Genetics of premenstrual syndrome: investigation of specific serotonin receptor polymorphisms

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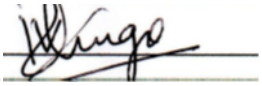
Signature of candidate …  …… Date ……02/06/2014

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ABSTRACT

Premenstrual dysphoric disorder (PMDD) is a distressing and disabling syndrome causing a significant degree of impairment on daily functioning and interpersonal relationships in 3-8% of the women.[1-3] With the convincing evidence that PMS is inheritable and that serotonin is important in the pathogenesis of PMS, and failure of initial studies to demonstrate significant associations between key genes controlling the synthesis, reuptake and catabolism of serotonin and PMDD, the main aim of this thesis was to target the functional polymorphisms of serotonin receptors.

Bioinformatics were used to identify the evolutionary relationship between the various serotonin receptors and their subtypes by drawing the phylogenetic tree and delineating the primary, secondary and tertiary structures of the receptors. We concluded that although close to the rest of the serotonin receptors in the evolution tree, 5-HT3 receptors constitute a separate family of receptors. Hence we hypothesize that the clad containing the 5-HT1,2,4,5,7 group of receptors comprises a series of homologous genes arisen by gene duplication and share common structural features.

Applying genomic techniques we analysed and looked for association between PMDD and the candidate genes $5HT_{1A}$, $5HT_{1B}$, $5HT_{2A}$, $5HT_{2C}$ and $5HT_{7}$, selected according to the following criteria: involvement of the genes in the function of serotonin; representative of the common receptor protein structure and equally spread around the evolutionary tree. The polymorphisms selected $5HT_{1A}$ C(-1019)G, $5HT_{1B}$ (A-161T), $5HT_{2A}$ (T102C), $5HT_{2C}$ (Cys23Ser) and $5HT_{7}$ (Pro279Leu) have been previously described and their suitability for genotyping assessed.

This is the first study linking the 5HT1A C(-1019) allele and PMDD. There was a marked over-representation of the C/C genotype of 5-HT1A C(-1019)G polymorphism in the

PMDD group. The presence of at least one C allele was associated with a 2.5-fold increased risk of PMDD. There were no significant associations between the other tested genotypes, allelic distribution and clinical category. These findings do not support a major role for common polymorphisms in contributing to susceptibility to PMDD.

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GLOSSARY & ABREVIATIONS

5-HT	5-Hydroxytryptamine
5-HIAA	5-Hydroxy-indol-acetic acid
AADC	Aromatic I- amio acid decarboxylase
ACTH	Adrenocorticotropic hormone
CGIS	Clinical Global Impression Scale
CHROMA	Chromatic representation of multiple alignments
CNS	Central nervous system
DOPA	Dihydroxyphenylalanine
DRSP	Daily Record of Severity of Problem
DSM	The Diagnostic and Statistical Manual of Mental Disorders
FSH	Follicle stimulating hormone
GABA	γ Aminobutyric acid
GAS	Global Assessment Scale
HPG axis	hypothalamic–pituitary–gonadal axis
HR-QOL	Health related quality of life
ISPMD	International Society for Premenstrual Disorders
LH	Luteinising hormone
MDQ	Menstrual Distress Questionnaire
OCP	Oral contraceptive pills
PAF	Premenstrual Assessment Form
PMD	Premenstrual Disorders
PMDD	Pre Menstrual Dysphoric Disorder
SSRI	Selective serotonin reuptake inhibitors
TPH	Tryptophan hydroxylase
VAS	Visual Analogue Scale

APPENDICES

CHAPTER 1:

PREMENSTRUAL SYNDROME/ PREMENSTRUAL DYSPHORIC DISORDER: LITERATURE REVIEW

1.1 INTRODUCTION

Majority of premenopausal women experience some level of premenstrual somatic, affective and behavioural symptoms[4]. The nature and severity vary from minimal to disabling, only 3-8% of these women suffer from extreme premenstrual symptoms to such a degree that it interferes with normal functioning and are diagnosed with premenstrual dysphoric disorder (PMDD). [1-3;5-7]

There was lack of credibility and interest among practitioners regarding premenstrual syndrome, but due to increasing social impairment and need for medical care raised concerns among psychiatrists. More recently with the diagnosis criteria generalization, the understanding, aetiology and response to medication has brought specialist from worldwide together to understand and treat this syndrome better.

Symptoms of Premenstrual Syndrome have been described from the time of Hippocrates, "the blood of females is subject to intermittent agitations and as a result the agitated blood make its way from the head to the uterus whence it is expelled."[8] Centuries passed before premenstrual symptoms were identified or referred to again. First time in 1931 Robert Frank, described Premenstrual Tension on paper at the Academy of Medicine, New York.[9] Frank brought it to the attention of the medical world the relationship of premenstrual symptoms to Ovarian Cycle. Later the recurrent and cyclical nature of premenstrual tension was highlighted by Karen Horney in 1931.[10] The word Premenstrual Syndrome was coined by Katharina Dalton in 1953,[11] the term is still widely used by women and practitioners. She published and reported many of her case studies where she showed that treatment with progesterone was invariably successful in the management of Premenstrual Symptoms.[11]

In 1983 in the United States, the National Institute of Mental Health conference devoted to this topic proposed the first diagnosis criteria, requiring a prospective and daily assessment of the symptoms. In 1987, the American Psychiatric Association, in the DSM III-R, introduced the Late Luteal Phase Dysphoric Disorder diagnosis that became in 1994 in the DSM IV the Premenstrual Dysphoric Disorder, with the same diagnosis criteria.[12]

Recently International Society for Premenstrual Disorders (ISPMD) group has developed an international universally acceptable multidisciplinary agreement regarding definition, quantification and clinical trial design of premenstrual disorders (PMD).[13]

Many theories have been proposed and investigated to establish the aetiology of Premenstrual Syndrome including Ovarian Hormone imbalance, water & electrolyte imbalance, deficiency of vitamins and minerals, allergy, hypoglycaemia, hyper-insulinaemia and psychology theories. Most of the studies diagnosed premenstrual syndrome randomly based on the patient's perception of her symptoms or the clinician's diagnosis from the history; hence a great effort has been put into evolving a Diagnostic Criteria and to Quantify Premenstrual Syndrome. In 1990 the DRSP (Daily Record of Severity of Problems) form was developed to aid in the diagnosis and evaluation of PMDD(Premenstrual Dysphoric Disorder)[13;14]

There is a wide range of treatment options available, and are being used to treat the PMDD symptoms worldwide. The research into the neurobiology of PMS/PMDD has been directly influenced by the successful management of these symptoms with the use of SSRI's (Selective Serotonin Reuptake Inhibitors).

Over these centuries research and studies have left no doubt that normal endocrine ovarian cycle provokes or triggers premenstrual symptoms in women with CNS sensitivity to these hormonal changes. Research using molecular genetics, endocrinology and brain

imaging will help explaining this sensitivity of neurotransmitters in CNS to endocrine changes.

1.2 THE SYNDROME/PMDD

Premenstrual syndrome (PMS) is a recurrence of negative behavioural (e.g. fatigue), psychological (e.g. irritability) and physical symptoms (e.g. headaches) that occur during the luteal phase of the menstrual cycle and remit by the follicular phase.[4]

1.3 PREVALENCE

In the literature, prevalence rates are very heterogeneous according to the diagnosis criteria used and to the populations studied. It has been sited that the incidence of the severe dysphoric symptoms necessitating the need for women to seek help and warrant treatment is 3-9%. [3;5-7;15]

Even though premenstrual symptoms are described in women from menarche to menopause, it is unclear whether symptoms remain stable or increase in severity with age.[2;16] Irritability has been identified as the most common premenstrual symptom in US and European samples.[3;16;17] Some cultures emphasize somatic rather than emotional premenstrual symptoms.[18] Symptom severity peaks on or just before the first day of menses.[17;19;20] Studies examining age, menstrual cycle characteristics, cognitive attributions, socioeconomic variables, lifestyle variables and number of children have not identified these variables as predisposing factors.[16;21] An elevated lifetime prevalence of major depressive disorder (MDD) in women with PMDD has been reported in several studies,[22] as has an elevated lifetime prevalence of postpartum depression.[23]

