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FACULTY OF GRADUATE AND
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GRADE / DEGREE

Department of Cellular and Molecular Medicine

FACULTÉ, ÉCOLE, DÉPARTEMENT / FACULTY, SCHOOL, DEPARTMENT

5-HT_{1A} Autoreceptor Regulation and Functional Polymorphisms that Modify the Serotonin System
and Their Association with Depression and Schizophrenia

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5-HT_{1A} AUTORECEPTOR REGULATION AND
FUNCTIONAL POLYMORPHISMS THAT MODIFY THE
SEROTONIN SYSTEM AND THEIR ASSOCIATION WITH
DEPRESSION AND SCHIZOPHRENIA

By

ARIEL BURNS

This thesis is submitted as a partial fulfillment of the M.Sc.
program in Neuroscience

August, 2006
Ottawa, Ontario

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Your file *Votre référence*
ISBN: 978-0-494-18403-5
Our file *Notre référence*
ISBN: 978-0-494-18403-5

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ABSTRACT

The serotonergic system, and in particular the 5-HT1A receptor, are involved in the etiology and treatment of depression. The hypothesis that antidepressants downregulate 5-HT1A autoreceptor gene expression was investigated by examining 5-HT1A agonist effects on 5-HT1A promoter activity using reporter construct assays and on 5-HT1A mRNA levels by quantitative real-time PCR in raphe primary cultures. 8-OH-DPAT had no effect on 5-HT1A autoreceptor expression in either paradigm. To elucidate the genetics of mental illness, monoaminergic receptor gene polymorphisms were analyzed. An association of the 5-HT2A A(-1438)G SNP with MDD was significant in males, and a trend was seen for the 5-HT1A C(-1019)G SNP with MDD. No association of these or a D2 SNP was detected with schizophrenia. Sequencing the macaque 5-HT1A promoter and human-primate comparisons revealed potentially functional novel polymorphisms and interspecies differences. These results contribute to our understanding of the 5-HT1A receptor's role in both normal affect and psychiatric disorders.

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LIST OF ABBREVIATIONS

-/-	knockout
5-HIAA	5-hydroxyindolacetic acid
5-HT	5-hydroxytryptamine, serotonin
5-HT1A	serotonin 1A receptor
5-HT2A	serotonin 2A receptor
5-HTT / SERT	5-hydroxytryptamine transporter / serotonin transporter
5-HTTLPR	5-hydroxytryptamine transporter gene-linked polymorphic region
8-OH-DPAT	8-hydroxy-2-(di- <i>n</i> -propylamino)tetralin
bp	base pairs
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
CNS	central nervous system
CSF	cerebral spinal fluid
D2	dopamine D2 receptor
DA	dopamine
DAT	dopamine transporter
dCTP	deoxycytosine triphosphate
Deaf-1	deformed epidermal autoregulatory factor-1
df	degrees of freedom
DIV	days <i>in vitro</i>
DNA	deoxyribonucleic acid

DNase	deoxyribonuclease
dNTP	deoxyribonucleotide triphosphate
DRE	dual repressor element
DSM-IV	diagnostic and statistical manual of mental disorders, 4 th edition
DR	dorsal raphe nucleus
E	embryonic day
FBS	fetal bovine serum
FRE	five-prime repressor element
Freud-1	five-prime repressor element under dual repression binding protein-1
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFAP	glial fibrillary acidic protein
GPCR	G protein-coupled receptor
HAMD	Hamilton depression rating scale
HVA	homovanillic acid
kb	kilobases
MAO	monoamine oxidase
MAOI	monoamine oxidase inhibitor
MDD	major depressive disorder
MnR	median raphe nucleus
mRNA	messenger ribonucleic acid
NE	norepinephrine
PBS	phosphate buffered saline
pCMV- β gal	plasmid cytomegalovirus promoter- β -galactosidase reporter gene

PCR	polymerase chain reaction
PET	positron emission tomography
PFC	prefrontal cortex
Q-PCR	quantitative real-time PCR
RNA	ribonucleic acid
SN	substantia nigra
SNP	single nucleotide polymorphism
SSRI	selective serotonin reuptake inhibitor
SV40	simian virus 40
TCA	tricyclic antidepressant
TPH	tryptophan hydroxylase
TRE	three-prime repressor element
VTA	ventral tegmental area
WAY-100635	N-(2-(4-(2-methoxyphenyl)-1-piperazinyl)ethyl)-N-2-pyridinyl- cyclohexanecarboxamide
χ^2	chi-squared

ACKNOWLEDGMENTS

To begin, I would like to thank my supervisor, Dr. Paul Albert, for his constant encouragement, guidance, and support that he has given me throughout my Master's degree. When my initial project did not work out as smoothly as expected, he provided me with several excellent opportunities to explore new avenues of research, which in the end resulted in the bulk of this thesis.

I would also like to thank our collaborators, Dr. Evgeny Rogaev from the University of Massachusetts Medical School in Worcester, MA, and Dr. Paul Roy from the Department of Psychiatry at the University of Ottawa, ON, for providing, respectively, the Russian and Canadian schizophrenic and control DNA extracts; Drs. Lisheng Du and David Bakish (formerly) of the Institute for Mental Health Research of the Royal Ottawa Hospital in Ottawa, ON, for contributing the depressed and control blood samples; and Dr. Cynthea Bethea from the Oregon National Primate Research Center in Beaverton, OR, for supplying the macaque DNA extracts.

This thesis would not have been possible without all the assistance I have received from Albert lab members, both past and present. Specifically, I would like to thank those who have worked to generate the human and rat promoter constructs and the cell lines that I have used, Sylvie Lemonde for performing some of the DNA isolation and 5-HT1A genotyping for the Canadian depressed and control samples and providing the NUDR protein, Christine Boudreau for her help with DNA isolation, and Kirsten Jacobsen for providing probes and assistance with EMSA. I would also like to thank Mireille Daigle for her excellent technical assistance with just about every part of this project, Anastasia

Rogaeva for always being available to teach me techniques and help me interpret results and for providing many Freud-1 EMSA components, and Federico Remes Lenicov, for providing primary culture samples, but more importantly for always being willing to lend a hand (especially with weekend media changes!), discuss experiments, and for being a good friend. Many thanks to Maya Arbach for being the best summer student one could ask for – hard working, conscientious and willing to do anything I ask! Lastly, thank you to Margaret Czesak for initially integrating me into the Albert lab, and for being so unbelievably helpful, friendly and good-hearted.

I am grateful to the members of my advisory committee, Drs. Stephany Bennett and Antonio Colavita, for their valuable input throughout my degree.

Finally, I would like to express my deepest thanks to my parents, Claudia and Bruce, for supporting me in every respect throughout my whole life, and to my brother, Andrew, for putting up with me. Last but not least, Tom, thank you for making these past couple of years the best of my life.

The research presented in this thesis was supported by a National Science and Engineering Research Council scholarship and by the Canadian Institutes of Health Research.

CHAPTER 1 – INTRODUCTION

1.1 Overview of the Serotonergic System

Serotonin (5-HT) is a biogenic amine which was initially identified and purified in 1948 as a vasoconstrictive substance present in the blood. Shortly thereafter, it was found to be present in the mammalian central nervous system (CNS) and recognized as a neurotransmitter (Green, 2006; Piñeyro and Blier, 1999). Dahlström and Fuxe (1964) were the first to identify and characterize serotonergic neurons and their projections. They found that the cell bodies of serotonergic neurons are located in nuclei in the brainstem, and divided them into nine groups, labeled B1-B9 (B1 is the most caudal, B9 the most rostral). These cell clusters are now referred to as raphe nuclei, and are anatomically and functionally separated into two (rostral and caudal) subsystems. The 5-HT neurons of the rostral raphe nuclei (B4-B9) have cell bodies in the midbrain and rostral pons, and project to the forebrain, including the cerebral cortex, limbic system, basal ganglia and diencephalons. The cell bodies of 5-HT neurons in the caudal raphe nuclei are primarily in the medulla oblongata, and project to the spinal cord, while both groups innervate the brainstem and cerebellum (Törk, 1990). The dorsal raphe nuclei (DR), and the median raphe nuclei (MnR) are both part of the rostral 5-HT system, and contain the largest and second largest populations of serotonergic neurons in the mammalian CNS (Piñeyro and Blier, 1999). The number of serotonergic neurons in the human DR is estimated to be ~235,000 (Baker *et al.*, 1990).

Although low in number (compared to the $\sim 10^{11}$ neurons in the human brain), 5-HT neurons innervate all regions of the brain through extensive axonal arborization (Celeda

et al., 2004). Through these projections, 5-HT is involved in regulating both physiological functions, such as locomotion, blood pressure, sexual activity, appetite, temperature, circadian rhythm and sleep, and cognitive/behavioural functions such as learning, memory, pain and mood (Mann, 1999; Pucadyil *et al.*, 2005).

Serotonergic neurons originate very early in development (in the rat, the first serotonergic neurons can be detected by 5-HT immunoreactivity at embryonic day 12 (E12)), and 5-HT is also critical for such developmental processes as neurogenesis and axonal branching (Aitken and Törk, 1988; Gaspar *et al.*, 2003; König *et al.*, 1988). Disruptions in the serotonergic system are implicated in the etiology of a large number of neuropsychiatric disorders, including major depressive disorder (MDD), bipolar disorder, suicide, obsessive-compulsive disorder, schizophrenia, eating disorders, and infantile autism (Pucadyil *et al.*, 2005).

As serotonin cannot cross the blood-brain barrier, it must be synthesized in raphe neurons from its precursor, the amino acid tryptophan. Tryptophan is primarily obtained from the diet, thus brain serotonin levels can be depleted by eliminating dietary sources of tryptophan (Cooper *et al.*, 2003). The synthesis of serotonin is regulated by the rate-limiting enzyme tryptophan hydroxylase (TPH), which generates 5-hydroxytryptophan. Decarboxylation by 5-hydroxytryptophan decarboxylase then yields serotonin (Kandel *et al.*, 2000). Recently a novel gene, TPH2, has been shown to be the major form expressed in raphe neurons (Walther *et al.*, 2003). Released serotonin acts by binding to 5-HT receptors, of which 14 are currently identified in mammals. These receptors are divided into seven families, 5-HT₁₋₇, on the basis of their amino acid sequence, second-messenger coupling and pharmacology. With the exception of the 5-HT₃ receptor, which

is a ligand-gated ion channel, the 5-HT receptors are seven-transmembrane domain, G-protein coupled (metabotropic) receptors (GPCRs). Different receptor families are coupled to different G-proteins and thus activate or repress different second-messenger pathways and cellular functions upon activation by 5-HT (Barnes and Sharp, 1999). Signaling is then terminated by reuptake of the neurotransmitter through the 5-HT reuptake transporter (5-HTT/SERT), and by the enzymatic degradation of serotonin via monoamine oxidase (MAO). Serotonin is degraded primarily by the MAO-A isoform of MAO, which deaminates it to produce 5-hydroxyindoleacetaldehyde, which is then oxidized to form the metabolite 5-hydroxyindoleacetic acid (5-HIAA) by an aldehyde dehydrogenase enzyme (Cooper *et al.*, 2003). Interestingly, MAO-B is more prevalent in 5-HT neurons; this may be to prevent degradation of the neurons' endogenous transmitter while facilitating elimination of monoamines taken up heterologously (Adell *et al.*, 2002).

1.1.1 The 5-HT_{1A} receptor

The 5-HT_{1A} receptor is expressed in the mammalian brain both post-synaptically as a heteroreceptor as well as pre-synaptically as a somatodendritic autoreceptor on serotonergic neurons. High levels of both the receptor mRNA and protein are detectable in limbic brain areas, specifically the raphe nuclei, hippocampus (on pyramidal and granular neurons), cortex (on glutamatergic pyramidal neurons), septum, amygdala and hypothalamus; while lower expression is seen in the basal ganglia, substantia nigra and cerebellum (Barnes and Sharp, 1999; Hoyer *et al.*, 1994; Pucadyil *et al.*, 2005). The 5-HT_{1A} gene is intronless and in humans is located on chromosome 5q11.2-13 (Albert and

Lemondé, 2004). The 5-HT_{1A} receptor couples to G_{i/o} proteins as indicated by immunoaffinity chromatography and by the pertussis toxin-sensitivity of the responses, except for in the hypothalamus, where toxin-insensitive G_z proteins mediate 5-HT_{1A}-dependent neuroendocrine processes (Hensler *et al.*, 2003). Through G_{ai} subunits, 5-HT_{1A} activation inhibits adenylyl cyclase, thus decreasing cyclic AMP (cAMP) formation, while G_{βγ} subunits mediate activation of potassium channels and inactivation of calcium channels (Raymond *et al.*, 1999). Therefore, when activated by 5-HT or by an exogenous agonist, 5-HT_{1A} autoreceptors mediate negative feedback inhibition of serotonergic neurons by hyperpolarizing the cell, which decreases firing rates and 5-HT release.

It should be noted that although the traditional view of neurotransmitter action is of release at an axon terminal followed by activation of receptors on a post-synaptic neuron, serotonin (and the other monoamines) are also released into the extracellular space around the cell bodies of their synthesizing neurons in the brainstem. Although the origin of this serotonin is still being debated, it may result from a combination of release from 5-HT somata and dendrites, and from the proximal portion of their axons and axonal terminals, as putative recurrent collaterals from 5-HT neurons form a fiber network in the raphe nuclei (Adell *et al.*, 2002; Piñeyro and Blier, 1999). Recently, a vesicular monoamine transporter 2-positive pool of vesicles has been associated with dendritic release of monoamines (Li *et al.*, 2005). The release of this serotonin is primarily impulse dependent, and thus when 5-HT neurons are activated by afferents, 5-HT is released both in projection areas and at the 5-HT neuronal cell bodies in the raphe nuclei. This of course leads to 5-HT_{1A} receptor activation, inhibition of raphe neuron firing and

reduced release of 5-HT in both target areas and in the brainstem. Interestingly however, although 5-HT_{1A} autoreceptors tightly control the activity of 5-HT neurons, 5-HT_{1A} antagonist treatment does not affect 5-HT neuron firing rate or 5-HT release in the raphe nuclei under basal conditions (Adell *et al.*, 2002; Piñeyro and Blier, 1999). Most studies of 5-HT_{1A} knockout mice have also shown that basal 5-HT concentration is not altered either pre-synaptically in the DR or post-synaptically in various brain regions (Ase *et al.*, 2000; Bortolozzi *et al.*, 2004; He *et al.*, 2001; but see Parsons *et al.*, 2001). However, Ase *et al.* (2000) also reported increased levels of the 5-HT metabolite 5-HIAA in both pre- and post-synaptic areas in the 5-HT_{1A} *-/-* mice, which they attributed to increased 5-HT turnover following increased basal 5-HT release. In contrast, using *in vivo* microdialysis, Bortolozzi *et al.* (2004) did not report any effect of 5-HT_{1A} disruption on 5-HT efflux in the DR, and argue that the increased metabolite concentrations may instead be due to increased cytoplasmic 5-HT turnover. Although still unresolved, it appears that serotonergic neurons may not be under tonic control by 5-HT_{1A} receptors. Instead, it may be that 5-HT_{1A} receptors act as sensors which detect excessive levels of serotonin around the serotonergic cell body, and respond by reducing 5-HT firing and release (Adell *et al.*, 2002).

Activation of post-synaptic 5-HT_{1A} receptors also generally inhibits neuronal activity, with 5-HT and 5-HT_{1A} agonists hyperpolarizing neurons in regions such as the hippocampus, cortex and septum. However, 5-HT_{1A} receptor agonists induce the release of acetylcholine, dopamine (DA) and norepinephrine (NE) in several brain regions including the cortex and hippocampus. These effects are blocked by selective 5-HT_{1A} antagonists, and although the pathways involved are not clear, they appear to involve

post-synaptic 5-HT_{1A} receptors (Barnes and Sharp, 1999; Blier and Ward, 2003). Thus, the 5-HT_{1A} receptor is an important regulator of both serotonergic neurotransmission and that of other neurotransmitters in the CNS. Through these pathways, activation of 5-HT_{1A} receptors influences physiological and behavioural responses such as corticosteroid and prolactin release, food intake, temperature regulation, and sexual behaviour. 5-HT_{1A} signaling is also involved in modulating cognitive functions such as learning and memory, and emotional responses including aggression, stress, anxiety and depression (Barnes and Sharp, 1999; Raymond *et al.*, 1999). Indeed, 5-HT_{1A} knockout mice show increased anxiety and decreased exploratory behaviour compared to wildtype mice (Gingrich and Hen, 2001; Zhuang *et al.*, 1999). Interestingly, this anxiety-like behaviour has been rescued in 5-HT_{1A} *-/-* mice by restoring 5-HT_{1A} receptor expression in the cortex and hippocampus during early post-natal life, but not in adulthood. This indicates that the 5-HT_{1A} receptor plays a critical role during development in establishing affective characteristics which persist into adulthood (Gross *et al.*, 2002).

1.1.2 The 5-HT_{2A} receptor

The 5-HT_{2A} receptor is a post-synaptic receptor highly expressed in cortical areas such as the neocortex, entorhinal and pyriform cortexes, and claustrum, as well as in the caudate nucleus, nucleus accumbens, olfactory tubercle and hippocampus. In the cortex, it is located on glutamatergic pyramidal neurons as well as on the GABAergic interneurons which control the activity of the pyramidal neurons in local circuits (Barnes and Sharp, 1999; Roth *et al.*, 1998). The 5-HT_{2A} gene is located on chromosome 13q14-q21, and contains three exons and two introns (Norton and Owen, 2005). 5-HT_{2A}

receptors are coupled via G_q proteins to the activation of phospholipase C. This leads to intracellular calcium mobilization, phosphoinositide hydrolysis, and the activation of protein kinase C (PKC). PKC in turn regulates many downstream pathways in the cell, including gene expression. 5-HT_{2A} receptor-mediated signaling leads to decreased potassium conductance, and thus neuronal excitation in the cortex, basal ganglia, hippocampus, and other brain regions (Barnes and Sharp, 1999). However, neuronal inhibition in the median prefrontal cortex (PFC) has also been reported in response to 5-HT_{2A} activation, likely resulting from increased GABAergic interneuron mediated inhibition of pyramidal neurons. Nevertheless, the overall effect of 5-HT_{2A} agonism on cortical pyramidal neurons appears to be excitatory (Celada *et al.*, 2004). The resulting physiological and behavioural effects of 5-HT_{2A} activation by agonists include hyperthermia, increased secretion of cortisol, renin and prolactin, and greater motor activity. 5-HT_{2A} receptors are also involved in respiration, non rapid eye movement sleep, working memory, and mood. Finally, 5-HT_{2A} agonism is thought to be involved in the action of hallucinogens, which has led to interest in the 5-HT_{2A} antagonistic properties of atypical antipsychotics (see 1.3.3) (Barnes and Sharp, 1999; Van Oekelen *et al.*, 2003; Popa *et al.*, 2005; Stahl, 1998).

1.1.3 The Serotonin Transporter

The 5-HTT is an evolutionarily conserved, 12-transmembrane domain, Na^+/Cl^- dependent, high-affinity transporter. The 5-HTT protein is expressed throughout the CNS, including the somata and dendritic arbours of serotonergic neurons in the midbrain and brainstem, as well as in their extensively projecting axonal terminals in the thalamus,

hypothalamus, striatum and cortex. mRNA expression, however, is limited to 5-HT neurons in the raphe nuclei, with highest levels being detected in the DR and MnR (Cooper *et al.*, 2003; Kaspar *et al.*, 2002; Murphy *et al.*, 2004; Piñeyro and Blier, 1999). Located on chromosome 17q11.2, the human 5-HTT gene encodes a protein of 630 amino acids (Lesch *et al.*, 1994). Its primary function is to clear 5-HT from the synapse after neuronal stimulation, allowing it to be repackaged for release or degraded. However, the 5-HTT can transport serotonin in either direction across the plasma membrane, depending on its concentration gradient. Therefore, the 5-HTT is essential in regulating the amplitude, length, and distribution of 5-HT receptor activation (Cooper *et al.*, 2003; Murphy *et al.*, 2004). Additionally, the recycling function of the 5-HTT in 5-HT homeostasis is critical. This is demonstrated in 5-HTT knockout mice. Studies have shown that 5-HT uptake was completely abolished in 5-HTT *-/-* mice, and total tissue 5-HT levels were reduced by 60-80% in several regions including the brainstem, striatum, hippocampus and cortex. Levels of the serotonin metabolite 5-HIAA were also decreased, though not to the same extent as 5-HT, which resulted in an increased 5-HIAA/5-HT ratio in the knockout compared to wildtype mice (Bengel *et al.*, 1998; Fabre *et al.*, 2000). Clearance of 5-HT was prolonged in the knockout mice, and thus basal extracellular 5-HT levels were increased several-fold in the striatum, cortex and basal ganglia (Holmes *et al.*, 2003b; Matthews *et al.*, 2004). Spontaneous firing activity of DR 5-HT neurons was also decreased by two-thirds in the 5-HTT *-/-* mice. This effect was partially blocked by the 5-HT_{1A} antagonist WAY-100635, indicating that increased synaptic availability of 5-HT in the 5-HTT^{-/-} mice overactivated inhibitory 5-HT_{1A} receptors to decrease neuronal activity (Gobbi *et al.*, 2001). These mice also exhibited a

downregulation of 5-HT_{2A} receptors, and pre-synaptic (but not post-synaptic) 5-HT_{1A} receptors, as well as increased anxiety- and depression-like behaviour (Fabre *et al.*, 2000; Gingrich and Hen, 2001; Holmes *et al.*, 2003a,b,c; Li *et al.*, 2000). This indicates that by disrupting 5-HTT function, other components of the serotonergic system, as well as serotonergic-mediated behaviours, are profoundly altered (see also 1.2.4).

1.2 Role of the Serotonergic System and the 5-HT_{1A} Receptor in Depression and Antidepressant Action

1.2.1 Overview of Depression

Psychiatric disorders are among the most common medical conditions in developed countries. The estimated lifetime prevalence of major depressive disorder (MDD) is 17% in the United States, with the occurrence in women being 1.5 to 2.5 times greater than that in men (Fava and Kendler, 2000). The World Health Organization reported that in 1990, MDD was the fourth most frequent cause of illness-related disability worldwide, and they estimate that by the year 2020, it will place second behind ischemic heart disease (Murray and Lopez, 1997a,b). This is because depression reduces quality of life and productivity, and also increases mortality. In addition, the average age of onset of MDD is in the mid-twenties (although it can occur at any age after early childhood), and about 70% of people who have suffered from one episode of MDD will experience a recurrence (Doris *et al.*, 1999; Kandel *et al.*, 2000). Thus, major depression has a severe and long-lasting negative impact on the lives of a significant portion of the population.

Although Hippocrates first described depression in the 5th century B.C., only recently have precise diagnostic criteria been developed for mood disorders. MDD, also called

unipolar depression, is the most common type of mood disorder (Kandel *et al.*, 2000). The Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV) is the most frequently used diagnostic manual in North America for psychiatric illness. Its criteria for MDD are: experiencing a depressed mood (dysphoria) and/or loss of interest or pleasure in activities (anhedonia) the majority of the time; and at least three of the following additional symptoms: significant weight change (usually weight loss and diminished appetite but can be weight gain); disturbed sleep (usually insomnia but sometimes hypersomnia); fatigue and/or loss of energy; feelings of guilt and worthlessness; restlessness (psychomotor agitation) and/or slowed thinking and movement (psychomotor retardation); difficulty in concentrating and making decisions; and recurrent thoughts of suicide and death. The above symptoms must be present nearly every day for at least two weeks, and must not result from other neuropsychiatric or neurological disorders, or recent traumatic events (such as death of a family member) (American Psychiatric Association, 1994).

In addition, MDD is divided into a variety of subtypes. Melancholic depression tends to be severe and recurrent, is present in 40-60% of MDD patients, and is characterized by symptoms which are worse in the morning, anorexia and significant weight loss, insomnia and early-awakening, psychomotor agitation, mental anguish, and anhedonia. Atypical depression, on the other hand, is somewhat less common, and has some symptoms opposite to those of melancholic depression; symptoms tend to be more chronic, patients feel worse later in the day, often gain weight, sleep more, and may be more responsive to positive life events. Lastly, dysthymia is characterized by milder depressive symptoms persisting for at least two years. Interestingly, these subtypes of

MDD appear to respond preferentially to different pharmacotherapies, indicating that the neurobiological abnormalities involved in each may differ somewhat (Kandel *et al.*, 2000). Additionally, other common mood disorders, which share many symptoms with MDD, include bipolar or manic-depressive disorder, psychotic depression, seasonal affective disorder, postpartum depression, and premenstrual dysphoric disorder (Blier, 2006). Of these, bipolar disorder is the best characterized, as up to one-quarter of depressed individuals will experience a period of mania. People who exhibit recurrent and alternating episodes of both mania and depression are classified as having bipolar disorder. A manic episode is distinguished by elevated, expansive or irritable mood for at least one week, and by additional symptoms which may include hyperactivity, increased energy and decreased sleep, pressure of speech, socially intrusive behaviour, grandiosity, flight of ideas and distractibility, increased libido, reckless spending, and in severe cases, hallucinations. Interestingly, bipolar disorder is equally prevalent in men and women and has an earlier age of onset than MDD (Kandel *et al.*, 2000). It should be noted that there is considerable variation between sources as to which disorders are considered subtypes of MDD versus related but separate mood disorders. Nevertheless, the variability in the clinical presentation of MDD, as well as the numerous subtypes and related disorders with overlapping symptom sets, has led many scientists and clinicians to conclude that major depression should be considered a heterogeneous set of illnesses with potentially distinct etiologies and pathologies (Nestler *et al.*, 2002a). This is significant in terms of variable results in studies attempting to decipher the genetic and neurobiological correlates of depression and the best treatment options (see below).

1.2.2 Evidence for the Role of the Serotonergic System in Depression

Decreased activity of the serotonergic and/or norepinephrine systems in the brain was first proposed to be related to depression over 50 years ago, with the discovery that a common treatment for hypertension, reserpine, resulted in depressive symptoms in about 15% of patients (as well as in animal models). Reserpine inhibits the uptake of biogenic amines (5-HT, and the catecholamines NE and DA) into synaptic vesicles, so that instead of being released they are degraded in the cytoplasm, thus decreasing brain levels of these transmitters. Shortly thereafter, monoamine oxidase inhibitors (MAOIs) such as isoniazid, which was developed to treat tuberculosis, were discovered to have antidepressant properties, and to increase catecholamine and 5-HT levels in the brain by inhibiting their degradation. This was followed by the discovery that the tricyclic drug imipramine, which was being developed as a potential antipsychotic, and which increases synaptic NE and 5-HT levels by blocking their respective reuptake transporters, is also an antidepressant. These findings solidified the view that antidepressants increase 5-HT and catecholamine (especially NE) availability at their receptors; and thus decreased signaling by these transmitters may be implicated in the etiology of depression (Blier, 2006; Cooper *et al.*, 2003; Kandel *et al.*, 2000). Initial work focused more on the role of NE in depression, and today continued research and considerable evidence still support this connection. Indeed, interactions between the 5-HT and NE systems in relation to depression are also being examined; however, details of these findings are not within the scope of this thesis (for review see: Blier, 2001a,b; Blier, 2006; Leonard, 2001; Young, 2001; Tremblay and Blier, 2006).

Over the last several decades, multiple lines of evidence have been reported which support the role of the serotonergic system in depression. Some studies found that levels of the 5-HT precursor tryptophan were lower in the plasma, and those of the 5-HT metabolite 5-HIAA were lower in the cerebral spinal fluid (CSF) of depressed patients, especially those with suicidal behaviour (although this finding may be correlated with impulsivity). In addition, in depressed patients prolactin release was decreased when stimulated by the serotonin-releaser fenfluramine. These findings are all indicative of 5-HT dysregulation or deficiency in depression (Arango *et al.*, 2003; Blier, 2006; Cooper *et al.*, 2003; Mann, 1999). One recent study directly measured the level of 5-HT in the CSF of MDD patients and controls, and found that it was decreased in patients who were the most suicidal; however, the group reported no difference between the levels of 5-HT in depressed patients versus controls (Hou *et al.*, 2006). Depleting 5-HT levels via the inhibition of tryptophan hydroxylase or dietary tryptophan restriction has been shown to result in the recurrence of depression in patients undergoing successful treatment or in remission, and to lower the mood of normal subjects (Mann, 1999; Moore *et al.*, 2000).

An increase in the number of 5-HT_{1A} autoreceptors present in the raphe nuclei of depressed suicide victims compared to healthy controls has been detected by receptor autoradiography (Stockmeier *et al.*, 1998). Using positron emission tomography (PET), Parsey *et al.* (2006) recently reported that while no difference in 5-HT_{1A} binding levels was found between MDD and control subjects across a large number of pre- and post-synaptic brain regions, when the depressed subjects were divided into antidepressant naïve versus antidepressant exposed, the drug naïve subjects had significantly higher 5-HT_{1A} binding in the raphe than either controls or drug treated MDD subjects. This

finding is especially interesting as one of the proposed mechanisms of antidepressant action is through downregulation of 5-HT_{1A} autoreceptors (see 1.2.5). However, other studies have either detected no difference or a decrease in autoreceptor levels between MDD patients or MDD suicides versus controls (Arango *et al.*, 2001; Drevets *et al.*, 1999; Sargent *et al.*, 2000). Interestingly, in patients with late life depression, Meltzer *et al.* (2004) reported lower DR 5-HT_{1A} binding levels compared to controls, but also a positive correlation between depression rating scores and binding levels (meaning that patients with more severe depression had more DR 5-HT_{1A}), and a trend towards shorter time to remission with paroxetine treatment being correlated with lower 5-HT_{1A} levels. The authors postulate that this may indicate a failure of adaptive mechanisms to decrease inhibitory 5-HT_{1A} levels in more severe depression, while lower autoreceptor levels might facilitate paroxetine action (see 1.2.4). Differences in study design (e.g. autoradiography with post-mortem brains versus PET imaging of live individuals, and also using different agonists and antagonists), heterogeneity in sample sets (e.g. including bipolar as well as MDD subjects, and antidepressant naïve and exposed subjects), and small sample sizes, may be responsible for these inconsistencies, as well as those reported below. Post-synaptic 5-HT_{1A} receptors have been shown to be decreased across multiple cortical regions (including the frontal, temporal and limbic cortex) in depressed patients compared to controls (Drevets *et al.*, 1999; Sargent *et al.*, 2000), although another group saw increases in antidepressant naïve subjects (Parsey *et al.*, 2006). In theory, greater inhibition of 5-HT neurons by 5-HT_{1A} autoreceptors and reduced activation at post-synaptic 5-HT_{1A} receptors would both result in decreased serotonergic neurotransmission.

Increased cortical and blood platelet 5-HT_{2A} receptors levels have also been repeatedly reported in depressed and suicidal individuals; however, some studies did not find any correlation between mood disorders and 5-HT_{2A} binding (Arango *et al.*, 2003; D'haenen, 2001; Mann, 1999; Oquendo *et al.*, 2006; Pandey, 1997). One study showed that the number of 5-HTT mRNA-expressing neurons in the DR was lower in depressed suicides compared to controls (Arango *et al.*, 2001). 5-HTT density has also been found to be decreased or unchanged in the brainstem and in serotonergic projection areas, as well as on blood platelets, in people with depression (Blier, 2006; Kasper *et al.*, 2002; Meyer *et al.*, 2004; Smith and Cavanagh, 2005). Increased 5-HT_{2A} and decreased 5-HTT levels in depression could potentially represent compensatory changes in reaction to hypoactive serotonergic neurotransmission.

