assay

The BCA assay is a biochemical assay used for the determination of total protein in a solution. Total protein concentration is exhibited by a colour change sample solution from green to purple according to protein concentration. The change in colour can be measured using colourimetric techniques.

Protein standards were made from a 20µM stock solution of BSA. In a 96 well plate 100µl of each standard was pipetted in triplicate. 200µl of BCA and copper-2-sulphate solution is added. Samples were either added neat (20µl) and diluted (1/2, 1/5 and 1/10). The 96 well plate was left to incubate at room temperature for 30 minutes before absorbances were recorded at 570nm on a Wallac Victor² 1420 multi-label counter (PerkinElmer Ltd, Turku, Finland). Absorbance values were used to calculate a standard curve, which was used to determine protein concentration of sample using the slope.

3.2.5.4- SDS-PAGE gel

Samples were run on a 12.5 percent separating gel (12.5% Bis-acrylamide solution, 0.39M Tris, 4.9mls water, 0.1% SDS, 0.1% APS and 15µl TEMED) and a 5 percent stacking (5% Bis-acrylamide solution, 0.12M Tris, 3.4mls water, 0.1% SDS, 0.16% APS and 8µl TEMED) SDS-PAGE gel. 1X SDS running buffer (Trizma base, glycine, SDS and water pH 8.3) was poured into tank before loading the gel. 7µl of colorBurst (Sigma-aldrich, UK) molecular weight marker was loaded onto the gel. A maximum of 30µl of sample was loaded with 10µl of sample buffer approximately 3µg/ml. The sample was heated at 100°C for 5 minutes with SDS sample buffer (Trizma base 62.7mM, glycerol 137mM,

SDS 79.7mM and bromophenol blue 0.1%) before loading sample onto gel. Gel was run at 150 volts for 40 minutes to 1 hour.

The gel was transferred on to a PVDF membrane (Amserham, UK) using transfer buffer (Glycine 40mM, Tris.HCL 20mM and methanol 20%) for 1 hour at 100 volts. The second gel was Coomassie blue stained (Coomassie blue 1.2mM, methanol 50% and acetic acid 20%).

3.2.6.5- Western Blot

The membrane was blocked with 3 percent blocking solution (BSA) (Sigma, UK). The primary antibody, SR-1A (Santa-cruz, USA), was used at a 1/200 dilution and aviva 5-HT_{1A} antibody (1/1000 dilution) incubated for 1hr. Secondary antibody Alexa-flura goat anti-rabbit (Invitrogen, UK) was used at a 1/1000 dilution and biotinylated secondary antibody incubated for 1hr. The detection method used was enhanced chemiluminescence (ECL), (Amserham, UK).

3.3- Results

3.3.1- Time-points of SH-SY5Y RA differentiated cells

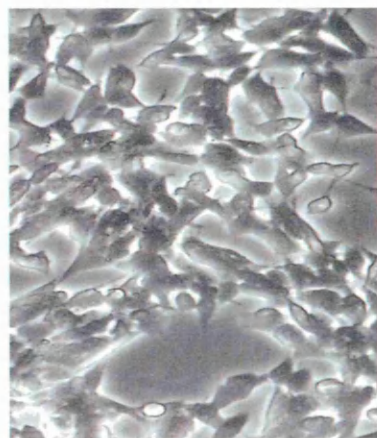
The vitamin A metabolite, retinoic acid (RA) plays an essential role in the nervous system development, including neuronal patterning, survival and neurite outgrowth (Clagett-Dame, 2005). Cells exposed to physiologic concentrations of RA increases the number of cells bearing neuritic processes and increased length of these processes. This is often accompanied by inhibition of cell proliferation (Clagett-Dame, 2005).

SH-SY5Y cells were grown in DMEM media for a period of 0, 3, 5, 7, 10 and 14 days in the presence of RA 10^{-5} M. SH-SY5Y cells treated for 5 days and more appear more neuronal in their phenotype, cells are more extended and process neurites compared to undifferentiated cells (Figure 3.1).

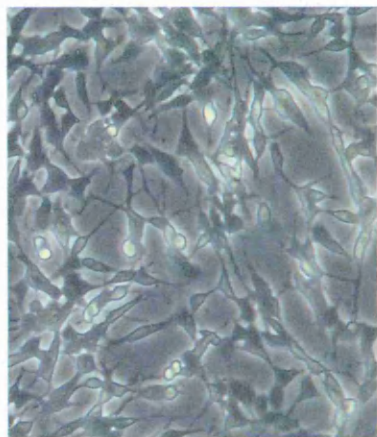
3.3.2- Time-points of SH-SY5Y NGF and aphidicolin differentiated cells

SH-SY5Y neuroblastoma cells treated with NGF alone result in limited neurite extension but did not inhibit proliferation. To increase neuronal differentiation and slow proliferation aphidicolin (a DNA polymerase α and δ inhibitor) is added with NGF to the cells (LoPresti et al, 1992). When SH-SY5Y cells are treated with a combination of NGF and aphidicolin cells are thought to irreversibly differentiate (Jensen, Zhong and Shooter, 1992).

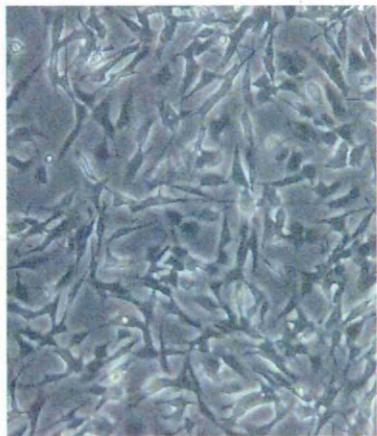
NGF and aphidicolin treated SH-SY5Y cells for 8-10 days show an increased neuronal phenotype displaying extending neurites and processes when compared to undifferentiated cells (Figure 3.2).



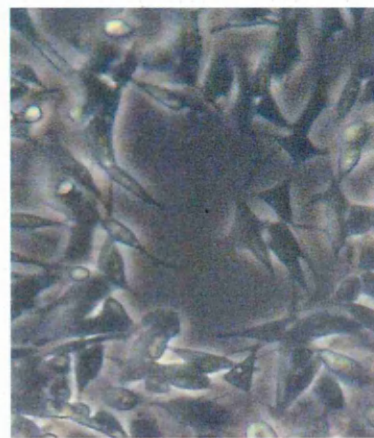
Undifferentiated



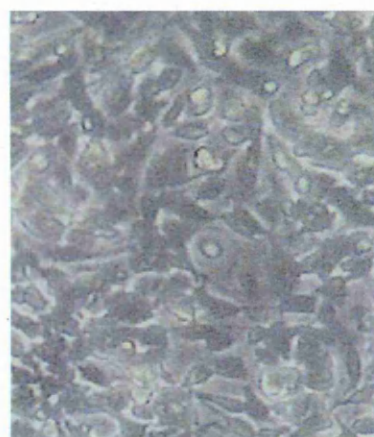
Time-point 3



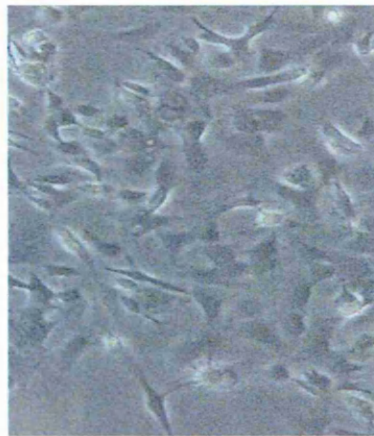
Time-point 5



Time-point 7

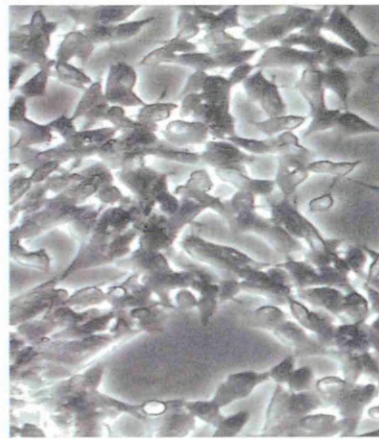


Time-point 10

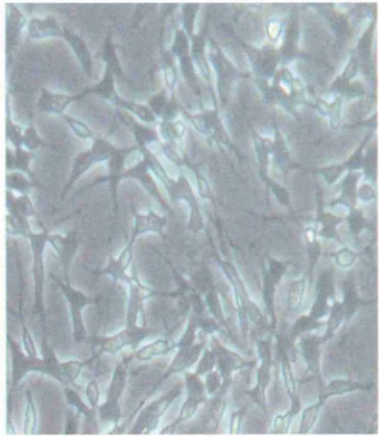


Time-point 14

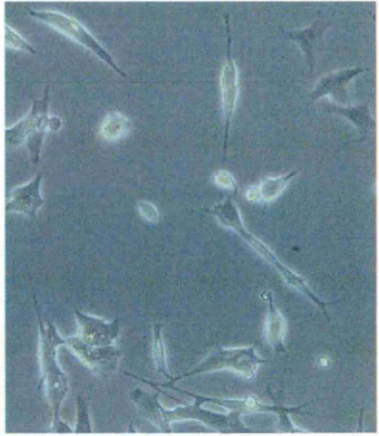
Figure 3.1- SH-SY5Y differentiated cells treated with RA over a time period of 0-14 days
Magnification X 100



Undifferentiated



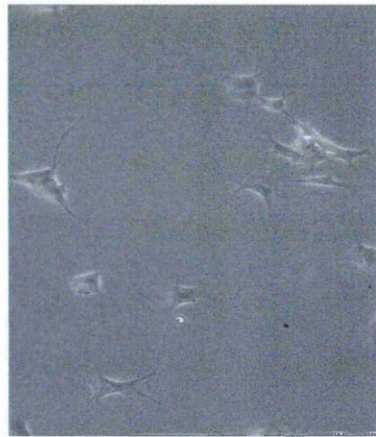
Time-point 3



Time-point 5



Time-point 7



Time-point 10



Time-point 14

Figure 3.2- SH-SY5Y cells differentiated with NGF and aphidicolin over a time period of 0-14 days
Magnification X100

3.3.3- SH-SY5Y cell line genotype

Specifically designed primers with a single base-pair substitution (see section 2.3.1) were used for genotyping RA differentiated SH-SY5Y cell line (time point 5 days) for the 5-HT_{1A} receptor promoter polymorphism C-1019G. Post mortem tissue sample 324 (G/C genotype) was used as a positive control. PCR bands were present at the expected product size (174bp). The SH-SY5Y genotyping PCR reaction was repeated 4x showing that one band was present with the G reverse primer, therefore, this cell line has a GG genotype (Figure 3.3).

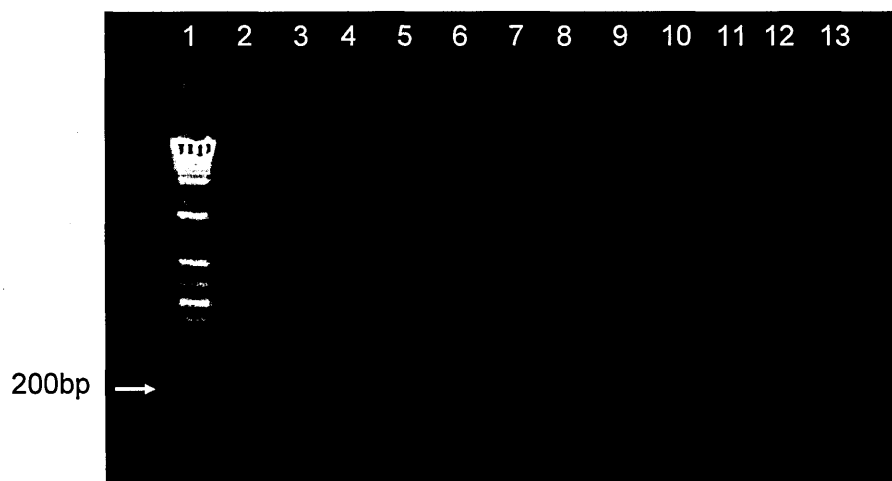


Figure 3.3- 5-HT_{1A} receptor genotype in SH-SY5Y cell line

1- DNA ladder 2-TP5 rev C 3- TP5 rev G 4- TP5 rev C 5-TP5 rev G
6- TP5 rev C 7-TP5 rev G 8- TP5 rev C 9- TP5 rev G 10- negative control rev C
11- negative control rev G 12- 324 rev C 13- 324 rev G

3.3.4- Real-time PCR gene expression

3.3.4.1- RNA

RNA was extracted from SH-SY5Y cells at different time-points (0, 3, 7, 10 and 14 days) after induction of RA or NGF and aphidicolin using a GenElute RNA extraction kit (Sigma-aldrich, UK).

All the time-point samples from RA and NGF and aphidicolin differentiated cells have both 18S and 28S rRNA bands present and show good quality RNA (Figure 3.3).

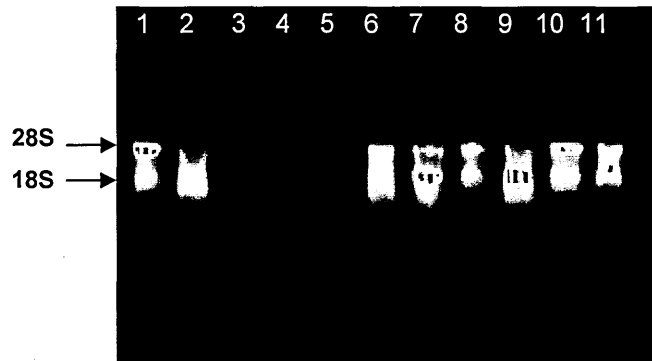


Figure 3.4- Example RNA agarose gel

1- TP0 2- TP3 RA 3- TP5 RA 4- TP7 RA 5-TP10 RA
6- TP14 RA 7- TP3 NGF 8- TP5 NGF 9- TP7 NGF 10- TP10 NGF
11- TP14 NGF

3.3.4.2- Housekeeping gene validation

The comparative method was used to calculate expression of housekeeping genes from C_t values. GeNorm software was then used to determine the two most stable housekeeping genes (Figures 3.5 and 3.6).

Change Data	Bactin	RPL13A	SDHA	UBC	YWHAZ	Normalisation Factor
0	7.07E-01	8.12E-01	6.37E-01	6.16E-01	1.49E-01	3.7818
3 RA	3.79E-01	7.58E-01	2.77E-01	4.67E-01	1.65E-01	2.6928
3 NGF	8.71E-01	1.00E+00	6.60E-01	5.36E-01	2.03E-01	4.2844
5 RA	9.81E-02	3.47E-02	4.42E-02	4.58E-02	1.07E-02	0.2791
5 NGF	8.54E-02	3.30E-01	3.92E-01	2.18E-01	1.02E-01	1.4133
7 RA	2.87E-01	4.35E-01	9.47E-02	2.77E-01	8.25E-02	1.4430
7 NGF	1.77E-01	1.05E-01	1.71E-01	2.97E-01	8.54E-02	1.1322
10 RA	1.00E+00	9.01E-01	1.00E+00	1.00E+00	1.00E+00	7.3060
10 NGF	7.93E-04	6.44E-04	4.81E-03	2.17E-03	5.81E-04	0.0093
14 RA	7.18E-02	7.43E-02	9.81E-02	1.25E-01	3.13E-02	0.5430
14 NGF	2.33E-01	5.63E-02	1.83E-01	2.33E-01	6.25E-02	0.9587
M < 1.5	1.051	1.157	1.079	0.810	0.899	

Figure 3.5 - geNorm data of 5 housekeeping genes

The relative expression ratios of each housekeeping gene were imported into the geNorm software. The M value for each gene was calculated and the gene with the highest M value (Shaded in red) is deemed the least stable and was removed. The most stable housekeeping gene is shaded in green. Housekeeping genes are removed until the two most stable genes remain.

Change Data	UBC	YWHAZ	Normalisation Factor
0	6.16E-01	1.49E-01	2.8598
3 RA	4.67E-01	1.65E-01	2.6224
3 NGF	5.36E-01	2.03E-01	3.1186
5 RA	4.58E-02	1.07E-02	0.2089
5 NGF	2.18E-01	1.02E-01	1.4053
7 RA	2.77E-01	8.25E-02	1.4299
7 NGF	2.97E-01	8.54E-02	1.5062
10 RA	1.00E+00	1.00E+00	9.4539
10 NGF	2.17E-03	5.81E-04	0.0106
14 RA	1.25E-01	3.13E-02	0.5909
14 NGF	2.33E-01	6.25E-02	1.1415
M < 1.5	0.609	0.609	

Figure 3.6- geNorm data of the 2 most stable housekeeping genes

Through a process of elimination according to the M values of the housekeeping genes the two remaining stable housekeeping genes are UBC and YWHAZ both have an M value of 0.905.

3.3.4.3- Primer efficiency

A dilution series of cDNA was tested with both housekeeping gene primers and the 5-HT_{1A} primers and NUDR primers (Figure 3.7).

A slope of -3.295 represents a 100 percent efficient primer.