1.4 BURDEN OF THE ILLNESS ON WORK AND FAMILY

Hellman et al in 1987 have reported an increase in the number of sick days taken by women suffering from PMS.[24] The diagnosis of PMDD requires the confirmation of luteal-phase impairment of social and/or work functioning that is due to premenstrual symptoms. The functional impairment reported by women with PMDD is similar in severity to the impairment reported in MDD and dysthymic disorder.[25;26] One study identified anxiety, irritability and mood lability as the premenstrual symptoms most associated with functional impairment.[27] The burden of illness of PMDD results from the severity of symptoms, the chronicity of the disorder and the impairment in work, relationships and activities.[28] It has been estimated that women with PMDD cumulatively endure 3.8 years of disability over their reproductive years.[25] A study of 1194 women who prospectively rated their symptoms reported that women with PMDD were more likely to endorse hours missed from work, impaired productivity, role limitations and less effectiveness.[29] Borenstein and colleagues[30;31] have published studies examining functioning and health service use in 436 women who prospectively charted their symptoms for 2 cycles. Women with confirmed PMS reported significantly lower quality of life, increased absenteeism from work, decreased work productivity, impaired relationships with others and increased visits to health providers, compared with control women. These authors also reported that, given a 14% absenteeism rate and a 15% reduction in productivity, PMDD was associated with US$4333 indirect costs per patient per year.[32] The economic burden associated with PMDD is more related to self-reported decreased productivity than to direct health care costs.[29;32] However, women with PMDD do report increased health services use, with visits to health care providers and use of prescription medications and alternative therapies.[29;31] Small studies of women with

prospectively confirmed PMDD have also reported decreased interpersonal and work functioning and reduced quality of life in comparison with women without PMDD.[33;34] Larger studies of women diagnosed retrospectively according to PMDD criteria have also reported substantial functional impairment in work and interpersonal roles.[2;35-37] Recently Heinemann LA; has reemphasised in his recent web based study that moderate-to-severe PMS/PMDD seems to be associated with work productivity impairment and increased absenteeism, and thus poses a potential economic burden.[38;39] They have also shown that moderate-to-severe PMS/PMDD has a negative impact on HR-QOL(health related quality of life), hobbies and social activities, and relationships with others.[40]

1.5 CLINICAL PRESENTATION

Millions of women of reproductive age have recurrent emotional, cognitive, and physical symptoms related to their menstrual cycles. These symptoms often recur discretely during the luteal phase of the menstrual cycle and may significantly interfere with social, occupational, and sexual functioning. Symptoms last for several days to two weeks before the menses and remit during the menstrual flow. More than 300 type of symptoms have been identified in the premenstrual phase.[25] In addition to increases in dysphoric mood during the luteal phase, women with PMDD also report impairments in cognitive abilities such as concentration, memory and motor coordination that interfere with their productivity and efficiency.[12;41] However, the extent to which various aspects of psychomotor or cognitive performance are actually impaired during the luteal phase in women with PMDD has not been extensively studied and the results have been inconsistent, with studies reporting no differences[42] or only subtle differences on isolated tasks.[43-45] For instance, although Keenan and colleagues[46;47] assessed a range of

neuropsychological tasks, women with PMDD were only impaired on a verbal learning task compared to a group of control women and this impairment was not related to menstrual cycle phase. In another well-controlled study that evaluated a range of tasks, the only performance impairment observed was that women with PMDD showed more psychomotor slowing during the luteal phase compared to control women.[45] Another study done by Morgan and Rapkin in 2002,[48] also assessed a full series of neurocognitive tasks reported no performance differences between women with PMDD and control women, despite a relatively large sample size. Evans in his study,[49] assessed changes in mood and performance as a function of menstrual cycle phase in women with confirmed PMDD after placebo or alprazolam administration. When placebo was administered, motor coordination (via a balance task) and performance on the Digit Symbol Substitution Task (DSST) were impaired in the luteal phase. Limitations of that study included only administering placebo one day in each phase and the lack of a control group. Regardless, one major distinction between the Evans study[49] and all the other studies mentioned above is that task performance was assessed multiple times over the day, not just once. The fact that most previous studies only assessed performance on a single occasion each phase may be one reason for the inconsistencies observed across studies.

Women with PMDD also report changes in appetite, food intake and specific food cravings during the luteal phase[12] and these changes appear to be correlated with premenstrual mood changes, primarily depression.[50-52] Most studies relied on retrospective reports of PMDD and retrospective food cravings, typically using a single question that did not specify food type.[50;53] Evans also assessed food cravings[54] and food intake in 19 women with PMDD and showed that craving for foods, specifically those containing fat, were significantly increased in the luteal[55;56] phase compared to the

follicular phase, while desires for CHO alone did not change as a function of menstrual cycle phase. In that same study, when placebo was administered there was no corresponding increase in actual food consumption at lunch during the luteal phase. Reed in his study found that women with PMDD in their luteal phase had increased dysphoric mood; had impaired cognitive performance; had an increased desire for food items high in fat (both savory and sweet); and ate more calories (particularly fat) at lunch compared to when they were in their follicular phase and/or compared to women without PMDD.[57]

Premenstrual exacerbation and co-morbidities need to be identified if present. Many symptoms of other gynaecological problems may be exacerbated or present just before menses for example; primary dysmenorrhea due to release of prostaglandins from the secretory endometrium, secondary dysmenorrhea, dyspareunia etc in case of endometriosis. Menstrually related migraines last longer and resistant are to routine medication, but are related to ovulatory cycles.[58-61]

64% of premenopausal women report of worsening pre-existing depression symptoms premenstrualy. [62] Many women with anxiety or depression seek treatment for PMS[63;64] but these symptoms are not limited to luteal phase. Premenstrual exacerbation of several conditions, are dealt with treating the primary condition and increasing the doses during the luteal phase if premenstrual exacerbation exists.

Recently International Society for Premenstrual Disorders (ISPMD) group in Montreal in September 2008 has developed an international universally acceptable multidisciplinary agreement regarding definition, quantification and clinical trial design of premenstrual disorders (PMD).[13;65]

ISPMD has classified Premenstrual disorder into two types Core PMD and Variant PMD (Figure 1)[65]

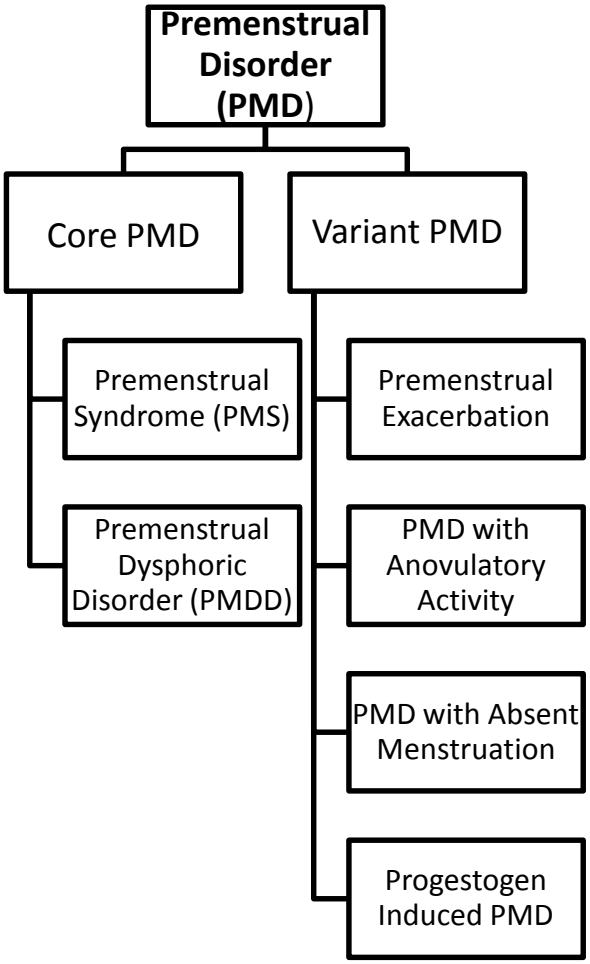


Figure 1: Classification of Premenstrual Disorder according to the ISPMD

1.6 AETIOLOGY

Currently, there is no consensus on the cause of PMDD. The aetiology of severe PMS and PMDD is largely unknown, but the current consensus seems to be that, normal ovarian function (rather than hormone imbalance) is the cyclical trigger for the premenstrual related biochemical events within the central nervous system and other target organs.[66]

1.6.1 Normal Physiology of Menstrual cycle

The menstrual cycle extends from the first day of period to the onset of next menstrual bleeding and comprises of two phase, Follicular phase and Luteal phase. The Hypothalmic- pituitary- Ovarian axis is responsible for the menstrual cycle, which remains

blocked throughout childhood until the age of 11-12yrs. The pulsatile release of Gonadotrophic releasing hormone (GnRH) from the hypothalamus regulates the synthesis and release of Luteinising hormone (LH) and Follicle stimulating Hormone (FSH) from the pituitary. LH and FSH are responsible for the ovarian activation. LH stimulates the synthesis of androstenedione and testosterone from the well vascularised theca cells which are the precursors for the formation of oestrogen.