1.2.3 Overview of Antidepressant Therapies

Thus, numerous studies indicate that serotonergic neurotransmission is dysregulated in depression. However, some of the strongest evidence implicating 5-HT in depression is that an increase in serotonergic neurotransmission is believed to be involved in the success of a majority of different antidepressant therapies

Electro-convulsive therapy (ECT) has been used as an antidepressant treatment for over 50 years, and is still considered to be the treatment of choice for extremely severe depression, as controlled trials have repeatedly shown that it produces full remission or significant symptom reduction in up to 85% of patients. ECT works by inducing generalized brain seizures, and a standard ECT course involves six to eight treatments given under general anesthesia over two to four weeks (Doris *et al.*, 1999; Kandel *et al.*,

2000). Although the mechanism by which CNS seizures improve mood is not known, one of the many neurobiological alterations which occur with ECT is 5-HT_{1A} receptor sensitization in the hippocampus (5-HT_{1A} autoreceptor sensitivity in the raphe is not affected), thus increasing net 5-HT neurotransmission (Blier and de Montigny, 1994). However, because ECT often has cognitive side effects such as post-ictal confusion and memory loss, it is generally only used after pharmacotherapy fails (Doris *et al.*, 1999).

Three classes of antidepressant drugs have been repeatedly shown to be effective and thus are most frequently used: tricyclic antidepressants (TCAs), MAOIs, and selective-serotonin reuptake inhibitors (SSRIs) (Kandel *et al.*, 2000). In addition, newer atypical antidepressants are also beginning to gain acceptance (see 1.3.5.2). It should also be noted that the efficacy of several psychological therapies have also been demonstrated, especially when administered in combination with pharmacotherapy (Doris *et al.*, 1999); however, details are not within the scope of this thesis. As mentioned above, the older classes of drugs, TCAs and MAOIs (inhibition of MAO-A is responsible for the antidepressant action), inhibit serotonin reuptake and increase the responsiveness of post-synaptic 5-HT_{1A} receptors in the hippocampus, or inhibit 5-HT catabolism and desensitize somatodendritic 5-HT_{1A} autoreceptors, respectively (Blier and de Montigny, 1994; Blier and de Montigny, 1999). Although TCAs and MAOIs are both effective treatments for depression, their use is limited by side effects. Due to their non-selective monoaminergic reuptake inhibition, TCAs can produce sedation, weight gain, anti-cholinergic symptoms, hypotension and potentially lethal cardiac arrhythmias. MAO-A also metabolizes tyramine, which is found in many foods, and the excessive tyramine accumulation in patients taking MAOIs can cause hypertensive crises through extensive

NE release (Blier, 2006). Therefore, due to both their safety and efficacy, the current first-line therapeutics for depression are SSRIs such as citalopram, fluoxetine, fluvoxamine, paroxetine and sertraline (Fava and Kendler, 2000). About 80% of antidepressants currently available are SSRIs, and their response rate (defined as 50% reduction in symptom severity) is about 60% at six weeks (Celada *et al.*, 2004). Compared to TCAs and MAOIs, SSRIs have considerably fewer serious side effects. The side effects are frequently drug-specific, and include anti-cholinergic effects, gastrointestinal problems, and sexual dysfunction (Blier, 2006).

1.2.4 Mechanism of Action of SSRI Antidepressants

At a basic level, SSRIs block the 5-HTT reuptake of released serotonin. Acute treatment with SSRIs preferentially increases extracellular 5-HT levels at serotonergic cell bodies. This rise in somatodendritic 5-HT activates 5-HT_{1A} autoreceptors, initiating a negative feedback loop which decreases serotonergic neuronal activity and negates the SSRI-mediated increase in 5-HT signaling. It has been proposed that upon chronic drug administration, desensitization of 5-HT_{1A} autoreceptors decreases the feedback inhibition on serotonergic neuronal firing, thus permitting a stable increase in synaptic 5-HT and clinical improvement. This mechanism explains the observation that while SSRIs can rapidly (within minutes) increase extracellular 5-HT levels, there is a two to three week delay before a patient's mood improves (Figure 1.1) (Albert *et al.*, 1996; Albert and Lemonde, 2004; Blier *et al.*, 1998; Piñeyro and Blier, 1999; Stahl, 1998).

Considerable evidence supports this hypothesized mechanism of SSRI action. Acute administration of therapeutically relevant doses of SSRIs to rats did not considerably

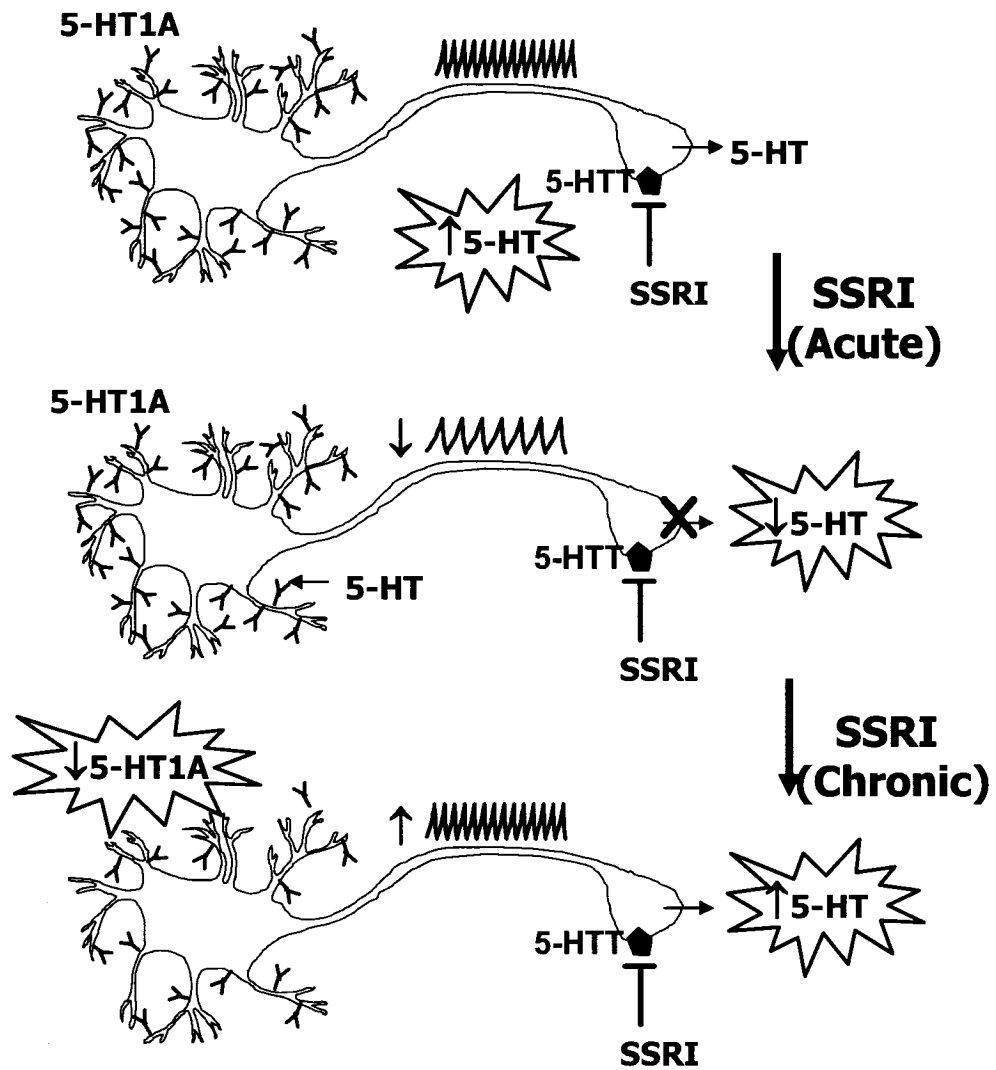


Figure 1.1. Mechanism of action of SSRI antidepressants.

Acutely, SSRIs block the 5-HTT, increasing extracellular 5-HT levels at synapses and at raphe neuron cell bodies. This activates somatodendritic 5HT1A autoreceptors, resulting in decreased neuronal activity and 5-HT release, thus negating the drugs' effect. However, with chronic treatment 5HT1A receptors desensitize, allowing the SSRIs to stably increase synaptic serotonin levels, which may elevate mood and relieve depression. (Adapted from Albert and Lemonde, 2004).

increase extracellular 5-HT levels in cortical areas (extremely high doses have been shown to increase 5-HT in projection areas); however, 5-HT levels were increased in the raphe (Adell *et al.*, 2002; Piñeyro and Blier, 1999). As mentioned above, this preferential increase in somatodendritic 5-HT stimulates inhibitory 5-HT_{1A} autoreceptors, the effect of which was demonstrated by a significant decrease in serotonergic neuronal firing activity in rats after two days of treatment with different SSRIs (Blier and de Montigny, 1994). In addition, directly applying SSRIs or the 5-HT_{1A} agonist 8-OH-DPAT to the DR decreased 5-HT release in the striatum and hippocampus, supporting the role of the 5-HT_{1A} autoreceptor in this effect (Piñeyro and Blier, 1999). In the above SSRI-treated rats, serotonergic firing returned to normal by the end of two weeks of treatment. Through application of either lysergic acid diethylamide (LSD) (a 5-HT autoreceptor agonist) or 5-HT directly onto serotonergic neurons, it was demonstrated that after the two week SSRI treatment, somatodendritic 5-HT_{1A} autoreceptors were desensitized (Blier and de Montigny, 1994). Multiple *in vivo* and *in vitro* electrophysiological studies also support the conclusion that 5-HT_{1A} autoreceptor desensitization is responsible for the restoration of 5-HT neuron firing activity (for comprehensive review see: Piñeyro and Blier, 1999). Thus, with chronic SSRI administration to rats, it was shown that extracellular 5-HT levels in projection areas such as the cortex underwent a significant and stable increase (Bel and Artigas, 1993). Interestingly, upon long-term SSRI treatment, post-synaptic 5-HT_{1A} receptors did not desensitize, which resulted in a tonic activation of forebrain 5-HT_{1A} receptors (Haddjeri *et al.*, 1998). Therefore, with chronic SSRI treatment, this differential desensitization of pre- and post-synaptic 5-HT_{1A} receptors, and the resulting increase in 5-HT neurotransmission through normo-sensitive

5-HT_{1A} receptors in areas such as the prefrontal cortex and the hippocampus, may be responsible for symptom amelioration (Adell *et al.*, 2002; Albert and Lemonde, 2004; Stahl, 1998).

Serotonin transporter knockout mice have been proposed as a model of life-long SSRI treatment. As described above (see 1.1.3), observations of the adaptive changes in the serotonergic system of these mice also support the proposed mechanism of SSRI action. Briefly, 5-HTT ^{-/-} mice exhibited decreased pre-synaptic 5-HT_{1A} receptor mRNA levels and labeling density in the raphe nuclei, while post-synaptically, a small increase (in the hippocampus) or no change in receptor levels has been observed (Fabre *et al.*, 2000; Li *et al.*, 2000). In addition, the inhibition of neuronal firing by acute SSRI and 5-HT_{1A} receptor agonist treatment was markedly decreased in the raphe nuclei of knockout mice compared to wildtype, while post-synaptic response was not affected (Mannoury la Cour *et al.*, 2001). These findings indicate that life-time blockade of 5-HT reuptake reduces the level of 5-HT_{1A} autoreceptors and thus their ability to inhibit serotonergic neuronal activity when extracellular 5-HT levels are elevated. In contrast, in mice lacking the 5-HT_{1A} receptor, fluoxetine treatment resulted in a greater acute increase in synaptic 5-HT concentration than in wildtype mice. This indicates that in these knockouts there is an inhibition of the normal SSRI-induced negative feedback response of serotonergic neurons (He *et al.*, 2001; Parsons *et al.*, 2001).

In accord with the above, 5-HT_{1A} agonists possess antidepressant properties. As with SSRIs, the chronic increased activation of 5-HT_{1A} autoreceptors with long-term administration of these agents is believed to decrease pre-synaptic 5-HT_{1A} receptor mediated negative feedback. However, post-synaptic 5-HT_{1A} receptors do not

desensitize, and signaling through them is increased by the combined action of the agonist and the augmented 5-HT release (Blier and de Montigny, 1994; Blier and Ward, 2003). The efficacy of 5-HT_{1A} agonist monotherapy, while promising in animal studies, has not been as consistent in human trials. Although it has been reported as effective in individual cases, the results of two placebo-controlled trials have been inconclusive (Blier, 2005). This may be due to the fact that these drugs have extremely short half-lives and thus are very difficult to dose properly. However, clinical trials indicate that they do have good tolerability profiles, likely due to their selective activation of post-synaptic 5-HT_{1A} receptors (many side effects are believed to result from overstimulation of post-synaptic 5-HT₂ and 5-HT₃ receptors (Stahl, 1998)). Currently, only one 5-HT_{1A} agonist antidepressant, buspirone, is on the market; however, pharmacokinetic improvement of this class of antidepressants may increase their use (Blier and Ward, 2003).

As mentioned above, the time required for 5-HT_{1A} receptors to desensitize is hypothesized to play a role in the several week delay of the therapeutic action of SSRIs. In 1993, Francesc Artigas proposed that 5-HT_{1A} antagonists could accelerate and potentially augment SSRI action by preventing the initial 5-HT_{1A} autoreceptor mediated negative feedback on the serotonergic system, allowing for a more rapid and stable increase in 5-HT neurotransmission (Celada *et al.*, 2004). In animal studies, pretreatment with the 5-HT_{1A} antagonist WAY-100635 was shown to potentiate both the increase in cortical 5-HT levels and the antidepressant effects of SSRIs (Schechter *et al.*, 2005). In humans, this hypothesis was tested using the 5-HT_{1A}/β-adrenoreceptor antagonist pindolol. Important to the action of pindolol is that it has been shown by several PET studies to preferentially antagonize pre-synaptic 5-HT_{1A} autoreceptors compared to post-

synaptic receptors, as a strong blockade of post-synaptic receptors would not potentiate 5-HT neurotransmission (Blier and de Montigny, 1999; Celada *et al.*, 2004). It is now known that pindolol is a weak partial 5-HT_{1A} agonist in some models, especially when administered alone, and this has raised questions about its mechanism of action. However, it appears that when administered in conjunction with SSRIs, its antagonistic properties predominate (Artigas *et al.*, 2001; Artigas *et al.*, 2006). In terms of efficacy, a recent meta-analysis of 15 placebo-controlled clinical trials supported pindolol's ability to significantly accelerate SSRI action, bolstering the involvement of 5-HT_{1A} autoreceptor activation in antidepressant delay (Ballesteros and Callado, 2004). Although the analysis did not find that pindolol augments SSRI action, this may be due to methodological discrepancies between studies, including using MDD patients with comorbid psychiatric conditions, different definitions of antidepressant response, and inadequate dosage or inappropriate timing of pindolol administration. Therefore, under proper conditions and in certain patients, pindolol may also enhance SSRI efficacy (Segrave and Nathan, 2005). Interestingly, in a study where the 5-HT_{1A} agonist buspirone and pindolol were co-administered, depressed patients improved rapidly, likely through the same mechanism of bypassing 5-HT_{1A} autoreceptor desensitization while stimulating post-synaptic 5-HT_{1A} receptors, as is hypothesized for SSRI-pindolol combination therapy (Blier and Ward, 2003). Recently, several companies have synthesized and are testing dual-acting SSRI/5-HT_{1A} antagonists as potential next-generation antidepressants (Schechter *et al.*, 2005).

Therefore, evidence from a large variety of experimental paradigms, involving electrophysiology, microdialysis, knockout mice, and pharmacology, support the

aforementioned mechanism of SSRI action through 5-HT_{1A} autoreceptor desensitization followed by increased post-synaptic 5-HT neurotransmission.

It should also be noted that terminal 5-HT_{1B/D} autoreceptors are also desensitized with chronic SSRI treatment, allowing greater 5-HT release upon serotonergic neuronal firing (Blier and de Montigny, 1994). Recently, it has been shown that chronic (3 week) treatment of rats with the SSRIs paroxetine and fluvoxamine desensitized their 5-HT_{2A} receptors. This was assessed as an attenuated elevation of corticosterone levels in response to the non-selective 5-HT receptor agonist *m*-chlorophenylpiperazine (*m*CPP) in SSRI-treated compared to control rats, an effect which was selectively blocked by the 5-HT_{2A} antagonist SB242084 (Yamauchi *et al.*, 2006). This supports previous animal studies which showed that 5-HT_{2A} levels were downregulated by chronic, but not acute treatment with both SSRI and MAOI antidepressants (Celada *et al.*, 2004; Van Oekelen *et al.*, 2003). This is interesting as 5-HT_{2A} agonists have been shown to inhibit 5-HT neuronal activity in the DR. This effect may be mediated by 5-HT_{2A} receptors on GABAergic interneurons and pyramidal cells in the medial prefrontal cortex which project back to the raphe, forming reciprocal connections, or on local GABAergic interneurons in the raphe (Adell *et al.*, 2002; Celada *et al.*, 2004). In either case 5-HT_{2A} desensitization by SSRI-treatment would again have a stimulatory effect on 5-HT neuronal firing and neurotransmitter release. However, brain imaging studies in humans have been very inconclusive (showing increases, decreases, and no change) with regard to the effect of SSRI-treatment on 5-HT_{2A} receptor binding (D'haenen, 2001).

1.2.5 Mechanism of SSRI-induced 5-HT1A autoreceptor desensitization

Although SSRI-induced functional desensitization of 5-HT1A autoreceptors is a well-established theory, the mechanism by which it occurs is considerably more controversial. Functional desensitization of GPCRs such as the 5-HT1A receptor can occur via several mechanisms. Agonist-induced phosphorylation of G-proteins coupled to the receptor rapidly terminates signaling; receptor sensitivity quickly returns to normal after removal of the agonist (Raymond *et al.*, 1999). Longer term desensitization can occur through receptor internalization. Studies have shown that acute administration of the SSRI fluoxetine or the 5-HT1A receptor agonist 8-OH-DPAT resulted in the internalization of 5-HT1A receptors in the dendrites of rat raphe neurons, as measured by both *in vivo* PET and *in vitro* immunohistochemistry. Pretreatment with the 5-HT1A antagonist WAY-100635 blocked the above effects, indicating the indirect activation of 5-HT1A autoreceptors by fluoxetine. Interestingly, hippocampal 5-HT1A heteroreceptors did not internalize, paralleling the selective desensitization of 5-HT1A autoreceptors observed upon chronic SSRI or 5-HT1A agonist treatment (Riad *et al.*, 2001; Riad *et al.*, 2004; Zimmer *et al.*, 2004).

Long-term desensitization of GPCRs can also be mediated by changes in receptor-G-protein interaction. This can be assayed by [³⁵S]GTPγS autoradiography, which detects receptor activation by G-proteins by measuring GDP exchange for [³⁵S]GTPγS. Using this technique, it was shown that after 14 days of treatment with either the SSRI fluoxetine or 8-OH-DPAT, 5-HT1A stimulated [³⁵S]GTPγS binding was significantly decreased in both the DR and MnR, but not in the hippocampus, cortex or septum of rats. This indicates that 5-HT1A desensitization may involve a diminished capacity of the

receptor to activate G-proteins (Hensler and Duram, 2001; Hensler, 2002; Hensler, 2003). In addition, Li *et al.* (1996) observed a parallel desensitization of 5-HT_{1A} mediated neuroendocrine responses and a reduction in midbrain G_o and G_{i2} proteins and hypothalamic levels of G_{i1} and G_{i3} proteins after chronic fluoxetine injections; G_i and G_o proteins in the frontal cortex were not affected.

An even more prolonged desensitization of 5-HT_{1A} autoreceptors, as is believed to occur with chronic antidepressant treatment, can be achieved by a downregulation of receptor gene expression. Changes in gene transcription and subsequently protein levels, occurs over hours to days and thus may account for the delay observed for antidepressant action (Albert *et al.*, 1996; Albert and Lemonde, 2004). A study by Welner *et al.* (1989) found that chronic treatment (21 days) with both the SSRI fluoxetine and the 5-HT_{1A} agonist gepirone significantly reduced 5-HT_{1A} binding in the rat DR as measured by autoradiography, but no change in hippocampal binding sites was observed. Another group found that chronic (21 day) but not shorter treatments with the 5-HT_{1A} agonist ipsapirone also robustly decreased 5-HT_{1A} binding in the DR, while binding in most post-synaptic areas was not affected (binding in the entorhinal cortex and interpeduncular nucleus was modestly decreased) (Fanelli and McMonagle-Strucko, 1992). A decrease in raphe 5-HT_{1A} receptor mRNA levels was also detected by competitive RT-PCR in rats treated for three weeks with fluoxetine, and functional desensitization of the receptors was determined by a decreased inhibition of neuronal firing in response to 8-OH-DPAT; however, no change in 5-HT_{1A} receptor binding sites was observed by autoradiography. Again, mRNA levels and agonist response in the hippocampus or cortex did not change with treatment (Le Poul *et al.*, 2000). Therefore, these studies generally indicate that the

effect of SSRIs is specific to pre-synaptic receptors. And as described above (1.2.4), 5-HTT knockout mice exhibit reduced 5-HT1A autoreceptor mRNA and protein levels. However, it should be noted that while most studies in wildtype rodents have shown functional desensitization of 5-HT1A receptors with chronic SSRI treatment (Piñeyro and Blier, 1999), whether or not the desensitization is mediated by a downregulation of receptor expression remains controversial. For instance, Hervas *et al.* (2001) reported no difference in either 5-HT1A receptor mRNA levels or density in the raphe nuclei of rodents chronically treated with SSRIs, while autoreceptor desensitization was still observed as a decreased response to an acute fluoxetine challenge that was prevented by co-administration of the 5-HT1A receptor antagonist WAY-100635. Also, in the chronic SSRI and 5-HT1A agonist treatment experiments in which decreased 5-HT1A autoreceptor activation of G-proteins was observed, no change in receptor numbers was detected (Hensler and Duram, 2001; Hensler, 2002).

In vivo studies in humans examining the 5-HT1A receptor have been limited (Smith and Cavanaugh, 2005), and very few have examined 5-HT1A binding levels before and after SSRI treatment. One study using PET scans with the 5-HT1A antagonist [¹¹C] WAY-100635 did not find any change in post-synaptic 5-HT1A receptor binding, but reported a non-significant trend towards decreased pre-synaptic 5-HT1A levels in depressed patients upon SSRI treatment (Sargent *et al.*, 2000). As mentioned above, another study found a decrease in pre-synaptic 5-HT1A receptor levels when they compared antidepressant untreated versus treated MDD patients; however, they did not directly assess the effect of treatment on the same individuals (Parsey *et al.*, 2006). Therefore, the hypothesis that 5-HT1A receptor desensitization upon SSRI and 5-HT1A

agonist treatment is mediated by downregulation of the 5-HT1A gene is supported by some animal and human studies, but is still under considerable debate.

1.2.6 Transcriptional Regulation of the 5-HT1A receptor

Downregulation of a protein such as 5-HT1A receptor could be mediated at several levels, including mRNA transcription, translation, or post-translation (including protein turnover) (Alberts, 2002). The transcriptional regulation of the 5-HT1A receptor gene has begun to be elucidated. In the rat, transcriptional initiation is TATA-dependent; however, murine and human promoters are TATA-less (Parks *et al.*, 1996; Storrington *et al.*, 1999). A tandem repeat of an upstream repressor element (dual repressor element, DRE), which is composed of two adjacent elements (5'-repressor element, FRE, and 3'-repressor element, TRE), decreases gene transcription in both neuronal and non-neuronal cells (Ou *et al.*, 2000). In 5-HT1A receptor positive cells, the transcription factor Freud-1 binds the FRE, and is thought to control basal receptor expression, while in receptor negative cells, additional transcription factors are believed to completely silence the gene (Ou *et al.*, 2003). A 26bp palindrome in the human 5-HT1A promoter is bound by the transcription factor NUDR [nuclear deformed epidermal autoregulatory factor (DEAF-1)]/suppressin (Lemondé *et al.*, 2003). A single nucleotide polymorphism (SNP) in the minimum consensus binding site of this element, C(-1019)G (Wu and Comings, 1999), differentially affects NUDR-binding. This results in a repression of receptor expression with the C-allele which is abrogated for the G-allele. Thus, the G-allele derepresses 5-HT1A receptor expression, which may result in increased autoreceptor inhibition of serotonergic neural activity (Figure 1.2) (Lemondé *et al.*, 2003).



Figure 1.2. Effect of the 5-HT1A C(-1019)G SNP on NUDR binding and 5-HT1A gene expression.

The transcription factor NUDR binds to a 26bp palindrome in the human 5HT1A promoter to repress 5HT1A expression in raphe neurons. A C/G polymorphism is found in the minimum consensus sequence of this element (bracket). The G-allele prevents NUDR binding, disinhibiting 5HT1A autoreceptor expression, and is associated with depression and suicide. (Adapted from Lemonde *et al.*, 2003)

1.3 Role of Genetic Polymorphisms in Mental Illness

1.3.1 Overview of schizophrenia

Schizophrenia is a devastating psychiatric disorder characterized by psychotic episodes (referred to as positive symptoms), during which patients may exhibit a loss of reality testing (they cannot realistically compare what they perceive and are thinking to events in the real world), delusional or incoherent thinking, disordered memory, confusion, and disorganized speech and/or behaviour. Patients may also experience hallucinations (especially auditory). These discrete psychotic episodes are separated by periods of social isolation, flattened affect, and lack of motivation (negative symptoms). Unlike the transitory psychotic aspects of the positive symptoms, the cognitive symptoms of impaired learning, memory and concentration, slowed psychomotor processing, and difficulties in problem solving and abstract thinking, tend to be long-lasting (Kandel *et al.*, 2000; Mueser and McGurk, 2004; Walker *et al.*, 2004). The DSM-IV criteria for diagnosis of schizophrenia require that at least some of these symptoms be present for at least 6 months (for detailed diagnostic criteria see: American Psychiatric Association, 1994). The impairment of both higher-order thinking and social functioning which characterizes schizophrenia leads to patient difficulties in work and school, independent living, and personal relationships. In addition, it has a relatively early age of onset (usually between 16 and 30 years), and symptoms often persist to some degree for the remainder of an individual's life. For these reasons, schizophrenia is the most disabling psychiatric disorder. Although it only affects about 1% of the population, it accounts for about 50% of psychiatric hospital admissions, has treatment costs of several billion

dollars each year in Canada alone (\$2.35 billion in 1996), and is one of the top ten causes of disability-adjusted life-years worldwide (Mueser and McGurk, 2004).

1.3.2 Role of the Dopamine System in Schizophrenia

DA neurons projecting from the midbrain substantia nigra (SN) to the striatum (nigrostriatal pathway) help to control motor behaviour; Parkinson's disease primarily involves this pathway. DA neurons in the ventral tegmental area (VTA) project to the limbic areas (mesolimbic pathway), and are involved in emotions, memory and reward, while those projecting to cortical (especially prefrontal) regions (mesocortical pathway) help regulate cognition, planning, attention and social behaviour. Finally, DA neurons in the arcuate and periventricular nuclei of the hypothalamus innervate the pituitary stalk (the tuberoinfundibular pathway) to inhibit prolactin release. As with the serotonergic system, DA also signals through GPCRs. The five receptor subtypes currently identified are grouped into D1-like (D1 and D5) and D2-like (D2, D3 and D4), with the former and latter positively and negatively coupling to adenylyl cyclase, respectively. Like the 5-HT_{1A} receptor, the dopamine D₂ receptor is expressed both as an inhibitory autoreceptor on DA neurons, and as a post-synaptic heteroreceptor (Kandel *et al.*, 2000; Marsden, 2006).

In concert with the monoamine hypothesis of depression, the dopamine hypothesis of schizophrenia was developed about 50 years ago based on similarities between amphetamine-induced psychosis and schizophrenia, coupled with the discovery that amphetamines act by increasing catecholamine, and especially dopamine signaling (amphetamines block reuptake and also stimulate DA release through the dopamine

transporter). L-dihydroxyphenylalanine (L-DOPA) and cocaine also increase DA levels and can induce psychotic episodes (Cooper *et al.*, 2003; Marsden, 2006). In 1976, Philip Seeman demonstrated that the antipsychotic drug dose effective for treating positive symptoms was almost perfectly correlated with the drug's affinity for the D2 receptor (Seeman *et al.*, 1976). Almost all current antipsychotics display some degree of D2 receptor antagonism (see 1.3.3). Thus, the initial hypothesis was that schizophrenia results from excessive dopaminergic neurotransmission. Because of the role of the mesolimbic dopamine system in mood and memory as well as similarities between psychosis and certain forms of limbic system epilepsy, psychotic symptoms are thought to result from overactivity of this pathway (Kandel *et al.*, 2000). This is supported by several studies which showed that amphetamine challenge resulted in greater dopamine release in subcortical (limbic) brain regions of schizophrenic patients compared to controls, and that in schizophrenics, this also induced an increase in positive symptom severity which was positively correlated with DA levels (Abi-Dargham, 2004). A single photon emission computed tomography study also demonstrated increased baseline occupancy of striatal D2 receptors in schizophrenics versus controls (Abi-Dargham *et al.*, 2000). In addition, a number of studies have found increased D2 receptor densities in schizophrenics; although these results are confounded by medication status and post-mortem issues (Abi-Dargham, 2004; Walker *et al.*, 2004). However, mesolimbic DA hyperactivity does not account for the cognitive and negative symptoms. Evidence of ventricular enlargement coupled with a reduction in grey matter volume in cortical brain areas in schizophrenics, and observations that dopamine depletion in animals induces cognitive impairment, led to a revised theory of schizophrenia: hyperactivity of the

mesolimbic DA pathways results in positive symptoms, while hypoactivity of the mesocortical DA pathways is responsible for the negative symptoms (Abi-Dargham, 2004; Kandel *et al.*, 2000; Walker *et al.*, 2004). Interestingly, because DA neurons in the VTA are under both excitatory (glutamatergic) and inhibitory (GABAergic) control from PFC reciprocal feedback circuits, developmental disruption of cortical DA signaling may enhance mesolimbic DA signaling, possibly resulting in schizophrenia (Marsden, 2006).