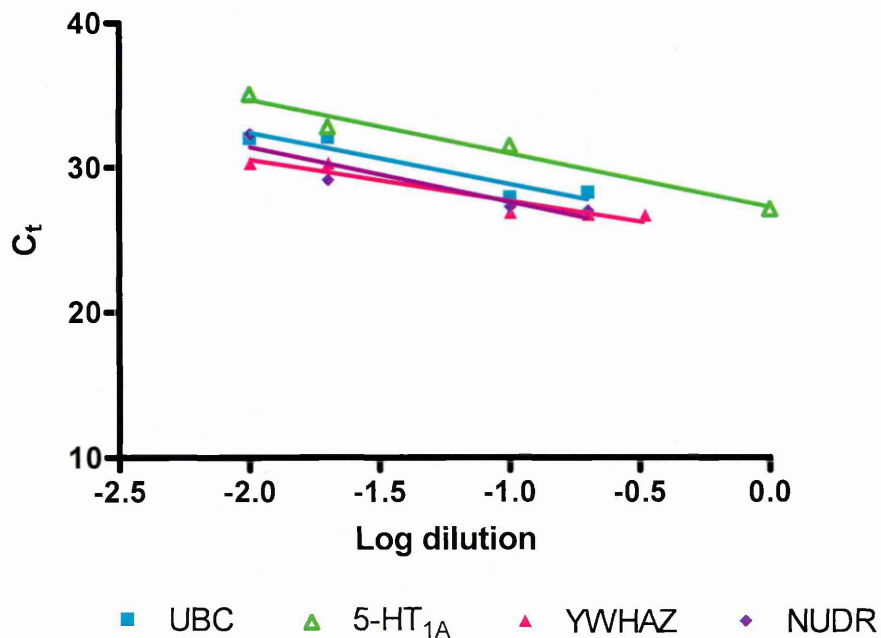


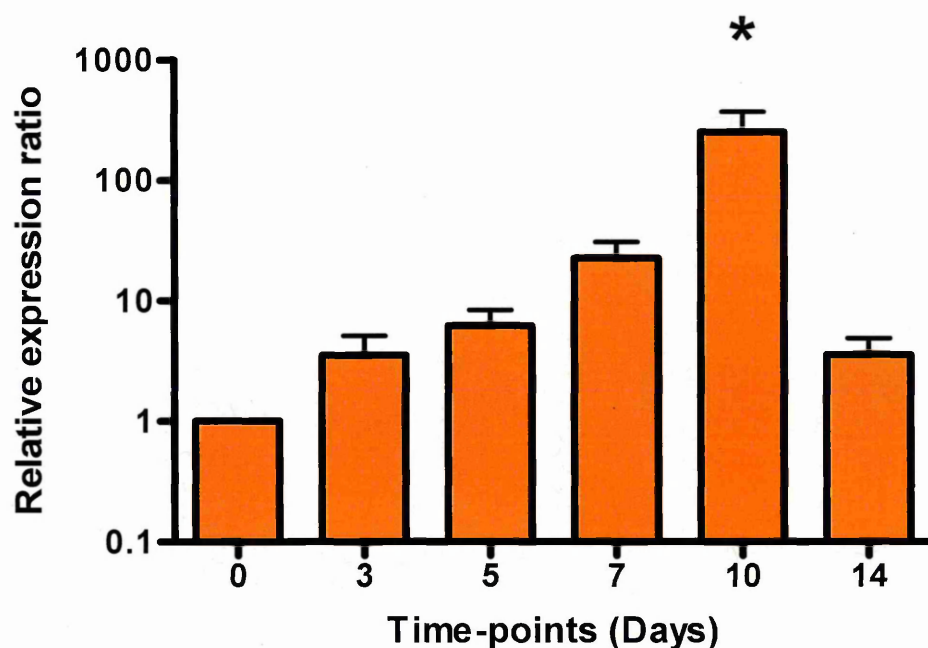
Figure 3.7- Efficiency of the primers UBC, YWHAZ, 5-HT_{1A} and NUDR

A dilution series of cDNA was tested with the primers UBC, YWHAZ, 5-HT_{1A} and NUDR. The C_t values for each dilution were used to calculate slope of the line. Percentage of efficiency is calculated using $E = 10^{(-1/\text{slope})} - 1$. UBC, YWHAZ, 5-HT_{1A} and NUDR have a slope of -3.537, -2.829, -3.710 and -3.754 and 91.74, 125, 86.01 and percentage efficiencies 84.6 percent respectively.

3.3.4.4- 5-HT_{1A} receptor expression in RA and NGF and aphidicolin SH-SY5Y differentiated cells.

The mean C_t values of each sample were imported into Excel and the Pfaffl method was used to calculate the relative gene expression ratios (Pfaffl, 2001) equation explained in section 2.2.9.2. These results demonstrate that SH-SY5Y cells treated with NGF and aphidicolin for 10 days had a 252-fold increase of 5-HT_{1A} receptor expression compared to undifferentiated cells (p<0.01) (Figure 3.8). 5-HT_{1A} receptor expression peaked at 10 days of treatment with NGF and aphidicolin followed by a decrease in 5-HT_{1A} receptor mRNA expression.

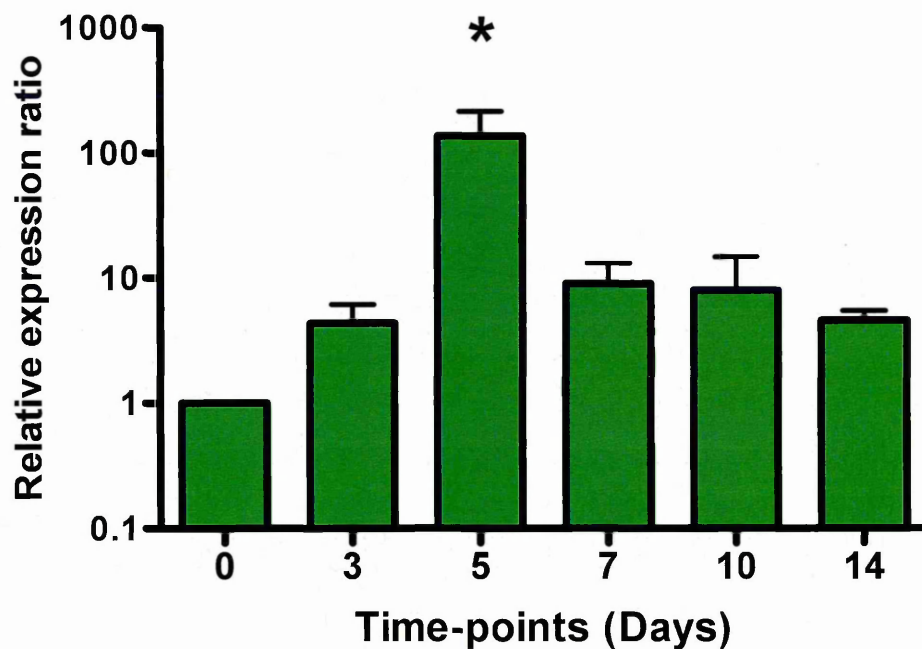
SH-SY5Y cells treated with RA for 5 days had a significant 136-fold increase in 5-HT_{1A} receptor expression compared to undifferentiated cells (p<0.05) (Figure 3.9). Data was analysed using a One-way ANOVA followed by a Dunnet's post t-test.



TIME-POINTS (DAYS)	0	3	5	7	10	14
Average Relative Expression Ratio	1.0±0.4	3.5±1.3	6.2±2.3	22.4±8.5	252.9±95.6	3.6±1.5

Figure 3.8- 5-HT_{1A} receptor mRNA expression in SH-SY5Y cells differentiated with NGF (100ng ml⁻¹) for different lengths of time (0-14 days).

Data is presented as the relative expression ratio compared to undifferentiated cells (time point 0 days), (n=6). Data presented as mean±SEM. One-way ANOVA with Dunnett's test, *p<0.05 indicates differences in expression at each time point relative to undifferentiated cells.



TIME-POINTS (DAYS)	0	3	5	7	10	14
Average Relative Expression Ratio	1.0±0.4	4.3±1.8	136.2±55.6	9.0±3.7	7.9±3.2	4.6±1.9

Figure 3.9- 5-HT_{1A} receptor mRNA expression in SH-SY5Y cells differentiated with RA (10⁻⁵M) for different lengths of time (0-14 days).

Data is presented as the relative expression ratio compared to undifferentiated cells (time point 0 days), (n=6). Data presented as means±SEM. One-way ANOVA with Dunnett's test, *p<0.05 indicate differences in expression at each time point relative to undifferentiated cells.

3.3.4.5- NUDR mRNA expression in RA and NGF and aphidicolin SH-SY5Y differentiated cells.

The results demonstrate that SH-SY5Y cells treated with NGF and aphidicolin for 10 days had a 34-fold increase of expression compared to undifferentiated cells ($p < 0.05$) (Figure 3.10). SH-SY5Y cells treated with RA for 5 days had a 4-fold increase in NUDR mRNA expression compared to undifferentiated cells (Figure 3.11). After 14 days of treatment of RA cells showed a 6-fold increase in NUDR expression.

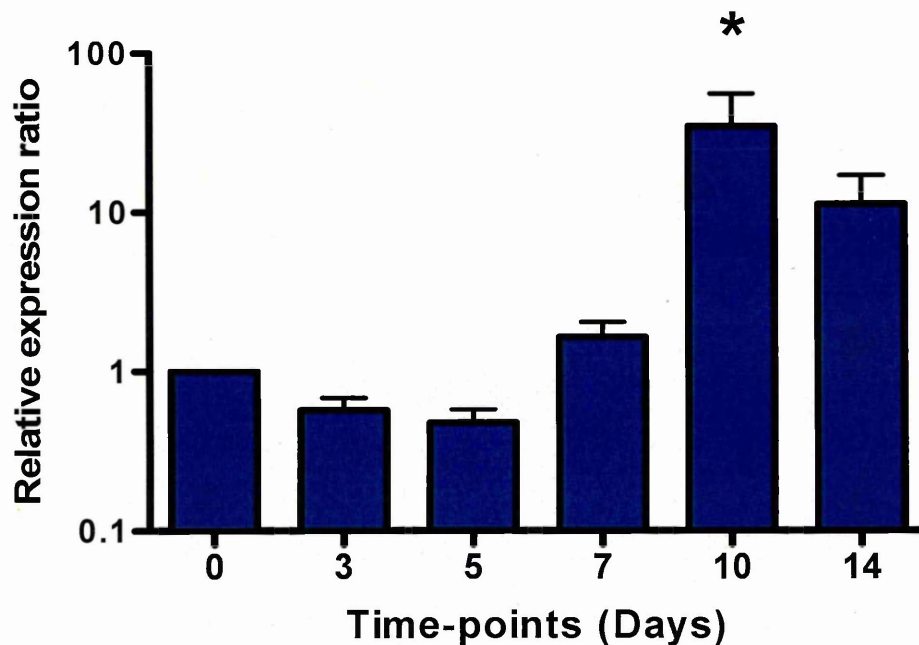


Figure 3.10- NUDR mRNA expression in SH-SY5Y cells differentiated with NGF (100ng/ml) for different lengths of time (0-14 Days).

Data is presented as the relative expression ratio compared to undifferentiated cells (time point 0 days), ($n=6$). Data presented as means \pm SEM. One-way ANOVA with Dunnett's test, * $p < 0.05$ indicate differences in expression at each time point relative to undifferentiated cells.

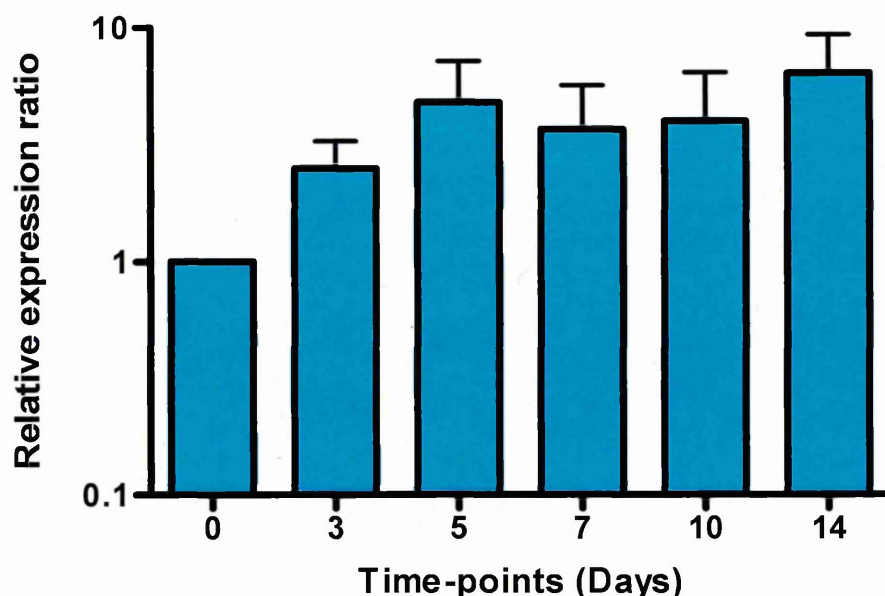


Figure 3.11 - NUDR mRNA expression in SH-SY5Y cells differentiated with RA (10^{-5}) for different lengths of time (0-14 days).

Data is presented as the relative expression ratio compared to undifferentiated cells (time point 0 days), (n=6). Data presented as means \pm SEM. One-way ANOVA with Dunnett's test indicated no significant differences in expression at each time point tested relative to undifferentiated cells

3.3.5- Immunocytochemistry

SH-SY5Y cells were grown in chamber slides in the presence of RA for 5 days. The SR-1A primary antibody and the alexa-fluor goat anti-rabbit secondary antibody were used to detect the presence of the 5-HT_{1A} receptor in both differentiated and undifferentiated cells.

Immunocytochemistry results show the presence of the 5-HT_{1A} receptor in SH-SY5Y cells treated with RA for 5 days. An increase in fluorescence is observed in cells differentiated with RA compared to undifferentiated cells (Figure 3.12).

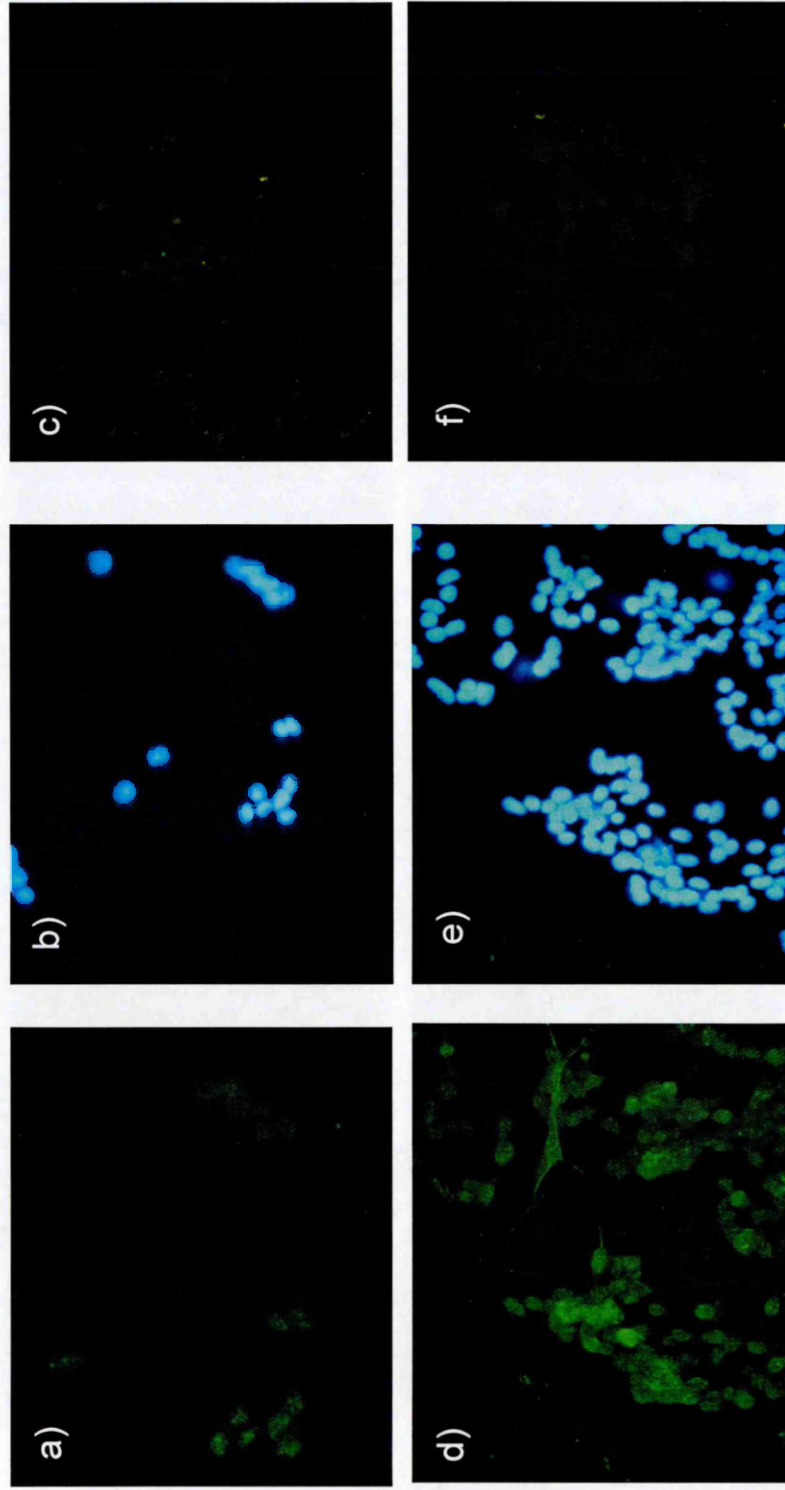


Figure 3.12- 5-HT_{1A} expression in SH-SY5Y cells using immunocytochemistry

Undifferentiated SH-SY5Y cells (a) show low levels of fluorescence compared to 5 day RA differentiated cells (d). Cells were counterstained with DAPI, undifferentiated cells (b) and differentiated cells (e). Negative controls no primary antibody, undifferentiated (c) and (f) differentiated cells. Magnification X 200.

3.3.6- Western blot

SH-SY5Y cells treated in the presence of RA or NGF and aphidicolin for different time-points (0, 3, 5, 7, 10 and 14 days). The SR-1A primary antibody and a goat anti-rabbit biotinylated secondary antibody were used to detect the presence of the 5-HT_{1A} receptor.