FSH acts on the granulosa cells increasing the mitosis of these cells and increasing their surface LH, FSH receptors and also acquisition of aromatase activity. The aromatase is responsible for conversion of androgens to oestrogen, which is released into the circulation. Oestrogen and Inhibin B has a feedback effect on the hypothalamus pituitary level augmenting the LH level and inhibiting the FSH release, critical to achieve monofollicular development. The LH surge last approximately for 24-48hrs and ovulation occurs 36hrs[67] after the peak level of serum LH is achieved. LH surge resumes the meiosis step before leading to series of inflammatory events causing follicle to rupture. High levels of LH also lead to decidualization of the granulosa cells which produce the progesterone. This production of progesterone by corpus luteum slows the release of hypothalamic GnRH secretion via the effect on γ- aminobutyric acid (GABA), noradrenergic and opiodergic systems. Once corpus luteum collapses FSH levels rise causing development of new follicles.[68]

Estradiol levels in the blood gradually rise in the follicular phase and abruptly fall for 24-48hrs following follicular rupture; this may lead to various symptoms including transient PMS like symptoms, mittelschmerz or bleeding. Steroidogenic activity of the corpus luteum, causes oestrogen and progesterone levels to rise again for 10-12 days. This function of the corpus luteum is maintained by the release of LH for 10-12 days following

which the function can be prolonged by hCG if conception takes place. There is wide fluctuation in the progesterone levels secondary to the fluctuation in the levels of LH on the corpus Luteum.

Serial measurements of testosterone and free testosterone levels has shown no difference in the women suffering with PMS or controls.[69]

1.6.2 Pathophysiology

Several reviews exist of pathophysiological hypotheses of PMS and PMDD and the evidence for them.[25;55;70;71] Most studies do not identify consistent abnormalities in hormones of the hypothalamic-pituitary-gonadal (HPG) axis, although a few studies have suggested altered luteinizing hormone (LH) pulse.[72] As well, studies have not identified clear abnormalities in thyroid hormones, cortisol, prolactin, glucose, prostaglandins, β-endorphins, vitamins or electrolytes.[25;70] Since specific abnormalities in the HPG axis have not been identified in women with PMDD when compared with control subjects, it is thought that premenstrual symptoms occur as a result of a differential sensitivity to the mood-perturbing effects of gonadal steroid fluctuations in women with PMS and PMDD.[73] It is probable that the aetiology of the "differential sensitivity" is multifactorial and in part genetically determined.[74] The recently identified allelic variation in the oestrogen receptor α gene in women with PMDD may underlie the neurotransmitter and neuropeptide differential sensitivity owing to oestrogen receptor influence on synthesis, receptors, transporters and cell signalling.[75]

Although the specific neurotransmitter, neuroendocrine and neurosteroid abnormalities in women with PMDD are not known, serotonin, norepinephrine, γ-aminobutyric acid (GABA), allopregnanolone (ALLO, an anxiolytic metabolite of progesterone that acts at the

$GABA_A$ receptor), endorphins and factors involved in calcium homeostasis may all be involved. Women with PMDD are more sensitive to the anxiogenic properties of carbon dioxide inhalation, lactate infusion, cholecystokinin tetrapeptide and flumazenil.[25] Increased adrenergic receptor binding may reflect abnormal noradrenergic function.[25] Women with PMDD have abnormal melatonin secretion and other circadian system abnormalities.[76] It has been proposed that women with PMDD have altered affective information processing and regulation during the luteal phase, with abnormal activation patterns in specific brain regions.[74] Imaging studies have demonstrated altered functional magnetic resonance imaging responses to negative and positive stimuli in the orbitofrontal cortex, amygdala and ventral striatum in women with PMDD compared with control subjects.[77] A recent study reported significantly increased negative bias in the recognition of emotional facial expressions during the luteal phase in women with PMDD when compared with control women.[78] Physiological, psychological, environmental and social factors all seem to play a part. Genetic influences mediated phenotypically through neurotransmitters and neuroreceptors seem to play a significant role in the aetiology. That PMS and PMDD are physiological (rather than psychological or psychosocial) phenomena is primarily underscored by recent, convincing evidence of the heritability of premenstrual symptoms[79] and the elimination of premenstrual complaints with suppression of ovarian cyclicity[73] or surgical menopause.[80;81] This viewpoint encourages investigation of the neuroendocrine-modulated central neurotransmitters and the role of the hypothalamic-pituitary-gonadal (HPG) axis in PMDD. Increasing evidence suggests that, of all the neurotransmitters studied to date, serotonin (5-HT) may be important in the pathogenesis of PMDD.[82;83] PMDD also shares many of the features of other mood and anxiety disorders that have been linked to serotonergic dysfunction.[84-86] In addition,

reduction in brain 5-HT neurotransmission is thought to lead to poor impulse control, depressed mood, irritability and increased carbohydrate craving - all mood and behavioural symptoms associated with PMDD. Animal studies have established reciprocity between fluctuations in ovarian steroid levels and serotonergic function, showing that oestrogen and progesterone influence central serotonergic neuronal activity. In the hypothalamus, oestrogen induces a diurnal fluctuation in 5-HT[87] whereas progesterone increases the turnover rate of 5-HT[88] Several challenge tests have also suggested abnormal 5-HT function in women with premenstrual dysphoria. A blunted growth hormone and cortisol response to tryptophan[89] as well as a blunted or delayed prolactin response to fenfluramine and buspirone challenges have been reported by most[90-92] but not all[93] investigators. These studies imply, at least in part, a recurrent, cyclic 5-HT dysfunction in women with premenstrual mood changes.

Taken together, most research indicates that women with PMDD may be behaviourally or biochemically sub- or supersensitive to biological challenges of the serotonergic system. It is not yet clear whether these women present with a trait or state marker of PMDD.

Acute treatment with SSRIs increases synaptic serotonin without the down-regulation of serotonin receptors needed for improvement in overt depression. This finding suggests that PMDD is possibly caused by altered sensitivity in the serotoninergic system in response to phasic fluctuations in female gonadal hormone. Other studies also favour the serotonin theory as a cause of PMDD. In particular, the efficacy of L-tryptophan,[94] a precursor of serotonin, and of pyridoxine,[95] which serves as a cofactor in the conversion of tryptophan into serotonin, also favours serotonin deficiency as a cause of PMDD.

Carbohydrate craving, often a symptom of PMDD, is also mediated through serotonin deficiency.

Because PMDD only affects women of reproductive age, it is reasonable to assume that female gonadal hormones play a causative role, possibly mediated through alteration of serotoninergic activity in the brain. Oestrogen and progesterone seem to modulate levels of monoamines, including serotonin. Eliminating the effect of ovarian gonadal hormones through the use of a gonadotropin-releasing hormone (GnRH) agonist relieves PMDD symptoms.[96] Subsequent administration of oestrogen and progesterone causes symptoms to return in women with PMS but not in those without PMS symptoms.

1.7 GENETICS OF PMS

Evidence for a genetic component in a complex and phenotypic traits can be shown by various studies:

1. Family Studies: the proportion of common genes shared may be predicted by the degree of relatedness.
2. Adoption Studies: Studies have shown there is little change in the rate of recurrence of a disease regardless of whether a child is reared by its adopted or biological parents in different or same environment.
3. Twin studies: concordance is the parameter used in the twin studies.

Inheritance plays a major role in behaviour as shown by selection and strain studies for animal behaviour and by twin and adoption studies for human behaviour. The conclusion of these studies in behavioural genetics can be difficult and controversial. The main areas of concern are that early twin studies were small, moreover it is difficult to define personality traits and disorders. Also the adoption and twins reared apart may not have a

very different environment because the adoption agencies tend to match the original environment. Unlike simple Mendelian characteristics, genetic variance for behavioural dimensions and disorders rarely accounts for more than half of the phenotypic variance, and multiple genes with small effects appear to be involved rather than one or two major genes.[97]

Genetic factors are also pertinent to the aetiology of PMDD : 70% of women whose mothers have been affected by PMS have PMS themselves, compared with 37% of women whose mothers have not been affected.[98] There is a 93% concordance rate in monozygotic twins, compared with a rate of 44% in dizygotic twins.[98] Genetic influences mediated phenotypically through neurotransmitters and neuroreceptors seem to play a significant role in the aetiology.