1.3.3 Atypical Antipsychotics and Role of the Serotonergic System in Schizophrenia

Recently, attention has also focused on the role of the 5-HT system in the neurochemistry of schizophrenia as well as its treatment (Meltzer, 1999). As mentioned above, traditional (typical) antipsychotics such as haloperidol act primarily as D2 receptor antagonists. This is beneficial in relieving the psychotic (positive) symptoms resulting from mesolimbic DA hyperactivity, but does not improve, and in fact may worsen, negative and cognitive symptoms which involve mesocortical DA hypoactivity. By blocking DA neurotransmission in the nigrostriatal pathway, these drugs are also known to result in extrapyramidal side-effects, which are Parkinsonian-like motor problems, and occasionally in the serious neurological condition, tardive dyskinesia (King, 1998; Mueser and McGurk, 2004; Owens, 1996). Members of a newer class of drugs, the atypical antipsychotics, which include clozapine, olanzapine, quetiapine, risperidone and ziprasidone, have been shown repeatedly to result in reduced motor side-effect profiles (although this may in part be due to excessive doses of typical antipsychotics used in the past (Kapur and Remington, 2001)) and are often more effective in improving negative and cognitive symptoms. For these reasons, atypical

antipsychotics now represent 90% of the market share of schizophrenic pharmacotherapies (Bender *et al.*, 2005; Marek and Merchant, 2005; Miyamoto *et al.*, 2005; Woodward *et al.*, 2005). However, it should be noted that up to three-quarters of patients discontinue these drugs due to intolerability and/or inefficacy (Lieberman *et al.*, 2005). Interestingly, clozapine appears to have uniquely high efficacy in treating both psychotic and negative symptoms, but it is rarely used as a first-line therapy due to the risk of agranulocytosis, a potentially fatal decrease in white blood-cell levels (Mueser and McGurk, 2004).

Much of the recent interest in serotonin's role in schizophrenia is due to the success of its treatment by atypical antipsychotics, which generally have lower affinity for the D2 receptor than typical antipsychotics, but also possess strong 5-HT2A antagonist and in some cases 5-HT1A agonist properties (in addition, many other neurotransmitter receptors and transporters are targeted by particular drugs) (Meltzer, 1999; Meltzer *et al.*, 2003; Miyamoto *et al.*, 2005; Newman-Tancredi *et al.*, 2005). Briefly, the following paradigm has been proposed to explain their actions. Serotonergic neurons project to the SN and VTA and to their DA terminals in the striatum, cortex and limbic areas. Serotonergic signaling inhibits the firing of DA neurons through the stimulation of post-synaptic 5-HT2A receptors. It appears that atypical antipsychotics disinhibit (enhance) DA neurotransmission preferentially in the frontal cortex compared to the nucleus accumbens, possibly through a combination of 5-HT1A agonism (which inhibits the 5-HT neurons) and 5-HT2A antagonism (Bantick *et al.*, 2001; Kuroki *et al.*, 1999; Miyamoto *et al.*, 2005; Rollema *et al.*, 2000). The involvement of 5-HT1A receptors in stimulating PFC dopamine release has been demonstrated by the lack of effect of atypical

antipsychotics in 5-HT1A knockout mice (Diaz-Mataix *et al.*, 2005), and when administered in conjunction with the 5-HT1A antagonist WAY-100635 (Chung *et al.*, 2004). The latter study also showed that clozapine resulted in a 5-HT1A-dependent increase in hippocampal DA release, which may also be involved in cognitive improvement. In addition, multiple feedback circuits connect the 5-HT1A and 5-HT2A receptor-containing glutamatergic and GABAergic neurons in the PFC with both DA and 5-HT nuclei in the brainstem. The involvement of both PFC 5-HT1A and 5-HT2A receptors in PFC DA release has been indicated by several recent studies and they are likely critical to the actions of atypical antipsychotics; however, the interactions of these circuits with each other and with serotonergic-dopaminergic circuits are still being deciphered (Bortolozzi *et al.*, 2005; Diaz-Mataix *et al.*, 2005; Pehek *et al.*, 2006).

Whatever the exact mechanisms, the preferential increase in cortical DA levels in combination with the lower D2 occupancy of atypical antipsychotics at effective doses, which decreases nigrostriatal DA inhibition (Kasper *et al.*, 2002), as well as the anti-cataleptic properties of 5-HT1A agonism in rodents and primates (Bantick *et al.*, 2001), may explain the ability of these drugs to improve cognitive and negative symptoms while causing fewer extra-pyramidal side effects than first-generation antipsychotics (Melzer, 1999; Neuman-Tancredi *et al.*, 2005; Reynolds, 2004).

Additionally, a role for the 5-HT system in schizophrenia is indicated by the majority of post-mortem studies having showed increased 5-HT1A receptor density in cortical regions of drug-naïve schizophrenic compared to control individuals (Bantick *et al.*, 2001; Kasper *et al.*, 2002). A number of post-mortem studies also reported decreased cortical 5-HT2A levels in schizophrenics. However, no change was found in a PET

study of patients with established schizophrenia, but a decrease was shown in another PET study of individuals at risk for the disease (Hurlemann *et al.*, 2005; Meltzer *et al.*, 2003). However, it should be noted that selective 5-HT_{2A} antagonists as monotherapies for schizophrenia have not been efficacious in clinical trials, indicating that D₂ antagonism is also required (Marek and Merchant, 2005).

1.3.4 Evidence for Genetic Component of Schizophrenia

Schizophrenia has a relatively uniform lifetime risk of approximately 1% worldwide and in both sexes; however, women tend to have less severe illness with a later age of onset (Mueser and McGurk, 2004). The etiology of schizophrenia is complex and likely involves many factors. However, vulnerability to schizophrenia is heritable, as twin, adoption and family history studies all show that the more closely related a person is to a biological relative with the disorder, the greater the risk of schizophrenia (Walker *et al.*, 2004). This indicates a key role for genetics in its development. Studies estimate about 50% concordance in monozygotic twins and 17% in dizygotic twins, which indicates that susceptibility to schizophrenia is as much as 80% due to heritable factors (Riley and Kendler, 2006; Norton *et al.*, 2006). Pedigree studies indicate that schizophrenia does not follow classical Mendelian inheritance patterns, indicating that it is likely a polygenic disease, involving several to dozens of different genes. Hence, a combination of genetic polymorphisms, each conferring a degree of increased risk, in the context of environmental and developmental factors, may predispose individuals to developing schizophrenia (Kandel *et al.*, 2000). Considerable evidence exists in support of neurodevelopmental models of schizophrenia, in which genetics and environmental

factors result in abnormal neural development which later leads to schizophrenia (however, details are not within the scope of this thesis; for reviews see: Rapoport *et al.*, 2005; Walker *et al.*, 2004).

Association and linkage analyses have examined many candidate schizophrenic susceptibility genes and genetic loci. Given the polygenic and multifactorial etiology of schizophrenia, not surprisingly, no single gene or genetic polymorphism shows a robust, highly replicable association with schizophrenia. However, a number of gene variants conferring small effect sizes have been reported. Considerable research effort is being focused on both discovering new functional polymorphisms and in performing follow-up studies of those already reported (Riley and Kendler, 2006; Norton *et al.*, 2006). Several polymorphisms in the genes of the 5-HT1A, 5-HT2A and D2 receptors (the targets of atypical antipsychotics) have been associated with schizophrenia.

1.3.4.1 The 5-HT1A C(-1019)G SNP and Schizophrenia

Specifically, a single study has reported an association of the G-allele of the functional promoter 5-HT1A C(-1019)G SNP (see 1.2.6) with schizophrenia in an American cohort (Huang *et al.*, 2004). As the G-allele prevents binding of the transcriptional repressor NUDR to the 5-HT1A promoter and thus increases 5-HT1A expression, this correlates well with the finding that 5-HT1A expression may be increased in the cortex of schizophrenic individuals (see 1.3.2.3).

1.3.4.2 The 5-HT2A A(-1438)G and T102C SNPs and Schizophrenia

A promoter SNP in the 5-HT2A receptor, A(-1438)G, has been reported, and is in almost complete linkage disequilibrium with the well-characterized 5-HT2A T102C silent coding SNP (Spurlock *et al.*, 1998) (however, see 4.2). Although initial characterization found no effect of the A(-1438)G SNP on promoter activity (Spurlock *et al.*, 1998), a recent study reported that in cell lines endogenously expressing 5-HT2A and in the presence of an enhancer element, the G-allele showed decreased promoter activity compared to the A-allele. This has led to the hypothesis that the A(-1438)G SNP may be the functional variant responsible for findings made examining the T102C SNP (Parsons *et al.*, 2004). Supporting this, one study has shown an association between increased 5-HT2A binding levels in the brain and the 102T and -1438A alleles (Turecki *et al.* 1999); however, two larger studies did not replicate this result (Kouzmenko *et al.*, 1997; Kouzmenko *et al.*, 1999). One study also showed a correlation between the T102C TT-genotype and greater platelet 5-HT2A binding in healthy controls, but not in subjects with mood disorders (Khait *et al.*, 2005). In addition, by using an allelic expression assay, which compares the relative expression from each allele in heterozygous individuals, one study found that in both schizophrenic and control individuals, the ratio of T102C C-allele to T-allele expression was ~0.8, and that both 5-HT2A mRNA and protein levels were lower with the CC compared to the TT genotype. They also reported that in medication-free schizophrenics, 5-HT2A mRNA levels were lower than in controls, linking innately lower allelic expression to the disease (Polesskaya and Sokolov, 2002). However, using a different allelic expression assay, another group did not find differential expression from the T- and C-alleles (Bray *et al.*, 2004). Recently, the former

group reported finding varying degrees of methylation between individuals in both the T102C C-allele and the A-1438G G-allele, and that promoter methylation levels corresponded to 5-HT2A mRNA expression levels. Thus, they suggest that only when the C-allele is not methylated is its expression lower than the T-allele (expression may be about the same when it is methylated). Therefore, differential 5-HT2A SNP methylation status of individuals in 5-HT2A expression studies could hinder detection of an effect of these SNPs (Poleskaya *et al.*, 2006). In summary, a number of studies support a functional role for these SNPs in regulating 5-HT2A expression, although this conclusion is still under considerable debate.

Linkage of both the T102C C-allele and A(-1438)G G-allele to schizophrenia has been supported by a recent meta-analysis; however, another found a significant association only of the T102C C-allele (but not the A(-1438)G G-allele, see 4.1.2), and both only in European populations (Abdolmaleky *et al.*, 2004; Li *et al.*, 2006). An association of the C-allele of the T102C SNP with poorer response to the atypical antipsychotic clozapine was indicated by meta-analysis (Arranz *et al.*, 1998); however, if the initial positive study is removed from consideration, the effect disappears, which may indicate a chance finding (Norton and Owen, 2005).

1.3.4.3 The D2 Taq1A (C/T) SNP and Schizophrenia

The D2 Taq1A (C/T) SNP (Grandy *et al.*, 1993) has been extensively studied with regards to both neuropsychiatric illness and its treatment, and to D2 receptor expression in the brain (Noble, 2003). Located ~10kb downstream of the D2 coding sequence (the D2 gene is ~270kb), the T-allele (A1) is associated with lower D2 receptor density in

normal individuals, indicating that it is likely in linkage disequilibrium with an unknown functional allelic variant which affects D2 expression (Ritchie and Noble, 2003). Both alleles of the Taq1A SNP have been associated with schizophrenia in different populations (Dubertret *et al.*, 2001; Golimbet *et al.*, 1998; Golimbet *et al.*, 2003); however, most studies have found no association (Noble, 2003). Several studies have reported an association of the T-allele with better response of positive symptoms to antipsychotic treatment (Wilffert *et al.*, 2005); however, a recent study found the opposite association in African-Americans (Hwang *et al.*, 2005). The incidence of tardive dyskinesia, a serious side effect of antipsychotic treatment in some individuals, was found to be greater in female schizophrenics homozygous for the C-allele, than for those with a T-allele (Chen *et al.*, 1997).

1.3.5 Evidence for Genetic Component of Major Depressive Disorder

Like schizophrenia, the etiology of MDD likely involves the interaction of genetic, environmental and developmental factors. Depression has been shown to be highly heritable, with a monozygotic twin concordance of 40-50% and an estimated heritability of 33-50%. It should be noted that there is significant co-morbidity (up to 60%) and co-segregation in family pedigrees between depressive and anxiety disorders. Thus, there is likely overlap in the gene variants conferring susceptibility to both these affective disorders (Leonardo and Hen, 2006).

Again as with schizophrenia, depression appears to be a polygenic disease, with a number of gene variants likely producing small effects to increase an individual's risk (Craddock and Forty, 2006; Fava and Kendler, 2000; Nestler *et al.*, 2002a). These

include genes involved in monoaminergic neurotransmission, with polymorphisms in the 5-HT1A, 5-HT2A and D2 receptors and the 5-HTT having been associated with depression and other mood disorders.

1.3.5.1 The 5-HT1A C(-1019)G SNP and Major Depressive Disorder

The G-allele of the C(-1019)G SNP in the 5-HT1A receptor promoter has been associated with severe depression and suicide (Lemonde *et al.*, 2003), as well as with depression- and anxiety-related personality traits in otherwise healthy individuals (Strobel *et al.*, 2003), and also panic disorder with agoraphobia (Rothe *et al.*, 2004). Recently, a strong trend ($p = 0.0630$) for overtransmission of the G-allele in suicide attempters with previous traumatic and/or stressful life events prior to the suicide attempt was observed by Wasserman *et al.* (2006). The G-allele prevents binding of the transcriptional repressor NUDR to the 5-HT1A promoter and thus increases 5-HT1A expression (see 1.2.6), which has been hypothesized to be the functional mechanism by which this polymorphism predisposes individuals to depression (Figure 1.2) (Albert and Lemonde, 2004). Interestingly, preliminary data from a study which examined 5-HT1A binding levels in antidepressant naïve versus treated MDD subjects (see 1.2.2) showed that in both MDD subjects and controls, the presence of at least one G-allele was associated with higher binding potential in the DR. In addition, this group reported that the G/G genotype was three times more prevalent in MDD individuals compared to controls; however, this result was only at a trend level of significance ($p = 0.059$) due to small sample size (Parsey *et al.*, 2006). Also indicating a functional role for the polymorphism, Domschke *et al.* (2005) found that panic disorder patients with the G/G

genotype showed decreased right PFC activation with fearful stimuli, while responding to happy faces increased activation of the amygdala. However, some studies have also found no association of the SNP with major depression or suicide attempt (Arias *et al.*, 2002; Huang *et al.*, 2004; Koks *et al.*, 2006). In addition, no association was found with depression-related personality traits or suicide attempts in alcoholics (Koller *et al.*, 2006).

Interestingly, it has recently been shown that the 5-HT1A C(-1019)G SNP may contribute to antidepressant response: patients with the G/G genotype compared to C/C genotype improved significantly less when treated with the 5-HT1A agonist flibanserin, and when treatment groups were pooled, the likelihood of not responding to flibanserin, the SSRI fluoxetine or the norepinephrine reuptake inhibitor nefazodone, was greater in G/G patients than in patients homozygous for the C-allele (Lemondé *et al.*, 2004b). Hong *et al.* (2005) also reported a poorer response in patients carrying the G-allele compared to the C/C genotype to fluoxetine. The group then extended their sample size and noted that while the effect was still significant, it was entirely due to poorer response in female G-allele carriers, demonstrating a gender-specific effect (Yu *et al.*, 2006). The combination of the G/G genotype with the 5-HTTLPR S/S genotype (see 1.3.3.5) was also associated with poorer outcome of treatment with the SSRI citalopram, although no single gene effect was detected for the 5-HT1A SNP (Arias *et al.*, 2005). However, another study showed no interaction between the two polymorphisms and response to the SSRI fluvoxamine (Serretti *et al.*, 2004). The latter study also showed that patients with bipolar (but not unipolar) depression exhibited a better response to the SSRI fluvoxamine if they carried the 5-HT1A C-allele versus the G-allele.

1.3.5.2 Interactions between the Serotonergic and Dopaminergic Systems and Atypical Antipsychotics in Depression

Just as serotonergic projections influence dopaminergic activity (see 1.3.3), the reverse also holds true, with dopaminergic projections from the VTA, SN, and hypothalamus innervating the DR and MnR. DA signaling stimulates the 5-HT system. Systemic administration of apomorphine, a non-selective DA receptor agonist, increased 5-HT firing and release in the DR; this effect was inhibited by both D1 and D2 receptor antagonists. Although D2 receptors are present on 5-HT somata, local application of D1 and D2 agonists in the DR had little effect, indicating that the DA receptors responsible were not solely located in the raphe (Adell *et al.*, 2002; Tremblay and Blier, 2006). Also demonstrating the interconnectivity between the two monoaminergic systems is the finding of increased levels of DA and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in several sub-cortical regions of 5-HT1A knockout mice (Ase *et al.*, 2000).

As discussed above (see 1.2.2), evidence for the involvement of the monoamines 5-HT and NE in depression is considerable. However, as mesocorticolimbic DA pathways are involved in motivation and reward, both of which can be altered in affective disorders, a role for DA in depression is also becoming a focus of research (Esposito, 2006). Anhedonia, or inability to obtain pleasure from stimuli, is a characteristic symptom of depression which has been found to be associated with dysfunction in the DA-innervated ventromedial PFC, amygdala and ventral striatum (Keedwell *et al.*, 2005). Another common feature of depression and other affective disorders is social aversion, which in a mouse model was found to involve neuronal activation and brain derived

neurotrophic factor (BDNF)-mediated signaling in the mesolimbic DA system (Berton *et al.*, 2006). More direct evidence includes observations in some studies DA turnover and plasma levels were lower in MDD and suicidal patients, as were the CSF levels of the DA metabolite HVA. In addition, both DA and HVA levels have been inversely correlated with depression severity. Increased D2/D3 receptor binding in the basal ganglia and amygdala, and decreased striatal and amygdaloid dopamine transporter (DAT) binding, have been reported in depressed subjects compared to controls, although some studies showed no difference (Esposito, 2006; Papakostas, 2006). The changes in DAT binding are interesting in the context of recent findings by Zhou *et al.* (2005), who showed that when extracellular 5-HT levels were elevated by exogenous 5-HT or SSRI treatment, striatal DATs (which normally have a low affinity for 5-HT) took-up the excess 5-HT. Co-release of 5-HT and DA was then observed from DA terminals, indicating that SSRIs alter the relationship between these two monoamines in the striatum. Although many contradictory results have been reported, the above suggests that a hypodopaminergic state may exist in depression, and that this is reflected by compensatory changes in DA receptor and transporter levels. Supporting this, animal studies have shown DA receptor hypersensitivity after antidepressant treatment (D'Aquila *et al.*, 2000; Dremencov *et al.*, 2006; Esposito, 2006; Papakostas, 2006). In addition, an interaction between prostate apoptosis response 4 (Par-4) protein and the D2 receptor to inhibit cAMP signaling in DA neurons in low calcium conditions was reported; this was relieved by calcium influx mediated activation of calmodulin, which displaced Par-4. Interestingly, mice lacking the Par-4/D2 interaction domain exhibited depression-like behaviour as well as increased cAMP signaling, suggesting that Par-4

may represent a molecular connection between altered DA neurotransmission and depression (Park *et al.*, 2005).

Additional evidence for a role of both the D2 receptor and the 5-HT_{2A} receptor (see 1.2.2 and 1.2.4) in depression is the increasing usage of atypical antipsychotics and other pharmacotherapies which act on these receptors as adjuncts to conventional antidepressants in treatment resistant patients. Around half of depressed patients will not exhibit full remission (defined as having an absence of or very minimal residual depressive symptoms) after their first course of antidepressants (Blier, 2005a; Keller, 2005). Several trials have demonstrated the efficacy of atypical antipsychotic addition to antidepressants (in particular SSRIs) for both MDD and bipolar disorder. The antipsychotics are administered at lower doses than are therapeutic for schizophrenia, which reduces the incidence of serious side effects, and are effective in the absence of psychotic symptoms (Blier, 2005b; Nemeroff, 2005). As with schizophrenia, the mechanism by which atypical antipsychotics act in conjunction with SSRIs to relieve depression is far from understood. However, recent animal studies have showed that an experimental SSRI/5-HT_{2A} antagonist compound enhanced (rather than inhibited as with SSRIs alone) NE release (Blier and Szabo, 2005). As decreased NE levels are implicated in depression (see 1.2.2), this effect may enhance treatment efficacy. In addition, as described above (1.3.3), atypical antipsychotics also increase DA release in the PFC, which may relieve DA hypoactivity in that region, and some act as 5-HT_{1A} agonists, which also have antidepressant properties (see 1.2.4).

Bupropion, a catecholamine (NE and DA) reuptake inhibitor and releaser, is also effective both as a monotherapy and in combination therapy with SSRIs. Small studies

have also shown that D2 agonists are useful as antidepressants (as an effective monotherapy in one study, but primarily as adjuncts), as are psychostimulants which block DA reuptake and stimulate DA release (Blier, 2005a; Papakostas, 2006; Tremblay and Blier, 2006). While these results appear incongruous with successful combination therapy using the D2-antagonizing atypical antipsychotics, it is important to note that the latter interact with numerous receptors and exhibit numerous effects (see 1.3.3).

Therefore, evidence indicates that the DA system is also important in depression, and that both 5-HT_{2A} and D2 receptors may be implicated in its treatment.

1.3.5.3 The 5-HT_{2A} A(-1438)G and T102C SNPs and Major Depressive Disorder

Both 5-HT_{2A} SNPs have also been associated in individual studies with a variety of psychiatric disorders other than schizophrenia, including anorexia nervosa, bipolar disorder, obsessive-compulsive disorder and seasonal affective disorder; however, when meta-analyses have been performed they do not support an association (Norton and Owen, 2005). With regard to MDD, studies have been inconsistent, and a large meta-analysis did not support an association (Anguelova *et al.*, 2003a; Choi *et al.*, 2004; Du *et al.*, 2000; Khait *et al.*, 2005; Koks *et al.*, 2006). Several meta-analyses have been performed to examine the relationship between 5-HT_{2A} gene variants and suicidal behaviour. Interestingly, while none supported an association with the T102C SNP, the most recent study did find an association of the A(-1438)G G/G genotype and suicidality (Anguelova *et al.*, 2003b; Arango *et al.*, 2003; Li *et al.*, 2006). One study also reported a strong association between intolerance to the SSRI paroxetine and the T102C C/C genotype, while another showed increased gastro-intestinal side effects with fluvoamine

treatment in patients with the A(-1438)G G-allele (Murphy *et al.*, 2003; Suzuki *et al.*, 2006). Studies examining genotype and SSRI efficacy have been inconclusive, and include a large, multi-center trial which did not see an effect of either the T102C or A(-1438)G SNPs (McMahon *et al.*, 2006; Serretti *et al.*, 2005).

1.3.5.4 The D2 Taq1A (C/T) SNP and Major Depressive Disorder

The D2 Taq1A T-allele has been linked with lower neuroticism-anxiety scores in men (Wacker *et al.*, 2005); however, the majority of studies have not found an association of the SNP with affective disorders, including MDD and bipolar disorder (Noble, 2003).

1.3.5.5 The 5-HTTLPR and Major Depressive Disorder

Genetic variation in the 5-HTT may also contribute to susceptibility to anxiety and depression, as well as to antidepressant response. 5-HTT transcription is controlled by several regulatory elements, and also by the 5-HTT gene-linked polymorphic region (5-HTTLPR), which is a variable length repetitive element located about 1.4kb upstream of the transcription start site. In humans, each repeat is 20-23bp, and the most common 5-HTTLPR alleles are the 16-repeat long (L) and 14-repeat short (S) forms, although super long alleles (up to 20 repeats) can occur (Murphy *et al.*, 2004). Importantly, the L- and S-variants differentially affect 5-HTT transcription in a variety of *in vitro* models, with the L-allele being associated with higher 5-HTT promoter activity, mRNA, protein, and 5-HT uptake levels (Heils *et al.*, 1996; Lesch *et al.*, 1996; Mortensen *et al.*, 1999; Murphy *et al.*, 2004). Importantly, a recent study has also reported an A/G SNP within

one of the repeats that is present in the L-allele (forming L_A and L_G alleles) but deleted in the S-allele. It was shown that while the L_A-allele expression in cell lines was significantly higher than the S-allele, the L_G-allele expression was similar to the S-allele. Given the moderate prevalence of the L_G-allele (~15%) compared to the L_A-allele (~50%) in U.S. Caucasians, this finding may explain some of the variation between studies of the 5-HTTLPR described below (Hu *et al.*, 2006).

Neuroimaging and post-mortem human brain studies have been inconclusive regarding an association of the 5-HTTLPR with 5-HTT binding in healthy individuals (Kasper *et al.*, 2002; Murphy *et al.*, 2004; Neumeister *et al.*, 2004). In addition, reduced gray matter volume in brain areas involved in negative emotions, and alterations in amygdala activation and neural fear circuits have been found in S-allele carriers (Hariri *et al.*, 2002; Pezawas *et al.*, 2005). Interestingly, it has also been reported that both pre- and post-synaptic 5-HT_{1A} binding levels were lower in healthy individuals with the S-allele compared to homozygous L/L individuals (David *et al.*, 2005). However, another study found the opposite effect post-synaptically, and no effect in the raphe of healthy women (Lee *et al.*, 2005). These studies indicate several potential functional mechanisms by which this polymorphism may affect the 5-HT system and mood.

As mentioned above, some studies have associated the low-activity S-allele with neuroticism-anxiety, depression, suicide and other neuropsychiatric illnesses; however, many studies found no or an opposite association (Arango *et al.*, 2003; Neumeister *et al.*, 2004), including a massive study with a total sample size of over 100 000 (Willis-Owen *et al.*, 2005). A recent meta-analysis of ten association studies (not including that of Willis-Owen *et al.* (2005)) concluded that a small but significant association exists

between unipolar depression and the S/S genotype (Lotrich and Pollock, 2004). In addition, Caspi *et al.* (2003) showed in a large, prospective study that the S-allele acted in combination with stressful life events to predispose people to depression and suicidality, indicating that the 5-HTTLPR may interact with an individual's environment to influence mood and psychopathology. Several other studies have either succeeded fully or in part (only in women), and in two cases failed to replicate these results (Wilhelm *et al.*, 2006).

The association of the low-expressing 5-HTTLPR S-allele with depression may initially appear paradoxical, as SSRIs work to relieve depression by inhibiting the 5-HTT. However, a possible explanation may be found in 5-HTT knockout mice, which as previously discussed (see 1.1.3) exhibit a depression-like phenotype that again is opposite to expectations. A recent study addressed this issue by examining the effects of early post-natal SSRI treatment on mouse behaviour, under the hypothesis that the depression- and anxiety-like characteristics are due to the effects of 5-HTT deletion in development. Indeed, fluoxetine treatment for post-natal days 4 to 21 resulted in similar adult behavioural phenotypes in wildtype mice as in untreated 5-HTT *-/-* mice (Ansorge *et al.*, 2004; Leonardo and Hen, 2006). Therefore, it is possible that effects of the 5-HTTLPR on depression in adulthood may also be due to serotonergic dysregulation in development.

Additionally, the 5-HTTLPR may also influence response to SSRIs. The majority of studies to date have shown that S/S depressed patients respond more poorly and more slowly to a variety of SSRIs than L/L or L/S individuals (Lesch and Gutnecht, 2005; Murphy *et al.*, 2004; Serretti *et al.*, 2005).

1.4 Primates as Models of the Effects of Serotonergic Gene Polymorphisms on Depression

1.4.1 Overview of Primates as Models of Affective Disorders

In their assessment of how best to further our understanding of mood disorders, a workgroup for the National Institutes of Mental Health identified developing better animal models of these disorders as one of the most critical areas of study. They note that ideally, an animal model of disease is theory-driven, such as one based on exposing the animal to factors which cause depression in humans, followed by examination of the symptoms which result. While these models are not yet possible as the etiology of depression is incompletely understood, non-human primates, such as rhesus (*Macaca mulatta*) and cynomolgus macaques (*M. fascicularis*), offer many advantages over rodents in examining both the effects of potential risk factors and the biological basis of depression-related symptoms (Nestler *et al.*, 2002b).

Firstly, other members of the Order Primates are evolutionarily the most closely related species to humans. Chimpanzees (*Pan troglodytes*) are our closest relatives, our lineages having diverged from a common ancestor about 6-7 million years ago (Stewart and Disotell, 1998). Thus, our DNA similarity is approximately 98.5-99% (Page and Goodman, 2001). However, this extremely high degree of genetic relatedness between humans and chimpanzees, combined with their endangered status, makes them unsuitable as a research model (Rogers *et al.*). The lineage of old world monkeys, which includes the macaque genus, separated from that of humans and other great apes about 25 million years ago (Stewart and Disotell, 1998), and the human - rhesus macaque DNA sequence similarity is 92.5-95% (Page and Goodman, 2001). It should be noted that in the wild,

rhesus macaques are much more widespread and numerous than chimpanzees (Rogers *et al.*). In comparison, primate and murid (rat and mouse) lines diverged about 75 million years ago, and the completion of the mouse and rat genome sequences has revealed that only about 40% of rodent and human euchromatic DNA can be aligned (Mullins and Mullins, 2004). The recent release of the draft rhesus macaque assembly (v.1.0, Mmul_051212) in January 2006 (Birney *et al.*, 2006), will allow researchers to benefit maximally from the use of this species as well as the very closely related cynomolgus macaque as model systems.

In addition to sharing a high degree of genetic relatedness, macaques are also metabolically and physiologically very similar to humans. With regards to modeling neuropsychiatric processes and diseases, they possess a well-developed prefrontal cortex and strong interconnectivity between their amygdala and orbitofrontal cortex, which allow them (like other primates) to engage in complex cognitive and emotional processes. Their neuroendocrine functions, including the female estrous cycle, are also akin to those of humans, allowing for relevant studies examining the effects of stress and reproductive hormones on mood. In addition, macaques can provide brain and endocrine samples, allowing for molecular experiments not possible in humans (Kalin and Shelton, 2003; Shively and Bethea, 2004).

Like humans, macaques form complex social structures and depend on social bonds. Mother-infant attachments are necessary for proper infant physical, social and emotional development. If these are lost, the emotional and physiological changes in the mother are similar to human depression, such as sleep disturbances, and increased heart rate and cortisol levels (Kalin and Shelton, 2003; Shively and Bethea, 2004). In addition, social

stress is thought to be associated with depression. In humans, these interactions are difficult to evaluate due to the complexity of controlling and quantifying stressors and in randomizing subjects; however, social stress also results in depressive responses in some monkeys which can be closely studied in a laboratory setting. Social status hierarchies are very important in macaque societies, and it has been found that subordinate female cynolmolgus macaques were subject to more aggression and exhibited increased diet-induced atherosclerosis, decreased ovarian function, and increased cortisol secretion. This indicates that social subordination is stressful and negatively impacts on health in these animals (Shively *et al.*, 1997). In order to examine the relationship between social stress and depression, a series of experiments was performed with female cynolmolgus macaques in which their social status was manipulated, such that half of the previously dominant monkeys became subordinate, and half the previously subordinate individuals became dominant. The current subordinates were groomed less, and spent more time alone, including an increased amount of time spent fearfully scanning their environment, compared to current dominants. Also, collapsed posture and unresponsiveness to environmental stimuli, which are used as indicators of behavioural depression, were observed almost exclusively in the current subordinates. Interestingly, current subordinates who were also previously subordinate were more susceptible to behavioural depression than former dominants turned subordinate. However, 35% of the current subordinates did not display any behavioural depression in response to their stressful social situation, indicating that susceptibility to depression may involve some combination of genetic predisposition, previous life experiences, and other factors (Shively *et al.*, 1997). Recently, the same group found lower body fat levels, decreased

activity levels, increased heart rate, disturbed stress axes, suppressed ovarian function and increased mortality in the monkeys exhibiting behavioural depression. These characteristics are also symptoms of human depression, indicating that both behaviourally and physiologically, these monkeys provide relevant models for adult depression in humans (Shively *et al.*, 2005). Additionally, the macaques with behavioural depression exhibited reduced 5-HT1A binding potential across their raphe, amygdala, hippocampus and anterior cingulate cortex, as measured by PET (Shively *et al.*, 2006). This correlates nicely with studies in humans which showed decreased 5-HT1A binding in a number of cortical regions (Drevets *et al.*, 1999, Sargent *et al.*, 2000), and is an indication of similarities in the neuropathological etiology of depression in macaques and humans. Interestingly, an opposite effect (increased 5-HT1A levels) was reported in the DR of depressed human suicides (Stockmeier *et al.*, 1998), but this finding is controversial (see 1.2.2). However, in the macaque study dilution of the binding signal due to the diffuse nature of the raphe nuclei was noted as a limitation by Shively *et al.* (2006); future studies may reveal if this represents a true interspecies difference.