Western blots results show the presence of the 5-HT_{1A} receptor in SH-SY5Y cells treated with RA and NGF and aphidicolin. An increase in band intensity is observed in cells differentiated with RA or NGF and aphidicolin compared to undifferentiated cells (Figure 3.13).



Figure 3.13- Western blots of RA and NGF and aphidicolin differentiated SH-SY5Y cells for 0-14 days

3.4- Discussion

The results confirm that SH-SY5Y cells can be differentiated in the presence of RA or NGF and aphidicolin to produce cells of a more neuronal phenotype. Real-time PCR demonstrated the presence of 5-HT_{1A} receptor mRNA in both RA and NGF and aphidicolin differentiated SH-SY5Y cells. In differentiated SH-SY5Y cells western blots and immunocytochemistry showed the presence of 5-HT_{1A} receptor protein.

3.4.1- Time-points of Retinoic acid differentiated SH-SY5Y cells

The results show that SH-SY5Y cells treated for 5 days and more appear more neuronal in their phenotype compared to undifferentiated cells (Figure 3.1). These results were comparable with Lombet et al (2001), whose study found that SK-N-SH cells differentiated with RA (3 μ M) for 7 days, cells became flatter and bipolar. Grynspan et al (1997), similarly found that within 3 days of RA treatment of SH-SY5Y cells most of the cells had extended one or more neurites that could be clearly distinguished from normal filipodia present in control cells, between 7 and 10 days these neurites lengthened and arborized. It has also been observed that RA induced differentiation results in growth inhibited adherent cells which have long neuritic cell processes (Pahlman et al, 1995).

3.4.2- Time-points of NGF and aphidicolin differentiated SH-SY5Y cells

The results obtained in this study show that NGF and aphidicolin treated SH-SY5Y cells for 8-10 days show an increased neuronal phenotype displaying extending neurites and processes when compared to undifferentiated cells (Figure 3.2). These observations are in agreement with Jensen, Zhong and Shooter, 1992. They observed that SH-SY5Y differentiated cells induced with NGF for 5 weeks attain a high degree of morphological, physiological, and biochemical differentiation. Cells that have differentiated have gradually extended neural processes and clustering into ganglionic structures over the 5 week treatment with NGF (Jensen, Zhong and Shooter, 1992). In addition, a

study by LoPresti et al (1992) showed that a time course of differentiation with NGF-aphidicolin treatment induces a commitment to differentiation. 6 percent of the SH-SY5Y treated for 5 days with NGF-aphidicolin displayed long neurites. After 4 days of treatment with NGF 60-70 percent of the cells assumed an altered morphology. SH-SY5Y cells have a more rounded appearance and extended neurites up to >400µM long. After 8 days of treatment with NGF aggregates of differentiated cell bodies were observed.

3.4.3- Real-time gene expression PCR

3.4.3.1- RNA

RNA used in this study had clear sharp bands present at 28S and 18S on an agarose gel showing good quality RNA (Figure 3.4).

RNA integrity assessment is an essential first step in obtaining meaningful gene expression data. It is therefore essential that appropriate measures are taken such as validating housekeeping genes for real-time PCR reactions into consideration.

3.4.3.2- Housekeeping gene validation

Housekeeping genes are normally expressed in moderately abundant levels and therefore, they are good genes to use for comparing expression levels (Warrington, 2000).

In this study the housekeeping genes deemed the most stable according to their M value where UBC and YWHAZ (Figure 3.6). The appropriate validation of internal references is crucial to avoid misinterpretation of gene expression data (Dheda et al, 2004). Therefore, housekeeping genes should be run alongside every PCR reaction performed.

3.4.3.3- Efficiency of primers

Efficiency of each primer set used in this study was determined to enable quantitative analysis of relative gene expression by the Pfaffl method (Pfaffl, 2001).

UBC, YWHAZ, 5-HT_{1A} and NUDR primer sets had efficiencies of 91.7, 125, 86.1 and 84.6 percent respectively (Figure 3.7).

3.4.3.4- 5-HT_{1A} receptor mRNA expression in RA or NGF and aphidicolin differentiated SH-SY5Y cells

Differentiation with NGF and aphidicolin significantly increased 5-HT_{1A} mRNA levels at 10 days producing an significant ($p < 0.01$) 252.9 fold increase in expression relative to control, (Figure 3.8). At 14 days stimulation of NGF and aphidicolin, 5-HT_{1A} receptor mRNA expression had declined to a 3.5 fold change in expression relative to control (undifferentiated cells).

The timing observed of 5-HT_{1A} receptor mRNA expression in response to NGF and aphidicolin is consistent with the results of LoPresti who showed that SH-SY5Y cell bodies are more rounded and extended neurites are present after 8 days of treatment with NGF (LoPresti et al, 1992).

At day 5 following stimulation with RA a significant 136.2 fold increase ($p < 0.01$) in 5-HT_{1A} receptor mRNA was determined relative to control (Figure 3.9). After day 5, 5-HT_{1A} receptor mRNA levels declined and change in expression observed was not significantly different relative to control.

The timing of 5-HT_{1A} mRNA receptor expression in response to RA is consistent with previous work by Ammer and Schulz, (1994) who showed that RA induced differentiation of SH-SY5Y cells markedly increased the abundance of all G-protein subunits investigated. The study showed that a RA time-course had a marginal effect on G protein levels after 2 days of exposure, whereas 4 and 6 days of treatment produced half-maximal and maximal G protein changes (Ammer and Schulz, 1994). A large increase in G₂ α during RA-induced

differentiation of SH-SY5Y cells was observed. The tissue distribution of $G_{2\alpha}$ suggests that this G protein subunit has a specialised function in neuronal tissue (Ammer and Schulz, 1994; Casey, 1990). Immunohistochemical studies have shown $G_{2\alpha}$ to be present in most neurons of the hippocampus and cerebral cortex (Hinton, 1990). This observation made by Ammer and Schulz can be explained because RA treatment is known to induce pronounced neurite growth in SH-SY5Y cells (Pahlman et al, 1984).

When SH-SY5Y cells are induced with RA or NGF and aphidicolin for long period of time (14 days) a decrease in 5-HT_{1A} receptor mRNA expression is observed. This observation could be explained by the phenomenon of transdifferentiation or by the fact that the cells have become neuronal at this time point and could therefore have stopped gene expression.

The SK-N-SH parental cell-line comprises at least two morphologically and biochemically distinct phenotypes, neuroblastic (N-type) and substrate adherent (S-type), which can undergo transdifferentiation (Ross, Spengler and Bledler, 1983). Although SH-SY5Y cell-line is derived from a neuroblastic subclone it still retains a low proportion of S-type cells which do not have a neuronal phenotype. Transdifferentiation between N and S-types seems to be common to the majority of neuroblastoma cell-lines (Jensen, 1987; Hill, 1987). It is considered that the frequency of a given phenotype in a continuous neuroblastoma cell-line is a consequence of slower rates of conversion rather than due to a loss of potential to generate the other phenotype (Sadee et al, 1987). When SH-SY5Y cells are cultured for longer periods of time without being passaged they may contain less neuronal N-type cells and more S-type cells which tend to remain adherent to the bottom of the cell culture flask and therefore decrease in the 5-HT_{1A} receptor expression is observed could be due to a higher population of non- neuronal S-type cells.

3.4.3.5- NUDR mRNA expression in RA or NGF and aphidicolin differentiated SH-SY5Y cells

At day 10 cells differentiated with NGF and aphidicolin a 34.6 fold increase in NUDR expression was observed ($p < 0.05$) (Figure 3.10). NUDR expression was not significantly affected by the length of time cells were treated with RA (Figure 3.11).

An increase in expression of NUDR mRNA is present at the same time as there is an increase in 5-HT_{1A} receptor expression when SH-SY5Y cells are differentiated with NGF and aphidicolin (TP10). A decrease in NUDR expression is observed after time-point 10 in NGF and aphidicolin differentiated cells. An explanation for this observation could be due to the C-1019G promoter polymorphism of the 5-HT_{1A} receptor which, is thought to regulate 5-HT_{1A} gene expression *in vivo* through depression of the 5-HT_{1A} promoter in pre-synaptic raphe neurons leading to a reduced serotonergic transmission due to impaired binding of a transcriptional regulator protein NUDR that acts as a repressor (Huang et al, 2004; Lemonde et al, 2003). This cell line is homozygous for the -1019G allele, as previously described NUDR binds to the -1019C allele leading to depression of 5-HT_{1A} receptor expression (Lemonde et al, 2003). Therefore, in SH-SY5Y cells NUDR would not prevent the transcription of the 5-HT_{1A} receptor, this is supported by the evidence that NUDR expression in SH-SY5Y cells is not modulated by the length of time, cells are differentiated with RA. However, NUDR expression was increased at a time occurring prior to an increase in 5-HT_{1A} receptor expression when SH-SY5Y cells were differentiated with NGF and aphidicolin. This can only occur when the -1019G allele is present as was determined in SH-SY5Y cells. For future work it would be of interest to investigate further the changes in NUDR expression observed.

3.4.4- Immunocytochemistry

Immunocytochemistry results show the presence of the 5-HT_{1A} receptor in SH-SY5Y cells treated with RA for 5 days. An increase in fluorescence is observed in cells differentiated with RA compared to undifferentiated cells (Figure 3.12).

No staining was observed on negative controls that were stained in the absence of primary antibody SR-1A. 4' 6-diamino-2-phenylindole (DAPI) a fluorescent stain that binds strongly to DNA and is often used to stain both live and fixed cells by labelling the cell nuclei. DAPI staining showed that all cells stained with alexa-fluor 488 antibody were viable.

3.4.5- Western blots

Western blots results show the presence of the 5-HT_{1A} receptor in SH-SY5Y cells treated with RA and NGF and aphidicolin. An increase in intensity of band is observed in cells differentiated with RA or NGF and aphidicolin compared to undifferentiated cells (Figure 3.13). No bands were present at 48KDa at time-points 7 with both RA and NGF and aphidicolin. At time-point 7 the absence of a band could be explained by experimental error or by the fact that could be someother gene regulation taking place at this time point.

Non-specific bands were present on blots which could be due to phosphorylated, splicing variant, primary antibody binding to a different member of the same family, or a cross reaction between primary antibody and a non-related protein.

Charest et al (1993), found that SN-48 neuroblastoma fusion cell-line when differentiated with 10µM RA for 24-96 hours expresses 5-HT_{1A} receptor RNA mouse species detected by the northern blot method.

3.4.6- Conclusion

Real-time PCR gene expression studies showed that 5-HT_{1A} receptor and NUDR mRNA was present in the SH-SY5Y cell line. Significant levels of 5-HT_{1A} receptor mRNA were present at 5 days with cells differentiated with RA and at 10 days with NGF. Both immunocytochemistry and western blots showed the presence of 5-HT_{1A} receptor protein.

The SH-SY5Y cell-line may provide a model for promoter studies, divergent from non-neuronal cell-lines in which to investigate neuronal gene expression (Hill and Reynolds, 2007). The SH-SY5Y cell-line is known to express the 5-HT_{2C} receptor mRNA (Flomen et al, 2004) and may therefore contain some of the regulatory elements required for neuronal expression.

SH-SY5Y cells that stably express 5-HT_{2A} and 5-HT_{2C} receptors have been shown to represent a useful model system for the study of these receptors within a neural cell environment (Newton et al, 1996). Parsons et al, (2004) also used SH-SY5Y cells to look at promoter polymorphisms in the 5-HT_{2A} receptor. 5-HT_{2A} receptor mRNA was found to be expressed in this cell-line.

From the results obtained in this study it has been demonstrated that the neuroblastoma SH-SY5Y cell line can be used as a model for studying the 5-HT_{1A} receptor.

Chapter 4

5-HT_{1A} second messenger signalling

4.0- Aim

To investigate whether the 5-HT_{1A} receptor is a functional receptor in the SH-SY5Y cell line by studying calcium signalling using fura-2AM assays on a flow cytometer.

4.1-Introduction

Calcium acts as a universal second messenger in a variety of cells. Several functions of all cell types are mediated by calcium to some degree (Takahashi et al, 1999). The first reliable measurements of intracellular calcium were performed by Ridgeway and Ashley, (1967). The photoprotein aequorin was injected into the giant muscle fibre of the barnacle. The development of a variety of chemical fluorescent indicators began in the 1980's by Tsien et al, (1985). The development of these fluorescent chemicals has revolutionised the measurement of intracellular Ca^{2+} levels in living cells.

4.1.2- Calcium signalling and 5-HT_{1A} receptor

G proteins are a large family belonging to the G-protein coupled receptor superfamily (GPCR) known for their characteristic 7 transmembrane structure and their involvement in second messenger pathways. G proteins can function as "molecular switches" that alternate between an inactive guanine diphosphate (GDP) and activated guanine triphosphate (GTP) bound state ultimately regulating downstream cell processes.

There are two distinct families of G-proteins; heterotrimeric G-proteins activated by GPCR's and made up of alpha (α), beta (β) and gamma (γ) subunits. The other family is the Ras superfamily, which bind GTP and GDP and are involved in signal transduction.

When a ligand activates a GPCR, GDP is exchanged for GTP on the G_α subunit from the $G_{\beta\gamma}$ dimer and receptor hence activating several different signalling cascades and effector proteins. This reaction can be terminated by the eventual hydrolysis of the attached GTP to GDP by the G_α subunit allowing the re-association with $G_{\beta\gamma}$ starting a new cycle.

Heterotrimeric G proteins are coupled to various signal transduction systems for example, adenylyl cyclase (AC) or phospholipase C use G proteins to transduce and amplify their signal to change the activity of effector enzymes (Lui, 1991; Gutland, 1998).

Forskolin is derived from an Indian coleus plant and is commonly used to increase levels of cAMP. Forskolin is thought to resensitise cell receptors by activating AC and therefore increasing intracellular levels of cAMP.

The activation of AC or phospholipase C leads to the generation of intracellular second messengers (Birnbaumer, Abramowitz and Brown, 1990; Gilman, 1987; Ross, splenger and Biedler, 1989). In the case of AC, cAMP is the second messenger which activates protein kinase A (Lui and Albert, 1991).

When 5-HT_{1A} is present and activated by an agonist such as, 8-hydroxy-2-(di-n-propylamino)tetralin HBr (8-OH-DPAT) AC is inhibited (DeVivo and Maayani, 1986) which leads to decreased calcium conductance (Pennington et al, 1991) and increased potassium conductance (Andrade, Malenka and Nicoll, 1986) via pertussis toxin-sensitive G proteins for example, G_i/G_o (Figure 4.1). The 5-HT_{1A} antagonist p-MPPI reverses the effect observed by the agonist 8-OH-DPAT. Additionally, the 5-HT_{1A} receptor may couple to the pertussis toxin-insensitive G protein G_z to increase secretion of some neuroendocrine hormones (Serres et al, 2000).

4.1.3- Measurement of intracellular calcium

The most widely used calcium indicators are chemical fluorescent probes as their signal is quite large for a given change in calcium concentration compared with other types of calcium indicators (Takahagi, 1999).

The most popular chemical fluorescent calcium indicators are UV-excitable and are used as quantitative ratiometric calcium indicators for example, Indo-1 and fura-2AM (Takahagi et al, 1999).

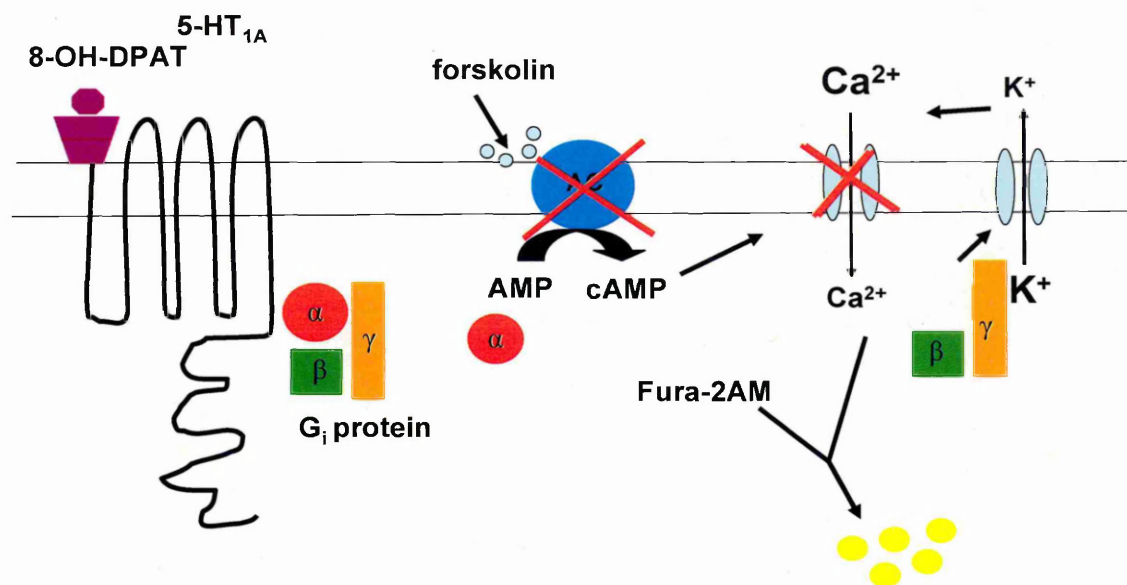


Figure 4.1- Schematic of Intracellular calcium assay

Treating SH-SY5Y cells with forskolin activates the enzyme adenylyl cyclase (AC), which converts AMP to cyclic AMP leading to an increase in Ca^{2+} . The dye FURA-2AM binds to Ca^{2+} and increases fluorescent signal.

When 5-HT_{1A} agonist 8-OH-DPAT is added the G_i protein subunit of 5-HT_{1A} receptor is activated. The β and γ subunits activate K^+ channels leading to an increase in K^+ and reduction in Ca^{2+} . The α -subunit inhibits AC producing a reduction in Ca^{2+} .