A recent study is the first to demonstrate allelic variation on the oestrogen receptor α gene in women with PMDD when compared with control women.[75] In addition, the allelic variation was only significant in women who had a valine/ valine genotype for the catechol-*O*-methyltransferase enzyme. This significant study may identify a source of abnormal oestrogen signalling during the luteal phase that leads to premenstrual affective, cognitive and somatic symptoms.[75] Previous studies had failed to identify gene polymorphism differences between women with PMDD and control subjects in regard to the serotonin transporter,[99;100] the transcription factor activating protein 2,[101] tryptophan hydroxylase and monoamine oxidase A promoter[100] genes. Overlap of PMDD with genetic liability for MDD, seasonal affective disorder and personality characteristics has been suggested.[102;103]

Thus, while there is strong evidence for an inherited contribution to the mood disorders, the mode of genetic transmission and the genes involved remain unclear. It is likely that a

number of genetic factors contribute to increase vulnerability to the illness, and that these factors vary among families, greatly increasing the difficulty of identifying genetic factors. In addition, environmental factors also play a critical etiologic role, further complicating genetic and epidemiologic analyses.

1.8 DIAGNOSIS AND QUANTIFICATION

Many women with a premenstrual disorder go undiagnosed, because of underreporting by the women or due to difficulty in diagnosing the disorder by the clinicians.[104]. To diagnose the syndrome after taking the complete personal and family history 2 months of prospective symptom ratings, employing 1 of a number of validated instruments should be used.

Premenstrual disorders are characterized by numerous moods, somatic and behavioural symptoms that occur during the late luteal phase of a woman's menstrual cycle and abate soon after the onset of menses. Diagnostic criteria for premenstrual syndrome (PMS) were published by the American College of Obstetricians and Gynecologists in 2000; the American Psychiatric Association had previously established very specific diagnostic criteria for premenstrual dysphoric disorder (PMDD). Both diagnoses require 2 months of prospective symptom ratings, which can be accomplished using forms designed by individual clinicians or employing 1 of a number of validated instruments. The patient will need to provide a complete family and personal history of mental disorders and medical diseases. A thorough physical examination and certain basic laboratory tests should either identify or rule out many potential causes of the symptoms. The diseases, disorders or situations considered in the differential diagnosis of PMS and PMDD will depend on the woman's presenting symptoms. Many women with a bothersome or debilitating

premenstrual disorder go undiagnosed, either because they do not report their symptoms to a clinician or because the clinician has difficulty diagnosing the disorder.

In the *Diagnostic and Statistical Manual of Mental Disorders*, 4th ed. (DSM-IV), PMDD is classified as "depressive disorder not otherwise specified" and emphasizes emotional and cognitive-behavioural symptoms.[12] At least five of the 11 specified symptoms must be present for a diagnosis of PMDD (Table 1). These symptoms should be limited to the luteal phase and should not represent amplification of pre-existing depression, anxiety, or personality disorder. In addition, they must be confirmed prospectively by daily rating for at least two consecutive menstrual cycles. A symptom-free period during the follicular phase of the menstrual cycle is essential in differentiating PMDD from pre-existing anxiety and mood disorders.

Researchers have developed a reliable and valid self-reporting scale called the Daily Symptom Report.[105] Patients rate each symptom on a five-point scale, from zero (none) to 4 (severe). The scale provides guidance for scoring the severity of each symptom and may be used in the office setting by primary care physicians for diagnosis and assessment of PMDD.

The psychiatric, medical and psychosocial screens, together with verification of the timing of symptoms, enable the clinician to make a diagnosis. Possible diagnoses after screening include the ISPMD classification of PMD (Figure 1):[65] 1) Core PMD as PMS or PMDD; 2) Variant PMD either as Premenstrual Exacerbation, PMD with anovulatory ovarian activity, PMD with absent menstruation or progestogen-induced PMD; 3) Other psychiatric or medical illness only; or, 5) no diagnosis (situational, psychosocial stressors).

1.8.1 Diagnosis and Statistical Manual of Mental Disorders (DSM IV)

There is little question that the creation of diagnostic criteria has improved the generalisability of findings in psychiatric research by assuring greater cross-study sample homogeneity. While this has certainly been true for PMS, questions can be legitimately raised about the stringency of the criteria and the means of their operationalisation.

a. In most menstrual cycles during the past year, five (or more) of the following symptoms were present for most of the time during the last week of the luteal phase, began to remit within a few days after the onset of the follicular phase, and were absent in the week post-menses, with at least one of the symptoms being either (1), (2), (3), or (4):
    1. markedly depressed mood, feelings of hopelessness, or self-depreciating thoughts
    2. marked anxiety, tension, feeling of being 'keyed up' or 'on edge'
    3. marked affective lability (e.g. feeling suddenly sad or tearful or increased sensitivity to rejection)
    4. persistent and marked anger or irritability or increased interpersonal conflicts
    5. subjective sense of difficulty in concentrating
    6. lethargy, easy fatigability, or marked lack of energy
    7. marked change in appetite, overeating, or specific food cravings
    8. hypersomnia or insomnia
    9. a subjective sense of being overwhelmed or out of control
    10. other physical symptoms, such as breast tenderness or swelling, headaches, joint or muscle pain, a sensation of 'bloating', weight gain.

Note: In menstruating females, the luteal phase corresponds to the period between ovulation and the onset of menses, and the follicular phase begins with menses. In non-menstruating females (e.g. those who have had a hysterectomy), the timing of luteal and follicular phase may require measurement of circulating reproductive hormones.

b. The disturbance markedly interferes with work or school or with usual social activities and relationships with others (e.g. avoidance of social activities, decreased productivity and efficiency at work or school).
c. The disturbance is not merely an exacerbation of the symptoms of another disorder, such as major depressive disorder, panic disorder, dysthymic disorder, or a personality disorder (although it may be superimposed on any of these disorders).
d. Criteria A, B and C must be confirmed by prospective daily ratings during at least two consecutive symptomatic cycles. (The diagnosis may be made provisionally prior to this confirmation.)

Table.1. Diagnosis and Statistical Manual of Mental Disorders IV Criteria

Unfortunately the DSM IV does not specify for how many days the symptoms should exist, nor does it specify that how soon the symptoms should remit. For clinical trials, inclusion criteria usually specify 'severe symptoms for at least 4 days' or average severity during 7 days.[106] The possibility that different lengths of premenstrual period may be

associated with different phenotypes and underlying mechanisms has not been fully elucidated.

Many of the scales listed in (Table.2) have been used to make the distinction between premenstrual dysphoric disorder alongside other disorders, in particular the Beck Depression Inventory,[107] Profile Of Mood States[108] and Hamilton Depression Scale.[109] Other scales which can be administered to assess the women's underlying psychiatric morbidity include the Hospital Anxiety Depression Scale,[110] General Health Questionnaire,[111] Self Reporting Questionnaire and the Structured Clinical Interview for DSM IV (SCID).[12]

1.8.2 Methods of Measurement Reported to Date

Early research projects attempted to use rating scales that were essentially established and designed to quantify other psychiatric and psychological conditions. Examples of this are the use of the Hamilton Rating Scale for depression[109] and the Beck Depression Inventory.[107] The lack of specificity of these techniques to quantify PMS led to the development of new "bespoke" techniques.