1.4.2 Serotonergic Polymorphisms in Primates

In addition, rhesus macaques carry an analogous length variation of the 5-HTTLPR to that found in humans (Lesch *et al.*, 1997). Interestingly, a recent study did not detect the short-allele of the 5-HTTLPR in a small sample of the closely related cynomolgus macaque (all 12 monkeys were homozygous for the long-allele) (Bethea *et al.*, 2005). Two studies have examined the interaction between environmental and genetic factors on

CNS 5-HT function and on stress-related behaviour in rhesus macaques, using a model of differential early rearing (Bennett *et al.*, 2002; Champoux *et al.*, 2002; Lesch and Gutknecht, 2005). Infant macaques were either mother-reared or peer-reared, assessed by neurobehavioural testing, and later, as juveniles or adults, tested for CSF 5-HIAA levels. Effects of genotype and interactions between genotype and rearing were observed in behavioural tests. Heterozygous (L/S) monkeys demonstrated increased affective responding (such as aggressiveness) compared to homozygous L/L monkeys, and peer-reared but not mother-reared L/S macaques scored lower than their counterpart L/L macaques on orientation tests (Champoux *et al.*, 2002). Only peer-reared animals showed a genotype effect on CSF 5-HIAA levels, with lower concentrations detected in L/S compared to L/L monkeys, indicating that both the lower-activity S allele and early life experiences contribute to adult 5-HT functioning in macaques (Bennett *et al.*, 2002). The 5-HTTLPR has been associated with altered serotonergic function and affective traits in humans (see 1.3.5.5). Therefore, it may be that the role of genetic variation in the 5-HTT in determining in conjunction with environmental factors, individual differences in these characteristics, is evolutionarily conserved (Lesch, 2004).

Polymorphisms in the 5-HT_{1A} receptor of macaques have yet to be reported. Due to the benefits of using macaques as models of emotional regulation and psychopathology, as outlined above, the identification of SNPs in the macaque 5-HT_{1A} gene (analogous or not to those found in humans) would allow for further investigation of how these traits are influenced by genotype and genotype-environment interactions.

1.5 Hypotheses and Approaches

In order to further elucidate the mechanism of action of the current first-line therapy for depression, SSRI antidepressants, I will investigate the model hypothesizing that both SSRIs and 5-HT_{1A} receptor agonists act through the downregulation of serotonergic neuron 5-HT_{1A} autoreceptors. I hypothesize that treatment with the 5-HT_{1A} receptor agonist 8-OH-DPAT will decrease 5-HT_{1A} promoter activity as measured by reporter assays in a rat serotonergic cell line, and will also decrease the level of 5-HT_{1A} receptor mRNA transcription as measured by real-time quantitative PCR in rat primary raphe neurons.

While the role for genetics in the etiology of schizophrenia and depression is well established, the genes and variants that contribute to illness susceptibility and the mechanisms by which they do so are poorly understood. I hypothesize that functional (or potentially functional) polymorphisms in monoaminergic receptor genes such as the 5-HT_{1A} promoter C(-1019)G, the 5-HT_{2A} promoter A(-1438)G and the D2 Taq1A (C/T) SNPs, as well as the serotonin transporter 5-HTTLPR variants, may be associated with mental illnesses such as schizophrenia and depression, possibly through transcriptional dysregulation. I will genotype DNA samples from schizophrenic and major depressive disorder patients, as well as control sets of individuals, in order to investigate associations between the above allelic variants and these disorders.

Finally, the 5-HT_{1A} receptor plays a critical role in both normal and pathological behaviour and mood. Analyzing the 5-HT_{1A} promoter sequence of other primates may identify novel and potentially behaviourally-relevant polymorphisms and may also provide insight into the regulation of this receptor. I will accomplish this by manually

sequencing the 5-HT1A promoter of two species of macaque monkeys, focusing on the regions surrounding the known NUDR and Freud-1 regulatory elements, in order to identify any polymorphisms as well as interspecies differences in comparison to human and chimpanzee sequences. Furthermore, I will assess the ability of the NUDR and Freud-1 transcription factors to interact with their respective macaque elements by electrophoretic mobility shift assays.

CHAPTER 2 – MATERIALS AND METHODS

2.1 Cell Line Culture

Rat raphe RN46A cells (White *et al.*, 1994) and 5-HT1A stably-transfected DP8 cells derived from the RN46A line (Kushwaha and Albert, 2005) were cultured in Neurobasal medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Wisent), 1mM L-glutamine (Wisent) and 1% Pen-Strep (1000IU penicillin, 1000µg/mL streptomycin) (Wisent) at 33°C in 5% CO₂. In order to differentiate both the RN46A and DP8 cells, the culturing media was changed to F12/DMEM (Invitrogen) supplemented with 0.5mM L-glutamine, 1% N-2 Supplement (Invitrogen), 750mg/L pyruvic acid (Sigma), 55mg/L ovalbumin (Sigma) and 1% FBS for 5 days at 39°C in 5% CO₂, and media was changed daily. Upon differentiation, RN46A cells develop a neuronal morphology and the 5-HT1A receptor as well as other serotonergic markers are upregulated (White *et al.*, 1994; Eaton *et al.*, 1995).

2.2 Plasmids

The 5-HT1A promoter constructs were all previously designed in the Albert lab. Briefly, progressive 5' deletions of rat and human 5-HT1A promoter sequence were inserted upstream of the luciferase reporter gene in the promoterless pGL3-Basic vector (pGL3B, Promega) (Storring *et al.*, 1999, Lemonde *et al.*, 2004a). Specifically, rat -1174bp and -426bp and human -1128bp and -723bp luciferase constructs were assayed. Thus, constructs both contained and were deleted for the C(-1019)G SNP region

(applicable constructs contained the wildtype C-allele). Diagrammatic representations of the 5-HT1A promoter constructs are shown in Appendix I.

2.3 Transfection Assays

Differentiated RN46A or DP8 cells grown to ~50-75% confluence on Primaria 6-well plates (Falcon) were transfected with the indicated rat and human 5-HT1A promoter-luciferase constructs or as controls, the pGL3B and/or the SV40 promoter-containing pGL3-Promoter (pGL3P, Promega) vectors and pCMV- β gal vector. Specifically, for each 6-well transfection, 1 μ g of pCMV- β gal, 5 μ g of the promoter construct or control vector, 8 μ L of Plus Reagent (Invitrogen) and 150 μ L of Opti-MEM Media (Invitrogen) were combined and incubated at room temperature for 15min. Next, 6 μ L of Lipofectamine Transfection Reagent (Invitrogen) was combined with 150 μ L of Opti-MEM Media, and this was combined with the DNA mixture and incubated at room temperature for 15min. The differentiation media was replaced with 1mL of Opti-MEM media per well, and 100 μ L of the DNA/lipid transfection mixture was added to each well in the 6-well plate. Cells were then incubated at 37°C for 6hr for optimal transfection. The transfection media was then changed to the differentiation media. The media for half of the wells (3 wells per construct) contained 1 μ M 8-OH-DPAT for the differentiated RN46A cells, and 10 μ M 8-OH-DPAT for the differentiated DP8 cells; control wells contained only differentiation media as water is the carrier for 8-OH-DPAT. Cells were then incubated at 37°C for 48hr.

Cells from each well were collected on ice in 350 μ L of 1X phosphate buffered saline (PBS) and pelleted by centrifugation at 4°C for 5min at 5000rpm. The pellet was

dissolved in 200 μ L of 1X Reporter Lysis Buffer (Promega) and then frozen at -20°C overnight. The cell lysate was vortexed until clear, and cell debris was pelleted by centrifugation at 4°C for 10min at 16000rpm.

For the RN46A luciferase assay, 30 μ L of each lysate sample was aliquoted into a cuvette, and in the dark 100 μ L of 47 μ M luciferin (Molecular Probes) in luciferase buffer (20mM tricine, 2.67mM MgSO₄, 1.07mM (MgCO₃)₄Mg(OH₂)₅H₂O, 0.1mM EDTA, 33.3mM DTT, 270 μ M Co-enzyme A, 530 μ M ATP) was added, and luciferase activity was determined by measuring the luminescence for 10sec with the BioOrbit 1250 luminometer. β -galactosidase activity was determined for each sample by combining 30 μ L of the lysate with 30 μ L of 4-methylumbelliferyl β -D-galactoside (MUG) substrate (Sigma) in 15mM Tris-HCl, pH 8.8, and incubating the mixture at 37°C for 30min. The reaction was stopped by adding MUG stop solution (300mM glycine; 15mM EDTA, pH 11.2). After the addition of 2mL of Z-buffer (60mM Na₂HPO₄; 40mM NaH₂PO₄; 10mM KCl, 1mM MgSO₄), the conversion of the substrate to the fluorescent methylumbelliferone molecule was measure for each sample using the Perkin-Elmer LS50 spectrophotometer at 350nm excitation and 450nm emission.

For the DP8 luciferase assay, 30 μ L of each lysate sample was aliquoted into the wells of a 96 well Microlite 1+ microtiter plate (Thermo Labsystems). 100 μ L of luciferin in luciferase buffer was added to each well, and luciferase activity was determined by measuring the luminescence at 595nm with the SpectraMax M2 Multi-Detection Microplate Reader (Molecular Devices). β -galactosidase activity was determined for each sample by combining 30 μ L of the lysate with 200 μ L of chlorophenol red- β -D-galactopyranoside (CRPG) substrate (0.4mg/mL CRPG (CalBiochem) in 1X PBS with

0.26% β -mercaptoethanol and 1mM MgSO_4) in the wells of a 96 well Cell Culture Cluster polystyrene plate (Corning). The plate was incubated at 37°C for ~30min, or until the colorimetric reaction of the yellow CRPG being metabolized into phenol red had proceeded to the point that the wells were noticeably red, at which point the reaction was quantified using the L-MaxII Luminescence Microplate Reader (Molecular Devices).

The ratio of luciferase to β -galactosidase activity was then determined for the triplicate samples for both assays.

2.4 Primary Raphe Dissection and Culture

All animal work was in accordance with the experimental protocol (NSI-53) approved by the University of Ottawa animal care facility. Pregnant Sprague-Dawley rats (Charles River) were rendered unconsciousness under 5psi CO_2/O_2 , and then were euthanized by increasing CO_2 levels to 10psi and decreasing O_2 to 0psi. Untimed E14-15 or timed E14 embryos (as indicated) were removed via c-section and placed in Hank's Balanced Salt Solution without Calcium (HBSS w/o Ca^{2+}) (Wisent). Rostral raphe neurons were dissected according to Lautenschlager *et al.* (2000), with modifications as follows. Dissections were performed in HBSS w/o Ca^{2+} using a dissecting microscope (Zeiss KL 1500 LCD). The embryos' heads were severed and the meninges were removed. The rhombencephalon/diencephalon was separated from the head and the neural tube was opened ventrally and flattened. A ~1mm wide strip of midline tissue corresponding to the dorsal and median (B4-B9) raphe nuclei (König *et al.*, 1988) was then dissected out. Approximately 30 embryos from two rats were combined per culture. Collected raphe tissue was triturated in 5mL of HBSS w/o Ca^{2+} . The dissociated cells

were passed through a 70 μ M cell strainer (BD Biosciences) to eliminate large tissue fragments. To restore neuronal firing activity, 5mL of HBSS with Calcium (Wisent) was added to the cell filtrate. Cells were pelleted by centrifugation for 5min at 850rpm (Jouan C3i centrifuge, Perkin Elmer). The pellet was resuspended in 5mL of Neurobasal media containing 2% B27 supplement (Invitrogen), 1% Pen-Strep and 0.8mM L-glutamine. Cells were counted (Fuchs-Rosenthal counting chamber, Hausser Scientific) and plated at a density of 3 000 000 cells per well in poly-D-lysine (Sigma)-coated Primaria 6-well plates. Cells were cultured for 12 days at 37°C in 5% CO₂, with one-half the volume of media being changed every two days. In initial cultures, the mitotic-inhibitor arabinosylcytosine (AraC) (10 μ M) was added to the media in order to eliminate non-neuronal dividing cells. However, this treatment resulted in considerable neuronal cell death (AraC concentrations as low as 2 μ M were evaluated) and was discontinued.

The raphe cultures have recently been extensively characterized in our lab. After 7 days *in vitro* (DIV), about 7-8% of the cultured neurons were serotonergic as identified by TPH2 and 5-HT immunohistochemistry (the percent of positive cells did not change after 7 DIV). Less than 5% of the cultured cells were positive for glial fibrillary acidic protein (GFAP), a glial cell marker. In addition, the neuronal identity of the remaining cells was confirmed by staining for the neuron specific beta III Tubulin antibody-1 (TUJ-1) and Neuronal Nuclei (NeuN) markers. NeuN is a marker for post-mitotic neurons, and ~90% of neurons were NeuN positive, indicating that the cultured neurons have a mature phenotype at 7 DIV when treatments are initiated. In addition to serotonergic neurons, approximately 55% of these cells were glutamate decarboxylate (GAD) 65/67 positive, indicating a GABAergic phenotype, while tyrosine hydroxylase (TH), which is indicative

of catecholamine synthesis, was expressed in 0.9% of neurons (Czesak *et al.*, in preparation).

For the 5-HT_{1A}-agonist treatments, after 5 DIV, half of the wells received culture media containing 1 μ M 8-OH-DPAT (control wells received culture media alone as water is the vehicle for 8-OH-DPAT) for 24hr or 7 days as indicated, with media changes containing fresh drug (\pm 8-OH-DPAT) being performed every 48hr during the 7 day treatments for both treated and control cells. For the 5-HT_{1A}-antagonist treatments, after 7 DIV, half of the wells received culture media containing 1 μ M WAY-100635 (control wells received culture media alone as water is the vehicle for WAY-100635) for 5 days, with media changes (\pm WAY-100635) being performed every 48hr for both treated and control cells.

2.5 RNA Isolation, DNase Treatment and cDNA Synthesis

Wells containing cultured cells were examined under the microscope prior to RNA isolation to assess cell differentiation and death. The approximate cell density was used to determine the number of control, 8-OH-DPAT- or WAY-100635-treated wells which were pooled together per sample (the minimum number necessary to obtain an RNA pellet, between 2 and 4). Cultured cells were washed twice with 1X PBS to remove residual media. Total RNA was isolated from the cells using Trizol reagent (Invitrogen). Briefly, 200 μ L of Trizol was added to each well, and cells were incubated on ice for 5min. The Trizol mixture from like-treated wells was then pooled as necessary, and all samples were treated separately from this point. Samples were then incubated at room temperature with 40 μ L of chloroform per 200 μ L of Trizol for 2min, followed by cold

(4°C) centrifugation at 12 000g for 15min. The aqueous (top) layer containing the RNA was transferred to a new tube, while the organic protein-containing layer was discarded. The aqueous layer was mixed with 100µL of isopropanol per 200µL of Trizol and left overnight at -20°C to enhance RNA precipitation. After cold centrifugation at 12 000g for 15min, the supernatant was removed, and the RNA pellet was washed with 70% ethanol (in DEPC water) by vortexing followed by cold centrifugation at 7500g for 5min. After removing the supernatant, the pellet was air-dried for 10min on ice, and then was resuspended in ~20µL of DEPC water. The sample was dissolved by 10min incubation at 55°C and then was stored at -80°C. RNA concentration and purity were determined by spectroscopy (DU 640 Spectrophotometer, Beckman). RNA concentration was calculated using the equation: absorbance at 260nm (A₂₆₀) X dilution factor X RNA absorbance factor (40). Purity was assessed by the A₂₆₀/A₂₈₀ ratio.

Samples were DNase treated using TURBO DNA-*free* reagent (Ambion). Briefly, the RNA samples (to a maximum of 5µg) were brought to 24µL with nuclease free water. Next, 2.8µL of 10X TURBO DNase buffer and 1µL TURBO DNase (2U/µL) was added, and the mixture was incubated at 37°C for 1hr. The DNase was then inactivated by adding 3µL DNase Inactivation Reagent, and incubating at room temperature for 2min with frequent vortexing. The inactivating reagent and enzyme were pelleted by centrifugation at 10 000g for 1.5min, followed by the removal of supernatant (DNase-treated RNA). The RNA concentration and purity were again determined as above.

Reverse-transcription (RT) of the DNase-treated RNA to cDNA was performed using the Cells-to-cDNA II kit (Ambion). For each sample, both an RT+ and RT- reaction was performed, in order to evaluate the amount of genomic DNA still remaining in the

DNase-treated RNA. This control was particularly important as the 5-HT1A gene is intronless, and thus cDNA specific primers cannot be designed. For all reactions, 0.5µg of RNA, 2µL of 2.5mM dNTPs, and 1µL of 50µM random decamer primers were brought to a final volume of 8µL with nuclease-free water. The mixture was heated at 70°C for 3min, cooled on ice for 1min, and then 1µL of 10X RT Buffer, 0.125µL of 40U/µL RNase Inhibitor and 0.125µL of 40U/µL M-MLV Reverse Transcriptase (or nuclease-free water for RT- control) were added. The mixture was then incubated at 42°C for 1hr, followed by incubation at 95°C for 10min to inactivate the RT enzyme. The cDNA samples were stored at -20°C.

2.6 Quantitative Real-Time PCR (Q-PCR) Analysis

Quantification of 5-HT1A expression levels in the control, 8-OH-DPAT- or WAY-100635-treated cDNA samples was performed by multiplex Q-PCR analysis using TaqMan (Applied Biosystems) chemistry. For each sample, 1µL of cDNA, 5µL of TaqMan Universal PCR Master Mix, 0.5µL of rat 5-HT1A FAM-labeled probe (TaqMan Gene Expression Assay, Rn00561409), 0.5µL of rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) VIC-labeled probe (TaqMan Endogenous Control, 4352338E), and 3µL of nuclease-free water were combined. All samples were run in duplicate. The Q-PCR run was performed using the Rotor-Gene 3000 Cycler (Corbett Research). The thermocycler program was as follows: 95°C for 10min (enzyme activation step); 40 cycles: 95°C for 15sec, 60°C for 1min; hold at 25°C. Additionally, duplicate no template controls (nuclease-free water instead of cDNA) were run in order to detect contamination of the reaction mixture. A sample of cDNA from the 5-HT1A stably-transfected DP8

cell line was run in duplicate as a standard. All Q-PCR data was analyzed using Rotor-Gene software (version 6) using the Quantitation Analysis function. Standard curves were generated for both 5-HT1A and GAPDH using serial dilutions of DP8 cDNA. The 5-HT1A expression levels were obtained by normalizing the concentration obtained to the house-keeping gene GAPDH for each duplicate of all samples. Any sample with significant amplification in its RT- control (concentration >1% that of the of the RT+ sample) was eliminated. The duplicates for each sample were averaged, and the averages of different treatment conditions were then determined as indicated.

2.7 Clinical Samples

The Canadian MDD and control blood samples were obtained as described in Lemonde *et al.* (2003). Briefly, the control samples were obtained from normal healthy volunteers with no personal or family history of mental illness either randomly selected or recruited by advertising. MDD patients were attending the Royal Ottawa Hospital Psychopharmacology Unit as described in Du *et al.* (2000). All patients were diagnosed using the DSM-IV criteria for major depression and bipolar patients were excluded. All had scores of 18 or greater on the 17 item Hamilton Rating Scale for Depression (HAM-D), which indicates at least moderately severe depression. The 182 depressed patients were 59% female, age 43 ± 10 years, and 96% Caucasian; the 213 controls were 57% female, age 35 ± 12 years, and 94% Caucasian. All genotype distributions of the samples were in Hardy-Weinberg equilibrium.

Russian control and schizophrenic DNA samples were obtained from Dr. Evgeny Rogaev (University of Massachusetts Medical School, Worcester, Massachusetts).

Controls had no previous personal or family history of mental illness, while schizophrenic subjects were recruited from a psychiatric hospital in Moscow (Research Center of Mental Health (RCMH) of the Russian Academy of Medical Sciences) and were diagnosed using both the International Classification of Disorders 10 and DSM-III-R criteria for schizophrenia as described in Chumakov *et al.* (2002) and Golimbet *et al.* (2004). The 151 schizophrenic patients were 53% female, age 31 ± 10 years; the 160 control individuals were 42.5% female, age 36 ± 9 years. All genotype distributions of the samples were in Hardy-Weinberg equilibrium.

Canadian control and schizophrenic blood samples were obtained from Dr. Paul Roy (Department of Psychiatry, University of Ottawa) and as part of an ongoing collaboration. The patients are all first-episode schizophrenics and were not medicated at the time of recruitment. Thus far, only 13 control and 12 schizophrenic individuals are included in the study (recruitment is ongoing). All genotype distributions of the samples were in Hardy-Weinberg equilibrium.

Post-mortem human brain samples (n=277) were obtained from Dr. Craig Stockmeier (Department of Psychiatry, University of Mississippi Medical Center, Jackson, Mississippi). The subjects were either diagnosed with a mental illness or were healthy controls, and their brains have been characterized for morphology and a variety of markers; however, our lab was blinded to the identity of the samples.

2.8 DNA Isolation from Human Blood Samples

DNA from the Russian schizophrenic and control blood samples was previously isolated (Golimbet *et al.*, 2004). DNA from a portion of the Canadian MDD and control

blood samples was also previously prepared (Lemonde *et al.*, 2003). DNA was isolated from the remaining Canadian MDD and control blood samples, as well as all of the Canadian schizophrenic and control blood samples, using the QIAamp DNA Blood Mini Kit (Qiagen). The protocol was followed exactly, with the final elution being performed with 100 μ L of buffer AE. All DNA samples were stored at -20°C.

2.9 DNA Isolation from Human Brain Samples

DNA was isolated from the brain tissue using the QIAamp DNA Mini Kit (Qiagen). The protocol was followed exactly, starting with 25mg of frozen tissue and eluting the DNA with 50 μ L of buffer AE. All DNA samples were stored at -20°C.

2.10 Genotyping of 5-HT1A, 5-HT2A and D2 SNPs

Genotyping of all DNA samples for these SNPs was performed by Q-PCR using the following TaqMan SNP Genotyping Assays (Applied Biosystems): 5HT1A C(-1019)G, C_11904666_10; 5-HT2A A-(1438)G, C_8695278_10; D2 Taq1A (C/T), C_7486676_10. The entire Russian schizophrenic (n=151) and control (n=160) DNA sample sets were genotyped for all three SNPs, as were all of the Canadian schizophrenic (n=12) and control (n=13) DNA samples. All of the Canadian MDD (n=182) and control (n=213) DNA samples were genotyped for the 5-HT1A and 5-HT2A SNPs; as no trend towards an association was evident with the D2 SNP, genotyping was terminated at 155 MDD individuals and 129 controls. All human brain DNA samples were genotyped for the 5-HT1A SNP. The genotyping reaction was carried out in a 10 μ L final volume of reaction mixture containing: 1 μ L of DNA, 5 μ L of TaqMan Universal PCR Master Mix,

0.5 μ L of the appropriate 20X TaqMan SNP Genotyping Assay Mix, and 3.5 μ L of nuclease-free water. Additionally, duplicate no template controls (nuclease-free water instead of DNA) were run in order to detect contamination of the reaction mixture. The Q-PCR run was performed using the Rotor-Gene 3000 Cycler (Corbett Research). The thermocycler program was as follows: 95°C for 10min (enzyme activation step); 40 cycles: 92°C for 15sec, 60°C for 1min; hold at 25°C. Genotypes were then determined using the Allelic Discrimination function of the Rotor-Gene software (version 6). A diagrammatic explanation of TaqMan-based SNP genotyping and a sample genotype amplification plot are shown in Appendix II. The 5-HT1A assay was validated by genotyping 20 samples previously analyzed by manual sequencing (Lemondé *et al.* 2003). The accuracy of the 5-HT2A and D2 assays were verified with 6 samples for each by PCR amplification of the SNP region followed by enzymatic digestion, using the protocols described in Spurlock *et al.* (1998) and Grandy *et al.* (1993)(Appendix III). In all cases, the TaqMan assay genotype corresponded with the genotype determined by manual sequencing or enzymatic digestion.

2.11 Genotyping of the 5-HTTLPR

Genotyping of the human brain samples for the 5-HTTLPR was performed using a standard PCR-based protocol, modified from Heils *et al.* (1996). Briefly, oligonucleotide primers flanking the 5-HTTLPR (sense, 5'-GAG GGA CTG AGC TGG ACA AC and antisense, 5'-GCA GCA GAC AAC TGT GTT CAT C) were used to amplify the 585bp (S) and/or 629bp (L) fragments. PCR amplification was carried out in a final volume of 25 μ L, with the reaction mixture containing: 2 μ L of DNA, 2.5 μ L of 10X PCR Buffer

(Invitrogen), 0.5 μ L of 10mM dNTPs (Invitrogen), 0.5 μ L of 100pmol/ μ L sense and antisense primers (IDT), 0.4 μ L of 50mM MgCl₂ (Invitrogen) and 0.25 μ L of 2U/ μ L Phusion High-Fidelity DNA Polymerase (Finnzymes). The amplification was performed using the Peltier Thermal Cycler PTC-200 (MJ Research) as follows: 98°C for 10min (enzyme activation step); 50 cycles: 98°C for 30sec, 61°C for 45sec; 72°C for 1min, 84°C for 10sec; 72°C for 10min; hold at 4°C. Samples were run on a 2% agarose gel containing ethidium bromide and amplification products were visualized under ultraviolet (UV) light.

2.12 Dideoxy Sequencing of Macaque 5-HT1A Promoter

Rhesus macaque (*Macaca mulatta*) (n=20) and cynomolgus macaque (*M. fascicularis*) (n=13) genomic DNA extracts were obtained from Dr. Cynthia Bethea (Oregon National Primate Research Center, Beaverton, Oregon). The rhesus and cynomolgus macaque 5-HT1A promoters were amplified between -879bp and -1709bp (relative to the human ATG) using the primers: upper1Apro: 5'-CTT GTC TTT TAA TAA CTG TCT TCC TCT-3' and lower1Apro: 5'-TTC TTA AAT CGT GTC AGC ATC C-3'. The amplified 831bp region included both the NUDR-binding site (containing the C(-1019)G SNP) and the tandem DREs which bind FREUD-1. PCR amplification was carried out in a final volume of 50 μ L, with the reaction mixture containing: 2 μ g of DNA, 10 μ L of 5X High Fidelity PCR Buffer (Finnzymes), 1 μ L of 10mM dNTPs, 1 μ L of 100pmol/ μ L of each primer and 0.5 μ L of 2U/ μ L Phusion High-Fidelity DNA Polymerase. The amplification was performed using the Peltier Thermal Cycler PTC-200 as follows: 98°C for 10min (enzyme activation step); 38 cycles: 98°C for 30sec, 62°C for

45sec; 72°C for 1min, 82°C for 30sec; 72°C for 10min; hold at 4°C. Samples were run on a 1% agarose gel containing ethidium bromide and amplification products were visualized and the 831bp band was excised under ultra-violet (UV) light. The gel bands were purified using the GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences). The protocol was followed exactly, with the DNA being eluted in a final volume of 50µL of nuclease-free water. The purified PCR products were then manually sequenced using the T7 Sequencing Kit (Pharmacia Biotech), with the following modifications. Sequence from -962 to -1121 and from -1468 to -1640 relative to the ATG codon was obtained using the above two primers. Briefly, to anneal the primer to the template, 14µL of the purified PCR product was boiled for 2min, followed by flash-freezing in a dry-ice/ethanol bath for 1min. Immediately, 2µL of annealing buffer (200mM Tris-HCl pH 7.5, 100mM MgCl₂, 250mM NaCl) and 1µL of one of the above primers (reactions are done separately with each primer) were added, and the mixture was spun down briefly, and then incubated at RT for 30min. For the labeling reaction, 2µL of diluted T7 DNA Polymerase (0.5µL of the 8U/µL T7 DNA Polymerase diluted with 2µL Enzyme Dilution Buffer), 3µL of Labeling Mix-dCTP (containing dATP, dGTP, and dTTP), and 0.5µL of 250µCi/µL ³²P-labeled dCTP (1µL if not fresh), were incubated with the annealed template/primer for 45sec at room temperature. For the termination reaction, tubes with 2.5µL of the 'G', 'A', 'T' and 'C' Mix-Short (containing the indicated dideoxynucleotide and non-limiting concentrations of all four dNTPs) were pre-warmed at 37°C. Next, 4.5µL of the labeling reaction mixture was added to each of the dideoxynucleotide mixes, and the tubes were incubated at 37°C for 5min. Finally, 5µL of Stop Solution (containing formamide and DNA loading dyes) was added to the

sequencing reactions, which were then frozen at -20°C. The sequencing reaction products were then electrophoresed on a thin 6% polyacrylamide gel under denaturing conditions. The gel was pre-warmed at 65W for 45min, and then the samples were run at 70W for 2-3hr (depending on the region being examined). The gel was then dried, and autoradiography was used to detect the separated fragments using BioMax MR Film (Kodak) exposed at room temperature for 24-72hr. DNA sequences were read manually.

2.13 Cloning and Automated Sequencing of Macaque 5-HT1A Promoter

The purified PCR products obtained from two of the cynomolgus macaque DNA samples were subcloned into the pGEM-T-Easy Vector (Promega), transformed into DH5 α *Escherichia coli*, and several positive colonies were selected by blue/white screening following the manufacturer's instructions. Colonies were cultured and plasmid DNA was isolated using the Wizard SV Miniprep Kit (Promega) according to the manufacturer's instructions. Samples were sequenced by the Ontario Genomics Innovation Centre using the T7 Promoter, according to their specifications.