The majority of chemical fluorescent indicators are cell impermeant. Therefore to load cells with these indicators it is necessary to adopt special biochemical techniques. Presently, several of the fluorescent calcium indicators are derivatised with an AM that is cell permeable (Tsien and Rink, 1980). The AM form can passively diffuse across cell membranes, and once inside cell esterases remove the AM group leading to a cell-impermeant indicator.

In many types of cells, indicators can leak from the cytosol to extracellular medium (McDonough and Button, 1989). This type of leakage can be regulated in part by anion transport systems which can be inhibited or suppressed by probenecid (DiVirgilio, Fasolato and Steinberg, 1988).

4.1.4- Techniques for measuring calcium

Fluorescence microscopy permits the analysis of the distribution and dynamics of functional molecules within single intact living cells. Confocal laser scanning microscopy is often used in the measurement of intracellular calcium levels by scanning a point across the specimen and collecting the emitted fluorescence through a pinhole that is located at the confocal point of a scanned focus (Takahaghi, 1999).

Electrophysiology can be used to measure changes in intracellular calcium. An estimation of intracellular calcium can be determined by monitoring the currents generated by calcium dependent ion channels located in the plasma membrane. A technique known as "patch cramming" involves a patch micropipette containing the channel in a membrane patch. This is inserted into a recipient cell where the channel locally "senses" the intracellular messenger (Kramer, 1990; Hamill et al, 1981).

Flow cytometry is the measurement of fluorescence and or light intensity emitted by whole cells, which are suspended in a flowing stream of solution (Rieseburg et al, 2001). The single file stream of cells is passed through a laser beam, which can be used for fluorophore excitation or for probing the size and structure of cells by light scattering. Light is detected with photomultipliers, which convert the light into electrical signals for display and storage in computer based systems.

Laser light, which is scattered by cells in the forward direction, is proportional to the cell size. Light scattered at right angles is proportional to granularity; the more complex the internal structure of the cell the more light is scattered.

Using a plate reader to measure intracellular calcium can provide a sensitive and cost effective fluorimetric assay to quantify measurements of rapid calcium responses using a multi-well plate format (Lin, 1999).

4.2- Materials and Methods

4.2.1- Cell culture

The SH-SY5Y cells were grown in DMEM containing 10% FCS and penicillin (100U ml^{-1}) and streptomycin ($100\mu\text{g ml}^{-1}$). Cells were differentiated with retinoic acid (RA, 10^{-5} M) for 5 days.

4.2.2- Plate-based assay

SH-SY5Y cells 1×10^5 per well were grown in 96 well-plates in the presence of RA (10^{-5}M) for 7 days. After 7 days, cells were washed with Krebs buffer (mM: HEPES 20, NaCl 103, KCl 4.77, CaCl_2 0.5, KH_2PO_4 1.2, NaHCO_3 25, Glucose 15, pH 7.2) then incubated with FURA-2AM ($5\mu\text{M}$), probenecid (2.5mM) and pluronic acid (0.2%) for 40 minutes at 37°C in the dark.

After incubation cells were washed once with Krebs buffer containing probenecid (2.5mM) and then stimulated with different concentrations of forskolin ($50\mu\text{l}$) (0, 5, 10, 20, 50, 100, 150 and $200\mu\text{M}$). $100\mu\text{l}$ of Krebs was then added to each well, cells were then treated with and without 8-OH-DPAT ($2\mu\text{M}$) ($50\mu\text{l}$).

Levels of fluorescence at 535nm were detected using a Wallac Victor² 1420 multi-label counter (PerkinElmer Ltd, Turku, Finland).

4.2.3- Flow cytometry

SH-SY5Y cells were grown in T75 flask in the presence of RA (10^{-5}M) for 5 days. After 5 days cells were trypsinised then counted. 10^6 cells per ml were pelleted at 1000rpm for 5 minutes. The pellet was re-suspended in Krebs buffer (mM: HEPES 20, NaCl 103, KCl 4.77, CaCl_2 0.5, KH_2PO_4 1.2, NaHCO_3 25, Glucose 15, pH 7.2). $200\mu\text{l}$ of re-suspended cells were added to each tube. $100\mu\text{l}$ of Fura-2AM ($5\mu\text{M}$), probenecid (2.5mM), pluronic acid (0.2%) and Krebs buffer was added and tubes were incubated at 37°C for 40 minutes in the dark.

After 40 minutes cells were pelleted at 400g for 5 minutes. Cells were re-suspended in $400\mu\text{l}$ Krebs buffer.

Before samples were analysed on the flow cytometer, forskolin (20 and 50 μ M) was added and 2 μ M 8-OH-DPAT or 5-HT (100 μ M) or 4-(2'-methoxy-phenyl)-1-[2'-(n-2"-pyridinyl)-p-iodobenzamido]-ethyl-piperazine (p-MPPI) (0, 10 and 100 μ M).

4.3- Results

4.3.1- Plate-based assay

SH-SY5Y RA differentiated cells were treated in the presence and absence of 8-OH-DPAT (a 5-HT_{1A} agonist) at different concentrations of forskolin (0, 5, 10, 20, 50, 100, 150 and 200 μ M). 8-OH-DPAT decreased levels of intracellular calcium compared to cells treated in the absence of 8-OH-DPAT.

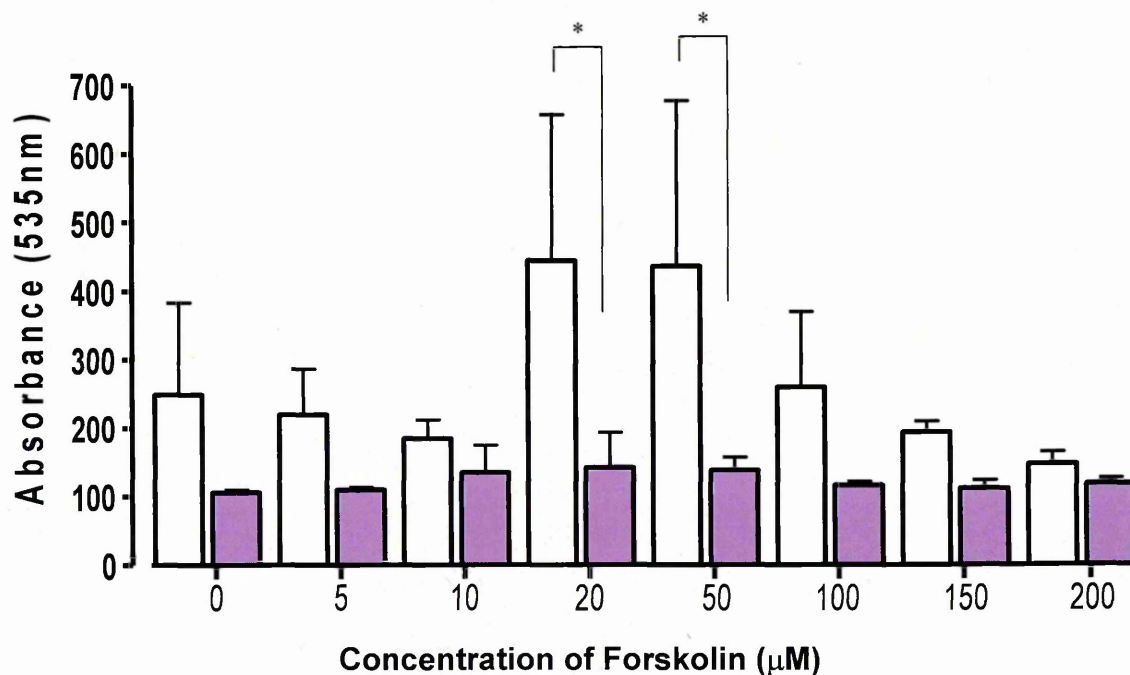


Figure 4.2- Effects of 8-OH-DPAT on intracellular Ca²⁺ levels in SH-SY5Y cells

RA differentiated SH-SY5Y cells treated with 8-OH-DPAT (2 μ M) (■, n=3) evoked a decrease in intracellular Ca²⁺ compared to untreated cells (□, n=3). Data presented as means \pm SEM. A significant decrease (Student's un-paired t-test, p < 0.05) in fluorescence was observed at 20 μ M and 50 μ M forskolin treated cells with 8-OH-DPAT compared to 20 μ M and 50 μ M forskolin treated cells without 8-OH-DPAT.

4.3.2- Flow cytometry

Flow cytometric data can be displayed using either a linear or a logarithmic scale. The use of a logarithmic scale is indicated when there is a broad range of fluorescence being used. A linear scale is used when there is not such a broad range of signalling implemented, for example, DNA analysis and calcium flux measurement.

Flow data is often represented as dot plots and histograms. Dot plots plot one dot or point on the display related to the amount of parameter x and y for each cell passed through the instrument. Histograms quantitate intensities of scatter or fluorescence one parameter per histogram. Gating in flow cytometry is used to select subpopulations of cells for analysis. A gate is a numerical or graphical boundary that can be used to define the characteristics of particles to include for further analysis. Gating is critical for subsequent analysis in order to select the population, free of debris and unrelated cells (Byrne, Reinhart and Hayek, 2000) Dead cells and cell debris are usually present at the lower left area of the dot plot (Figure 4.3).

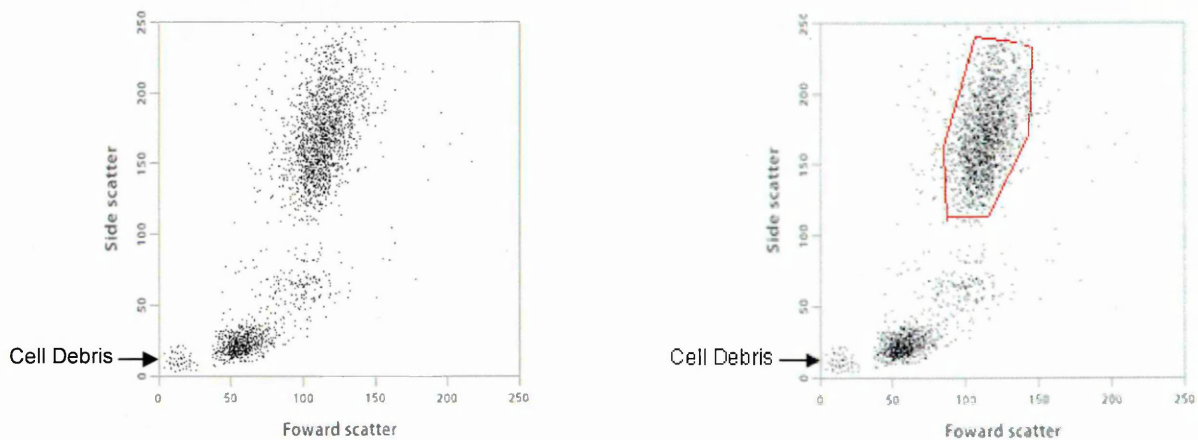


Figure 4.3 - Forward and side scatter dot plot

The dot plot on the left shows a mixed population of cells plotted according to their shape and size. Cell debris and dead cells are often represented at the bottom left of the dot plot. The dense cell population of interest has been marked with the red shape and have been gated for further analysis.

To quantify flow cytometric data the measures of the distribution of a population need to be observed. The mode is the channel with the most events in it. This is

rarely used as it is subject to errors. The median is the central value. The mean can be used as a measure to quantitate cellular fluorescence. In a linear distribution the mean is easily calculated. When comparing absolute fluorescence values it is best to use linear values as these can be directly compared.

4.3.3- Undifferentiated SH-SY5Y cells treated with and without 8-OH-DPAT

For each experiment performed two controls were run at the same time. The first control run was Krebs buffer to provide a base-line reading. The second control contained no forskolin or 8-OH-DPAT. As 8-OH-DPAT is dissolved in methanol and forskolin is dissolved in DMSO the second control contained both of these to rule out any effect they may have on levels of intracellular calcium.

Samples were gated on the basis of forward scatter (FSC-H) and side scatter (SSCH) signals which eliminates cellular debris and non-viable cells. Distinct cell populations present in the sample were identified using FL-1 (fluorescence wavelength of fura-2AM dye) and SSCH.

Intracellular calcium levels were quantified by measuring the mean value of FL-1 fluorescence. The distribution of fluorescence was always close to a normal gaussian distribution, thus the mean value of fluorescence histogram was a good representative parameter.

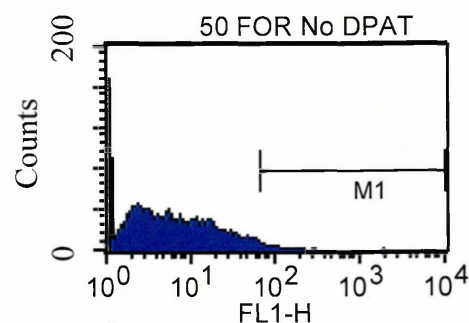
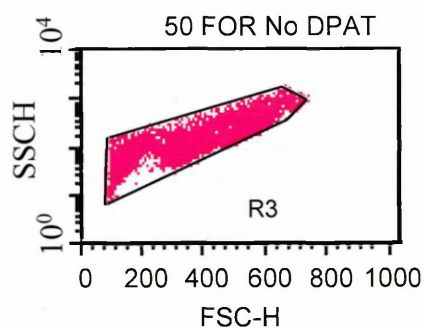
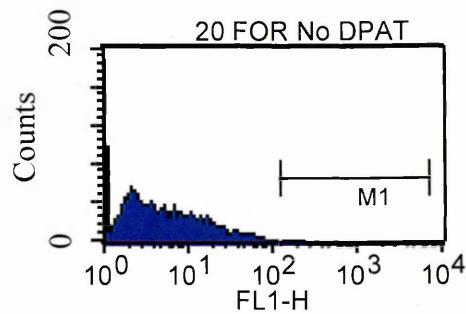
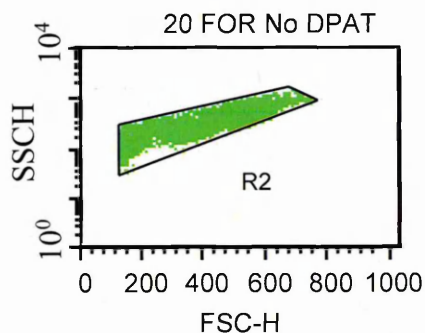
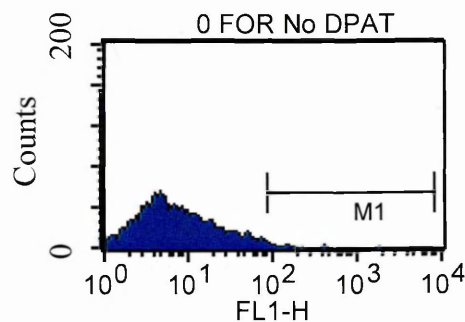
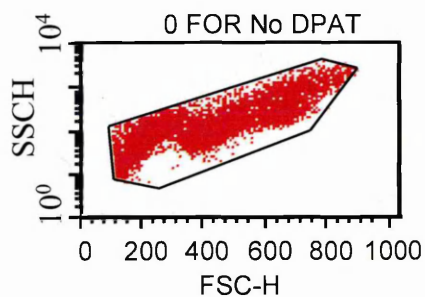
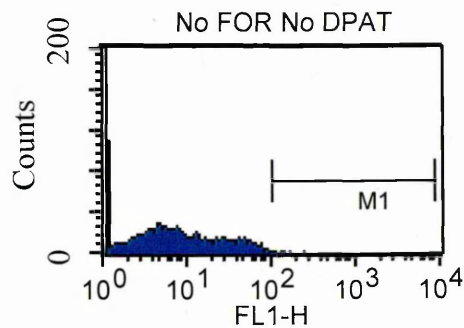
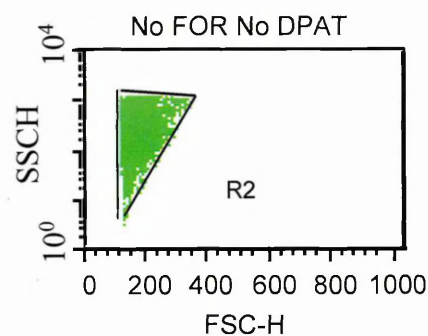


Figure 4.4 - Undifferentiated SH-SY5Y cells treated with forskolin (FSK) in the absence of 8-OH-DPAT

The mean values of fluorescence were calculated using CellQuest software (BD) from the M1 value.

The control had a mean of 106. SH-SY5Y cells were treated with 0, 20 and 50 μ M FSK and in the absence of 8-OH-DPAT, had means of 120.89, 120.95 and 114.04 respectively. The experiment was repeated in triplicate.