The earliest published measure specific to premenstrual symptoms was the Moos' Menstrual Distress Questionnaire (MDQ)[112] which used a 47 item 0-6 rating scale. The first use of visual analogue scales (VAS) was published as the Premenstrual Mood Index which was used for the first time within the context of a randomised clinical trial of spironolactone at University of Nottingham,UK.[113]

Visual Analogue Scale

VAS techniques demonstrate admirably the character and cyclicity of symptoms. It is likely that visual analogues scales (VAS) are the most sensitive measure of PMS/PMDD as

they allow a continuous rating. The VAS can be used to look at individual scores and their response to therapy or a Global score (Figure2). Visual analogue scales have been found to be an effective tool in measuring the change in premenstrual symptoms over time and their validity and reliability have since been well documented.[113-115] The 100mm line has both positive and negative mood adjectives with the midline being "a normal day". The anchors are 0 = "not at all" (that is the way you normally feel when you don't have premenstrual symptoms) and 100 = "extreme symptoms" (that is, the way you feel when your premenstrual symptoms are at their peak)

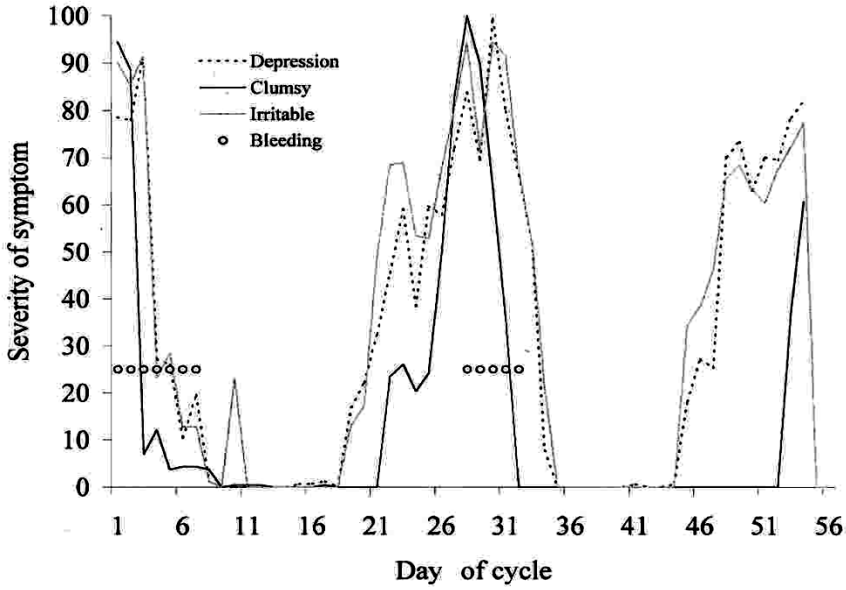


Figure.2.Daily visual analogue scale scores for psychological symptoms

Other Rating Scales

Newer techniques rate from zero to 3,4,5,6 or 7 using categorical rating scales. Early scales included Clinical Global impression Scale (CGIS), the Global Assessment Scale (GAS),[116] the Steiner Self Rating Scale,[117] Later; the more widely used scales included Premenstrual Assessment Form (PAF),[118] Prospective Record of the Impact of Menstrual

Symptoms (PRISM),[119] and the Calendar of Premenstrual Experiences (COPE).[120] These and many other tools used over the past 30 years are summarised in the following table2.

Reference	Method	Comment
* Hamilton 1960[121]	Hamilton rating Scale for depression (HAM-D)	Observer-rated instrument to assess 17/21 items in premenstrual mood disturbance.
* Beck et al 1961[107]	Beck Depression Inventory (BDI)	21-item self-report questionnaire; symptoms rated 0-3 to give an overall depression score
Moos 1968[112]	Moos' Menstrual Distress Questionnaire (MDQ)	47 symptom rated on a six-point scale
* McNair et al 1971[108]	Profile of Mood States (POMS)	65 symptoms rated on 0-4 scale combined to give summary scores for five dimensions
* Weissman and Bothwel 1976[122]	Social Adjustment Scale (SAS)	Self-report instrument containing 56 questions in seven different sections
Endicott et al 1976[116]	Global Assessment Scale (GAS)	Has not been used extensively
* Guy 1976[123]	Clinical Global Impression Scale (CGIS)	Seven-point observer/patient-rated global scale
* Derogatis et al 1977[124]	Symptom Checklist-90 (SCL-90)	general index of psychological and physical symptoms plus additional nine subscales
O'Brien et al 1979[113]	Visual Analogue Scale (VAS). Premenstrual Mood Index	100-mm line at either end of which are opposing adjectives representing the symptoms
Steiner et al 1980[117]	Self-rating Scale for Premenstrual Syndrome	A 36 item yes/no rating scale
Steiner et al 1980[117]	Premenstrual Tension Syndrome-Observer (PMTS-O) and Self Rating (PMTS-SR)	Assess symptoms in 10 different domains. 36 symptom with severity ranging from 0 to 4
Halbreich et al 1982[118]	Premenstrual Assessment Form (PAF)	Retrospective questionnaire based on psychological and behavioural symptoms
Reid 1985[119]	Prospective Record of the Impact and Severity of Menstrual Symptoms (PRISM)	Daily chart records a large number of symptoms rated 1-3
Rubinow et al 1984[115]	Visual Analogue Scale (VAS)	100-mm line at either end of which are opposing adjectives representing the symptoms
Magos and Studd 1986[125]	Modified Moos' Menstrual Distress Questionnaire (MDQ)	Ten items derived from MDQ, usually subjected to Trigg's trend analysis
Casper and Powell 1986[114]	Visual Analogue Scale (VAS)	100-mm line at either end of which are opposing adjectives representing the symptoms
Magos and Studd 1988[126]	Premenstrual Tension-Cator (PMT-Cator)	Five symptoms rated 0-3S
Mortola el 1990[120]	Calendar of Premenstrual Experiences (COPE)	
Endicott & Harrison 1990[127]	Daily Record of Severity of Problems	22-item rated 0-6 specifically for symptoms of PMDD
* Rivera-Tovar and Frank 1990[128]	Daily Assessment Form (DAF)	33-item symptom checklist rated from 0 (none) to 6 (extreme)
Steiner et al 2003[35]	Premenstrual Screening Tool (PSST)	Retrospective 0-3 Scale. Retrospective for PMDD

Table.2. Techniques used to quantify premenstrual syndrome.

***=methods originally designed for diagnoses other than PMS or PMDD.**

The Daily Record of Severity of Symptoms

During the evolution of these various methods, the DSM III and DSM IV criteria[12] were developed for LLPDD and then PMDD. In line with that, in 1990, Endicott and Harrison[127] published the somewhat simple tool named Daily Record of Severity of Problems (DRSP). DRSP was developed to help individual women and their therapist assess the nature, severity and timing of onset and offset of problems which may[129] develop during specific phases of the menstrual cycle. The feelings and behaviours which are to be rated each day are those which make up the diagnostic criteria for PMDD. Completion of such rating is essential to determine the nature of the problem being experienced[127]

Daily ratings made for several menstrual cycles helps to establish when specific symptoms first appear or become more severe, how severe they become, how much impairment in functioning they cause, and when they go away or become less severe. The pattern of change in the symptoms helps the women and her therapists determine which of the following conditions are most likely to be present (Jean Endicott, PhD, discusses the DRSP):

1. Premenstrual worsening of her on going condition which is present throughout her cycle
2. PMDD with patterns of changes that clearly meet criteria
3. PMS which is clearly present but does not meet the severity or impairment criteria for PMDD
4. Symptoms and impairment which show no evidence of being linked to phases of the menstrual cycle.

The reason such a diagnostic evaluation is important is that it will guide the treatment of the condition (Figure 3).

DAILY RECORD OF SEVERITY OF PROBLEMS
Name or Initials----------Month/Year----------
Each evening note the degree to which you experienced each of the problems listed below. Put an "x" in the box which corresponds to the severity: 1 - not at all, 2 - minimal, 3 - mild, 4 - moderate, 5 - severe, 6 - extreme.

BLEEDING																			
Cycle Day	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	40
Felt depressed, sad, "down", or "blue"																			
Felt hopeless																			
Felt worthless or guilty																			
Felt anxious, tense, "keyed up" or "on edge"																			
Had mood swings (eg suddenly felt sad or tearful)																			
Was more sensitive to rejection or my feelings were easily hurt																			
Felt angry, irritable																			
Had conflicts or problems with people																			
Had less interest in usual activities																			
Had difficulty concentrating																			
Felt lethargic, tired, fatigued, or had a lack of energy																			
Had increased appetite or overate																			
Had cravings for specific foods																			
Slept more, took naps, found it hard to get up when intended																			
Had trouble getting to sleep or staying asleep																			
Felt overwhelmed or that I could not cope																			
Felt out of control																			
Had breast tenderness																			
Had breast swelling, felt "bloated", or had weight gain																			
Had headache																			
Had joint or muscle pain																			
Impairment as demonstrated by interference with normal work, school or home activities or interference with usual social activities and relationships with others																			
At work, at school, at home, or in daily routine, at least one of the problems noted above caused reduction or productivity or inefficiency																			
At least one of the problems noted above interfered with hobbies, or social activities (eg avoid or do less)																			
At least one of the problems noted above interfered with relationships with others																			

Figure 3. Daily record of severity of problem

The Premenstrual Symptoms Screening Tool (PSST)

PSST is a simple user friendly screening tool devised by Steiner et al 2003[35] to identify women who suffer from severe PMS/PMDD and who are likely to benefit from treatment. The PSST reflect and translates categorical DSM IV criteria into a rating scale with degrees of severity and impact of premenstrual symptoms. It is less time consuming and more practical than

two cycles of prospective charting, hence, an important starting point for further assessment (figure 4).