2.14 Electromobility Shift Assay (EMSA)

EMSAs were performed as described in Ou *et al.* (2000) with minor modifications. Briefly, for the NUDR element EMSAs, complementary oligonucleotides corresponding to the human -1019 C-allele (26bp-C: 5'-GGA ACG AAG ACA CAC TCG GTC TTC TT-3'), the human -1019 G-allele (26bp-G: 5'-GGA AGG AAG ACA CAC TCG GTC TTC TT-3') and the monkey A-deletion (25bp-M: 5'-GGA ACG A-G ACA CAC TCG GTC TTC TT-3'), and for the Freud-1 element EMSAs, complementary oligonucleotides

corresponding to the human 5'DRE (5'HDRE: 5'-AGA TGG CAC TCT AAA ACA TTT GCC AGA-3'), the human 3'DRE (3'HDRE: AGG TGG CGA CAT AAA ACC TCA TTG CTT AGA ACT-3'), the macaque 5'DRE (5'MDRE: 5'-AGA CGG CAC TCT AAA ACA TTT GCC AGA-3') and the macaque 3'DRE (3'MDRE: AGG TGG CGA CAT AAA CCC TTA TTG CTT AGA GCT-3') were annealed by boiling for 10min, and then gradual cooling to room temperature. The GG-overhangs on the NUDR oligonucleotides, and the 5' and 3' overhangs on the Freud-1 oligonucleotides (the upper strand of each was labeled with a 5' SacI site and a 3' NheI site, not shown) were labeled with [³²P]α-dCTP (150μCi per reaction) using 2.5U Klenow fragment DNA polymerase (New England Biolabs) and purified using Sephadex G-50 beads. The appropriate protein (bacterially expressed, Ni-NTA agarose bead-purified human NUDR (3μg) or human Freud-1 (2μg)) (obtained from Sylvie Lemonde and Anastasia Rogaeva) or water for no-protein control, was incubated for 10min at room temperature with 0.25μg of Herring sperm DNA, and the maximum volume of DNA binding buffer (20mM HEPES, 0.2mM EDTA, 0.2mM EGTA, 100mM KCl, 5% glycerol and 2mM DTT) for an end reaction volume of 25μL. Next, specific (unlabeled probes) or unrelated competitor DNA (for NUDR EMSA, PEA3: 5'-GGG ATC CAG GAA GTG A-3'; for Freud-1 EMSA, E2F: 5'-CGG CAT AAG CAA GCC CTT ATT GCA CAG AGC T-3') at 50-fold excess was incubated with the appropriate reactions for 20min at room temperature. Finally, the labeled probes (50 000 cpm/sample) were added to the mixtures for 30min. Samples were then electrophoresed on a 5% native polyacrylamide/Tris glycine gel at 4°C. The gel was pre-warmed at 150V for 30min, and then the samples were run at 182V

for ~2hr. The gel was then dried, and autoradiography was used to detect the separated fragments using BioMax MR Film (Kodak) exposed with intensifying screens at -80°C.

2.15 Statistical Analysis

All statistical analyses were performed as indicated using GraphPad Prism software using 95% confidence intervals. For transfection assays and Q-PCR analysis, unpaired *t*-tests with two-tailed *p* values were used to compare treated versus control data sets. Genotype frequencies were compared by χ^2 analysis with two-tailed *p* values; allele frequencies were compared by Fisher's exact test with two-tailed *p* values.

CHAPTER 3 – RESULTS

3.1 Effect of 5-HT1A Agonist Treatment on 5-HT1A Expression

3.1.1 Effect of 5-HT1A Agonist Treatment on 5-HT1A Promoter Construct Transfection Assays

Previous studies indicate that chronic treatment with either SSRIs or 5-HT1A agonists desensitizes 5-HT1A receptors (see 1.2.4). In order to test whether 5-HT1A autoreceptor stimulated downregulation of 5-HT1A receptor gene expression could be a component of its desensitization (see 1.2.5), the action of chronic 5-HT1A agonist 8-OH-DPAT treatment on 5-HT1A promoter activity was tested using human or rat 5-HT1A promoter-luciferase constructs. Specifically, two constructs derived from the rat 5-HT1A promoter, beginning at -1174bp and -426bp from the initial ATG start codon (Storring *et al.*, 1999), and two human 5-HT1A promoter constructs starting at -1128bp and -723bp from the ATG (Lemondé *et al.*, 2004a) were examined (see Appendix I).

As described in 1.2.6, the rat 5-HT1A promoter contains a brain-specific transcription initiation site -967bp from the start codon, which is driven by a TATA box element 58bp upstream. Transcription is enhanced by a large region located between -1519bp and -426bp containing CCAAT, Sp1 and AP-1 transcription factor binding elements. In addition, TATA box independent transcription can be initiated from a C-G rich region containing AP-1 elements found between -426bp and -117bp (transcription from this region may not normally occur *in vivo*) (Storring *et al.* 1999). Additionally, NFκB response elements located at -64bp and -365bp enhance 5-HT1A transcription (Wissink *et al.*, 2000). Therefore, the -1128bp construct contains many of these enhancer

elements; most importantly, the TATA box and initiation site, while the -426bp construct contains only a few elements which drive alternate start sites. Conversely, the human 5-HT1A promoter does not contain a TATA box driven transcription start site. Instead, transcription appears to be initiated from multiple C-G rich sites which extend over 700bp upstream of the ATG. A ~200bp region flanking the start codon contains several binding sites for the MAZ and Sp-1 transcriptional activators. Also, the upstream NF κ B element is conserved in the human sequence (Parks and Shenk, 1996). Additionally, the human 5-HT1A gene is repressed by NUDR, which binds to a 26bp palindrome containing the C(-1019)G SNP (Lemonde *et al.*, 2003). Therefore, the human -1128bp construct, but not the -723bp construct contains the NUDR repressor element (with the C-allele) while both constructs contain most of the transcription initiation sites. It is important to note that although NUDR represses the rat 5-HT1A gene, its exact binding site in the rat sequence is still to be determined (Lemonde *et al.*, 2003). However, a putative binding site has been located in a region homologous to the human sequence, and thus is only in the rat -1174bp construct.

Initial experiments were luciferase/ β -galactosidase transfection assays performed using differentiated RN46A cells as well as in differentiated DP8 cells. These rat serotonergic cell lines provide a model for pre-synaptic 5-HT1A receptor gene regulation. RN46A cells were derived from medullary raphe cells dissected from Sprague-Dawley rat E13 embryos. The cells were then immortalized by infection with a retrovirus coding for a temperature-sensitive mutant of the SV40 large T-antigen. At 33°C, the cells are mitotically active, while at the non-permissive temperature of 39°C, the cells stop dividing and undergo differentiation, developing a neuronal morphology. While 5-HT

immunoreactivity is only detectable in differentiated RN46A cells, both undifferentiated and differentiated cells express the 5-HT1A autoreceptor, and its expression as well as that of other serotonergic markers is up-regulated upon differentiation (White *et al.*, 1994; Eaton *et al.*, 1995). DP8 cells are previously generated RN46A cells stably transfected with the rat 5-HT1A receptor (Kushwaha and Albert, 2005). These cells express considerably higher amounts of the receptor than wildtype RN46A cells, and thus may show a greater response to agonist treatment.

No significant effect of 8-OH-DPAT treatment was found on the promoter activity of any 5-HT1A promoter construct in either cell line (Figure 3.1). When comparing the activity of the different constructs, the SV40 promoter containing pGL3P control showed high levels of expression compared to the promoterless pGL3B and also to all of the rat and human 5-HT1A promoter constructs. The human constructs showed somewhat greater transcriptional activity than the rat constructs, although they were all within approximately one order of magnitude of each other. In addition, all 5-HT1A promoter constructs showed higher expression levels than the basal pGL3B vector (data not shown for activity in differentiated RN46A cells). The TATA box containing rat -1174bp construct showed significantly greater activity than the TATA box deleted -426bp construct in both cell lines. Interestingly however, the human NUDR element containing -1128bp construct and NUDR element deleted -723bp construct were each more active in the differentiated RN46A and DP8 cells respectively. This result was consistent among several experiments.

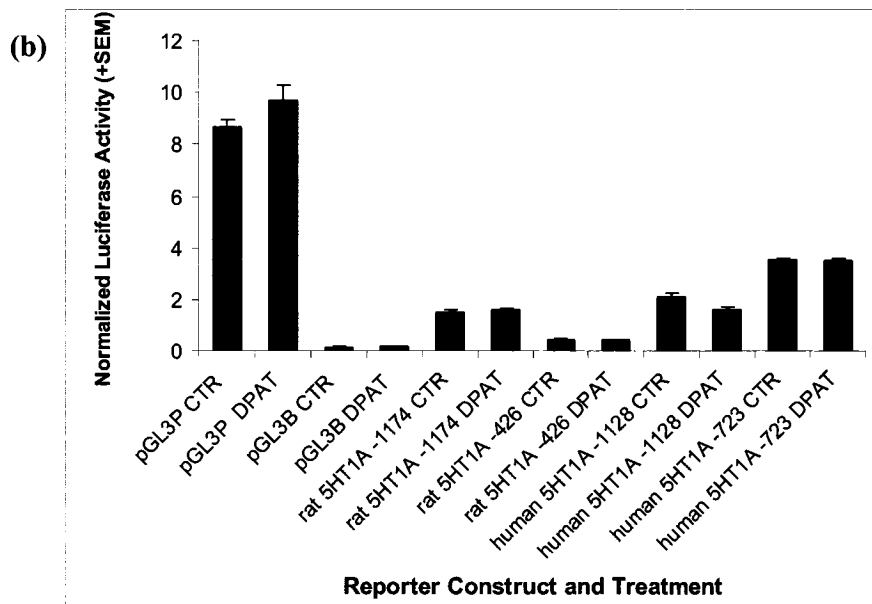
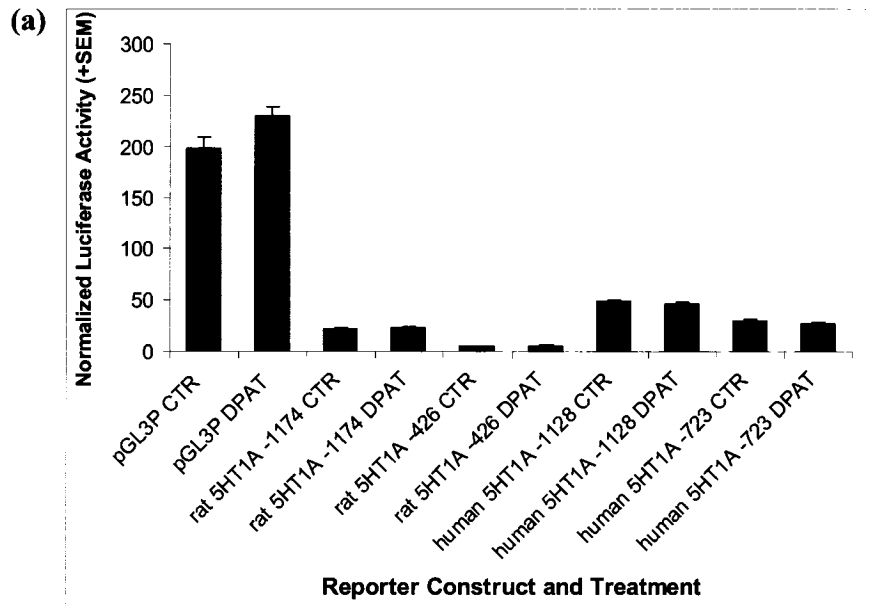


Figure 3.1. Effect of 5-HT1A agonist treatment on 5-HT1A receptor promoter activity in rat serotonergic cell lines.

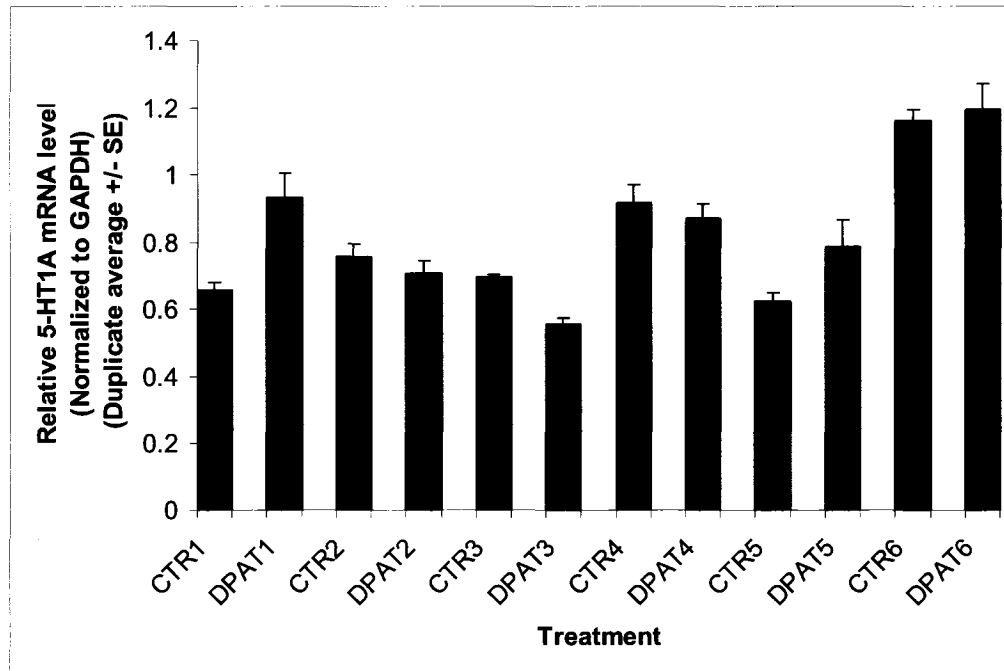
Cells were co-transfected with the indicated rat and human 5-HT1A promoter-luciferase constructs or vector controls (pGL3P or pGL3B) and pCMV- β gal. **(a)** Differentiated RN46A (wildtype) cells were cultured in 1 μ M 8-OH-DPAT (DPAT) or control media (CTR) for 48hr post-transfection. **(b)** Differentiated DP8 (rat 5-HT1A-stably transfected RN46A) cells were cultured in 10 μ M 8-OH-DPAT (DPAT) or control media (CTR) for 48h post-transfection. **(a, b)** Luciferase activity is normalized to β -galactosidase activity for each sample. Data are presented as mean + SE of triplicate transfections per experiment. Differences are not significant for any 5-HT1A promoter construct. Differences in scale between (a) and (b) are due to equipment changes (see 2.3). Results are representative of two independent experiments.

3.1.2 Effect of 5-HT1A Agonist Treatment on 5-HT1A Gene Expression in Raphe Primary Cultures

As no effect of 8-OH-DPAT on 5-HT1A promoter activity was observed, the effect of 8-OH-DPAT on 5-HT1A gene transcription was examined by Q-PCR in order to evaluate the hypothesis of 5-HT1A induced downregulation of 5-HT1A expression in a different experimental paradigm. Initially, differentiated RN46A cells were utilized. However, after months of trouble-shooting, including comparing two different Q-PCR chemistries (SYBR-green DNA-binding dye and TaqMan hydrolysis probes), several DNase treatment and cDNA synthesis kits, and various other optimization strategies, consistent levels of 5-HT1A mRNA were not detectable in differentiated RN46A cells. 5-HT1A expression is readily detectable in DP8 cells; however, as its expression is primarily plasmid-based, the effects of regulators on endogenous 5-HT1A mRNA levels cannot be assessed in these cells. Thus, primary cultures of E14-15 rat raphe neurons were utilized, as 5-HT1A expression is easily detectable in those cells. A representative Q-PCR run demonstrating the relative 5-HT1A expression levels of differentiated RN46A cells, differentiated DP8 cells, and cultured raphe neurons is illustrated in Appendix IV.

As shown in Figure 3.2 and Figure 3.3, no effect of 8-OH-DPAT treatment on 5-HT1A expression was detected for either the short-term 24hr or the long-term 7 day time course. Although individual sample sets show differences upon treatment, these were not consistent, and never exceeded a 2-fold effect. Some of this variability may be due to differences in the age of the embryos cultured (although the rats were ordered to be 14 to 15 days pregnant at the time of dissection, they were untimed, and the age of the embryos

(a)



(b)

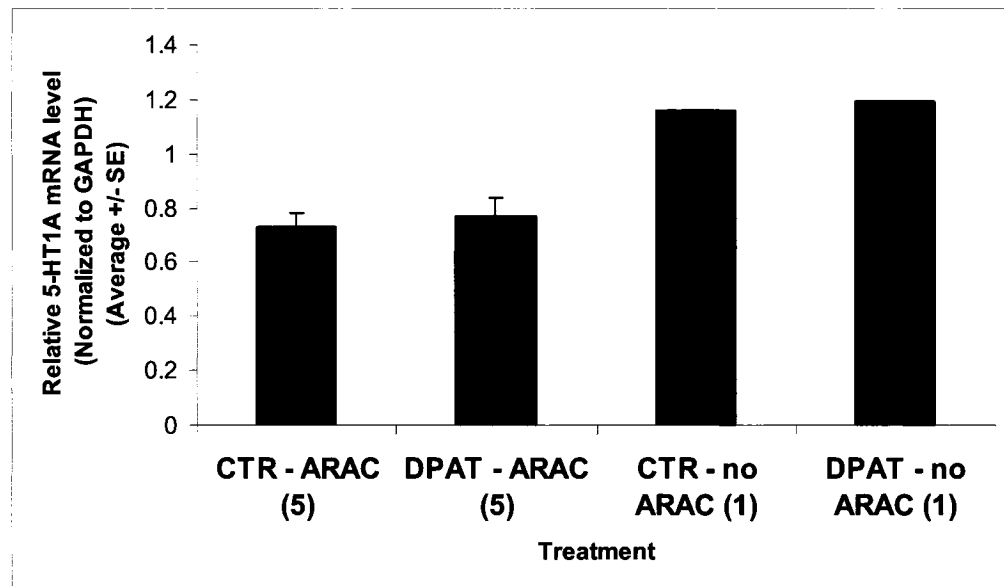


Figure 3.2. Effect of short-term 5-HT1A agonist treatment on 5-HT1A mRNA levels in primary raphe cultures.

E14-15 rat raphe neurons cultured 5 DIV were treated with 1 μ M 8-OH-DPAT for 24hr (DPAT); media was changed for control cultures (CTR). Treatment of cultures with 10 μ M AraC is indicated (ARAC). RNA extraction was performed using Trizol, followed by DNase treatment and reverse-transcription to generate cDNA. 5-HT1A mRNA levels were measured by Q-PCR using TaqMan probes, and normalized to GAPDH mRNA levels measured in multiplex. (a) Values for duplicates of each sample. (b) Combined data for AraC and non-AraC treated cultures. Number of samples is indicated in brackets. Data are presented as mean \pm SE. Differences are not significant for combined data.

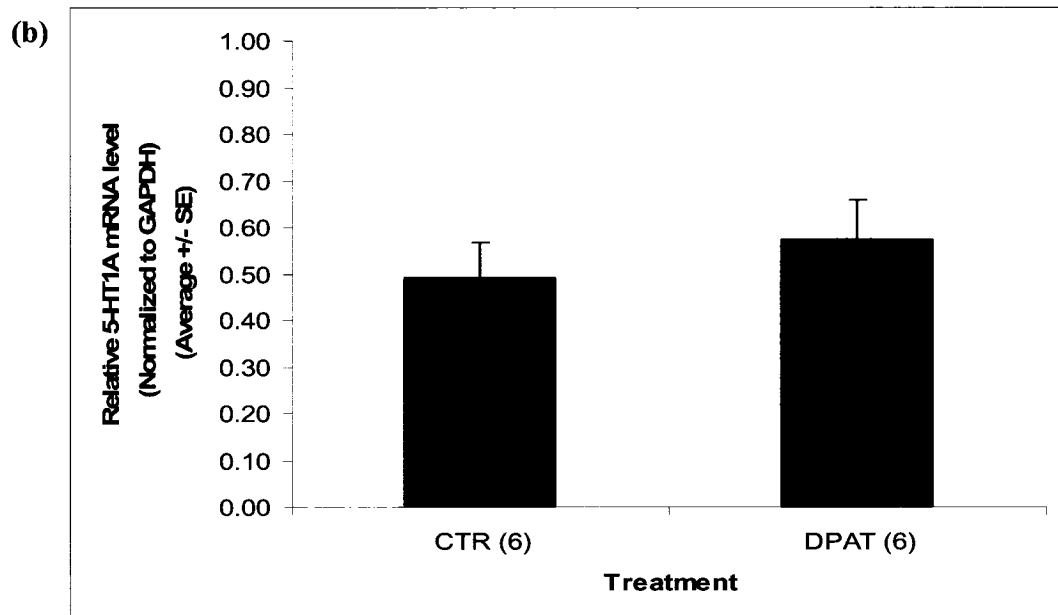
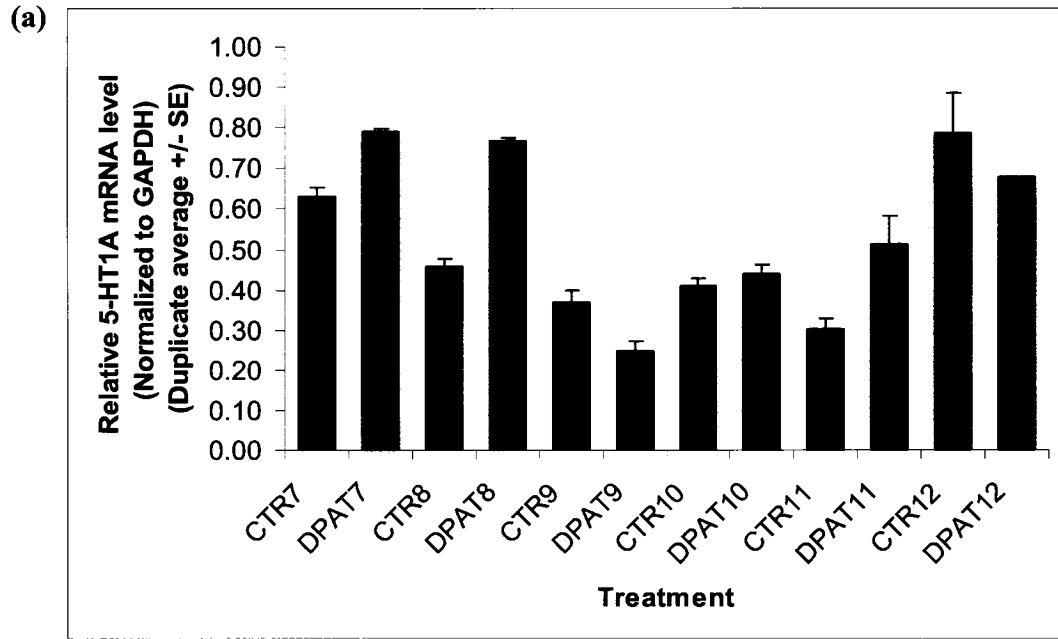


Figure 3.3. Effect of long-term 5-HT1A agonist treatment on 5-HT1A mRNA levels in primary raphe cultures.

E14-15 rat raphe neurons cultured 5 DIV were treated with 1 μ M 8-OH-DPAT for 7 days (DPAT); media was changed for control cultures (CTR). RNA extraction was performed using Trizol, followed by DNase treatment and reverse-transcription to generate cDNA. 5-HT1A mRNA levels were measured by Q-PCR using TaqMan probes, and normalized to GAPDH mRNA levels measured in multiplex. **(a)** Values for duplicates of each sample. **(b)** Combined data for all cultures. Number of samples is indicated in brackets. Data are presented as mean \pm SE. Differences are not significant for combined data.

was not always consistent), the relative cell density and cell viability (although the same number of cells were plated in each experiment, cell death varied considerably from culture to culture for unknown reasons). However, no consistent pattern was observed between the effect of 8-OH-DPAT in a particular sample set and any of these factors (data not shown).

In addition, AraC treatment killed a large percentage of the cultured cells. As the vast majority of the cultured cells were neurons (see 2.4) this was probably not due to the elimination of non-neuronal cells. Because serotonergic subgroups emerge and enlarge until E19 (Aitken and Törk, 1988; König *et al.*, 1988), it is likely that AraC, which targets mitotically-active cells, was toxic to the expanding neuronal population in the cultures. The stress on the surviving neurons due to this treatment may explain why 5-HT1A levels were lower in the AraC cultures (Figure 3.2).

The lack of effect of 8-OH-DPAT may be in part due to culturing conditions, as addition of the neuronal growth supplement B27 to the media results in a substantially higher than physiological concentration of L-tryptophan (80 μ M). The high levels of 5-HT then synthesized by the TPH2 enzyme may result in neuronal activity independent efflux of 5-HT from the cells (Liu *et al.*, 2005). This might mimic the addition of the 5-HT1A agonist in both control and treated cells, thus negating any effect.

This issue was addressed by examining the effect of a 5-HT1A antagonist (WAY-100635) on 5-HT1A gene expression in the raphe cultures. WAY-100635 had no effect on 5-HT1A expression (Figure 3.4). Though only two samples from one culture were analyzed in this experiment, no effect was seen on 5-HT1A levels in cDNA samples from three separate cultures (generously provided by Federico Remes Lenicov) (Appendix V).

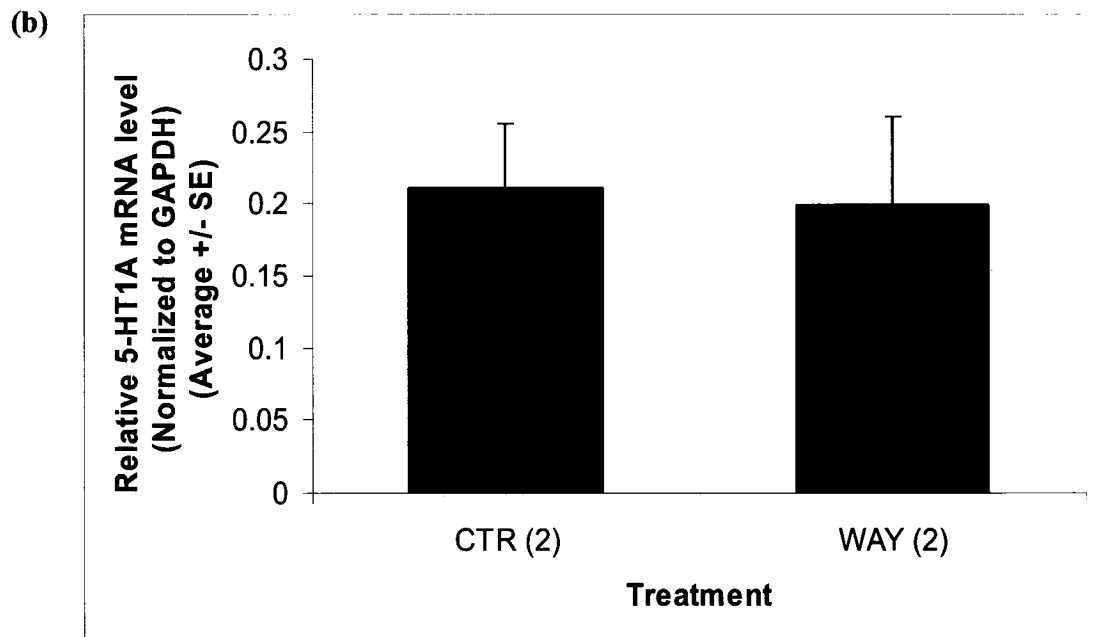
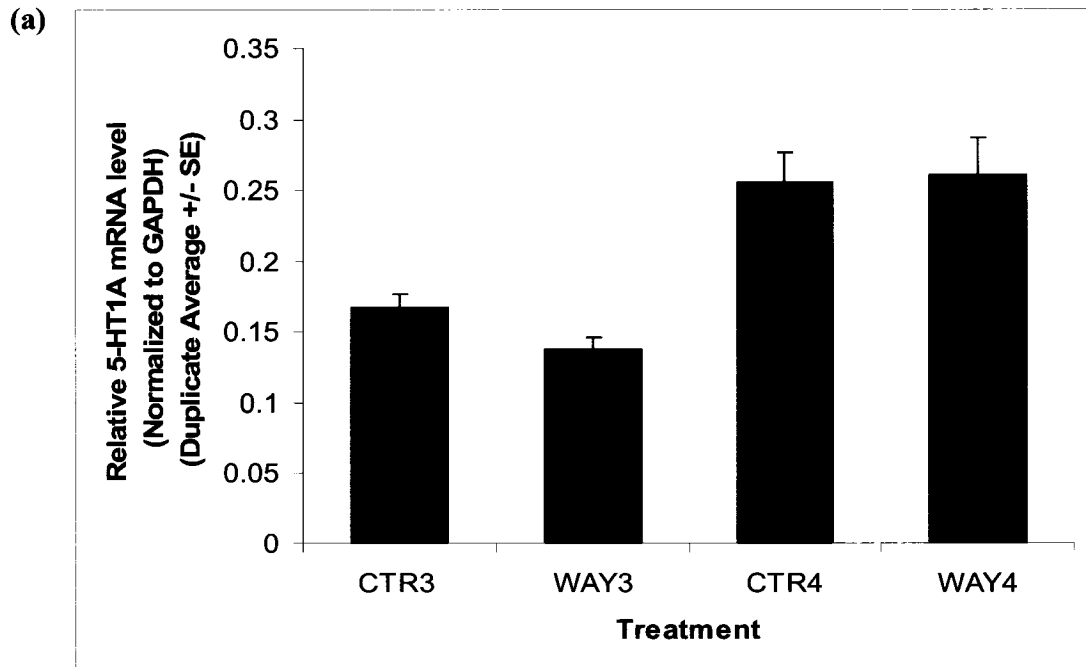


Figure 3.4. Effect, of long-term 5-HT1A antagonist treatment on 5-HT1A mRNA levels in primary raphe cultures.

E14-15 rat raphe neurons cultured 7 DIV were treated with 1 μ M WAY-100635 for 5 days (WAY); media was changed for control cultures (CTR). RNA extraction was performed using Trizol, followed by DNase treatment and reverse-transcription to generate cDNA. 5-HT1A mRNA levels were measured by Q-PCR using TaqMan probes, and normalized to GAPDH mRNA levels measured in multiplex. **(a)** Values for duplicates of each sample. **(b)** Combined data for all cultures. Number of samples is indicated in brackets. Data are presented as mean \pm SE. Differences are not significant for combined data.

3.2. Association Analysis of 5HT1A, 5-HT2A and D2 Receptor Polymorphisms with Schizophrenia and Major Depressive Disorder

In order to further understand the genes and variants that contribute to schizophrenia and depression susceptibility, polymorphisms in monoaminergic receptor genes, specifically the 5-HT1A promoter C(-1019)G, the 5-HT2A promoter A(-1438)G and the D2 Taq1A (C/T) SNPs, as well as the serotonin transporter 5-HTTLPR variants were examined for association with these neuropsychiatric illnesses. These particular polymorphisms were chosen based on previous reports indicating their functionality: the 5-HT1A promoter G-allele prevents binding of the transcriptional repressor NUDR and has been associated with increased 5-HT1A binding potential in the DR (see 1.2.6 and 1.3.5.1); the 5-HT2A promoter G-allele results in decreased *in vitro* promoter activity and may be associated with decreased allelic expression and 5-HT2A binding levels (see 1.3.4.2); the D2 T-allele is associated with lower D2 receptor density (see 1.3.4.3); and the 5-HTTLPR L-allele may result in higher 5-HTT promoter activity, gene expression, and 5-HT uptake levels (see 1.3.5.5). In addition, there is some evidence supporting an association with schizophrenia and depression for each polymorphism (see 1.3.4.1-3 and 1.3.5.1-5), potentially resulting from transcriptional dysregulation.

3.2.1 Association of the 5-HT1A C(-1019)G SNP with Schizophrenia

A large Russian cohort of DNA samples from 160 control and 151 schizophrenic individuals was first examined to assess an association between schizophrenia and the 5-HT1A C(-1019)G SNP. As described in 2.7, the schizophrenic patients were diagnosed using two standard sets of criteria, and control individuals did not have a history of

psychiatric illness (Chumakov *et al.*, 2002; Golimbet *et al.*, 2004). In this cohort, no association was found with the 5-HT1A C(-1019)G SNP genotypes ($\chi^2 = 2.679$, $p = 0.2620$, $df = 2$) or alleles ($p = 0.3338$).