Abbreviations: **SSCH**- Side scatter signals, **FSC-H**- Forward scatter and **FL1-H**- Fluorescence wavelength

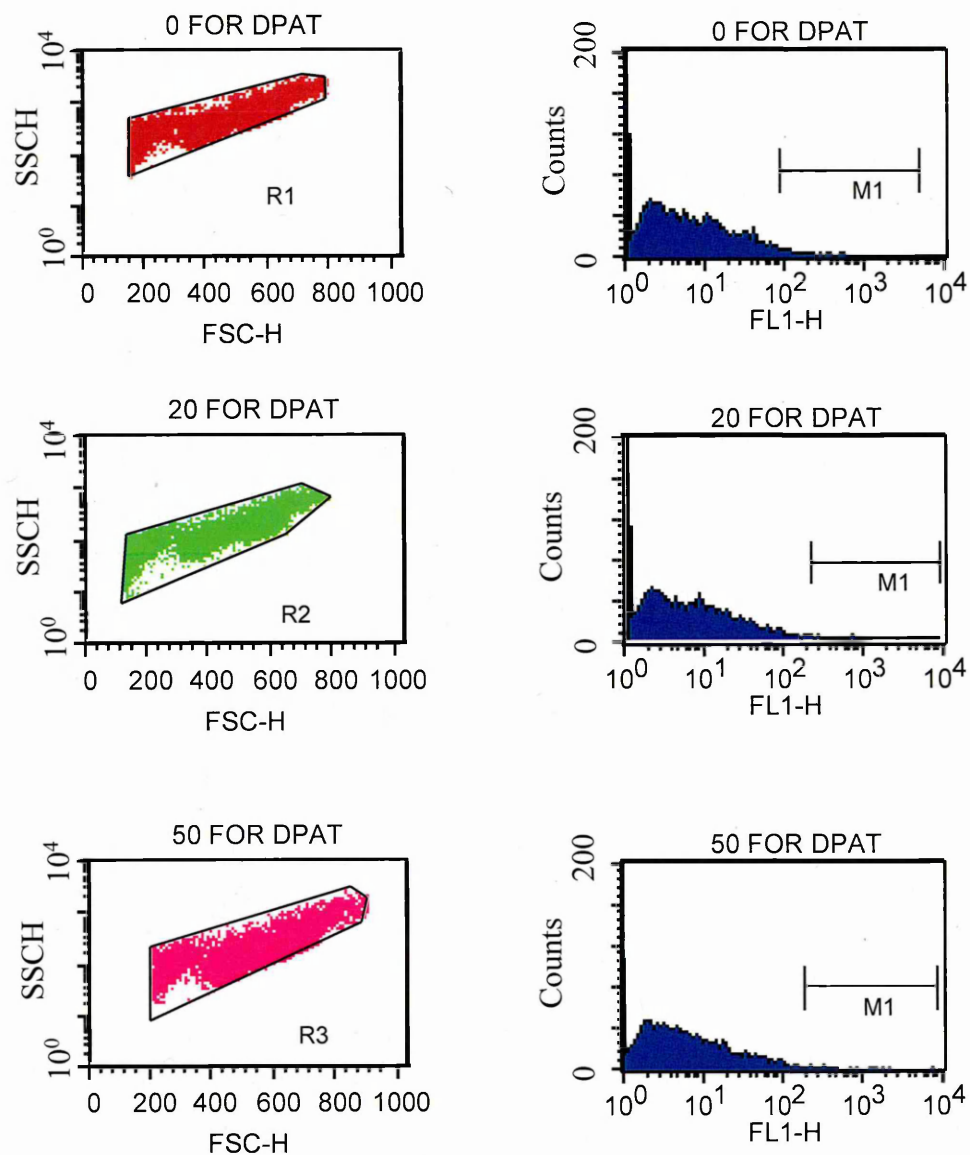


Figure 4.5- Undifferentiated SH-SY5Y cells treated with FSK and 8-OH-DPAT

Undifferentiated SH-SY5Y cells were treated in the presence of 8-OH-DPAT (2 μ M) and 0, 20 and 50 μ M FSK had means of 149.16, 141.47 and 216.44 respectively.

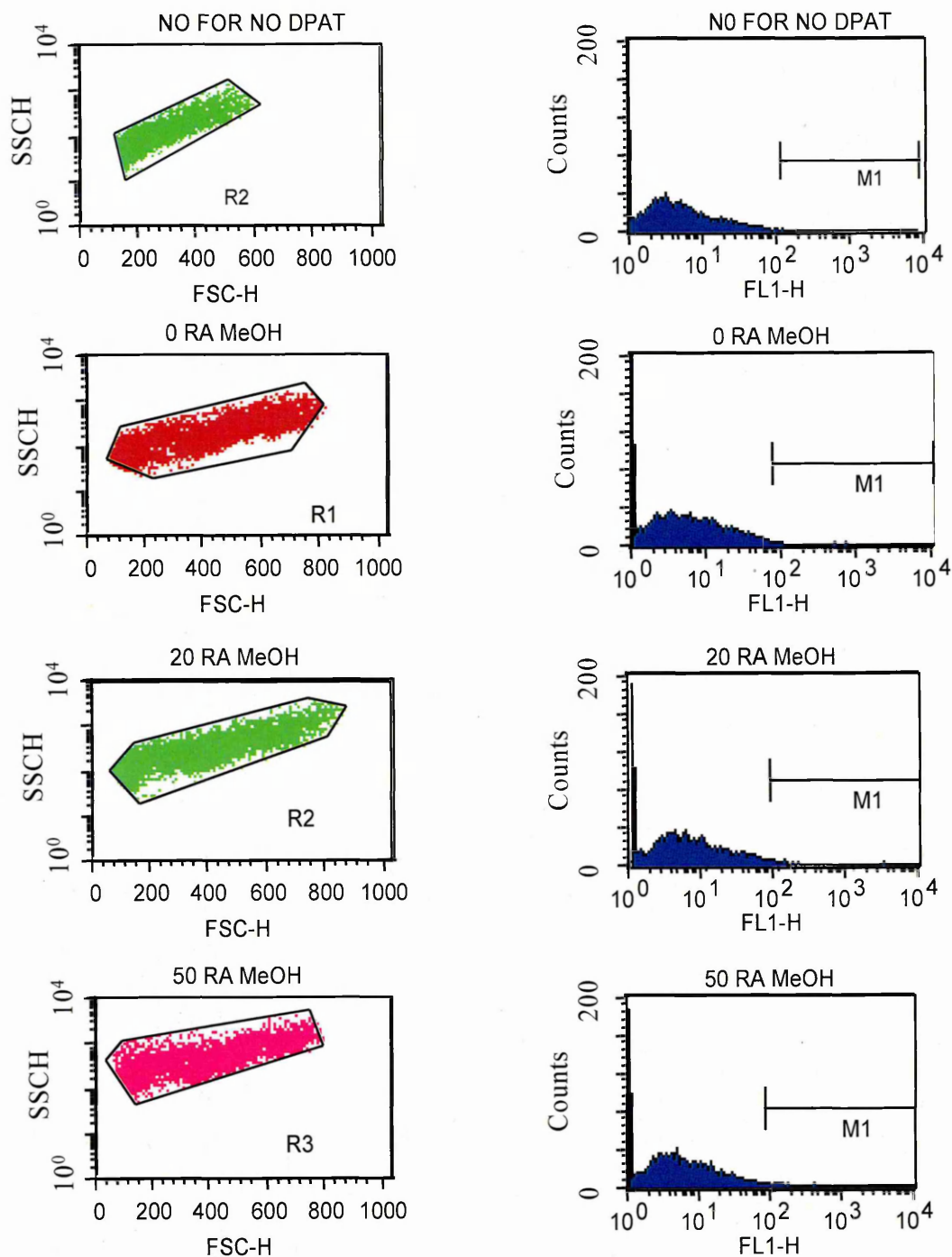


Figure 4.6 - SH-SY5Y RA differentiated cells treated with FSK

The control had a mean of 82.48.

SHSY-5Y differentiated cells treated with 0, 20 and 50 μ M FSK and in the absence of 8-OH-DPAT had means of 199.21, 160.11 and 254.83 respectively.

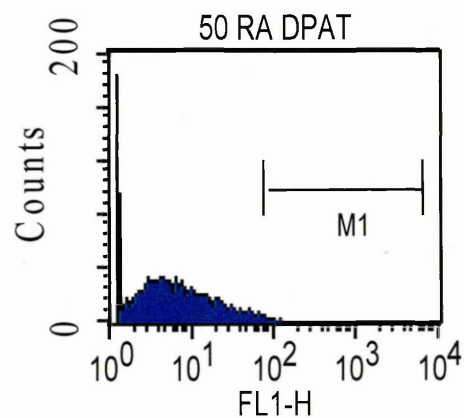
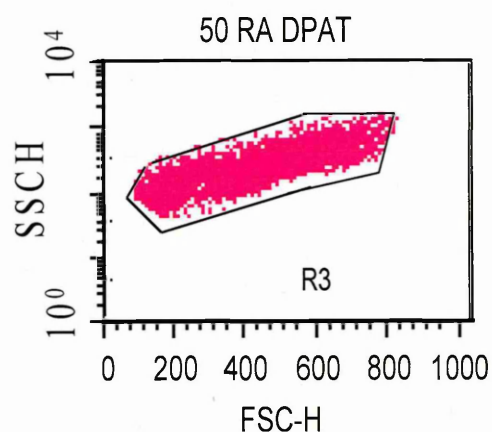
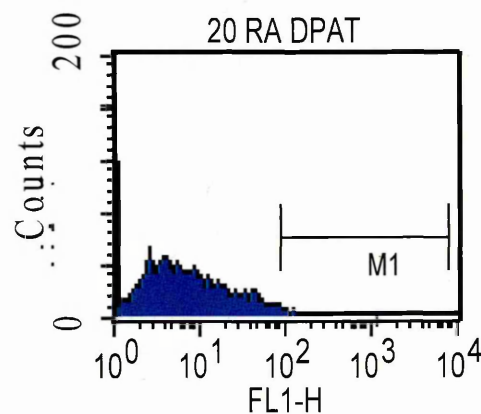
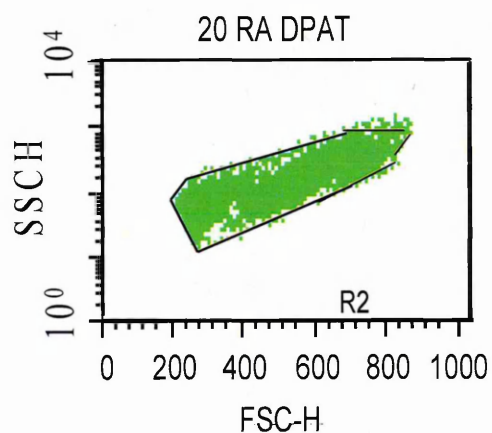
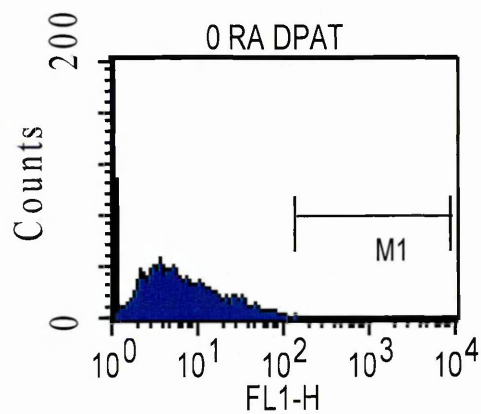
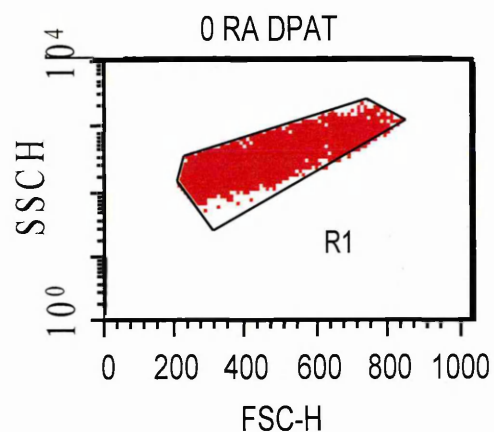


Figure 4.7 - RA differentiated SH-SY5Y cells treated with FSK and 8-OH-DPAT

Differentiated SH-SY5Y cells treated with 0, 20 and 50 μ M FSK and 8-OH-DPAT had means of 76.37, 82.46 and 96.33 respectively.

4.3.4- Undifferentiated SH-SY5Y cells treated in the presence and absence of 8-OH-DPAT

Undifferentiated SH-SY5Y cells were incubated with Fura-2AM, probenecid, pluronic acid and different concentrations of forskolin (0, 20 and 50 μ M).

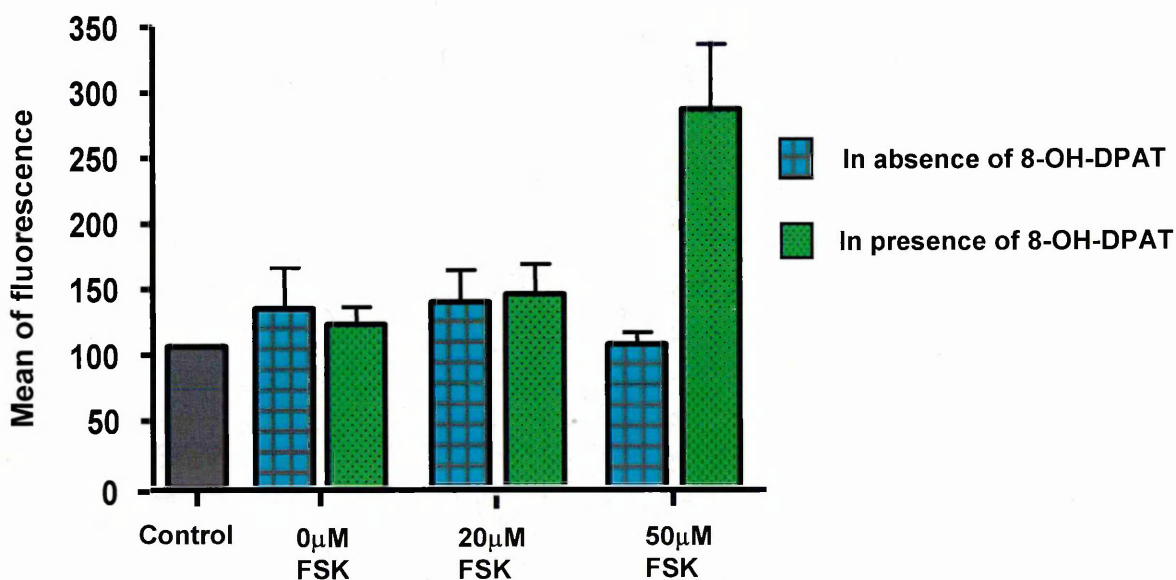


Figure 4.8 - Undifferentiated SH-SY5Y cells treated with and without 8-OH-DPAT

8-OH-DPAT (2 μ M), a 5-HT_{1A} receptor agonist had no effect on the intracellular Ca²⁺ levels in forskolin stimulated undifferentiated SH-SY5Y cells. Data presented as means \pm SEM, n=3, One-way ANOVA with Bonferroni's test, p>0.05. SH-SY5Y cells present in the control sample were treated in the absence of FSK and 8-OH-DPAT.

4.3.5- RA differentiated SH-SY5Y cells treated in the presence and the absence of 8-OH-DPAT

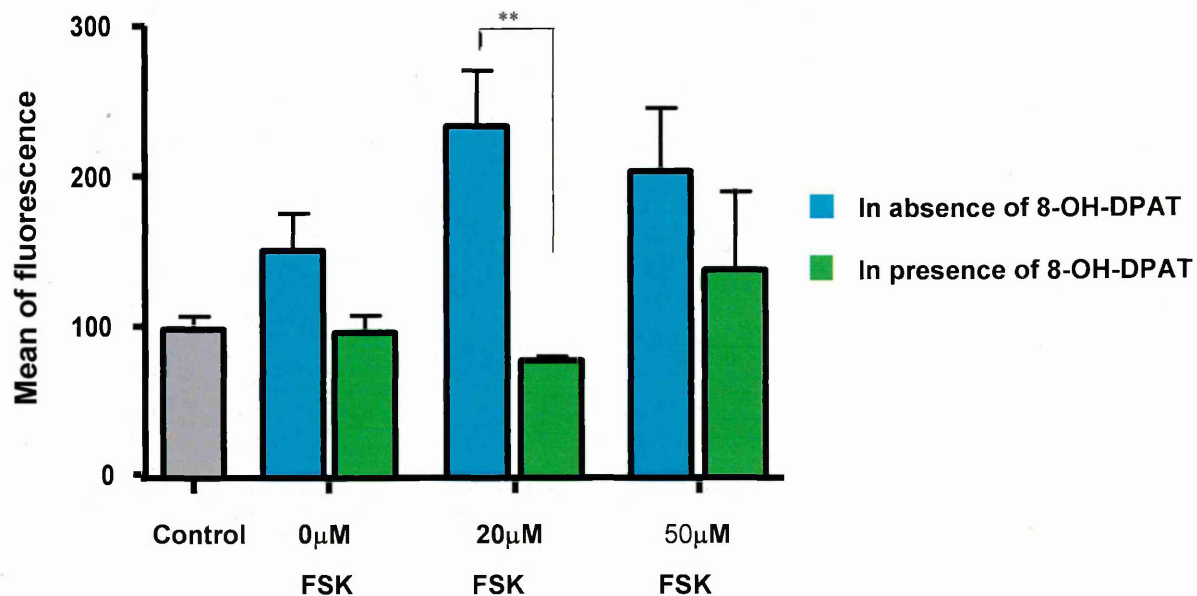


Figure 4.9 - RA differentiated SH-SY5Y cells treated with and without 8-OH-DPAT

A significant decrease in Ca^{2+} levels in SH-SY5Y cells treated with 20μM FSK and 8-OH-DPAT (2μM) compared to those treated in the absence of 8-OH-DPAT (One-way ANOVA with Bonferroni's, $**p < 0.01$) was observed ($n=3$).

4.3.6- 5-HT

5-HT is a neurotransmitter that when bound to 5-HT_{1A} receptor (G-protein) activates the receptor leading to a reduction in intracellular calcium.

For this experiment 5-HT was dissolved in 0.1M HCL, therefore, the control (No FSK and 8-OH-DPAT) used for this experiment contained 0.1M HCL.