Do you experience some or any of the following premenstrual symptoms which *start before* your period and *stop* within a few days of bleeding?
(please print and mark an "X" in the appropriate box)

SYMPTOMS	NOT AT ALL	MILD	MODERATE	SEVERE
1. Anger/irritability				
2. Anxiety/tension				
3. Tearful/Increased sensitivity to rejection				
4. Depressed mood/hopelessness				
5. Decreased interest in work activities				
6. Decreased interest in home activities				
7. Decreased interest in social activities				
8. Difficulty concentrating				
9. Fatigue/lack of energy				
10. Overeating/food cravings				
11. Insomnia				
12. Hypersomnia (needing more sleep)				
13. Feeling overwhelmed or out of control				
14. Physical symptoms: breast tenderness,				

Have your symptoms, as listed above, interfered with:

	NOT AT ALL	MILD	MODERATE	SEVERE
A. Your work efficiency or productivity				
B. Your relationships with coworkers				
C. Your relationships with your family				
D. Your social life activities				
E. Your home responsibilities				

Scoring

The following criteria must be present for a diagnosis of **PMDD**
1. at least one of #1, #2, #3, #4 is severe
2. in addition at least four of #1 - #1 4 are moderate to severe
3. at least one of A, B, C, D, E is severe

The following criteria must be present for a diagnosis of **moderate to severe PMS**
1. at least one of #1, #2, #3, #4 is moderate to severe
2. in addition at least four of #1 - #14 are moderate to severe
3. at least one of A, B, C, D, E is moderate to severe

Figure 4. Premenstrual symptoms screening tool

When using any chart for either research or clinical purposes, ideally symptoms should be rated prospectively in order to avoid the inaccuracies inherent in retrospective rating.

The National Institute of Mental Health definition states that premenstrual changes should show at least 30% increase from the intensity of symptoms measured in the follicular phase, that is, in days 5-10 of the menstrual cycle, compared with those measured in the premenstrual phase (on the 6 days before menstruation). The 30% change in the ratings of symptoms has been shown to be too liberal and a poor discriminator when comparing women with self-reported severe PMS, women using contraceptives whose natural cyclicity has been suppressed and women with normal cyclicity who report no premenstrual symptoms.[34;130] Gallant et al emphasise that what may be more clinically relevant is women's perceptions of variations in social and occupational functioning and "the way in which having PMS is meaningful in a women's life". The authors' concern is that ever more stringent criteria might result in the exclusion from studies or treatment of significantly troubled individuals. As early as 1986 Magos & Studd had applied Trigg's Trend analysis[125] to evaluating severity and cyclicity producing and produced quite a useful tool for this purpose.

Ekholm et al,[131] compared four different methods to assess the cyclicity and severity, based on the daily prospective symptoms rating. The methods compared were: a) the non-parametric Mann-Whitney U-test, b) effect size, c) Run-test and d) a 30% of change in symptom degree between the follicular and the luteal phases. They concluded that the three of the methods used seemed to correctly identify the same patients as having or not having cyclical changes. However some differences in the outcome of validity testing and the 30% change methods seemed less valid than the other three methods.

Menstrual Symptometrics

Various researchers have attempted to address the simplification of data collection. One of the simplest was the PMT-cator which was a gadget like an obstetric wheel calculator –

although novel, it was never validated nor used in any further research. There have been several attempts at data acquisition, documentation and transfer into a database by electronic means. Mini-Doc method has had limited use. At least one research study using this technique was terminated prematurely because of difficulties with the system; the authors have been unable to locate other publications using the technique of voice recognition. North Staffordshire Hospital/keele University and Nottingham University, investigated the Menstrual Symptometrics device which was developed and validated against paper based techniques. This method used a very early PDA (Amstrad Pen Pad) which is now obsolete. Visual analogue scales were used to record scores for symptoms of PMS, dysmenorrhoea and perception of blood loss. It also incorporated the Menstrual Pictogram which is a previously published pictorial method of measuring menstrual blood loss volume - hence all symptoms related specifically to disorders of the menstrual cycle could be measured.[95;132]

Menstrual Symptometrics is a simple 'palmtop' personal computer system which is programmed to collect the daily menstrual cycle symptoms of PMS, blood loss and pain and also to assess the women's quality - quality of life (using SF-36) and simple measure of underlying psychological disorder (GHQ) was also documented with other questionnaires incorporated into the system. It avoided the need to measure by hand the visual analogue scales, as the touch sensitive screen allowed the instant measuring of visual analogue scales. It has a high level of patient acceptability and can provide instant pictorial feedback on symptoms for patients and clinicians.

This method is now obsolete because of advances in PDA technology and is no longer valid because menstrual sanitary products acceptable to most women have changed dramatically

1.9 MANAGEMENT

A wide range of therapeutic interventions have been tested in the treatment of premenstrual symptoms. For women who do not meet criteria for PMDD or other physical and psychological disorders, conservative treatments are appropriate, and nonpharmacologic management should be encouraged. Unfortunately, there have been few randomized controlled trials to determine the efficacy of these more conservative interventions (Table 3); however, there is some evidence that these patients may best respond to individual or group cognitive-behavioural psychotherapy in combination with lifestyle changes. Recommended dietary changes (especially during the luteal phase) should include reducing or limiting intake of tobacco, chocolate, caffeine and alcohol. Some women report improvement as a result of eating small, frequent meals high in complex carbohydrates, as well as taking vitamins and minerals in moderation. A recent study identified the efficacy of a specially formulated carbohydrate-rich beverage compared with placebo.[133] Patients should be encouraged to decrease excess sodium in the diet when edema or fluid retention occurs and, if possible, to reduce their body mass index to less than 25 kg/M^2.[134] Regular exercise is important,[135;136] and particularly effective when combined with the regular practice of stress management techniques. Patients should also be taught to review their own monthly diaries and identify triggers that exacerbate symptoms. Most nonpharmacologic interventions that have been proven efficacious require a series of interventions. Cognitive-behavioural therapy in the form of 12 weekly individual sessions significantly improved symptoms and functional impairment in women with PMS randomly assigned to immediate treatment, compared with those in the waiting-list control group.[137] Ear, hand and foot reflexology administered once weekly

for 8 weeks by a trained reflexologist significantly decreased premenstrual symptoms in women who received treatment compared with those who received "sham" reflexology.[138]

Conservative	*Interventions to Treat PMS and PMDD*
Charting	Daily charting of symptoms
Diet	Reduction or elimination, especially in the luteal phase of, salt, chocolates, caffeine and alcohol. Small frequent meals high in complex carbohydrates, vitamins and minerals in moderation.
Exercise	Moderate, regular aerobic exercise
Stress reduction	Stress management counselling or courses if necessary; or both
Relaxation	Relaxation courses or audiotapes
Relationships	Assertiveness course or marital counselling if necessary; or both
Self help groups	If available
Education	Self help books

Table 3. Conservative Interventions to treat PMS and PMDD

Of the low-risk pharmacologic interventions that have been studied under controlled conditions (Table 4), calcium carbonate (1000 to 1200 mg daily) significantly improved affect and alleviated water retention, food cravings and pain after 3 treatment cycles.[139;140] Magnesium supplementation (200 mg or 360 mg daily during the luteal phase) significantly reduced premenstrual fluid retention after 2 cycles of treatment.[141;142] There have been enough studies of vitamin B6 to allow for a systematic review, which concluded that doses of up to 100 mg daily are likely to be of moderate benefit in treating premenstrual symptoms and depression.[95] Additional treatments that have demonstrated efficacy include nutritional supplements[143;144] and daily administration of vitamin E.[145] A systematic review of evening primrose oil concluded that this intervention was ineffective for this disorder.[146] Mefenamic acid given premenstrually alleviated fatigue, headache, aches and pains, and improved mood,[147] whereas

premenstrual administration of naproxen reduced pain in one study,[148] and daily naproxen administration improved menstrual migraine in another.[149] Daily or luteal-phase administration of spironolactone appears efficacious for somatic and physical symptoms, including weight gain and bloatedness.[113;150-152] For mastalgia, bromocriptine (1.25 to 7.5mg daily during the luteal phase) was helpful in 10 of 14 randomized trials.[153]