Next, a small Canadian cohort consisting of 13 controls and 12 first-episode, non-medicated schizophrenics was analyzed. This group may provide a more sensitive population in which to examine the genetics of both schizophrenia susceptibility and drug response as the patients are being evaluated and followed by a single psychiatrist and considerable data is being gathered with regards to their psychiatric outcomes. However, at present the cohort very small and thus results will likely be inconclusive; recruitment is ongoing. Preliminary data for the Canadian cohort indicate a non-significant trend towards an association of the C-allele ($p = 0.0863$) and C/C genotype ($\chi^2 = 4.033$, $df = 2$, $p = 0.1331$) with schizophrenia (Table 3.1).

3.2.2 Association of the 5-HT2A A(-1438)G SNP with Schizophrenia

Again, in the Russian samples, no association of the 5-HT2A A(-1438)G genotypes ($\chi^2 = 2.021$, $p = 0.3640$, $df = 2$) or alleles ($p = 0.3169$) was observed with schizophrenia. Similarly, no effect of genotype ($\chi^2 = 3.766$, $df = 2$, $p = 0.1521$) or allele ($p = 0.5573$) was found in the preliminary analysis of the Canadian samples (Table 3.2).

3.2.3 Association of the D2 Taq1A (C/T) SNP with Schizophrenia

For the D2 Taq1A (C/T) SNP, there was no effect of genotype ($\chi^2 = 2.676$, $p = 0.2624$, $df = 2$) in the Russian cohort; but there was a trend towards an association of the T-allele ($p = 0.1158$) with schizophrenia. Similarly, preliminary Canadian cohort data for

Table 3.1. Allele and genotype distribution of the human 5-HT1A promoter C(-1019)G polymorphism in schizophrenic and control subjects from Russian and Canadian cohorts.

Genomic DNA extracts were analyzed using a QPCR-based genotyping assay (ABI: C_11904666_10). Genotype frequencies were compared by χ^2 analysis with two-tailed p values; allele frequencies were compared by Fisher's exact test with two-tailed p values.

Subjects	N	Genotype frequencies (%)			Allele frequencies (%)	
		C/C	C/G	G/G	C	G
Russian Controls	160	37 (23.1)	78 (48.8)	45 (28.1)	152 (47.5)	168 (52.5)
Russian Schizophrenics	151	24 (15.9)	83 (55.0)	44 (29.1)	131 (43.4)	171 (56.6)
Canadian Controls	13	1 (7.7)	6 (46.6)	6 (46.6)	8 (30.8)	18 (69.2)
Canadian Schizophrenics	12	5 (41.7)	4 (33.3)	3 (25.0)	14 (58.3)	10 (41.7)

Russian control versus schizophrenic subjects: genotype, $\chi^2 = 2.679$, $df = 2$, $p = 0.2620$ (ns); allele: $p = 0.3338$ (ns). Canadian control versus schizophrenic subjects: genotype, $\chi^2 = 4.033$, $df = 2$, $p = 0.1331$ (ns); allele: $p = 0.0863$ (ns).

Table 3.2. Allele, and genotype distribution of the human 5-HT2A promoter A(-1438)G polymorphism in schizophrenic and control subjects from Russian and Canadian cohorts.

Genomic DNA extracts were analyzed using a QPCR-based genotyping assay (ABI: C_8695278_10). Genotype frequencies were compared by χ^2 analysis with two-tailed p values; allele frequencies were compared by Fisher's exact test with two-tailed p values.

Subjects	N	Genotype frequencies (%)			Allele frequencies (%)	
		A/A	A/G	G/G	A	G
Russian Controls	160	27 (16.9)	68 (42.5)	65 (40.6)	122 (38.1)	198 (61.9)
Russian Schizophrenics	151	17 (11.3)	69 (45.7)	65 (43.0)	103 (34.1)	199 (65.9)
Canadian Controls	13	1 (7.7)	6 (46.6)	6 (46.6)	8 (30.8)	18 (69.2)
Canadian Schizophrenics	12	4 (33.3)	2 (16.6)	6 (50.0)	10 (41.7)	14 (58.3)

Russian control versus schizophrenic subjects: genotype, $\chi^2 = 2.021$, $df = 2$, $p = 0.3640$ (ns); allele: $p = 0.3169$ (ns). Canadian control versus schizophrenic subjects: genotype, $\chi^2 = 3.766$, $df = 2$, $p = 0.1521$ (ns); allele: $p = 0.5573$ (ns).

Table 3.3. Allele and genotype distribution of the human D2 Taq1A (C/T) polymorphism in schizophrenic and control subjects from Russian and Canadian cohorts.

Genomic DNA extracts were analyzed using a QPCR-based genotyping assay (ABI: C_7486676_10). Genotype frequencies were compared by χ^2 analysis with two-tailed p values; allele frequencies were compared by Fisher's exact test with two-tailed p values.

Subjects	N	Genotype frequencies (%)			Allele frequencies (%)	
		C/C	C/T	T/T	C	T
Russian Controls	160	114 (71.3)	43 (26.9)	3 (1.9)	271 (84.7)	49 (15.3)
Russian Schizophrenics	151	96 (63.5)	49 (32.5)	6 (4.0)	241 (79.8)	61 (20.2)
Canadian Controls	13	11 (84.6)	2 (15.4)	0 (0)	24 (92.3)	2 (7.7)
Canadian Schizophrenics	12	7 (58.3)	4 (33.3)	1 (8.3)	18 (75.0)	6 (25.0)

Russian control versus schizophrenic subjects: genotype, $\chi^2 = 2.676$, $df = 2$, $p = 0.2624$ (ns); allele: $p = 0.1158$ (ns). Canadian control versus schizophrenic subjects: genotype, $\chi^2 = 2.520$, $df = 2$, $p = 0.2837$ (ns); allele: $p = 0.1319$ (ns).

genotype ($\chi^2 = 2.520$, $df = 2$, $p = 0.2837$) was not significant, while a trend towards a higher T-allele frequency ($p = 0.1319$) in schizophrenics was present (Table 3.3).

3.2.4 Association of the 5-HT1A C(-1019)G SNP with Major Depressive Disorder

In order to examine relationships between the above polymorphisms and MDD, a Canadian cohort of 182 MDD and 213 control blood samples was genotyped. As described in 2.7, control samples were from volunteers with no personal or family history of mental illness, while MDD patients were diagnosed using the DSM-IV criteria for major depression and had at least moderately severe depression (Du *et al.*, 2000). These samples represent an extension of the cohort of 129 MDD and 134 control individuals examined in Lemonde *et al.* (2003), and for which a significant association with the 5-HT1A C(-1019)G SNP was observed. In that study, not all samples were examined due to the technical difficulty of genotyping by manual sequencing. However, using the Q-PCR based TaqMan assays, the genotype of these samples was readily determined. Thus, in this extended cohort a trend towards an association of the G-allele with depression was observed ($p = 0.1329$); however, no effect of genotype was evident ($\chi^2 = 2.993$, $df = 2$, $p = 0.2239$). No differences between female and male genotype ratios were observed when samples were divided by gender, and no significant association with depression was observed for either genotype (female: $\chi^2 = 1.126$, $df = 2$, $p = 0.5695$; male: $\chi^2 = 2.284$, $df = 2$, $p = 0.3192$) or allele (female: $p = 0.3472$; male: $p = 0.2254$) frequency in the subsets (Table 3.4).

Table 3.4. Allele and genotype distribution of the human 5-HT1A promoter C(-1019)G polymorphism in all, female and male major depressive disorder (MDD) and control subjects from a Canadian cohort.

Genomic DNA extracts were analyzed using a QPCR-based genotyping assay (ABI: C_11904666_10). Genotype frequencies were compared by χ^2 analysis with two-tailed p values; allele frequencies were compared by Fisher's exact test with two-tailed p values.

Subjects	N	Genotype frequencies (%)			Allele frequencies (%)	
		C/C	C/G	G/G	C	G
Canadian Controls	213	63 (29.6)	113 (53.1)	37 (17.4)	239 (56.1)	187 (43.9)
Canadian MDD Subjects	182	46 (25.3)	92 (50.5)	44 (24.2)	184 (50.5)	180 (49.5)
Female Controls	121	37 (30.6)	64 (52.9)	20 (16.5)	138 (57.0)	104 (43.0)
Female MDD Subjects	108	28 (25.9)	57 (52.8)	23 (21.3)	113 (52.3)	103 (47.7)
Male Controls	92	26 (28.3)	49 (53.3)	17 (18.5)	101 (54.9)	83 (45.1)
Male MDD Subjects	74	18 (24.3)	35 (47.3)	21 (28.4)	71 (48.0)	77 (52.0)

Canadian control versus MDD subjects: genotype, $\chi^2 = 2.993$, $df = 2$, $p = 0.2239$ (ns); allele: $p = 0.1329$ (ns). Female control versus MDD subjects: genotype, $\chi^2 = 1.126$, $df = 2$, $p = 0.5695$ (ns); allele: $p = 0.3472$ (ns). Male control versus MDD subjects: genotype, $\chi^2 = 2.284$, $df = 2$, $p = 0.3192$ (ns); allele: $p = 0.2254$ (ns).

3.2.5 Association of the 5-HT2A A(-1438)G SNP with Major Depressive Disorder

Although only a weak trend was observed towards an association with depression of the 5-HT2A G/G genotype ($\chi^2 = 3.680$, $df = 2$, $p = 0.1588$) and the G-allele ($p = 0.1520$) in the total Canadian cohort, when the sample set was divided by gender, a clear association between both genotype ($\chi^2 = 7.339$, $df = 2$, $p = 0.0255$) and allele ($p = 0.0189$) frequencies were observed in males only, with the A/A genotype being 2.5 times more frequent in male controls, and the G/G genotype and G-allele overrepresentation in male MDD subjects. By contrast, in females, a trend towards an effect of genotype was observed ($\chi^2 = 4.524$, $df = 2$, $p = 0.1042$); however, this is the result of under representation of the C/G genotype and overrepresentation of both homozygous genotypes in the MDD subjects compared to controls. Unlike in males, allele frequencies were almost equal between controls and MDD subjects ($p = 1.000$) (Table 3.5).

3.2.6 Association of the D2 Taq1A (C/T) SNP with Major Depressive Disorder

In the subset of Canadian MDD and control samples analyzed (see 2.7), no association of either genotype ($\chi^2 = 1.518$, $df = 2$, $p = 0.4680$) or allele ($p = 0.3050$) frequency was observed for the D2 Taq1A SNP (Table 3.6). Because of this, no gender based analyses were attempted.

3.2.7 Analysis of 5-HT1A C(-1019)G SNP and 5-HTTLPR in Human Brain Samples

Additionally, human brain samples obtained from Dr. Craig Stockmeier were genotyped. Brain samples were obtained post-mortem from either control individuals or those with a psychiatric illness, but the diagnosis associated with each sample was

Table 3.5. Allele and genotype distribution of the human 5-HT2A promoter A(-1438)G polymorphism in all, female and male major depressive disorder (MDD) and control subjects from a Canadian cohort.

Genomic DNA extracts were analyzed using a QPCR-based genotyping assay (ABI: C_8695278_10). Genotype frequencies were compared by χ^2 analysis with two-tailed p values; allele frequencies were compared by Fisher's exact test with two-tailed p values.

Subjects	N	Genotype frequencies (%)			Allele frequencies (%)	
		A/A	A/G	G/G	A	G
Canadian Controls	213	47 (22.1)	111 (52.1)	55 (25.8)	205 (48.1)	221 (51.9)
Canadian MDD Subjects	182	37 (20.3)	82 (45.1)	63 (34.6)	156 (42.9)	208 (57.1)
Female Controls	121	26 (21.5)	64 (52.9)	31 (25.6)	116 (47.9)	126 (52.1)
Female MDD Subjects	108	31 (28.7)	42 (38.9)	35 (32.4)	104 (48.1)	112 (51.9)
Male Controls	92	21 (22.8)	47 (51.1)	24 (26.1)	89 (48.4)	95 (51.6)
Male MDD Subjects	74	6 (8.1)	40 (54.1)	28 (37.8)	52 (35.1)	96 (64.9)

Canadian control versus MDD subjects: genotype, $\chi^2 = 3.680$, $df = 2$, $p = 0.1588$ (ns); allele: $p = 0.1520$ (ns). Female control versus MDD subjects: genotype, $\chi^2 = 4.524$, $df = 2$, $p = 0.1042$ (ns); allele: $p = 1.000$ (ns). Male control versus MDD subjects: genotype, $\chi^2 = 7.339$, $df = 2$, $p = 0.0255$ (*); allele: $p = 0.0189$ (*)

Table 3.6. Allele, and genotype distribution of the human D2 Taq1A (C/T) polymorphism in major depressive disorder (MDD) and control subjects from a Canadian cohort.

Genomic DNA extracts were analyzed using a QPCR-based genotyping assay (ABI: C_7486676_10). Genotype frequencies were compared by χ^2 analysis with two-tailed p values; allele frequencies were compared by Fisher's exact test with two-tailed p values.

Subjects	N	Genotype frequencies (%)			Allele frequencies (%)	
		C/C	C/T	T/T	C	T
Canadian Controls	129	83 (64.3)	42 (32.6)	4 (3.1)	208 (80.6)	50 (19.4)
Canadian MDD Subjects	155	92 (59.4)	54 (34.8)	9 (5.8)	238 (76.8)	72 (23.2)

Canadian control versus MDD subjects: genotype, $\chi^2 = 1.518$, $df = 2$, $p = 0.4680$ (ns); allele: $p = 0.3050$ (ns).

initially not provided. All 277 human brain DNA samples were successfully genotyped for the 5-HT1A C(-1019)G SNP. Overall, 67 (24.2%) had the homozygous C/C genotype, 135 (48.7%) were C/G heterozygotes, and 75 (25.1%) were homozygous G/G. C- and G- allele frequencies were 48.6% and 51.4%, respectively. Although the psychiatric status of the individuals from which the majority of the samples were obtained has not yet been made available, data for 52 samples (26 MDD patients and 26 matched controls) has been released to our lab. Preliminary results indicate a slightly greater frequency of the 5-HT1A G/G genotype and G-allele in MDD subjects compared to controls in the American cohort; however, due to small sample size no significant effect is seen for genotype ($\chi^2 = 0.6222$, $df = 2$, $p = 0.7326$) or allele ($p = 0.5518$) frequency (Appendix VI).

Technical difficulties have prevented the completion of the 5-HTTLPR genotyping of the human brain DNA samples. Briefly, very poor or no amplification was obtained with the majority of the samples after lengthy optimization of the PCR conditions, likely due to the low yield of DNA obtained from the brain samples and the C-G rich and repetitive sequence of the 5-HTTLPR.

3.3 Sequence Analysis of the Primate 5-HT1A Promoter Region

3.3.1 Analysis of Macaque 5-HT1A Promoter Sequence in Region of NUDR-Binding Element

As described in 1.4.1-1.4.2, non-human primates are excellent models in which to study emotional regulation and psychopathology, and analyzing the macaque 5-HT1A promoter sequence may identify novel polymorphisms (5-HT1A receptor

polymorphisms, analogous or not to those found in humans, have yet to be reported) and lead to new insights about the receptor's regulation. In particular, determining whether the functional human C(-1019)G SNP is present in the macaque species was the initial objective. However, identifying any polymorphisms that could potentially be involved in genotype and genotype-environment interactions with depressive behaviours in primates was also a goal of this work.

All of the rhesus and cynomolgus macaque DNA samples were successfully sequenced. Three sites of intraspecies variation have been identified in the 5-HT1A promoter in the vicinity of the NUDR binding element, two of which were confirmed by automated sequencing of different clones of a PCR product from a heterozygous animal (Figure 3.5) (a homozygous animal was also sequenced for comparison (data not shown)). All three polymorphisms have been confirmed by repeated manual sequencing. Table 3.7 indicates the genotypes obtained for each macaque sample. For the A/G substitution at position -969, of 13 cynomolgus macaques, one (7.7%) was homozygous for the G-allele, one (7.7%) was an A/G heterozygote, and the remaining 11 (84.6%) were homozygous for the A-allele; all 20 rhesus macaques were homozygous for the G-allele. At position -1101, a variable insertion/deletion mutation (-/CAAA/AAAA) was present in the rhesus macaques. Five (25%) animals had the heterozygous -/AAAA genotype, two (10%) were heterozygous -/CAAA and the remaining 13 (65%) had the homozygous -/- genotype. In the cynomolgus macaques, one (7.7%) was the heterozygous -/CAAA genotype, 11 (84.6%) were homozygous -/-, and one macaque had a different insertion/deletion mutation near that position which could not be deciphered



Figure 3.5. Intraspecies variation in macaque 5-HT1A promoter sequence.

Shown is an alignment of automated sequences of obtained from 5 clones of cynomolgus macaque C13 PCR product. Indicated are two cynomolgus macaque polymorphisms detected by both automated and manual sequencing (red boxes). Also shown is a polymorphic four-nucleotide insertion (CAAA or AAAA) detected by manual sequencing in both rhesus and cynomolgus macaques (red triangle).

Table 3.7. Genotypes of rhesus and cynomolgus macaques for identified 5-HT1A promoter variants.

PCR products amplified from rhesus macaque (*Macaca mulatta*) (n=20) and cynomolgus macaque (*M. fascicularis*) (n=13) genomic DNA extracts were manually sequenced. Shown in bold are the sequence variants identified in each species at each position indicated (relative to the human start codon). The genotype of each macaque is indicated.

Monkey ID	Sample ID	Position		
		-969	-1101	-1117
Rhesus		G	-/AAAA/CAAA	AA(-/-)
19217	R1	G	-/-	-/-
19513	R2	G	-/-	-/-
19524	R3	G	-/-	-/-
20144	R4	G	-/AAAA	-/-
20303	R5	G	-/-	-/-
19455	R6	G	-/AAAA	-/-
19458	R7	G	-/CAAA	-/-
19596	R8	G	-/-	-/-
19621	R9	G	-/-	-/-
10755	R10	G	-/-	-/-
10832	R11	G	-/-	-/-
19875	R12	G	-/AAAA	-/-
19877	R13	G	-/-	-/-
19987	R14	G	-/-	-/-
20016	R15	G	-/-	-/-
20031	R16	G	-/-	-/-
20040	R17	G	-/CAAA	-/-
20042	R18	G	-/-	-/-
20159	R19	G	-/AAAA	-/-
20209	R20	G	-/AAAA	-/-
Cynomolgus		A/G	-/CAAA	AA(A/-)
19505	C1	A	-/-	-/-
19506	C2	A	-/-	-/-
19507	C3	A	-/-	-/-
19120	C4	A	-/CAAA	-/-
19131	C5	G	<i>a</i>	-/-
19122	C6	A	-/-	-/-
19124	C7	A	-/-	-/-
19127	C8	A	-/-	-/-
19128	C9	A	-/-	-/-
19492	C10	A	-/-	-/-
19493	C11	A	-/-	-/-
19495	C12	A	-/-	-/-
19497	C13	A/G	-/-	A/-

^aThe genotype at this position was not obtained due to difficulty in interpreting sequence

due to difficulty in reading the sequence in that area. Finally, one cynomolgus macaque had an -/A insertion/deletion mutation at -1117; this was not seen in the rhesus macaques.

A number of interspecies differences were observed when the two macaque sequences were aligned with the human and chimpanzee sequences (obtained from NCBI Genbank) (Figure 3.6). Interestingly, a base pair deletion in the NUDR core binding motif is present in both species of macaque examined (the human and chimpanzee sequence is AAGC while the macaque sequence is AGC) (Figure 3.6, Figure 3.7). All macaque DNA samples analyzed were homozygous C/C for the human C(-1019)G SNP. In order to confirm the deletion in the NUDR element, the macaque promoter was sequenced in the reverse orientation (data not shown). The results of automated sequencing also verified the deletion (Figure 3.5).

3.3.2 Binding Assays for NUDR and Putative Macaque NUDR-Binding Element

To determine whether the deletion in the macaque NUDR minimum consensus site affects the ability of NUDR to bind the macaque element in comparison to the human element, EMSA was performed (Figure 3.8). As previously reported (Lemonde *et al.*, 2003), NUDR bound more strongly to the human C-allele compared to the G-allele. Interestingly, NUDR bound the macaque probe with similar affinity to the human C-allele, and greater affinity than the G-allele. This indicates that the base-pair deletion in the macaque NUDR binding motif does not affect NUDR binding.

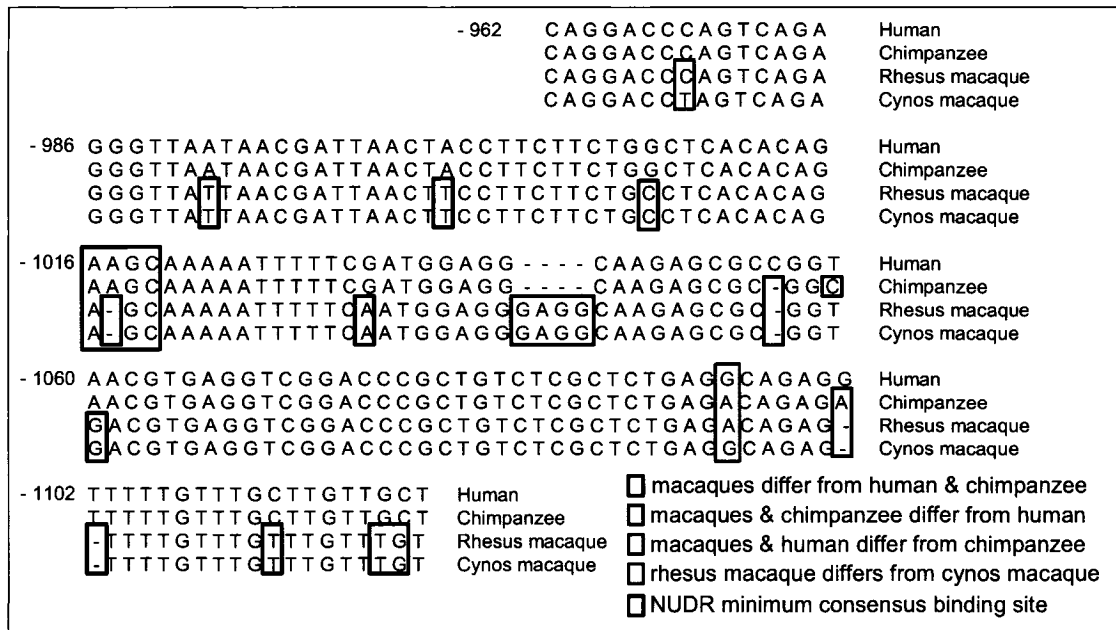


Figure 3.6. Alignment of primate 5-HT1A promoter sequence in region of NUDR-binding element.

PCR products amplified from rhesus macaque (*Macaca mulatta*) (n=20) and cynomolgus macaque (*M. fascicularis*) (n=13) genomic DNA extracts were manually sequenced. Most frequent alleles are shown when polymorphisms exist in macaque sequences. Sequences for humans (*Homo sapiens*) and chimpanzees (*Pan troglodytes*) were obtained from NCBI GenBank®. Interspecies mutations and the NUDR minimum consensus binding site are indicated. Numbering is relative to human start codon (+1).

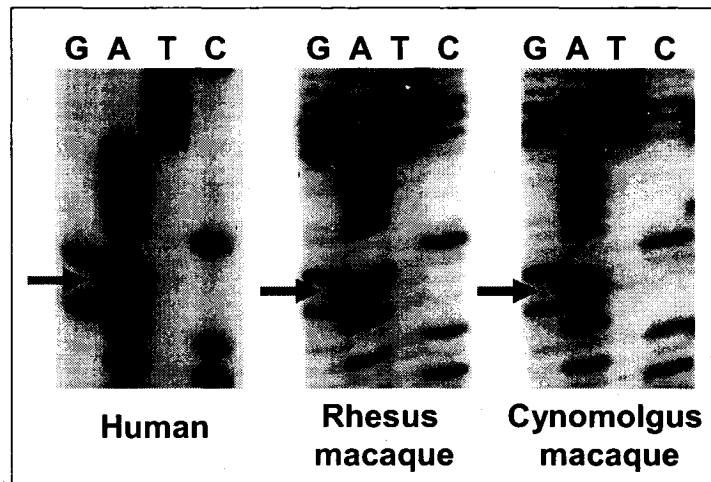


Figure 3.7. Base-pair deletion in the 5-HT1A promoter NUDR minimum consensus binding site in macaques.

Shown are partial sequences from human (Lemonde *et al.*, 2003), rhesus macaque and cynomolgus macaque genomic DNA samples. Arrows indicate the presence of two A nucleotides in humans and a single A in macaques.

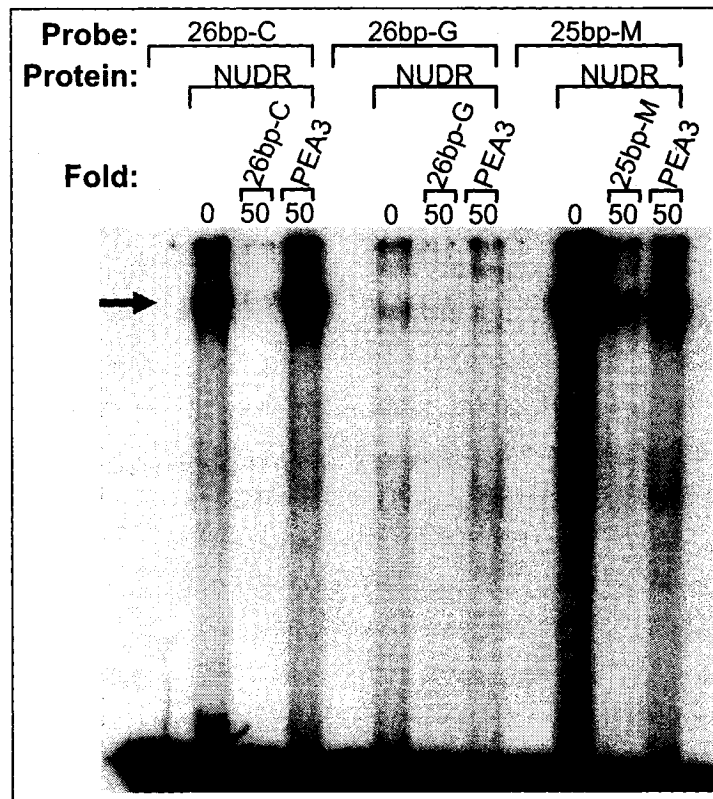


Figure 3.8. Deletion in macaque 5-HT1A promoter NUDR minimum consensus element does not prevent NUDR protein binding.

Electrophoretic mobility shift assay (EMSA) was performed using bacterially expressed, Ni-NTA agarose bead-purified NUDR protein (3ug) and 26bp human probes with either the C- (26bp-C) or G- (26bp-G) allele of the C(-1019)G polymorphic element, or a corresponding macaque (25bp-M) probe with the A deleted. Unlabelled specific or unrelated (PEA3) probes were added at 50-fold excess as indicated. Samples were electrophoresed on a 5% native polyacrylamide gel. Arrow indicates NUDR/probe binding as detected by autoradiography. These results are representative of two separate experiments.

3.3.3 Analysis of Macaque 5-HT1A Promoter Sequence in Region of Freud-1-Binding Elements

No intraspecies polymorphisms were detected in either macaque species in the 5-HT1A promoter sequence surrounding the Freud-1 binding elements (5'DRE and 3'DRE). Several interspecies variations were observed when comparing the macaques' sequences to those of humans and chimpanzees in the tandem DREs, as well as in another element, Repressor Element (RE)-1, which binds Repressor Element-1 Silencing Transcription factor (REST) (Lemonde *et al.*, 2004; Schoenherr *et al.*, 1996) (Figure 3.9).

In addition, 18 human genomic DNA samples were sequenced with the same primers as used for the macaque DNA, in an attempt to identify novel polymorphisms in the human DRE sequences. No polymorphisms were detected (data not shown).

3.3.4 Binding Assays for Freud-1 and Putative Macaque Freud-1-Binding Element

EMSA was performed to determine whether the variations in the macaque 5'DRE and 3'DRE sites affect the ability of Freud-1 to bind the macaque elements in comparison to the human elements, (Figure 3.10). Preliminary results (n=1 due to technical difficulties) indicate that Freud-1 bound specifically to both macaque DREs. While the binding affinity appears to be slightly weaker than to the human DREs, this must be confirmed by additional experiments.

- 1468	GGTCTCGGACGTTTGGAGACCCCCTTTGAACCACCTTAGG GGTCTCGGACGTTTGGAGACCCCCTTTGAACCACCTTAGG GGTCTCGGACGTTTGGAGACCCCCTTTGAACCACCTTAGG GGTCTCGGACGTTTGGAGACCCCCTTTGAACCACCTTAGG	Human Chimpanzee Rhesus macaque Cynos macaque
- 1508	GCCAAGCGATCGGCCGATCGGAGAGAATTAGAGTCTTTGA GCCAAGCGATCGGCCGATCGGAGAGAATTAGAGTCTTTGA GACCAAGCGATCGGCCGATCGGAGAGGATAGGGTCTTTGA GACCAAGCGATCGGCCGATCGGAGAGGATAGGGTCTTTGA	Human Chimpanzee Rhesus macaque Cynos macaque
- 1548	CCCAAGTCGTGGACCCTGTCAAAGATTTCGTTACTCCAAAAT CCCAAGTCGTGGACCCTGTCAAAGATTTCGTTACTCCAAAAT CCCAAGTCGTGGACCCTGTCAAAGATTTCGTTACTCCAAAAT CCCAAGTCGTGGACCCTGTCAAAGATTTCGTTACTCCAAAAT	Human Chimpanzee Rhesus macaque Cynos macaque
- 1588	ACAGCGGTGGAAGACCGTTTACAAAATCTCACGGTAGACC ACAGCGGTGGAAGACCGTTTACAAAATCTCACGGTAGACC ACAGCGGTGGAAGACCGTTTACAAAATCTCACGGTAGACC ACAGCGGTGGAAGACCGTTTACAAAATCTCACGGTAGACC	Human Chimpanzee Rhesus macaque Cynos macaque
- 1628	TTTGGGAATACG TTTGGGAATACG TTTGGGAATACG TTTGGGAATACG	Human Chimpanzee Rhesus macaque Cynos macaque

Figure 3.9. Alignment of primate 5-HT1A promoter sequence in region of Freud-1-binding element.

PCR products amplified from rhesus macaque (*Macaca mulatta*) (n=20) and cynomolgus macaque (*M. fascicularis*) (n=13) genomic DNA extracts were manually sequenced. No intraspecies polymorphisms were identified for either species of macaque. Sequences for humans (*Homo sapiens*) and chimpanzees (*Pan troglodytes*) were obtained from NCBI GenBank®. Interspecies mutations, the repressor element (RE)-1 and the dual repressor elements (DREs) are indicated. Numbering is relative to human start codon (+1).