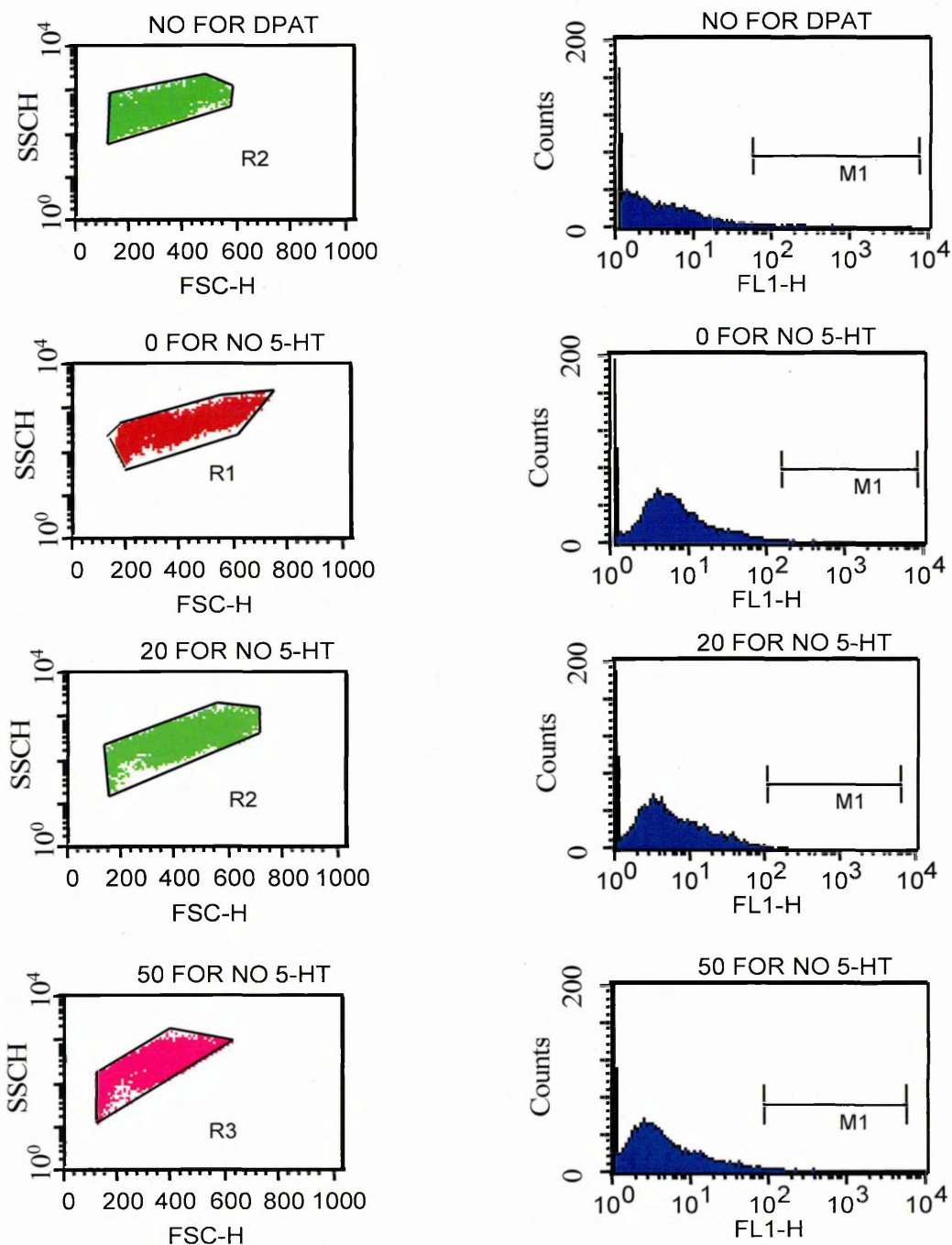


Figure 4.10 - SH-SY5Y RA differentiated cells treated without 5-HT

The mean of the control no FSK no 8-OH-DPAT was 110.

Differentiated cells treated without 5-HT (1mM) at 0, 20 and 50μM FSK had means of 185.07, 129.86 and 121.04 respectively.

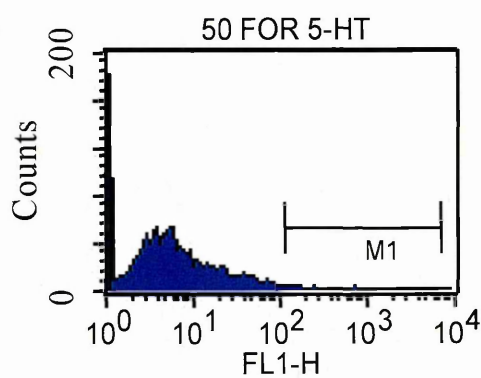
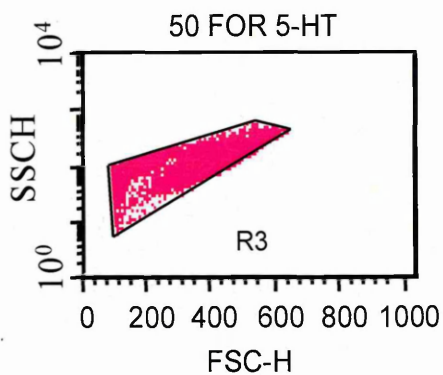
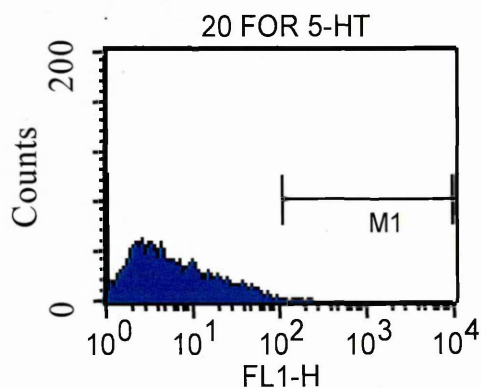
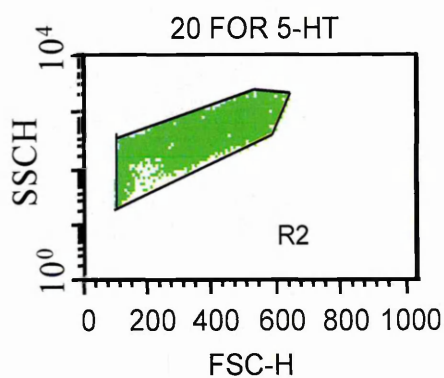
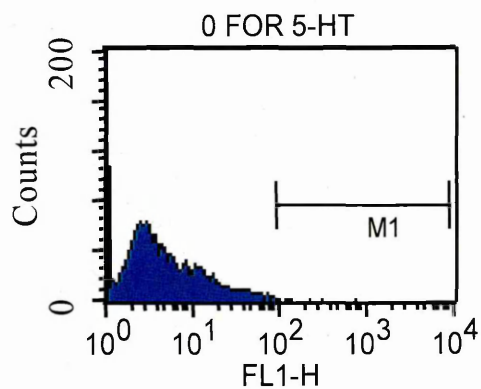
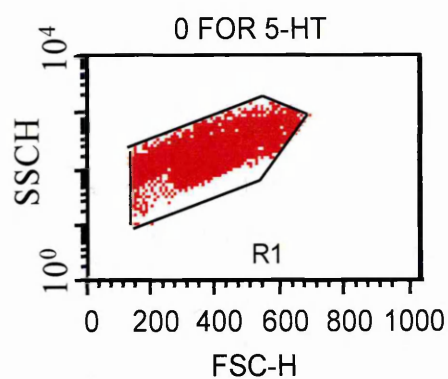


Figure 4.11 - SH-SY5Y RA differentiated cells treated with 5-HT

Differentiated cells treated with 5-HT (1mM) at 0, 20 and 50 μ M FSK had means of 116.86, 81.88 and 88.15 respectively.

4.3.7- RA differentiated SH-SY5Y cells treated in the presence and absence of 5-HT

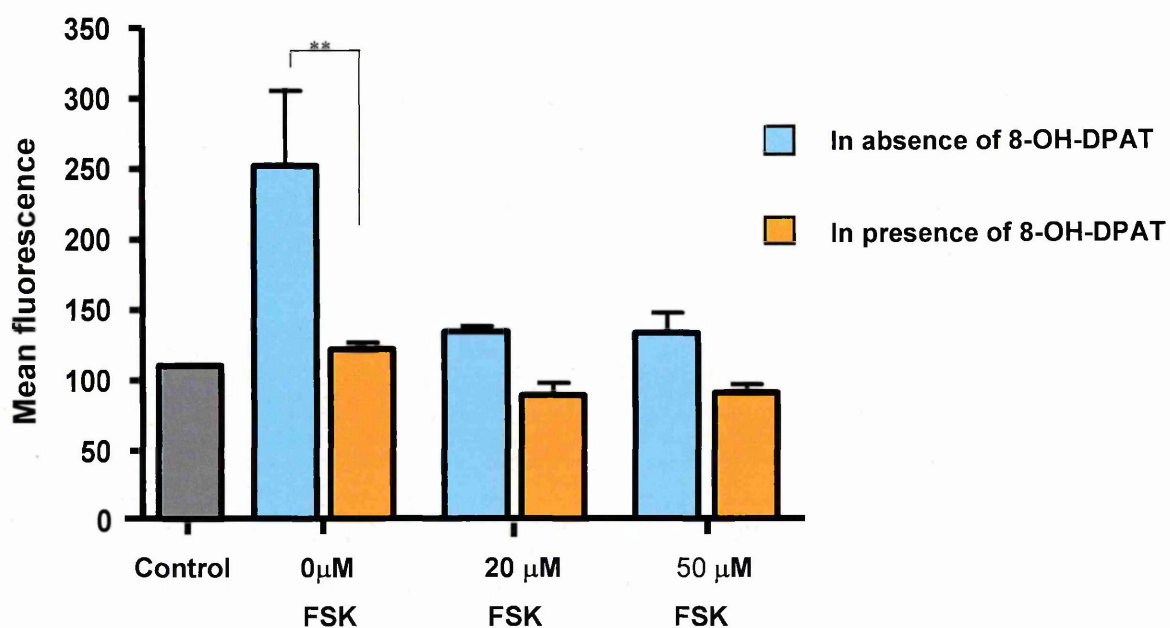


Figure 4.12 - RA differentiated SH-SY5Y cells treated with and without 5-HT

5-HT a 5-HT_{1A} receptor agonist significantly reduced Ca²⁺ levels when treated with no FSK compared to cells treated in the absence of 5-HT. Data presented as means±SEM, n=3, One-way ANOVA with Bonferroni's, **p<0.01.

4.3.8- p-MPPI

p-MPPI is a selective 5-HT_{1A} receptor antagonist has high binding affinity and receptor selectivity for the 5-HT_{1A} receptor. SH-SY5Y cells treated with both p-MPPI and 8-OH-DPAT will compete for the 5-HT_{1A} receptor. Cells treated with an increased concentration of p-MPPI a rise in intracellular calcium concentration should be observed.

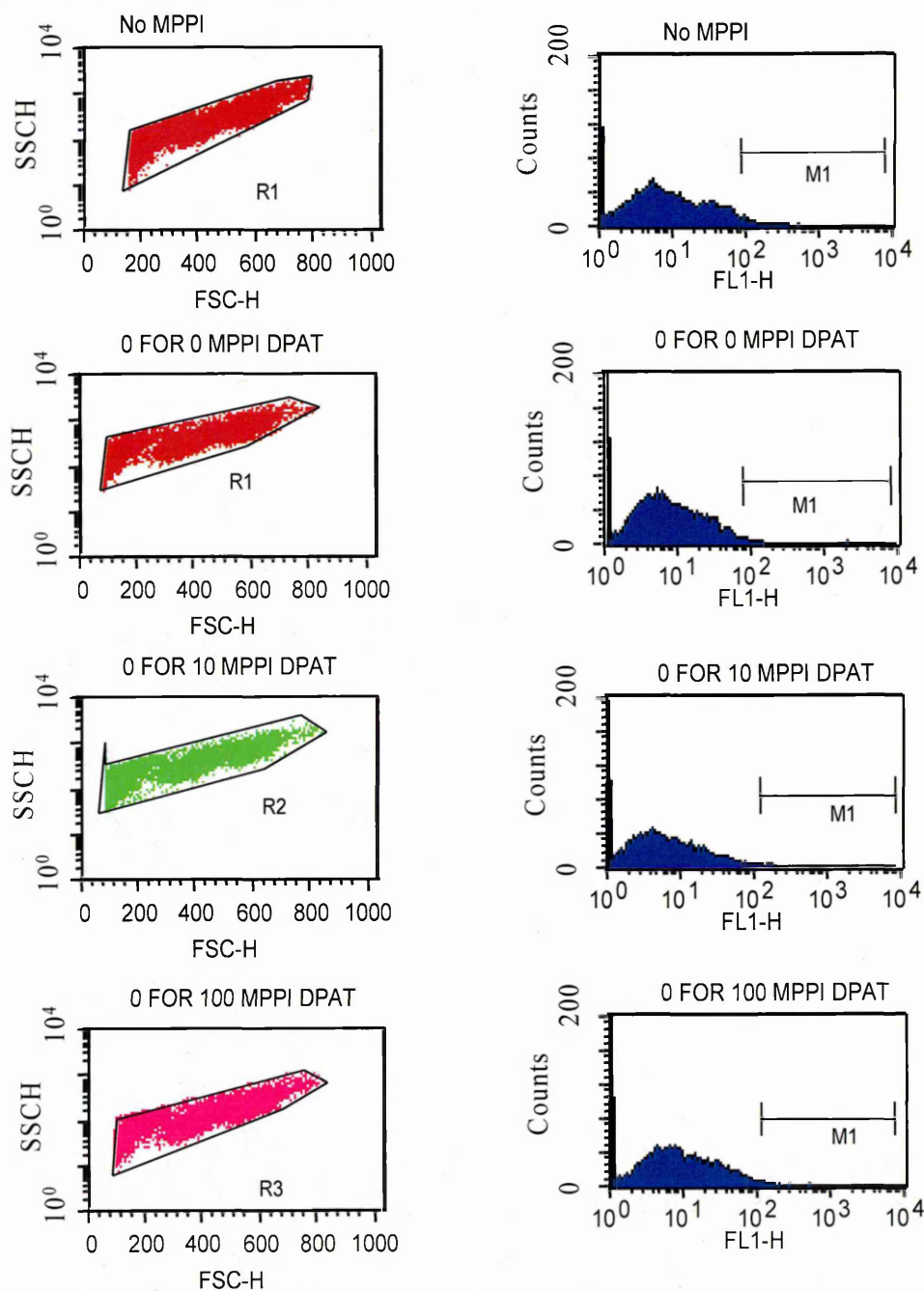


Figure 4.13 - RA differentiated SH-SY5Y cells treated with forskolin (0 μ M), p-MPPI (0 μ M, 10 μ M and 100 μ M) and 8-OH-DPAT (2 μ M).

SH-SY5Y differentiated cells treated with no FSK, 8-OH-DPAT and 0, 10 and 100 μ M MPPI had mean values of 141.87, 199.6 and 287.26 respectively. The no MPPI control had a mean value of 130.13.

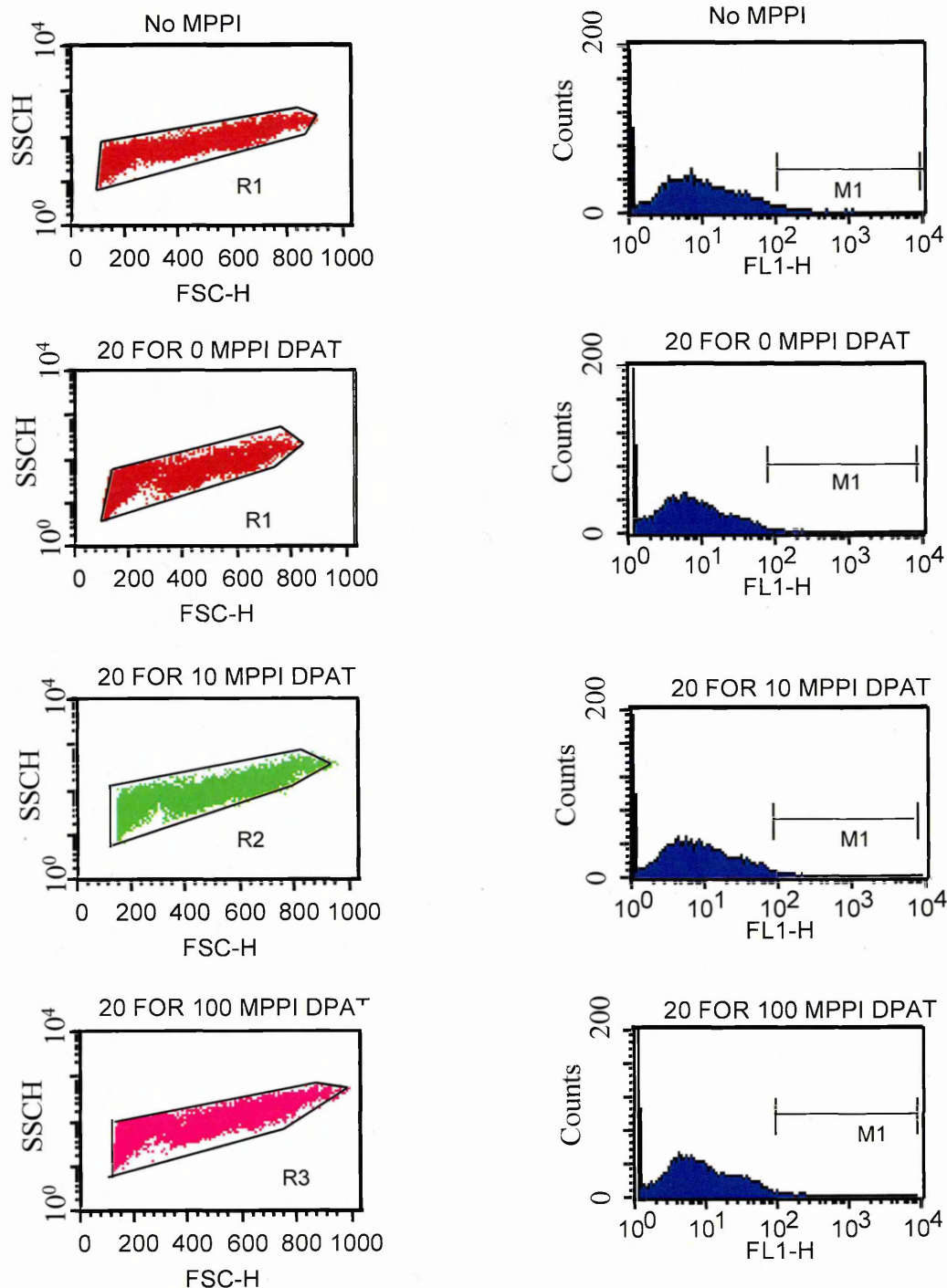
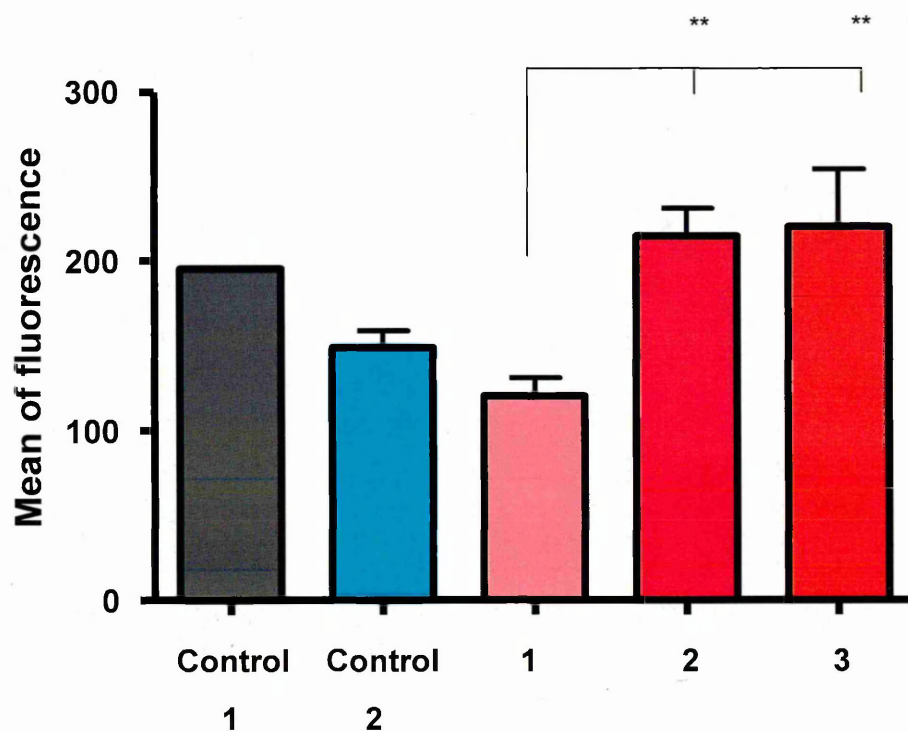