Supplement	*Dosage*
Vitamin B6	100mg daily
Calcium	1000 to 1200 mgs daily
Magnesium ions	200 0r 360 mgs daily (14 days before menses)
Vitamin E	400 IU daily

Table 4. Low Risk Pharmacological interventions supported by the evidence

The pharmacological approaches to PMDD and severe PMS include psychotrophic medications and hormonal interventions. The newer antidepressants in particular, including many of the selective serotonin reuptake inhibitors (SSRIs)[129;154-169]as well as clomipramine[170;171] and L-tryptophan,[94] have demonstrated excellent efficacy and minimal side effects in women with severe PMS and PMDD in whom conservative treatment has failed (Table 5). There is increasing evidence that intermittent low-dose SSRI treatment significantly improves both psychological and physical premenstrual symptoms within the first few cycles of treatment.[129;165-171] Intermittent treatment typically consists of starting medications 14 days before menstruation starts and continuing daily treatment until menstruation or shortly thereafter. In spite of considerable differences in chemical structure, all SSRIs appear to be effective for PMDD and PMS. SSRI treatment is not contraindicated for women taking oral contraceptives. The anxiolytics alprazolam[172-174] and buspirone[175] have also demonstrated efficacy in most trials; however, the magnitude of the therapeutic effect is less than that of SRIs,

while the side effect profile and potential for dependence are cause for caution. Remission rates of PMS are low on cessation of treatment with SSRI.[176;177]

Drug Class	*Drug*	*Dosage*
Antidepressant	Fluoxetine	20mg daily or during luteal phase
	Sertraline	50-150mg daily or during luteal phase
	Paroxetine	10-30mg daily or during luteal phase
	Citalopram	5-20mg daily or during luteal phase
	Clomipramine	25-75mg daily or during luteal phase
Anxiolytics	Alprazolam	
	Buspirone	
Ovarian Suppressants (GnRH aginists)	Buserline	400-900mg/day (intranasal)
	Leuprolide	3.75-7.5mg/month (intramuscular)
	Danazol	

Table 5. Pharmacological Interventions to treat PMS or PMDD Supported by Evidence

Oral contraceptives (OCs) have been commonly prescribed by gynecologists and primary care clinicians for the treatment of PMS even though there were few studies demonstrating their efficacy until recently. Two older randomized controlled trials (RCTs) in samples of women with prospectively confirmed PMS reported a lack of efficacy with monophasic and triphasic OCs.[178;179] Surveys of population cohorts without defined PMS or PMDD have reported that OCs do not alter mood in most women, but a subset of women report improvement of premenstrual symptoms, and another subset of women report the production of negative premenstrual symptoms.[180;181] After the introduction in the late 1990s of an OC containing ethinyl estradiol 30 µg and a unique progesterone, drospirenone 3 mg, improved mood and quality of life during the luteal phase began to be reported in nonclinical population cohorts.[182-184] In addition, a 6-month extended regimen of this OC was reported to be associated with fewer premenstrual emotional and physical symptoms than the normal 21/7 monthly administration in women not seeking

care for problematic premenstrual symptoms.[185] An RCT comparing the OC containing ethinyl estradiol 30 μg and drospirenone 3 mg with placebo in 82 women with PMDD reported that both the OC and placebo improved most premenstrual symptoms and that the OC was significantly more effective than placebo in decreasing food cravings, increased appetite and acne only.[186] However, 2 recent studies of YAZ (Bayer HealthCare), an OC containing ethinyl estradiol 20 μg and drospirenone 3 mg, administered as 24 days of active pills followed by a 4-day hormone-free interval (24/4), have reported superiority in reducing premenstrual emotional and physical symptoms when compared with placebo.[187;188]

Yonkers and colleagues[187] reported on a parallel design study in which YAZ or placebo was administered to 450 women with PMDD over 3 months. Pearlstein and colleagues[188] reported on a crossover design study in which YAZ or placebo was administered to 64 women with PMDD over 7 months with a middle washout cycle. Both studies reported that self-rated symptom, functioning and quality of life measures, along with clinician-rated symptom and functioning measures, all significantly improved with YAZ in comparison with placebo. In both studies, adverse effects that were more common with YAZ, compared with placebo, included nausea, intermenstrual bleeding and breast pain.[187;188] In 2006, YAZ received United States Food and Drug Administration (FDA) approval for the treatment of PMDD in women desiring oral contraception. The efficacy of this particular OC for reducing premenstrual symptoms may be due to its administration in a 24/4 regimen, which provides more stable hormone levels and reduces adverse symptoms that can occur during withdrawal bleeding.[189;190] Differential efficacy of this OC may also be due to the unique anti-mineralocorticoid and anti-androgenic properties of drospirenone.[184;187;188]

The second line of pharmacological treatment includes hormonal agents. In particular, gonadotrophin releasing hormone (GnRH) agonists can temporarily suppress the menstrual cycle (often called "medical ovariectomy" or "medical menopause"). In clinical trials, GnRH agonists have proven very successful in relieving physical symptom (Table 5). Unfortunately, the long-term use of GnRH agonists is limited by the occurrence of side effects that mimic menopause and the potential for hypo-oestrogenism and osteoporosis. Preliminary evidence suggests that "add-back" therapy with low-dose oestrogen and progesterone replacement therapy may prevent some of these side effects.[191] Intranasal buserelin[192;193] or intramuscular leuprolide,[194] are the most appropriate GnRH treatments for clinical use. In clinical trials, danazol has also been effective,[195-199] most recently in the treatment of premenstrual mastalgia.[200] The final line of treatment is ovariectomy. Two open studies have demonstrated the effectiveness of ovariectomy in the complete relief of severe premenstrual symptoms.[80;81] Oral contraceptives suppress ovulation while maintaining menstruation due to periodic withdrawal.

Women who manifest severe physical symptoms or a psychiatric disorder with premenstrual magnification should be treated for their primary condition. Premenstrual symptoms usually remit considerably with successful treatment of the primary condition, and residual symptoms can be treated as indicated. SSRIs and reproduction. Perhaps the most troublesome SSRI side effect for women is sexual dysfunction, defined as "normal libido and arousal with delayed or absent orgasm." This side effect can be reduced by reducing the dose, taking "drug holidays," substituting another agent or augmenting treatment with various agents.

To date, no single intervention has proven to be equally effective in treating all women with severe PMS or PMDD, although SSRIs have demonstrated tolerability and efficacy in

more than 60% of patients studied.[16;21;201] Patients should be assessed every 2 weeks (i.e., during both the follicular and luteal phases) within the first month of commencing therapy and instructed to continue to chart their symptoms daily. Dosage strategies vary; however, most recent investigations have demonstrated the efficacy of most therapeutic drugs at low dosages. If efficacy has not been attained after several increases in dosage, other treatment options should be considered. There is also evidence that response will be relatively immediate in women with PMS or PMDD; thus, if there is no change in symptoms within 2 to 3 menstrual cycles, an alternative therapy should be considered. Continued symptom charting helps to track efficacy, symptom response to changes in dosage, symptoms upon termination of therapy, and real versus perceived side effects. For example, women who report headaches or nausea as side effects are often surprised to see that they rated these symptoms as just as severe before commencing therapy. Investigators have yet to reach a consensus on how to define efficacy. Clinically, the easiest way to define efficacy is by the reduction of luteal symptoms (that is, the luteal symptoms remit significantly or the difference between the follicular and luteal phases is less than 30%). It has become obvious that intervention alone cannot predict efficacy, and more consideration is now being given to psychiatric history as well as to family psychiatric history, especially to mood disorders in the families of women with PMDD.

There are 3 major concerns regarding the prognosis in severe PMS or PMDD: 1) the average age of onset is around 26 years, 2) there is evidence that symptoms gradually worsen over time, and 3) there is evidence that symptoms recur when treatment is halted.[16;201] For these reasons, therapeutic goals must be set to ensure maximal safety and efficacy for the patient (Figure 5).