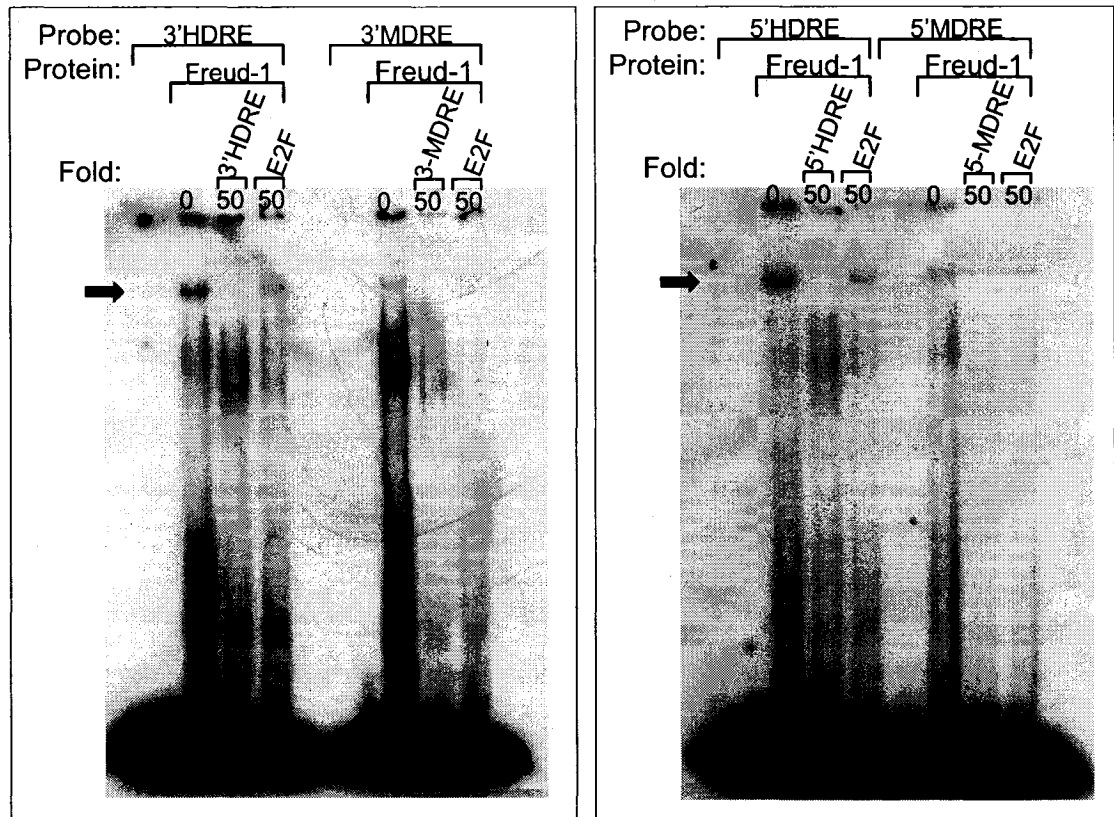


Figure 3.10. Macaque 5-HT1A promoter 3'DRE and 5'DRE bind Freud-1 protein.

Electrophoretic mobility shift assay (EMSA) was performed using bacterially expressed, Ni-NTA agarose bead-purified Freud-1 protein (2ug) and labeled probes corresponding to the human (H) and macaque (M) 3'DRE and 5'DRE. Unlabelled specific or unrelated (E2F) probes were added at 50-fold excess as indicated. Samples were electrophoresed on a 5% native polyacrylamide gel. Arrow indicates specific Freud-1/probe binding as detected by autoradiography.

CHAPTER 4 – DISCUSSION

4.1 Effect of 5-HT1A Agonist Treatment on 5-HT1A Expression

No effect of treatment with the 5-HT1A agonist 8-OH-DPAT was observed on the activity of any 5-HT1A promoter construct in either the RN46A or DP8 cell lines (Figure 3.1). The activity of all of the 5-HT1A promoter constructs was sufficiently high (at least several times that of the basal pGL3B) to detect any repression which might have occurred. In addition, the promoter constructs generally followed previously observed patterns of activity, indicating that the assay was functioning appropriately.

As was observed in Storrington *et al.* (1999), the rat -1174bp construct was considerably more active than the -426bp construct. This is likely due to the presence of a TATA box driven transcription initiation site in the longer construct, which is eliminated in the shorter construct. Although there is a putative NUDR element in the -1174bp construct, this did not appear to repress the construct to the same degree as the loss of the TATA site in the -426bp construct. However, again as previously reported, the -426bp construct did show greater activity than the basal pGL3B in these cell lines, indicative of the enhancer activity of the C-G rich promoter and AP-1 sites still present in this short construct.

Intriguingly, while the human constructs followed a previously reported pattern of activity in the RN46A cells (Lemondé *et al.*, 2004a), with the -1174bp construct being slightly more active than the -723bp construct, in the DP8 cells, which are 5-HT1A stably transfected RN46A cells, this pattern was consistently reversed. A key difference between these constructs is the binding element for the transcriptional repressor NUDR.

Although it might be expected that deleting this site would enhance transcription, this only occurs in the DP8 cells. However, there are also several transcriptional start sites in the human 5-HT1A promoter upstream of -723bp (Parks and Shenk, 1996) which could potentially enhance the activity of the longer construct. Therefore, it is possible that the relative effects of these elements on transcriptional activity may differ in the two cell lines. One possible explanation is that levels of the transcription repressor NUDR may be higher in the DP8 cells (resulting in greater repression of the longer construct), perhaps as an autoregulatory mechanism due to the high levels of exogenous 5-HT1A being transcribed in these cells. This hypothesis could be tested by quantifying the relative levels of NUDR in the two cell lines by Q-PCR and Western analysis.

A final point to note is that the rat and human constructs demonstrated relatively similar activities. Therefore, although the cell lines were derived from rat raphe neurons, the human constructs were able to activate transcription of the luciferase reporter gene in this heterologous system. This is likely because of the relatively high degree of homology between the rat and human promoter sequences (Storring *et al.*, 1999), especially in the region encompassed by the constructs. In addition, NUDR was found to repress both exogenous human 5-HT1A reporter constructs transfected into RN46A cells and endogenous 5-HT1A mRNA expression (Lemondé *et al.*, 2003), indicating that the RN46A system is capable of regulating human 5-HT1A promoter activity.

One major caveat of this work is that as 5-HT1A mRNA is not present at levels detectable by Q-PCR in the wild-type RN46A cell line (Appendix IV), and the 5-HT1A stably-transfected DP8 cells may not express 5-HT1A endogenously at sufficient levels to observe regulation, these cell lines may not be appropriate models for examining 5-

HT1A agonist effects on 5-HT1A promoter activity. Future experiments utilizing primary rat raphe cultures, which do express high levels of the 5-HT1A receptor endogenously, should be performed to address this issue. In addition, larger constructs containing the DRE and RE-1 elements could be tested, under the hypothesis that these are important for an effect of 8-OH-DPAT on 5-HT1A promoter activity. However, in cell lines these constructs have low basal activity due to the repressor elements (Lemondé *et al.*, 2004a), making any additional repression difficult to detect. It is possible that in cultured raphe neurons the activity might be sufficiently high to assay.

As the results from the transcriptional reporter assays with regards to 5-HT1A agonist treatment were negative, the hypothesis that 5-HT1A agonists downregulate 5-HT1A autoreceptor expression was evaluated by Q-PCR analysis of 5-HT1A mRNA expression in the primary raphe cultures. Again, no effect of 8-OH-DPAT treatment was found for either a short- or long-term time course (Figure 3.2, Figure 3.3). However, as discussed in 3.1.2, for the majority of the 24hr treatments, AraC was used which resulted in cultures that exhibited high levels of cell death, and the “unhealthy” state of the surviving cells could have influenced 5-HT1A mRNA expression response. Also, some variability in neuronal survival and differentiation was present from culture to culture in both treatments. Additionally, only about 5-10% of the neurons in these cultures were serotonergic, and this percentage varied somewhat, as did the relative percentages of the other neuronal subtypes (Czesak *et al.*, in preparation). These confounding factors may also make detection of subtle effects of 5-HT1A activation on gene transcription difficult to observe in this system. To address some of these issues, the level and responsiveness of 5HT1A receptors in the cultures should be verified by ligand binding studies and by

assaying 5-HT1A activation mediated responses such as inhibition of MAPK or of forskolin-stimulated cAMP activation (Kushwaha and Albert, 2005). Such studies would indicate whether the lack of 8-OH-DPAT response and variability in 5-HT1A mRNA levels are the result of insufficient 5-HT1A receptor function.

These results contrast with those of Welner *et al.* (1989) and of Fanelli and McMonagle-Strucko (1992), who reported that chronic 5-HT1A agonist treatment reduced 5-HT1A autoreceptor binding in the rat DR; however, it may be that the agonist acted at the translational or post-translational levels in those studies. Additionally, the results do not support the finding of decreased raphe 5-HT1A receptor mRNA levels in rats treated chronically with fluoxetine (Le Poul *et al.*, 2000); however, SSRIs may stimulate different or additional intracellular signaling pathways than the more specific 5-HT1A agonists. On the other hand, these results do support other work which found no change in raphe 5-HT1A mRNA and/or protein levels upon SSRI and/or 5-HT1A agonist treatment (Hensler and Duram, 2001; Hensler, 2002; Hervas *et al.*, 2001) (see 1.2.5).

Interestingly, a recent study examining the effects of ethanol on E14 rat rhombencephalic neurons cultured 5 DIV found that 6hr of treatment with the 5-HT1A agonist ipsapirone increased 5-HT1A expression as detected by Q-PCR. They also determined that GAPDH expression was not affected by 5-HT1A agonist treatment, indicating that it is a valid house-keeping gene in this experimental system (Druse *et al.*, 2006). The results of this study are not in agreement with the lack of effect of 8-OH-DPAT on 5-HT1A expression reported in this thesis. However, although the differences are not significant, the levels of 5-HT1A in both time courses are on average slightly higher in the 8-OH-DPAT treated cells. In both studies this could potentially be an effect

of the primary culture system, in which most of the regulatory feedback mechanisms involving different brain regions and cell types that might also influence serotonergic gene expression in a whole animal have been disrupted. The different results obtained in this study compared to that of Druse *et al.* (2006) (a significant increase in 5-HT1A versus a slight increase) may be due to important methodological differences such as using a relatively short treatment course (6hr) versus longer treatments (24hr or 7days), which may differentially affect transcriptional regulation and mRNA turnover. In addition, while in this thesis only rostral (DR and MnR) raphe nuclei were cultured, Druse *et al.* (2006) used both rostral and caudal raphe nuclei.

Another important consideration is the 5-HT1A agonist used in the experiments reported here. 8-OH-DPAT is the prototypical 5-HT1A agonist, due to its both its relatively high selectivity and high affinity ($K_d = 0.3-1.8\text{nM}$) for the 5-HT1A receptor (Pucadyil *et al.*, 2005). However, it should be noted that 8-OH-DPAT is also a weak agonist at 5-HT7 receptors, but these receptors are expressed post-synaptically rather than in the raphe nuclei (Barnes and Sharp, 1999).

Additionally, the effect of overstimulating 5-HT1A receptors due to high media tryptophan levels is a potential confounding factor in interpreting these results (see 3.1.2). However, the opposite experiment, blockade of the receptors with the potent 5-HT1A antagonist, WAY-100635 (Barnes and Sharp, 1999), did not affect 5-HT1A mRNA levels (Figure 3.4). 5-HT1A transcript upregulation would have indicated that baseline gene expression was being affected by overactivation of the receptors by 5-HT.

Also relevant in interpreting these results is that in the positive studies of 5-HT1A agonist or SSRI downregulation of 5-HT1A levels reported above, 5-HT1A in the DR of

adult rats was examined, while in this report, rostral raphe nuclei from E14-E15 embryos were cultured. Two electrophysiological studies which measured the inhibition of 5-HT neuronal firing indicated that DR 5-HT_{1A} autoreceptors were more sensitive than those of the MnR to the agonistic effects of 8-OH-DPAT (Adell *et al.*, 2002). In addition, another study examining 5-HT release following administration of an acute challenge of 8-OH-DPAT to rats 24hr after chronic 8-OH-DPAT treatment found that in the striatum, which is innervated by DR 5-HT neurons, the decrease in 5-HT release with acute treatment was diminished after 7 and 14 days, indicating autoreceptor desensitization, while in the MnR-innervated hippocampus, no effect was observed after 7 days, and after 14 days only a trend towards diminishment occurred (Kreiss and Lucki, 1997). However, the most recent electrophysiological study showed no differing effects among nuclei upon treatment with 8-OH-DPAT, another 5-HT_{1A} agonist, BAYX3702, or the SSRI paroxetine (Adell *et al.*, 2002). Therefore, it is not clear if co-culture of DR and MnR neurons might influence the results reported here. Additionally, although the primary raphe neurons were cultured 7 DIV before treatment, during which time they matured as indicated by axonal and dendritic branching and elongation (data not shown) and by positive staining for the post-mitotic neuronal marker NeuN (see 2.4), it is possible that 5-HT_{1A} gene expression is differentially regulated in development compared to adulthood, which would again contribute to discrepancies between this study and others.

Finally, Hillion *et al.* (1994) showed in cultured E14 raphe neurons that 5-HT_{1A} mRNA co-localized with 5-HT in neurons, indicating that 5-HT_{1A} was being expressed as an autoreceptor in this system, while staining for glial cells did not co-localize with 5-HT_{1A} receptor mRNA or protein. Additionally, culturing E14 rostral raphe nuclei for 12

DIV, Hery *et al.* (1999) could detect 5-HT1A and 5-HT1B receptors, and showed that their autoregulatory activity on 5-HT neurotransmission was mature at that point. This suggests that the 5-HT1A mRNA detected by Q-PCR is from serotonergic neurons, and that the 5-HT1A receptor functioning in the cultures examined here may be representative of mature serotonergic neurons. However, several groups have found by both electrophysiological characterization and 5-HT/5-HT1A co-immunoreactivity that in the adult rat DR, both serotonergic and non-serotonergic neurons expressed 5-HT1A receptors, although the majority appeared to be autoreceptors on 5-HT+ neurons (Beck *et al.*, 2004; Kirby *et al.*, 2003; Marinelli *et al.*, 2004). Therefore, a small percentage of the 5-HT1A receptors present in our cultures may also be post-synaptic. This could hinder the detection of an effect since unlike pre-synaptic receptors, post-synaptic 5-HT1A receptors generally do not desensitize with agonist treatment (see 1.2.5). Unfortunately, within the context of detection by Q-PCR, these two populations cannot be separated.

In summary, although many potential explanations exist, the results reported here do not support the hypothesis that chronic SSRI- or 5-HT1A agonist- induced 5-HT1A autoreceptor desensitization is mediated by decreased 5-HT1A gene transcription. Future studies addressing this hypothesis could directly stimulate the molecular signaling pathways thought to be involved in 5-HT1A agonist induced regulation. Inhibitors of specific signaling pathways such as H89 (protein kinase A), nifedipine (calcium channel blocker), chelerythrine (protein kinase C), U0126 or PD98059 (mitogen-activated protein kinase (MAPK)) or wortmannin (phosphoinositide-3 kinase (PI3K)) could be used to mimic 5-HT1A receptor action (Raymond *et al.*, 1999). These regulators may produce a more robust effect than 8-OH-DPAT activated 5-HT1A receptor signaling, which

involves multiple steps and is limited by the expression of the receptor and of downstream signaling molecules. 5-HT_{1A} receptor activation may act by decreasing calcium entry, either by hyperpolarizing the neuron or by inhibiting calcium channels (see 1.1.1). However, Kushwaha and Albert (2005) demonstrated that 5-HT_{1A} receptors also inhibited MAPK activity in RN46A cells, and this could be another pathway by which the 5-HT_{1A} receptor inhibits its transcription. Conversely, triggering cAMP-, calcium-, or PI3K/MAPK-dependent signaling pathways might upregulate 5-HT_{1A} receptor expression. Ou *et al.* (2003) showed that KCl/ionomycin increased 5-HT_{1A} receptor transcription in raphe RN46A cells by a calcium/calmodulin kinase-dependent pathway. The ability of 5-HT_{1A} receptor activation to inhibit the stimulated transcription could then be examined by Q-PCR in the raphe cultures.

4.2 Association Analysis of 5-HT_{1A}, 5-HT_{2A} and D2 SNPs with Schizophrenia and Major Depressive Disorder

Monoaminergic receptor polymorphisms which have previously shown some evidence of functionality and of conferring an increased risk of psychiatric illness were examined for an association with schizophrenia and MDD. The 5-HT_{1A}, 5-HT_{2A} and D2 receptors were chosen for analysis because of their involvement in the action of atypical antipsychotics (see 1.3.3) and in the etiology of depression and its treatment by conventional antidepressants and adjunct therapies (see 1.2.2 to 1.2.5 and 1.3.5.2).

No association was found between the 5-HT_{1A} C(-1019)G, 5-HT_{2A} A(-1438)G and D2 Taq1A (C/T) SNPs and schizophrenia in either a large Russian cohort or in a preliminary investigation of a Canadian cohort (Table 3.1, Table 3.2, Table 3.3).

The absence of an association of the 5-HT1A G/G genotype with schizophrenia differs from the association reported by Huang *et al.* (2004). This may be in part due to the higher frequency of the G-allele in this Russian control population (52.5%) compared to previously reported North American control groups (15% to 45%) (Huang *et al.*, 2004; Lemonde *et al.*, 2003), as this may hinder detection of any relationship between the SNP and the disease phenotype. Interestingly, a recent meta-analysis of the role of the 5-HTTLPR in mood disorders found that in Asian populations, although MDD subjects had a higher frequency of the S-allele compared to controls, both groups had a very high S-allele frequency, and thus unlike Caucasian studies, the results were not statistically significant (Lotrich and Pollock, 2004). The differences between the results in Russian and American cohorts may also be due to gene-gene and gene-environment interactions differentially influencing the degree of risk an allele confers for a disease in specific populations (see below). Thus, additional studies, including of the Canadian cohort of schizophrenic and control samples currently being assembled, will be necessary to confirm or refute an association between the C(-1019)G SNP and schizophrenia, either in general or in specific ethnic populations.

The absence of an association of the A(-1438)G 5-HT2A polymorphism with schizophrenia may also be in part explained by the ethnicity of the sample set – the association of this SNP and the 5-HT2A T(102)C SNP with schizophrenia have both been reported to be stronger in certain European compared to Asian populations (Abdolmaleky *et al.*, 2004; Li *et al.*, 2006). It is not evident whether a Russian cohort should be considered European or Asian; however, this variability may be indicative of the influence of genetic and/or environmental background on the results of association

studies (see below). In addition, the majority of association studies have been performed with the T(102)C SNP rather than the A(-1438)G SNP examined in this thesis. While all previous reports have indicated that these two variants are in almost complete linkage disequilibrium, a recent large meta-analysis did not find strong linkage disequilibrium structure, and reported a weak association of the T(102)C but not the A(-1438)G SNP with schizophrenia, while also detecting a significant association of the A(-1438)G but not the T(102)C SNP with suicidal behaviour (Li *et al.*, 2006). Additional analysis of the potential linkage disequilibrium between these two 5-HT2A SNPs is necessary to further our understanding of the role that each plays as a risk allele for schizophrenia. Stronger associations could also potentially be obtained by combining the different 5-HT2A alleles using haplotype analysis. However, it should be noted that large sample sizes are necessary when numerous loci are examined in order to maintain statistical power while correcting for multiple testing (Cardon and Bell, 2001).

Although an association of the D2 Taq1A T-allele with alcoholism has been confirmed by multiple meta-analyses, and many studies have reported linkages with abuse of other substances and with gambling, most studies have not reported an association of the D2 Taq1A polymorphism with schizophrenia (Noble, 2003). Thus, although a weak trend was seen for a higher frequency of the T-allele in schizophrenia, in general the results reported here support this conclusion.

Genetic analysis of a Canadian MDD and control cohort revealed a trend towards an association of the 5-HT1A C(-1019)G SNP, a significant association in males only of the 5-HT2A A(-1438)G SNP, and no association of the D2 Taq1A (C/T) SNP (Table 3.4, Table 3.5, Table 3.6).

The trend towards an association between the G-allele of the 5-HT1A SNP and depression represents the loss of the significant association previously detected with a subset of these samples (Lemonde *et al.*, 2003). When the number of control samples was increased from 134 in the previous study to 213, the control G-allele frequency rose from 37% to 44%. However, when the MDD sample size increased from 129 to 182, the G-allele frequency decreased only slightly (from 52% to 49.5%). Interestingly, these results are closer to those reported by Huang *et al.* (2004), of control and MDD G-allele frequencies of 45% and 52% respectively. Like in this study, those frequencies resulted in a non-significant trend towards an association. These discrepancies indicate the importance of accurately determining the allele frequency in different populations by genotyping large numbers of samples to ascertain what the expected control allele frequency is for a locus.

Further analysis of the data set by separating genders (Table 3.4) and eliminating non-Caucasian samples (5%) (data not shown), did not reveal a significant effect. The gender analysis is interesting in the context of a study of unmedicated Chinese MDD patients which found that the 5-HT1A C(-1019)G C/C genotype was associated with a significantly shorter auditory evoked potential P2 latency (which is thought to involve the 5-HT system), but that this result was only significant in males (Chen *et al.*, 2004).

Because a stronger association was reported between depressed suicide victims and the G-allele in Lemonde *et al.* (2003), the MDD subjects were also divided based on their Hamilton Rating Scale for Depression (HAM-D) suicidal ideation score (Item 3), with patients reporting no to low suicidal ideation (Item 3 < 2) versus moderate to severe suicidal ideation (Item 3 ≥ 2) as two groups. However, no significant effect of the SNP

on suicidality was observed. Surprisingly, compared to controls a trend towards an increased G-allele frequency in MDD subjects with a lower suicidal ideation score but not a higher score was found. This may be due to the smaller size of the high-score subset (Appendix VII).

Most theories of the genetics of depression postulate that a combination of susceptibility alleles act in concert (see 1.3.5). For the 5-HT1A C(-1019)G allele, this is supported by Arias *et al.* (2005), who found that the G/G genotype in combination with the 5-HTTLPR S/S genotype is associated with poorer response to citalopram. The 5-HT1A and 5-HT2A genotype combinations were analyzed for the Canadian MDD and control cohort; however, no association was found when comparing all genotype combinations (Appendix IX). Under the hypothesis that the presence of risk alleles for both genes (5-HT1A G-allele; 5-HT2A G-allele) in an individual may have a synergistic effect, genotype combinations were grouped into those that contain at least one risk allele for each gene versus those with the homozygous low-risk genotype (5-HT1A C/C; 5-HT2A A/A) for at least one gene. However, again no association was detected, indicating that in this sample set, the 5-HT1A and 5-HT2A SNPs examined do not act together to correlate with MDD. Upon the resolution of technical difficulties which have prevented genotyping the 5-HTTLPR, synergistic effects of the 5-HT1A C(-1019)G SNP and the 5-HTT polymorphism will also be examined in both the Canadian cohort and in the human brain samples.

The possibility that the larger sample set examined in this thesis differed significantly from the smaller subset of samples analyzed in Lemonde *et al.* (2003) in terms of demographics or illness severity was examined. In this thesis, the control population was

56.6% female, while in Lemonde *et al.* (2003) 56.1% of controls were female. The MDD individuals in this report were 59.3% female, had an average HAMD17 total score (an indicator of depression severity) of 23.85, and an average suicidal ideation (Item 3) score of 1.12; in Lemonde *et al.* (2003) they were 58.5% female, the average HAMD17 score was 24.0, and the average suicidal ideation score was 1.10. Additionally, all sample sets were ~95% Caucasian. Hence, it does not appear that the characteristics of the cohort examined previously are different from the larger cohort genotyped in this study.

Therefore, these results support only a trend-level association between the 5-HT1A C(-1019)G SNP and MDD. In the context of independent studies examining this association, positive results have been reported for the SNP and depressive personality traits and panic disorder with agoraphobia (Rothe *et al.*, 2004; Strobel *et al.*, 2003). Likely due to small sample size, a strong trend towards an association ($p = 0.059$) with MDD was observed in a recent study (Parsey *et al.*, 2006), while Huang *et al.* (2004), who reported a negative study, also saw a trend towards an association ($p = 0.091$). Importantly, in all cases, it is the G/G genotype and/or G-allele which is more prevalent in depression and other mood disorders. Preliminary results from genotype analysis of samples from MDD and control post-mortem brains (see 3.2.7) also follow this pattern. Further large studies and meta-analyses may therefore detect a significant association, as the trend appears consistent but the effect size is small.

In addition, the role of this polymorphism in mood disorders may also be elucidated by gene-environment interaction studies similar to those performed with the 5-HTTLPR (see 1.3.5.5). As mentioned in 1.3.5.1, a study of the transmission disequilibrium of the 5-HT1A C(-1019)G SNP in suicide attempter families found that the G-allele showed a

strong trend ($p=0.063$) of overtransmission with suicidality in a subset of individuals with high levels of stressful or traumatic life events, although no overtransmission was detected when all families were considered (Wasserman *et al.*, 2006). Future work should address interactions between life stresses and this SNP as potential risk factors for MDD.

Also, additional studies like those of Parsey *et al.* (2006) and Domschke *et al.* (2005), which examined the effect of the SNP on 5-HT1A binding and activation of brain regions implicated in MDD (see 1.3.5.1), may enhance our understanding of the mechanisms by which the G-allele predisposes certain individuals to mood disorders. Indeed, an analysis of genotype effects on 5-HT1A binding and other brain morphological characteristics is currently ongoing in collaboration with Dr. Craig Stockmeier using the post-mortem human brains from which the human brain DNA was obtained (data not shown).

The significant association of both the 5-HT2A A(-1438)G SNP G-allele and G/G-genotype with MDD in males, and the trend towards an association when both sexes are considered, are important findings of this work. These results are interesting in the context of a previous study which found a significant association between the 5-HT2A T102C SNP (which may or may not be strongly linked to the A(-1438)G SNP as described above) and MDD using a subset of the samples examined in this study (Du *et al.*, 2000). Several previous studies have shown an association of both SNPs with MDD, although this was not supported by meta-analysis (Anguelova *et al.*, 2003a; Choi *et al.*, 2004; Du *et al.*, 2000; Khait *et al.*, 2005; Koks *et al.*, 2006) (see 1.3.5.3). However, the number of studies examining the A(-1438)G SNP is relatively small, and future work should examine each of the SNPs in order to confirm or refute linkage disequilibrium

between them and determine which or both is a risk allele for mood disorders. Accordingly, the T(102)C SNP will be examined for the Canadian MDD and control cohort.

The finding that the 5-HT2A SNP is only associated with MDD in males is interesting in the context of twin and adoptee studies which have addressed gender differences in the genetics and heritability of MDD. Although the results of these studies are somewhat conflicting, a large study of almost 3000 twin pairs found that heritability was somewhat greater in females, although when stricter diagnostic criteria were used, this effect disappeared. Importantly, the study also found that the genetic risk factors for MDD in males and females were positively correlated (between +0.5 and +0.65) but not identical. This implies that there may be differences in the impact of certain loci on MDD susceptibility between the two sexes (Kendler *et al.*, 2001). Confirming that the A(-1438)G allele confers a greater risk to MDD in males will require further evaluation of different populations.

A recent large meta-analysis found that the A(-1438)G G/G genotype (but not the T102C SNP) was correlated with suicidality (Li *et al.*, 2006), and an association of the 102 C allele and C/C genotype with any suicidal ideation (Item 3 > 0) was reported in the previous analysis of a subset of the samples examined in this thesis (Du *et al.*, 2000). However, no association with suicidal ideation was found with the A(-1438)G SNP when the entire sample set was divided into no or low versus moderate or severe suicidal ideation (Appendix VIII). This discrepancy with previous results may be due to differences between studies in defining suicidality and in the 5-HT2A SNP examined.

Finally, the lack of association detected between the D2 Taq1A (C/T) SNP and MDD supports the conclusions of the majority of studies analyzing this SNP and mood disorders (Noble *et al.*, 2003). Interestingly, a single photon emission computed tomography study found significantly lower striatal D2 binding in MDD patients that responded to SSRI treatment compared to non-responders and controls, indicating that reduced D2 levels may be implicated in depression in the subset of patients who are treatment-responsive (Klimke, 1999). Given the evidence of lower D2 levels in T-allele carriers (Ritchie *et al.*, 2003), future studies should examine the association of this SNP with antidepressant response.

As mentioned above (1.3.4 and 1.3.5), no robust and highly replicable associations have yet been found between any genetic polymorphisms and either schizophrenia or major depression. Indeed, unlike rare disorders involving single gene defects, deciphering the genetic basis of most common complex disorders is extremely difficult for a number of reasons. The genes involved often confer a small increase in susceptibility to a disease, which can be modified by other genes, epigenetic factors and the environment (Becker, 2004). In addition, many common disorders, and especially psychiatric disorders, share many clinical features (e.g. the negative symptoms of schizophrenia overlap with MDD) (Bearden *et al.*, 2004). For this reason, it has been proposed that genetic variants with weak effects may be common and could confer susceptibility to many related disorders, and that manifestation of a particular disease is thus dependent on other genetic and environmental factors (Becker, 2004; Hirschhorn, 2005). This hypothesis validates the goals of this study, which examined several common variants in two related psychiatric disorders. However, it also emphasizes the

difficulty in detecting a significant association for individual variants with individual disorders, as the effect size is most often small, and influencing genetic and environmental factors may be present to varying degrees in different populations. Indeed, in a review of 166 different disease-associated genetic variants, only 6 were found to be replicated in at least 75% of subsequent studies. However, a number of additional variants showed some replication. These discrepancies between studies can be explained by the need for sample sizes in the thousands in order to have high enough power to detect a significant association of a gene that confers a small (e.g. 10%) risk for a disease (Hirschhorn *et al.*, 2002; Hirschhorn, 2005).

Several solutions to these problems exist, including examining phenotypes that are more directly related to the functional effect of the variant (e.g. brain morphology, receptor levels or behavioural traits), rather than particular disorders (Bearden *et al.*, 2004). Also, as mentioned above, meta-analyses of multiple studies can potentially better detect weak but consistent genetic effects. Finally, replicate studies examining several polymorphisms in concert or the effect of environment on genetic risk may help to resolve findings of population-specific genetic susceptibilities while decreasing the likelihood of false-positive results from multiple testing (Hirschhorn *et al.*, 2002).

Therefore, although a significant association of the 5-HT2A A(-1438)G SNP with MDD only in males was detected, examining the role of functional variants such as the 5-HT1A C(-1019)G and the D2 Taq1A (C/T) SNPs in the above frameworks may be revealing.