Figure 4.14 - SH-SY5Y RA differentiated cells treated with forskolin (20 μ M), p-MPPI (0 μ M, 10 μ M and 100 μ M) and 8-OH-DPAT (2 μ M).

SH-SY5Y differentiated cells treated with 20 μ M forskolin, 8-OH-DPAT and 0, 10 and 100 μ M MPPI had mean values of 157.99, 174.9 and 206.37 respectively. The no MPPI control had a mean value of 166.54.

4.3.9- RA differentiated SH-SY5Y cells treated with p-MPPI



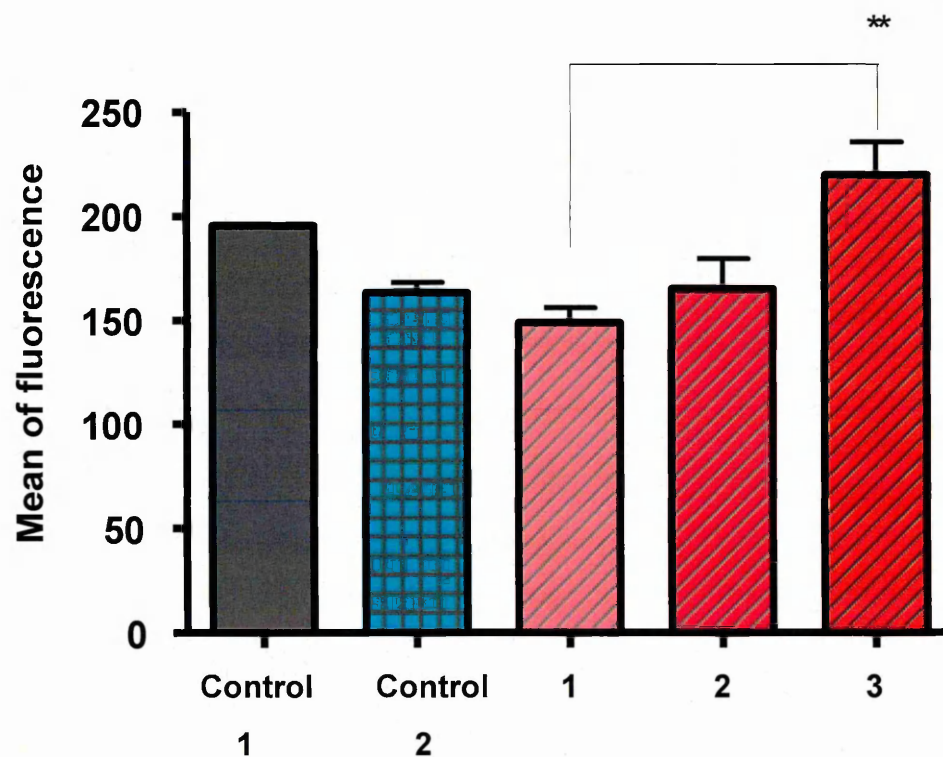
	1	2	3
Forskolin	0μM	0μM	0μM
8-OH-DPAT	2μM	2μM	2μM
p-MPPI	0μM	10μM	100μM

Control 1- No forskolin and no 8-OH-DPAT

Control 2- Control for MPPI, ddH₂O only

Figure 4.15 - RA differentiated SH-SY5Y cells treated with MPPI (0, 10 and 100μM) and 8-OH-DPAT (2μM) in the absence of forskolin

A significant increase in Ca²⁺ levels were observed when treated with 100 MPPI and 8-OH-DPAT compared to cells treated with 0μM MPPI and 8-OH-DPAT. The results show that in the absence of forskolin p-MPPI effectively increases levels of intracellular calcium to that of background levels. Data presented as means±SEM, n=3, One-way ANOVA with Bonferroni's **p<0.01.



	1	2	3
Forskolin	20μM	20μM	20μM
8-OH-DPAT	2μM	2μM	2μM
p-MPPI	0μM	10μM	100μM

Control 1- No forskolin and no 8-OH-DPAT

Control 2- Control for MPPI, ddH₂O only

Figure 4.16 - RA differentiated SH-SY5Y cells treated with 20 μ M forskolin and MPPI (0, 10 and 100 μ M) and 8-OH-DPAT (2 μ M)

An increase in Ca²⁺ level was observed in cells treated with 20 μ M forskolin, 8-OH-DPAT (2 μ M) and 100 MPPI compared to 20 μ M forskolin, 8-OH-DPAT (2 μ M) and 0 μ M MPPI. Data presented as means \pm SEM, n=3, One-way ANOVA with Bonferroni's **p<0.01.

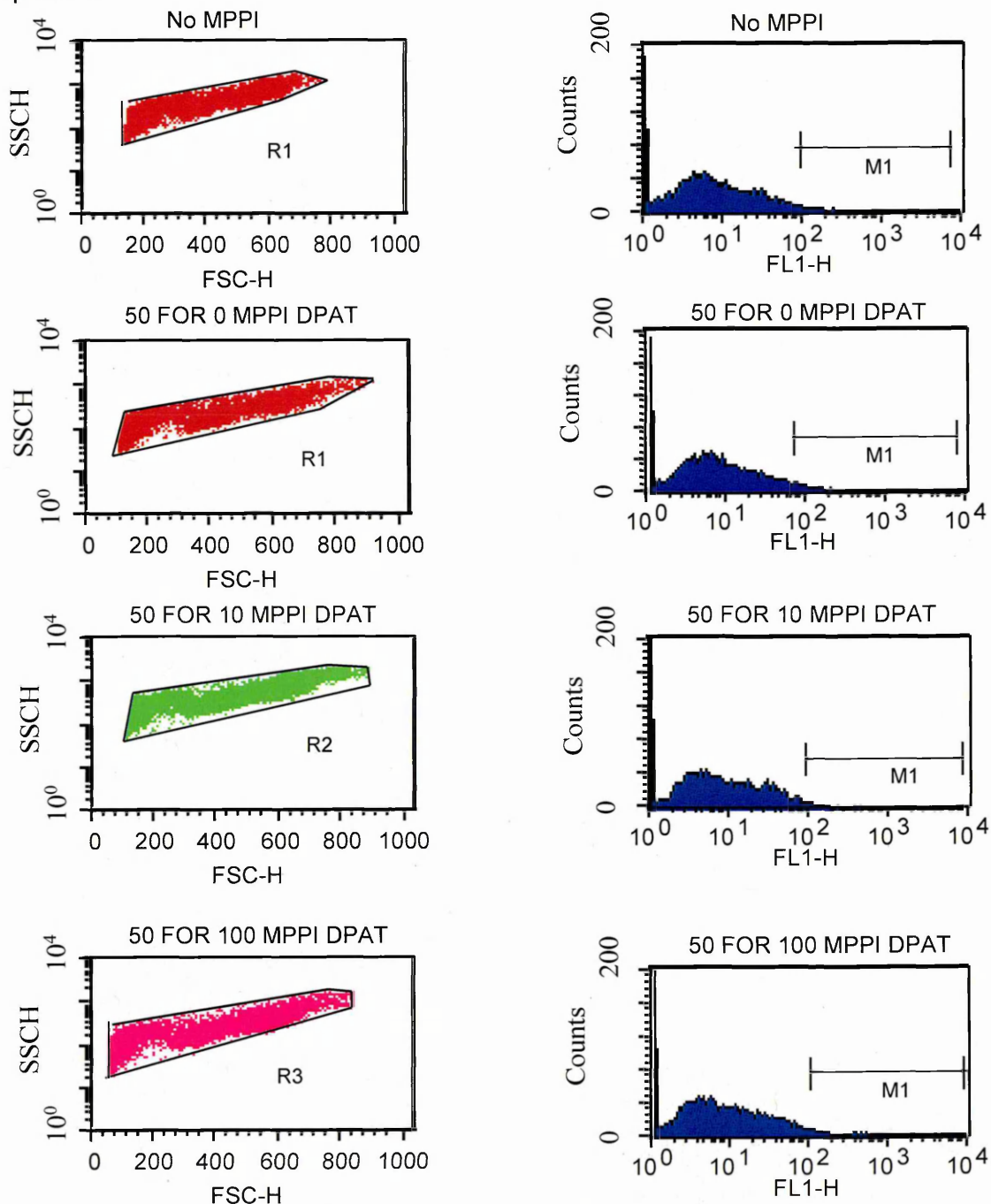
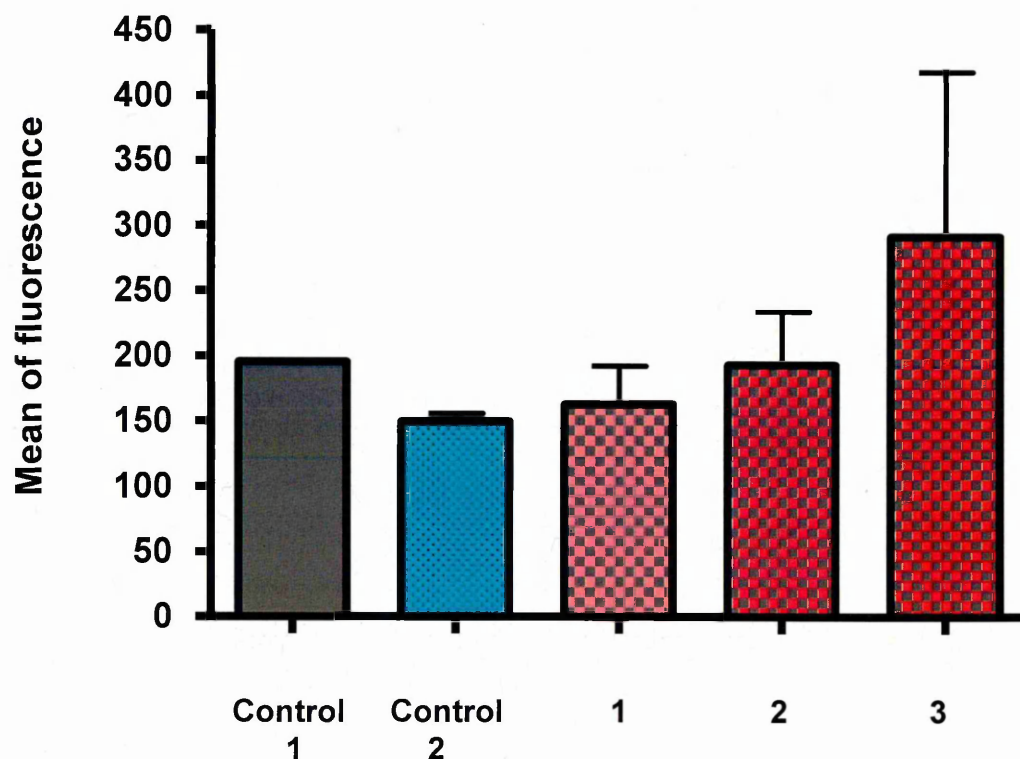


Figure 4.17 - RA differentiated SH-SY5Y cells treated with forskolin (50 μ M), p-MPPI (0 μ M, 10 μ M and 100 μ M) and 8-OH-DPAT (2 μ M).

SH-SY5Y differentiated cells treated with 50 μ M forskolin, 8-OH-DPAT and 0, 10 and 100 μ M MPPI had mean values of 131.45, 146.58 and 153.78 respectively. The no MPPI control had a mean value of 161.52.



	1	2	3
Forskolin	50μM	50μM	50μM
8-OH-DPAT	2μM	2μM	2μM
p-MPPI	0μM	10μM	100μM

Control 1- No forskolin and no 8-OH-DPAT

Control 2- Control for MPPI, ddH₂O only

Figure 4.18 - RA differentiated SH-SY5Y cells treated with 50μM forskolin and MPPI (0, 10 and 100μM) and 8-OH-DPAT (2μM)

No significant increase in Ca²⁺ levels was observed with cells treated with 50μM forskolin, 8-OH-DPAT (2μM) and 0, 10, and 100μM MPPI. Data presented as means±SEM, n=3, One-way ANOVA with Bonferroni's p>0.05.

4.4- Discussion

The results in this study show that 8-OH-DPAT did not diminish intracellular calcium levels in undifferentiated SH-SY5Y cells whereas, in RA differentiated SH-SY5Y cells forskolin-stimulated increase in intracellular calcium was efficiently reduced by 8-OH-DPAT (2 μ M). A similar effect was seen with 5-HT also a 5-HT_{1A} agonist, which diminished forskolin-stimulated increase in intracellular calcium in RA differentiated SH-SY5Y cells. A non-classical dose-response effect on calcium concentration was observed with 8-OH-DPAT using the plate based assay. The dose-response curve observed reached a maximum value where it peaked and then values declined back to baseline values representing a bell-shaped curve. This observation could be due to 5-HT_{1A} receptor desensitisation at higher concentrations of forskolin (100-200 μ M).

This indicates that 5-HT_{1A} receptor is functional as 8-OH-DPAT effectively reduces the levels of intracellular calcium in this cell line. The 5-HT_{1A} receptor agonists 5-HT and 8-OH-DPAT are known to explicitly activate G_{i/o} class of G-proteins which consequently diminish cAMP levels (Paila and Chattopadhyay, 2006).

Paila et al (2006) found that forskolin-stimulated increase in cAMP levels is efficiently inhibited by 8-OH-DPAT in a concentration dependent manner. The normal mechanism of 5-HT_{1A} signalling is via G proteins which inhibit AC.

Khan et al (1995) showed that 5-HT reduced intracellular calcium concentrations in a dose-dependent manner in K562 cells loaded with fura-2. Similarly, in LZD-7 fibroblast cells 5-HT inhibited AC reducing the forskolin-induced enhancement of cAMP levels by 50 percent (Liu and Albert, 1991). In this study 8-OH-DPAT was found to be a more potent agonist than 5-HT on 5-HT_{1A} receptors comparable with studies by De Vivo et al (1986) and Dumius et al (1988) who found that 8-OH-DPAT is a full agonist and is generally thought to be more potent than 5-HT on 5-HT_{1A} receptors that are negatively coupled to AC in adult rat hippocampus and mouse hippocampal neurons.

It is thought that the binding of 5-HT and 8-OH-DPAT to the 5-HT_{1A} receptor may either directly open "ligand gated" channels or they may modulate the channel functions directly via intracellular messengers (Khan et al, 1995).

Harrington et al (1991) showed that SH-SY5Y cell line expresses D₂ receptors and the D₂ receptor is functional in this cell line. 8-OH-DPAT is also thought to have a weak affinity for dopamine (D₂) receptors (Kleven and Koek, 1997). Dopamine (D₂) receptor interacts with G proteins particularly G_{i/o} G proteins and can inhibit AC (Hall and Strange, 1999) that leads to a decrease in cytosolic calcium concentration like that observed with 5-HT_{1A} receptors. In this study, to make certain that it was the 5-HT_{1A} receptor inhibiting AC and therefore leading to a decrease in calcium levels and not D₂ receptors, p-MPPI was used. p-MPPI has been shown to act as a selective and potent 5-HT_{1A} receptor antagonist both at somatodendritic and post-synaptic 5-HT_{1A} receptors (Allen et al, 1997; Bjorvatan et al, 1998 and Thielen and Frazer, 1995).