4.3 Sequence Analysis of the Primate 5-HT1A Promoter

Analysis of the 5-HT1A promoter sequence of 20 rhesus macaques and 13 cynomolgus macaques revealed both intraspecies polymorphisms and interspecies differences between macaques and humans in important regulatory elements (Figure 3.6, Figure 3.9, Table 3.7). Although a draft sequence of the rhesus macaque genome has been published (see 1.4), to my knowledge, the cynomolgus macaque 5-HT1A promoter sequence has not been reported, nor have any polymorphisms in the 5-HT1A promoter region of either species of macaques. In addition, comparative sequence analyses between the macaque and human 5-HT1A promoters do not appear to have been performed. Therefore, the intraspecies variants reported here are novel polymorphisms in the rhesus and cynomolgus macaque sequences. With regards to these variants, the A/G substitution at -969 in the cynomolgus macaques is of interest firstly because of its proximity to the 26bp palindrome to which NUDR binds. Also, it is one of very few sites of difference between the consensus rhesus (G) and the consensus cynomolgus (A) sequences. In addition, the A-allele eliminates a putative estrogen response element (ERE) which is present with the G-allele (determined using the Transcription Element Search Site (TESS), <http://www.cbil.upenn.edu/tess>) (data not shown). The consensus ERE is comprised of two inverted half sites separated by three nucleotides: GGTCAnnnTGACC (Nelson *et al.*, 1999). In comparison, the putative macaque ERE has one perfect half-site separated by three nucleotides from an imperfect half site: **GGTCA***gtc***TCCCA** (matches to the consensus ERE are in bold, and the A/G substitution site is in italics). While only two of five nucleotides of the imperfect half-site correspond to the consensus half-site, the same degree of correspondence is seen on occasion in the

EREs of known estrogen-responsive genes; for example, the mouse c-fos ERE is **GGTCAnnnCAGCC** (Klinge, 2001). It should be noted that the putative macaque ERE has been determined by sequence similarity to the consensus alone; further work (e.g. EMSA and transcriptional reporter assays) is necessary to determine if this element can actually bind to the estrogen receptor. If the putative ERE site does prove to be functional, it would be interesting to examine the effect of the polymorphism on serotonergic function and behaviour in macaques, in particular because of evidence that ovarian steroids affect the serotonin system and stress-related behaviour in this primate model (Bethea *et al.*, 2002; Shively and Bethea, 2004). To this end, additional cynomolgus macaques which have been extensively characterized with respect to both 5-HT1A expression and depressive behaviour (Shively *et al.*, 2005; Shively *et al.*, 2006) are currently being genotyped for this polymorphism. Preliminary data (not shown) indicates that the variant is present in this population of macaques.

The two additional sites of sequence variance (both insertion/deletion mutations at -1101 and -1117) observed in the macaque 5-HT1A promoter are within a large repetitive region which is poorly conserved between primate species, and the TESS search did not recover any transcription factor binding sites which may be potentially affected by these polymorphisms.

With regards to the interspecies differences observed when the rhesus and cynomolgus macaques' sequences were aligned with those of chimpanzees and humans, it is important to note that the two macaque species are closely related, and in a phylogenetic analysis based on mitochondrial DNA sequence, they clustered together independently from chimpanzees and humans, which also grouped together (Appendix

X) (Hayasaka *et al.*, 1988). The sequences obtained of the rhesus and cynomolgus macaque 5-HT1A promoters appear to support this phylogeny. This analysis is also of value as macaques can provide a valuable outgroup for use in chimpanzee-human sequence comparisons, which is useful when attempting to determine whether differences are due to mutations in the human or chimpanzee sequences (Rogers *et al.*).

It is also interesting that serotonin receptors are evolutionarily ancient. The primordial 5-HT receptor is thought to have evolved over 800 million years ago, while the 5-HT1A receptor is estimated to have separated from other 5-HT1 receptors at approximately the same time as vertebrates and invertebrates diverged – 650 million years ago. However, the rate of amino acid sequence evolution is approx 1% per 10 million years, and 5-HT receptor proteins are ~91% conserved between 10 mammalian species (Peroutka and Howell, 1994). This indicates that 5-HT1A receptor sequence homology between primate species may be quite high, especially in the coding regions, but also in functionally important non-coding regions such as promoter elements. This is supported by the fact that in the regions surrounding the NUDR-binding element and the Freud-1-binding DREs, 90% and 94% of nucleotides are conserved, respectively, between the four primate species.

Of the interspecies differences, the deletion in the putative macaque NUDR-binding site (in comparison to the derived consensus NUDR core binding motif) was of particular interest due to the regulation by NUDR of 5-HT1A expression. However, EMSA indicated that NUDR can still bind the macaque element (Figure 3.8), which may imply that NUDR regulates 5-HT1A expression in macaques similarly as in humans. This supports the previous finding that although the consensus core binding site for efficient

NUDR binding using *in vitro* screening was AAGC (present in ~75% of selected oligonucleotides), several oligonucleotides with variable insertions or deletions in the core sequence also bound NUDR (Huggenvik *et al.*, 1998). It should be noted, however, that EMSA only detects the ability of a protein to bind a DNA sequence *in vitro* but does not indicate functionality; in the future, co-transfection assays must be performed to ascertain the ability of NUDR to repress its respective macaques versus humans binding elements in an *in vivo* paradigm.

Lastly, the variation in the macaque DREs in comparison to the human sequence did not appear to eliminate Freud-1 binding (Figure 3.10), again potentially indicating similar mechanisms of 5-HT1A transcriptional regulation in humans and macaques. Additional EMSAs are required to evaluate the relative strength of binding to the different elements, and again, co-transfection assays are necessary to determine the ability of Freud-1 to repress transcription through the macaques' elements.

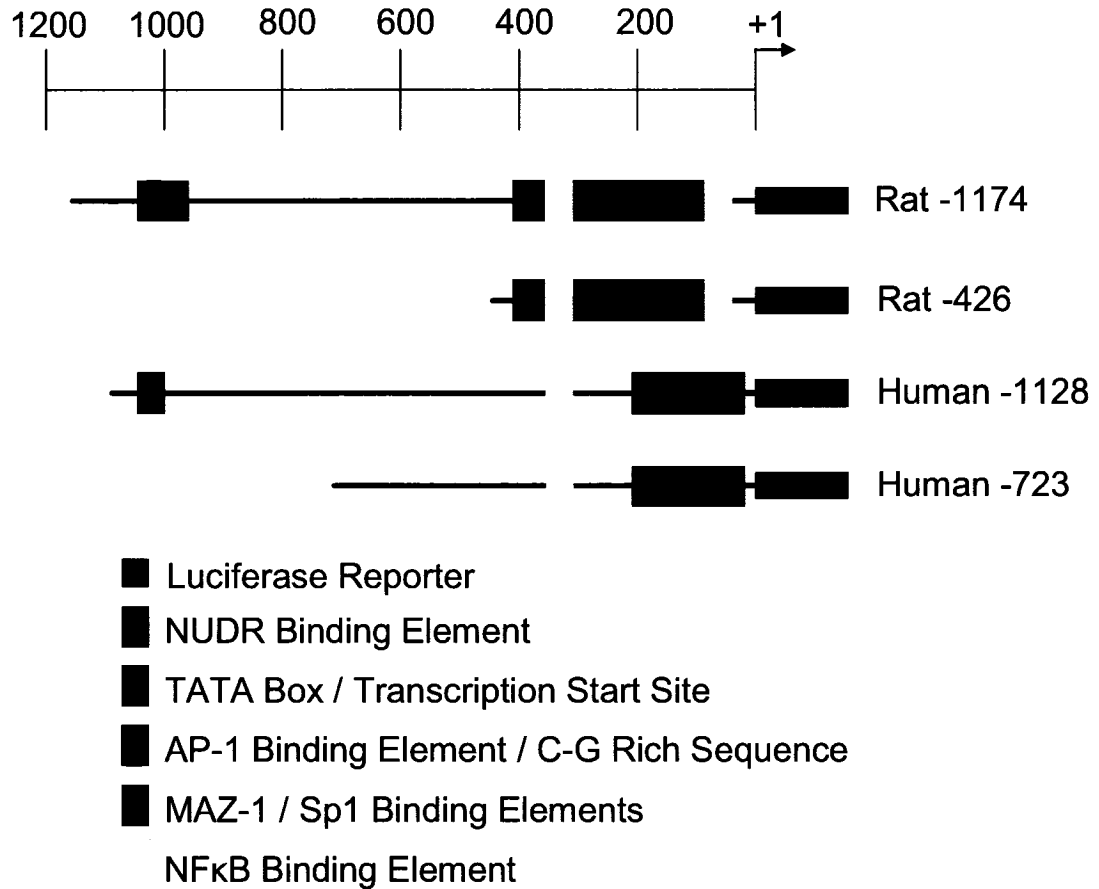
4.4 Conclusions

Results of the above studies have been negative regarding the hypothesis that 5-HT1A autoreceptor activation by agonist treatment downregulates 5-HT1A gene expression. However, many confounding issues such as very low levels of endogenous 5-HT1A receptor expression in the cell lines, assaying promoter constructs which lack certain potentially important elements located further upstream, inconsistencies in the health of the primary cultures as well as in their 5-HT1A mRNA levels and possibly their responsiveness, and the embryonic nature and heterogeneity of cell types in the cultures make it difficult to draw firm conclusions from these results. By further investigating the

effects of activators and repressors of 5-HT_{1A} signaling pathways on transcription of the 5-HT_{1A} autoreceptor, new insights may be gleaned regarding the mechanisms of SSRI antidepressant action. As SSRIs are the most widely used class of therapy for major depressive disorder, a prevalent and disabling disease, such knowledge could lead to improvements in the efficacy of current drugs, and to the development of new pharmacotherapies.

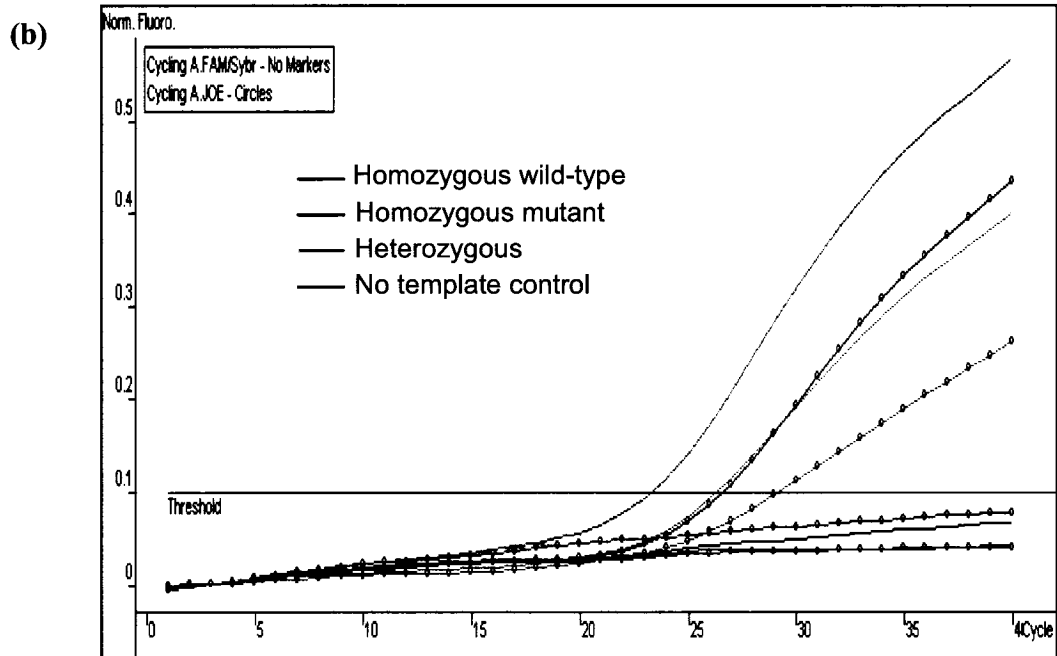
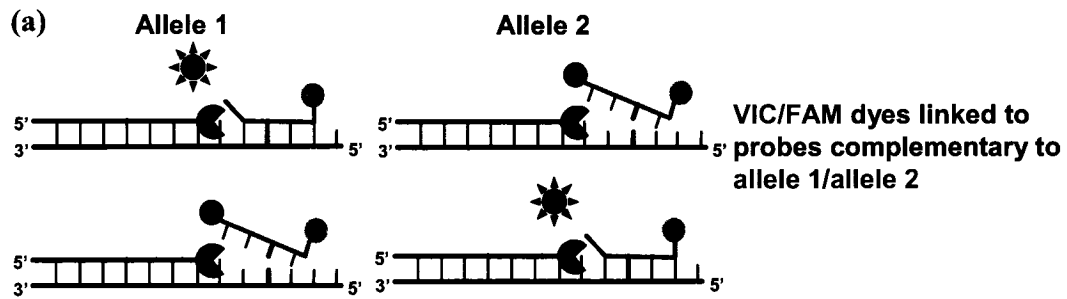
Although the only statistically significant association detected in this thesis was between the 5-HT_{2A} A(-1438)G SNP and MDD in males, the presence of a trend towards an association of the 5-HT_{1A} C(-1019)G SNP with MDD was also of interest and should be investigated further. Association analysis both of functional polymorphisms in the monoaminergic receptors targeted by atypical antipsychotics with schizophrenia, and of 5-HT receptor and transporter variants with major depression, may contribute to knowledge about the genetics of developing and responding to pharmacotherapy for these neuropsychiatric disorders.

This work identified novel genetic polymorphisms in the rhesus and cynomolgus macaque 5-HT_{1A} promoter sequences. Future studies examining the potential functionality of these variants could provide insight into how 5-HT_{1A} receptor regulation affects the serotonin system as well as behaviour in a primate model of depression. In addition, comparative analysis of the 5-HT_{1A} promoter sequence of humans and macaques has revealed interesting interspecies variations. Further examination of the potential functional consequences of these differences may bolster our understanding of the 5-HT_{1A} gene's regulation. This in turn may augment our understanding of the role of this important gene in both normal affect and pathological mood disorders.



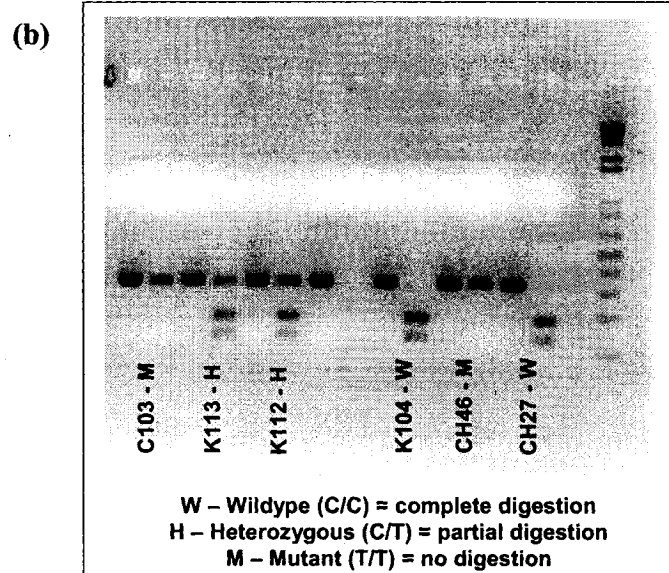
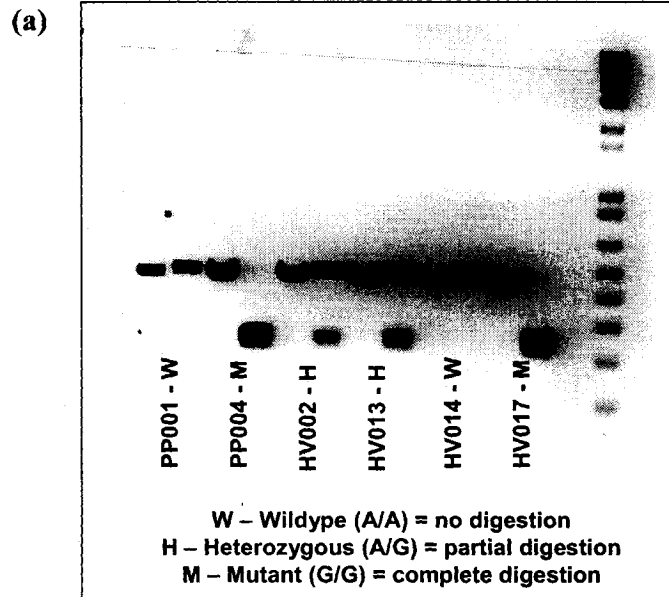
Appendix I. Structure of Rat and Human 5-HT1A Promoter Constructs.

Sequential 5' deletions of the rat and human 5-HT1A promoter sequence were inserted upstream of the luciferase reporter gene in the pGL3B vector as described in Storrington *et al.* (1999) and Lemonde *et al.* (2004a). The relative positions of known promoter elements are indicated. Note that the exact location of the NUDR binding element in the rat 5-HT1A promoter has not been determined. Scale at top indicates distance upstream from ATG start codon (+1).



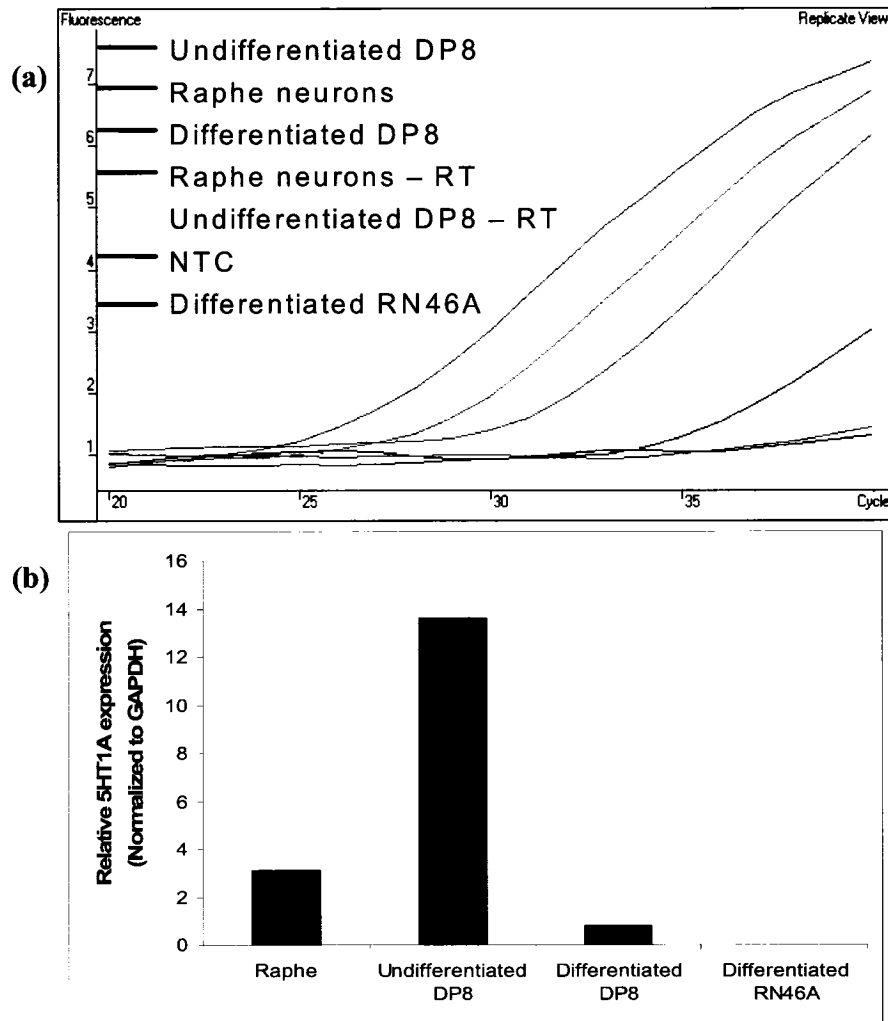
Appendix II. Genotyping using TaqMan SNP Genotyping Assays.

(a) The assay contains probes specific for each allele which are labeled with different 5' fluorescent reporter dyes (VIC/JOE (circles) and FAM (straight line)). Reporter dyes are quenched by proximity to a 3'quencher dye. DNA polymerase cleaves the reporter dye, which fluoresces upon release from the quencher dye. Only the complementary (matched) probe hybridizes strongly to its allele; unmatched probes do not hybridize or are displaced rather than cleaved by DNA polymerase. (b) Example of an allelic discrimination amplification plot. Genotype is determined by probes with fluorescence intensity (represented for each probe by straight or circled lines) above threshold.



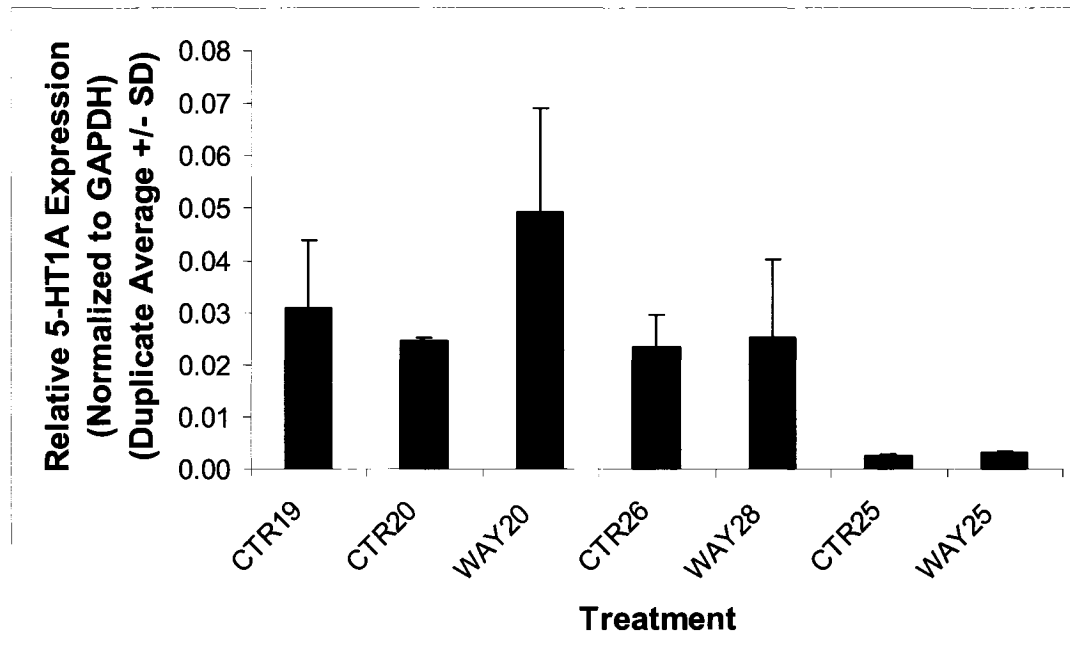
Appendix III. Verification of TaqMan SNP Genotyping Assays.

Assay accuracy was confirmed for six DNA samples by SNP-specific enzymatic digestion and gel electrophoresis of PCR amplification products, as described in Spurlock *et al.* (1998) and Grandy *et al.* (1993). (a) The 5-HT_{2A} A(-1438)G assay was verified by HpaII digestion. (b) The D2 Taq1A (C/T) assay was verified by TaqI digestion.



Appendix IV. Relative 5-HT1A mRNA levels in rat primary raphe cultures and serotonergic cell lines.

RNA was extracted from E14-15 raphe neurons cultured 7 DIV and from differentiated RN46A cells and differentiated and undifferentiated DP8 cells using Trizol, and then was DNase treated and reverse-transcribed to generate cDNA. 5-HT1A mRNA levels were measured by Q-PCR using TaqMan probes, and normalized to GAPDH mRNA levels measured in multiplex. Results are representative of several experiments. (a) 5-HT1A amplification plot. (b) Relative 5-HT1A mRNA levels in different cultures.



Appendix V. Effect of long-term 5-HT1A antagonist treatment on 5-HT1A mRNA levels in primary raphe cultures.

E14-15 rat raphe neurons cultured 7 DIV were treated with 1 μ M WAY-100635 for 5 days (WAY); media was changed for control cultures (CTR). RNA extraction was performed using Trizol, followed by DNase treatment and reverse-transcription to generate cDNA (Samples were obtained at this point from FRL). 5-HT1A mRNA levels were measured by Q-PCR using TaqMan probes, and normalized to GAPDH mRNA levels measured in multiplex. Data are presented as mean \pm SD for duplicates of each sample.

Appendix VI. Allele and genotype distribution of the human 5-HT1A promoter C(-1019)G polymorphism in major depressive disorder (MDD) and control subjects from American cohort.

Genomic DNA extracts were analyzed using a QPCR-based genotyping assay (ABI: C_11904666_10). Genotype frequencies were compared by χ^2 analysis with two-tailed p values; allele frequencies were compared by Fisher's exact test with two-tailed p values.

Subjects	N	Genotype frequencies (%)			Allele frequencies (%)	
		C/C	C/G	G/G	C	G
American Controls	26	10 (38.5)	12 (46.2)	4 (15.4)	32 (61.5)	20 (38.5)
American MDD Subjects	26	8 (30.8)	12 (46.2)	6 (23.1)	28 (54.8)	24 (45.2)

American control versus MDD subjects: genotype, $\chi^2 = 0.6222$, $df = 2$, $p = 0.7326$ (ns); allele: $p = 0.5518$ (ns).

Appendix VII. Allele and genotype distribution of the human 5-HT1A promoter C(-1019)G polymorphism in major depressive disorder (MDD) subjects divided by suicidality and control subjects from Canadian cohort.

Genomic DNA extracts were analyzed using a QPCR-based genotyping assay (ABI: C_11904666_10). Genotype frequencies were compared by χ^2 analysis with two-tailed p values; allele frequencies were compared by Fisher's exact test with two-tailed p values. Suicide is Item 3 on the Hamilton Rating Scale for Depression (HAMD) and is scored as follows: 0 = Absent; 1 = Feels life is not worth living; 2 = Wishes he were dead or any thoughts of possible death to self; 3 = Suicidal ideas or gesture; 4 = Attempts at suicide.

Subjects	N	Genotype frequencies (%)			Allele frequencies (%)	
		C/C	C/G	G/G	C	G
Canadian Controls	213	63 (29.6)	113 (53.1)	37 (17.4)	239 (56.1)	187 (43.9)
Canadian MDD Item 3 < 2	118	28 (23.7)	60 (50.8)	30 (25.4)	116 (49.2)	120 (50.8)
Canadian MDD Item 3 ≥ 2	64	18 (28.1)	32 (50.0)	14 (21.9)	68 (53.1)	60 (46.9)

Canadian control versus MDD Item 3 < 2 subjects: genotype, $\chi^2 = 3.448$, $df = 2$, $p = 0.1783$ (ns); allele: $p = 0.0883$ (ns). Canadian control versus MDD Item 3 ≥ 2 subjects: genotype, $\chi^2 = 0.7753$, $df = 2$, $p = 0.7170$ (ns); allele: $p = 0.6123$ (ns). Canadian MDD Item 3 < 2 versus MDD Item 3 ≥ 2 subjects: genotype, $\chi^2 = 0.5393$, $df = 2$, $p = 0.7636$ (ns); allele: $p = 0.5106$ (ns).

Appendix VIII. Allele and genotype distribution of the human 5-HT_{2A} promoter A(-1438)G polymorphism in major depressive disorder (MDD) subjects divided by suicidality and control subjects from Canadian cohort.

Genomic DNA extracts were analyzed using a QPCR-based genotyping assay (ABI: C_8695278_10). Genotype frequencies were compared by χ^2 analysis with two-tailed *p* values; allele frequencies were compared by Fisher's exact test with two-tailed *p* values. Suicide is Item 3 on the Hamilton Rating Scale for Depression (HAM-D) and is scored as follows: 0 = Absent; 1 = Feels life is not worth living; 2 = Wishes he were dead or any thoughts of possible death to self; 3 = Suicidal ideas or gesture; 4 = Attempts at suicide.

Subjects	N	Genotype frequencies (%)			Allele frequencies (%)	
		A/A	A/G	G/G	A	G
Canadian Controls	213	47 (22.1)	111 (52.1)	55 (25.8)	205 (48.1)	221 (51.9)
Canadian MDD Item 3 < 2	118	26 (22.0)	50 (42.7)	42 (35.6)	102 (43.2)	134 (56.8)
Canadian MDD Item 3 ≥ 2	64	11 (17.2)	32 (50.0)	21 (32.8)	54 (42.2)	74 (57.8)

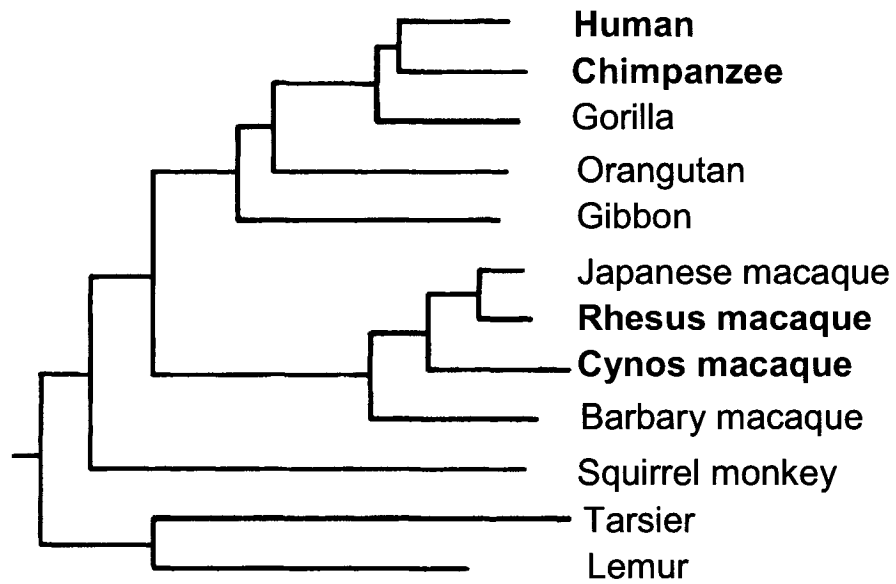
Canadian control versus MDD Item 3 < 2 subjects: genotype, $\chi^2 = 3.955$, *df* = 2, *p* = 0.1384 (ns); allele: *p* = 0.1944 (ns). Canadian control versus MDD Item 3 ≥ 2 subjects: genotype, $\chi^2 = 1.478$, *df* = 2, *p* = 0.4775 (ns); allele: *p* = 0.2264 (ns). Canadian MDD Item 3 < 2 versus MDD Item 3 ≥ 2 subjects: genotype, $\chi^2 = 1.108$, *df* = 2, *p* = 0.5747 (ns); allele: *p* = 0.9118 (ns).

Appendix IX. Combined genotype distribution of the human 5-HT1A promoter C(-1019)G polymorphism and the 5-HT2A promoter A(-1438)G polymorphism in major depressive disorder (MDD) and control subjects from Canadian cohort.

Genomic DNA extracts were analyzed using a QPCR-based genotyping assay (ABI: 5-HT1A, C_11904666_10; 5-HT2A, C_8695278_10). All genotype frequencies were compared by χ^2 analysis with two-tailed p values. Frequencies of presence versus absence of at least one risk allele for each gene was compared by Fisher's exact test with two-tailed p values.

Subjects	N	5-HT1A/5-HT2A Genotype frequencies (%)								
		CC/AA	CC/AG	CC/GG	CG/AA	CG/AG	CG/GG	GG/AA	GG/AG	GG/GG
Canadian Controls	213	14 (6.6)	33 (15.5)	16 (7.5)	26 (12.2)	58 (27.2)	29 (13.6)	7 (3.3)	20 (9.4)	10 (4.7)
Canadian MDD Subjects	182	6 (3.3)	26 (14.3)	14 (7.7)	23 (12.6)	34 (18.7)	35 (19.2)	8 (4.4)	22 (12.1)	14 (7.7)

Canadian control versus MDD subjects for all genotype combinations: genotype, $\chi^2 = 9.626$, $df = 8$, $p = 0.2923$ (ns). Canadian control versus MDD subjects for presence versus absence of at least one risk allele for each gene: $p = 0.6117$ (ns).



Appendix X. Primate phylogeny as determined by mitochondrial DNA sequence.

In red are species examined in this thesis. (Adapted from Hayasaka *et al.*, 1988).

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