SH-SY5Y cells treated with both MPPI a 5-HT_{1A} receptor antagonist, and 8-OH-DPAT demonstrated that cells treated with MPPI at higher concentration (100µM) significantly increased forskolin-stimulated intracellular calcium levels and hence effectively reversed the agonistic effect of 8-OH-DPAT. Increased concentration of forskolin (50µM) did not significantly augment intracellular calcium levels at higher concentrations of MPPI (100µM).

Kung et al (1994) demonstrated that p-MPPI completely antagonises the inhibition of forskolin-stimulated adenylyl cyclase activity induced by 8-OH-DPAT in hippocampal membranes. p-MPPI binding occurred in Sf9 cells regardless of the expression of a G protein subunit, as would be anticipated for an antagonist (Butkerait et al, 1995).

Therefore, these results suggest that pharmacological modulation of the 5-HT_{1A} receptor in these cells affects intracellular calcium levels showing that the 5-HT_{1A} receptor signals via second messenger pathways and is a functional receptor in this cell line.

Chapter 5

Final Discussion

5.0- Final Discussion and conclusions

The aims of the first part of this project were to investigate the effects of the C-1019G 5-HT_{1A} receptor promoter polymorphism on expression of the 5-HT_{1A} receptor in hippocampal post-mortem tissue by quantifying 5-HT_{1A} receptor mRNA expression using real-time PCR and 5-HT_{1A} receptor density using radioligand binding.

The aims of the second part the study were to validate differentiated SH-SY5Y cells as a model system for studying the 5-HT_{1A} receptor. Cells were differentiated with RA or NGF and aphidicolin to provide a neuronal cell subtype. Real-time PCR was used to quantify 5-HT_{1A} receptor and NUDR mRNA expression in this cell line. 5-HT_{1A} receptor protein was determined using immunocytochemistry and western blots. Intracellular calcium levels were measured to investigate whether the 5-HT_{1A} receptor was functional in this cell line using flow cytometry.

There is a substantial amount of information on the neuropharmacology of serotonin (5-HT) which implicates the serotonin system as an important modulator in a variety of central nervous system processes (Green, 2006). These processes include: anxiety, fear, depression and aggression; control of sleep and modulation of ingestive behaviours and the cardiovascular system (Gingrich and Hen, 2001; Hoyer et al, 2002).

Mood disorders are among the most prevalent forms of mental illness. Severe forms of depression affect approximately 14.8 million American adults (Kessler et al, 2005) and 20 percent of the American population are thought to suffer from milder forms of the illness. In the UK, depression affects 1 in 10 adults and the estimate of a life time prevalence of depression varies from 1 in 6 to 1 in 4 (National office of statistics; Hale, 1997). Mood disorders can be recurrent, life threatening and a major cause of morbidity worldwide (Blazer, 2000).

In many cases depression should not be viewed as a single disease but a heterogeneous disease comprised of many diseases of distinct cause and pathophysiologies. Several epidemiologic studies have shown that approximately 40-50 percent of the risk for depression is genetic (Saunders et

In many cases depression should not be viewed as a single disease but a heterogeneous disease comprised of many diseases of distinct cause and pathophysiologies. Several epidemiologic studies have shown that approximately 40-50 percent of the risk for depression is genetic (Saunders et al, 1999). Depression is thought to be a complex disease with several genes thought to be associated in the pathophysiology of this disease. However, the predisposition to depression is only partly genetic, with non-genetic factors also being an important consideration. These non-genetic factors include stress and emotional trauma among many other diverse factors which have all been implicated in the etiology of depression (Fava and Kendler, 2000). There are several studies which support the hypothesis that episodes of depression often occur in the context of some form of stress. Conversely, stress is not the sole cause of depression and it has been suggested that depression in the majority of people is due to the interactions between a genetic predisposition and some environmental factors (Nestler et al, 2002).

The 5-HT_{1A} receptor is of great interest due to its association in the pathogenesis and is also a target for the treatment of anxiety and depression (Veenstra-VanderWeele, Anderson and Cook, 2000). The 5-HT_{1A} receptor is present presynaptically as an autoreceptor on the soma and dendrites found mainly in the median and dorsal raphe nuclei and post-synaptically in the limbic regions of the brain (Jacobs and Azmitia, 1992). Activation of the postsynaptic 5-HT_{1A} receptors results in an inhibition of the activity of neurons of the limbic system.

The 5-HT_{1A} receptor promoter polymorphism C-1019G has been associated with depression. Lemonde et al (2003), observed that depressed patients were twice as likely as controls to have the homozygous -1019G genotype. Several transcription factors have been found to specifically bind to the -1019C allele, in particular the transcription factor NUDR, which is thought to suppress the transcriptional activity of the -1019C allele and therefore decreasing the expression of the 5-HT_{1A} receptor leading to an increase in firing rate, whereas with the G allele NUDR does not bind leading to an increase in 5-HT_{1A} receptor expression and a reduction in the firing rate.

The serotonergic system interacts with the HPA axis and glucocorticoid secretion (Dinan, 1994). Glucocorticoid receptors have an effect on 5-HT neurotransmission by down regulating 5-HT_{1A} receptor expression postsynaptically in the hippocampus (Chalmers et al, 1995).

The 5-HT_{1A} receptor couples to the G_{i/o} effector. Once the 5-HT_{1A} receptor is activated by an agonist the dissociation of the G protein occurs resulting in two subunits G_α and G_{βγ} subunits. These two subunits can activate different transduction pathways. The G_α subunit leads to the inhibition of AC producing a decrease in intracellular calcium levels. The neuroblastoma SH-SY5Y cell line is a well characterised cell line used in neurotransmitter studies and when differentiated with either RA or NGF and aphidicolin a more neuronal cell type is generated making this a suitable model system for studying the 5-HT_{1A} receptor.

5.1- Human post-mortem study

5.1.1- 5-HT_{1A} receptor genotype and expression

Human post mortem tissue was genotyped in this study for the 5-HT_{1A} receptor promoter polymorphism C-1019G. Real-time PCR was used to quantify mRNA levels of the 5-HT_{1A} receptor. The distribution of genotype was within the Hardy Weinberg equilibrium and agreed with studies by Lemonde et al, (2003), Arias et al, (2002), Parsey et al, (2006) and Huang et al, (2004).

The results obtained in this current study show that a significantly higher 5-HT_{1A} receptor expression was observed with the G allele compared to subjects with a C allele in control post-mortem hippocampal tissue which is in agreement with Lemonde et al, (2003). The present findings demonstrate that a similar presynaptic mechanism of gene regulation is also present postsynaptically. A plausible explanation for this is that some depressed subjects hypersecrete cortisol in response to stress, which is thought to down regulate 5-HT_{1A} receptor expression (Lopez et al, 1998) by lowering the availability of L-tryptophan leading to a reduction in 5-HT turnover and hence downregulating pre-synaptic 5-HT_{1A} receptors (Chalmers et al, 1993). Lopez et al (2004) demonstrated that there was a decrease in postsynaptic 5-HT_{1A} RNA in the hippocampus of post-mortem tissue from subjects with major depression which may be explained due to glucocorticoid secretion masking genotype.

Contrary, to the results obtained in this thesis Stockmeier et al (1998) found an upregulation of 5-HT_{1A} receptors in the raphe area and observed no change in expression of postsynaptic sites.

5.1.2- 5-HT_{1A} receptor density

In this study 5-HT_{1A} receptor density in hippocampal post-mortem tissue was determined by radioligand binding.

The results obtained in this study indicated a significant increase in postsynaptic 5-HT_{1A} receptor when correlated with genotype in control human post-mortem tissue samples, when analysed using radioligand binding. Higher 5-HT_{1A} receptor expression was observed with subjects who had the GG or G/C genotype compared to the CC genotype. However, other studies have demonstrated that the greater binding of the 5-HT_{1A} receptor is not correlated to genotype of the C-1019G polymorphism in pre-frontal cortex post-mortem brain tissue (Huang et al, 2004).

In the present literature there are several disagreements regarding the presence and the direction of 5-HT_{1A} receptor binding abnormalities in depression. Stockmeier et al (1998) have shown that there is an increase in 5-HT_{1A} receptors in the raphe in depressive and suicidal subjects. Whereas, other studies have reported a decrease in 5-HT_{1A} receptor binding in the hippocampal region of suicide samples (Gross-Isseroff et al, 1998) and reduced 5-HT_{1A} receptor binding in mesiotemporal cortex and raphe in depressives compared to controls (Drevets et al, 1999; Lopez et al, 1998; Arango et al, 2001). These abnormalities could be explained by differences in anatomical localisation and that some depressed subjects hypersecrete cortisol which can down regulate 5-HT_{1A} receptor expression (Lopez et al, 1998).

5.1.3- Conclusions of post-mortem tissue study

The aim of this post-mortem study was to provide a greater understanding of the 5-HT_{1A} receptor expression in control hippocampal human post-mortem tissue.

The relationship between the C-1019G 5-HT_{1A} receptor promoter polymorphism and 5-HT_{1A} receptor expression, where the G-1019 allele is associated with a predisposition to depression has only previously been reported at pre-synaptic sites, mainly in the raphe nuclei. Therefore, the findings presented in this study are novel and could suggest that gene regulation of the 5-HT_{1A} receptor is similar both pre- and post-synaptically. The findings in this study are however contradicted by those reported by Lemonde et al (2003). The study by Lemonde et al, used embryonic day 18 hippocampal and cortical primary cultures that were colocalised with the NUDR protein and the 5-HT_{1A} receptor regulating protein, their findings have suggested that NUDR, a 5-HT_{1A} transcription factor does not repress, but enhances 5-HT_{1A} transcriptional activity in hippocampal and septal cells.

5.2- SH-SY5Y cell line

5.2.1- Differentiation of the SH-SY5Y cell line

SH-SY5Y cells were differentiated with either RA or NGF and aphidicolin. After 5 days of RA treatment cells appeared more neuronal in their phenotype compared to undifferentiated cells. This was consistent with results obtained by Lombet et al, 2001. After 8 days of NGF and aphidicolin treatment cells showed an increased neuronal phenotype displaying extended neurites when compared to undifferentiated cells. These observations were in agreement with Jensen et al, (1991).

5.2.2- mRNA and protein expression of the 5-HT_{1A} receptor in differentiated SH-SY5Y cells

SH-SY5Y cells differentiated with RA or NGF and aphidicolin had significantly increased 5-HT_{1A} receptor mRNA levels compared to undifferentiated cells. RA induced SH-SY5Y cells, after 5 days of differentiation, showed a significant

increase relative to control. The timing of 5-HT_{1A} receptor expression is in agreement with the study by Ammer and Schulz, (1994) who showed that RA induced differentiation of SH-SY5Y cells considerably increased the abundance of G-protein subunits investigated. With NGF and aphidicolin differentiated SH-SY5Y cells, after 10 days significantly increased 5-HT_{1A} receptor mRNA levels, relative to control. These results are consistent with results obtained by LoPresti et al, (1992) who showed that SH-SY5Y cell bodies are more rounded and have extended neurites present after 8 days of treatment with NGF.

The presence of 5-HT_{1A} receptor protein in SH-SY5Y cells treated with RA for 5 days was determined by immunocytochemistry. Western blots also confirmed the presence of the 5-HT_{1A} receptor in SH-SY5Y cells treated with either RA or NGF and aphidicolin.

These results clearly demonstrate that this cell line when differentiated expresses the 5-HT_{1A} receptor.

5.2.3- NUDR mRNA expression in differentiated SH-SY5Y cells

The 5-HT_{1A} receptor transcription factor NUDR was detected in this cell line when cells were differentiated with NGF and aphidicolin. An increase in NUDR expression is observed at the same time as there is an increase in 5-HT_{1A} receptor expression in SH-SY5Y cells treated with NGF and aphidicolin. This is an interesting observation and may indicate that the C-1019G 5-HT_{1A} receptor promoter polymorphism, which is known to regulate 5-HT_{1A} receptor gene expression *in vivo* through the depression of the 5-HT_{1A} promoter in presynaptic raphe neurons leading to an reduction in serotonergic transmission (Huang et al, 2004; Lemonde et al, 2003) is operational in this cell line. The SH-SY5Y cell line was genotyped for the C-1019G 5-HT_{1A} receptor promoter polymorphism using the ASO method. This cell line was determined as homozygous for the -1019G allele, as previously described NUDR binds to the -1019C allele leading to depression of 5-HT_{1A} receptor expression (Lemonde et al, 2003). NUDR expression was increased at a time occurring prior to an increase in 5-HT_{1A} receptor expression when SH-SY5Y cells were differentiated with NGF and

aphidicolin. For future work it would be of interest to investigate further the changes in NUDR expression observed.

The presence of NUDR expression in this differentiated cell line demonstrates that the regulatory transcription factors of the 5-HT_{1A} receptor are present.

5.2.4- Second messenger signalling of the 5-HT_{1A} receptor

Levels of intracellular calcium were measured using flow cytometry by detecting the amount of SH-SY5Y cells that had bound to the Fura-2AM dye.

In this study it was hypothesised that 8-OH-DPAT a 5-HT_{1A} agonist would reduce the levels of intracellular calcium by activating the 5-HT_{1A} receptor G_i protein subunit leading to the dissociation of the $\beta\gamma$ subunit which activate K⁺ channels, therefore reducing calcium levels. The α -subunit inhibits adenylyl cyclase also leading to a reduction in calcium levels. 5-HT is also a 5-HT_{1A} agonist and hence it was assumed that a similar effect to that seen with 8-OH-DPAT would be observed.

When SH-SY5Y cells are treated with both p-MPPI (a 5-HT_{1A} antagonist) and 8-OH-DPAT (a 5-HT_{1A} agonist), p-MPPI will compete with 8-OH-DPAT to bind with the 5-HT_{1A} receptor. At higher concentrations of p-MPPI a gradual increase in calcium levels is observed. Therefore, these results show that 8-OH-DPAT is not binding to D₂ receptors in this cell line and is specifically binding to the 5-HT_{1A} receptor.

A function of the 5-HT_{1A} receptor is to inhibit adenylyl cyclase and thereby reduce the levels of cAMP. The results presented in the current study demonstrate that 8-OH-DPAT (a 5-HT_{1A} agonist) did not diminish intracellular calcium levels in undifferentiated SH-SY5Y cells, whereas, in RA differentiated SH-SY5Y cells forskolin- stimulated increase in intracellular calcium was efficiently reduced by 8-OH-DPAT. These results are in agreement with Pailia et al, (2006) and Khan et al, (1995). Pailia et al (2006) showed that forskolin-stimulated increase in cAMP levels is efficiently inhibited by 8-OH-DPAT in a concentration dependent manner. Khan et al (1995) found that 5-HT reduced

intracellular calcium concentration is a dose dependent manner in K562 cells loaded with fura-2.

Similarly, the 5-HT_{1A} agonist 5-HT was also shown to diminish forskolin-stimulated increase in cAMP in RA differentiated cells confirmed by Khan et al (1995).

SH-SY5Y cells treated with both p-MPPI (a 5-HT_{1A} receptor antagonist) and 8-OH-DPAT demonstrated that cells treated with MPPI at higher concentrations (100µM) significantly increased forskolin-stimulated intracellular calcium levels and therefore effectively reversed the agonistic effect of 8-OH-DPAT. p-MPPI has been verified to be a selective and potent 5-HT_{1A} antagonist (Allen et al, 1997; Bjorvatan et al, 1998 and Thielen and Frazer, 1995).

The results presented in the current study clearly show that the 5-HT_{1A} receptor is functional and capable of signalling via second messengers in differentiated SH-SY5Y cells.

5.2.5- SH-SY5Y cell line conclusions

The findings presented here demonstrate that the SH-SY5Y cell line when differentiated with either RA or NGF and aphidicolin is a useful model system for studying the 5-HT_{1A} receptor. These findings have not been previously described.

The development of a model system to study the 5-HT_{1A} receptor could be highly advantageous due to the unlimited availability of cells compared to post-mortem tissue which can often be in limited supply.

5.3 - Future work

The studies described in this thesis assessing the gene regulation of control postsynaptic 5-HT_{1A} receptor in human post-mortem tissue would merit more detailed investigation of post-mortem tissue from depressed subjects using real-time PCR to quantify 5-HT_{1A} receptor expression in these samples and correlate with genotype. This would clarify whether 5-HT_{1A} receptor genotype is upregulated or downregulated in postsynaptic post-mortem tissue in depressed subjects.

This study has focused on the 5-HT_{1A} receptor in hippocampal postmortem tissue samples. It would also be of interest to investigate the effect of the 5-HT transporter polymorphism on 5-HT_{1A} expression in post-mortem tissue (Zammit and Owen, 2006) as the 5-HT transporter is known to affect 5-HT_{1A} receptor expression.

The SH-SY5Y cell line has been shown to be successfully differentiated with RA or NGF and aphidicolin. It would be of interest to further investigate the presence of neuronal markers in this cell line when differentiated. The SH-SY5Y cell line is thought to consist of a mixed population of cells. It would, therefore, be useful to use flow cytometry to sort the SH-SY5Y cell line for the cells expressing the 5-HT_{1A} receptor to permit the sub cloning of the cell line for the 5-HT_{1A} receptor. The development of a sub-cloned 5-HT_{1A} receptor SH-SY5Y cell line could be a useful model to use in the assessment of the effects of antidepressant drugs on 5-HT_{1A} receptor expression.

Clear evidence from the work presented here supports the role of the SH-SY5Y cell line as a model system for studying the 5-HT_{1A} receptor. Further, investigation into the presence of other 5-HT receptors in this cell line using real-time PCR, immunocytochemistry and western blots could further validate this cell line as a model system.

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