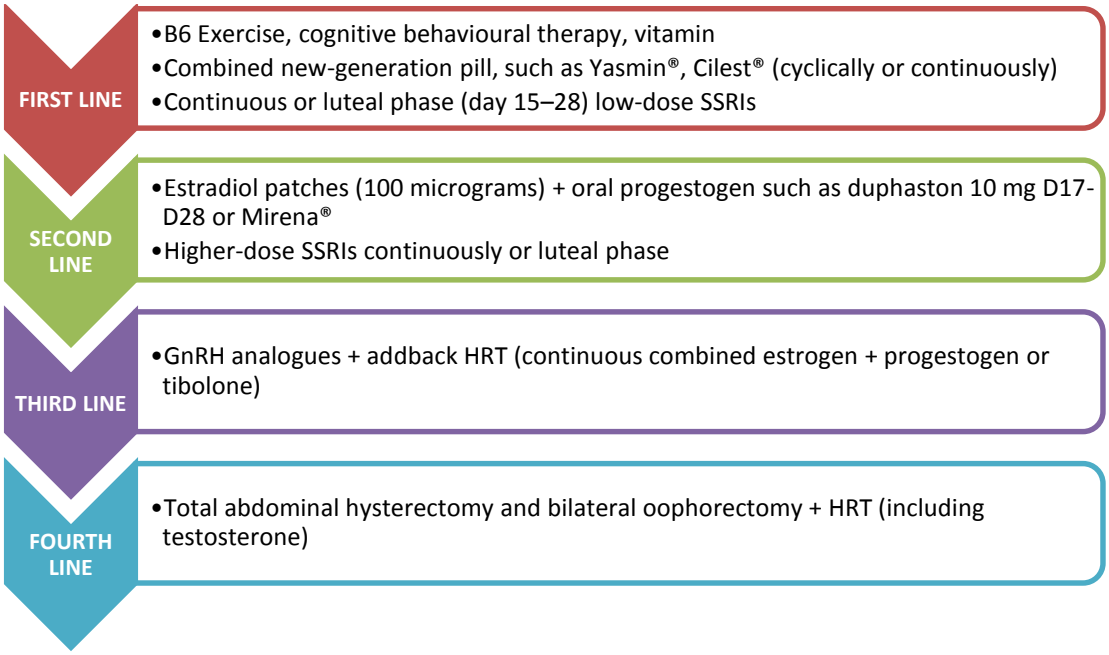


Figure 5. Possible Treatment Regimen for the Management of Severe PMS (Green top guideline no 48)

1.10 RELATIONSHIP OF PMDD TO DEPRESSION

Features of PMDD and depressive disorders, specifically atypical depression overlap considerably. Symptoms of atypical depression (i.e., depressed mood, interpersonal rejection hypersensitivity, carbohydrate craving, and hypersomnia) are similar to those of PMDD. Thirty to 76 percent of women diagnosed with PMDD have a lifetime history of depression,[202] compared with 15 percent of women of a similar age without PMDD. A family history of depression is common in women diagnosed with moderate to severe PMS.[16] There is significant co morbidity between depression and PMDD. Like most individuals with depression ladies with PMDD also complain of altered cognitive functioning during the symptomatic phase.[41] While there is growing evidence that PMDD and MDD (major depressive disorder) share similar biological underpinnings such as serotonergic dysregulation[158;159] low plasma gamma-aminobutyric acid levels[203], and

circadian desynchronization[98;204;205], it remains unclear whether observable cognitive and behavioural correlates of these substrates exist in PMDD.

Despite this relationship, many patients with PMDD do not have depressive symptoms; therefore, PMDD should not be considered as simply a variant of depressive disorder.[206] The effectiveness of selective serotonin reuptake inhibitors (SSRIs), administered only during the luteal phase of the menstrual cycle,[129;165-169] highlights the difference between PMDD and depressive disorder.

CHAPTER 2:

GENETICS OF PREMENSTRUAL SYNDROME: INVESTIGATION OF SPECIFIC SEROTONIN RECEPTORS POLYMORPHISM:

INTRODUCTION & HYPOTHESIS

INTRODUCTION

2.1 WHAT IS SEROTONIN

Serotonin or **5-Hydroxytryptamine (5-HT)** is a monoamine neurotransmitter that is primarily found in the gastrointestinal (GI) tract and central nervous system (CNS) of humans and animals. Serotonin was first isolated from blood in 1948 and shortly later associated with the nervous system.[207]

Approximately 95 percent of the human body's total serotonin is located in the enterochromaffin cells in the gut, where it is used to regulate intestinal movements.[208] Out of the remainder, 1% of total body stores is synthesized in serotonergic neurons in the CNS where it has various functions, including the regulation of mood, appetite, sleep, muscle contraction, and some cognitive functions including memory and learning. Modulation of serotonin at synapses is thought to be a major action of several classes of pharmacological antidepressants.

Serotonin secreted from the enterochromaffin cells eventually finds its way out of tissues into the blood. There, it is actively taken up by blood platelets, which store it. When the platelets bind to a clot, they disgorge serotonin, where it serves as a vasoconstrictor and helps to regulate hemostasis and blood clotting. Serotonin also is a growth factor for some types of cells, which may give it a role in wound healing.

Serotonin is eventually metabolized to 5-HIAA by the liver, and excreted by the kidneys.

2.2 SEROTONIN PATHWAY

Not all cells that contain 5-HT synthesize it. For example, platelets do not synthesize 5-HT; rather, they accumulate 5-HT from plasma by an active-transport mechanism found on the platelet membrane. Certain brain cells do synthesize 5-HT.

2.2.1 Biosynthesis:

Serotonin or 5-hydroxytryptamine is synthesized from L-tryptophan. The initial step in the synthesis of serotonin is the facilitated transport of the amino acid l-tryptophan from blood into brain. The primary source of tryptophan is dietary protein. The daily recommended allowance of tryptophan is 200mg. Only the tryptophan which is free and unbound to albumin crosses the blood brain barrier. This transport of tryptophan is limited by competitive transfer of other neutral amino acids, such as phenylalanine, leucine and methionine. The entry of tryptophan into brain is related not only to its concentration in blood but is also a competitive relation to the concentrations of other neutral amino acids as stated above. Consequently, lowering the dietary intake of tryptophan while raising intake of the other amino acids that tryptophan competes with, lowers the serotonin content in brain changing certain behaviours associated with serotonin function. This method of reducing the brain content of serotonin has been used clinically to evaluate the importance of brain serotonin in the mechanism of action of psychotherapeutic drugs.[209] Transformation of tryptophan into serotonin involves two steps (Figure 6):

1. Hydroxylation in 5-hydroxytryptophan catalysed by tryptophan hydroxylase, which is the rate limiting enzyme of the synthesis. This enzyme requires for its activity the presence of tetrahydrobiopterine, oxygen, NADPH2 and a metal, iron or copper.

Recently, it has been discovered that there are two TPH enzymes, TPH1 and TPH2, which define two independent 5-HT systems [210;211]. TPH1 generates more than 95% of the bodily 5-HT in the gut, from where it is transported by platelets to all organs except the brain since it cannot cross the blood-brain barrier. In the brain, TPH2 is exclusively responsible for the first step of 5-HT synthesis [212-214].

2. Decarboxylation of 5-hydroxytryptophan is catalysed by L-aromatic amino acid decarboxylase with pyridoxal-phosphate as coenzyme.

The combination of the hydroxyl group in the 5th position of the indole nucleus and a primary amine nitrogen serving as a proton acceptor at physiological pH makes 5-HT a hydrophilic substance. As such, it does not pass the lipophilic blood—brain barrier readily. Thus, its discovery in brain indicated that 5-HT is synthesized in brain, where it might play an important role in brain function. Various theories have been proposed linking abnormalities of 5-HT function to the development of a number of psychiatric disorders, particularly schizophrenia and depression. Psychotherapeutic drugs are now available that are effective in depression, anxiety disorders and schizophrenia; some of these drugs have potent, and in some cases selective, effects on serotonin neurons in brain.

The other enzyme involved in the synthesis of serotonin, aromatic l-amino acid decarboxylase (AADC), is a soluble pyridoxal-5'-phosphate-dependent enzyme which converts 5-HTP to 5-HT. It has been demonstrated that administration of pyridoxine increases the rate of synthesis of 5-HT in monkey brain, as revealed using position emission tomography. This presumably reflects an interesting issue of the use of pyridoxine supplementation in situations associated with 5-HT deficiency.

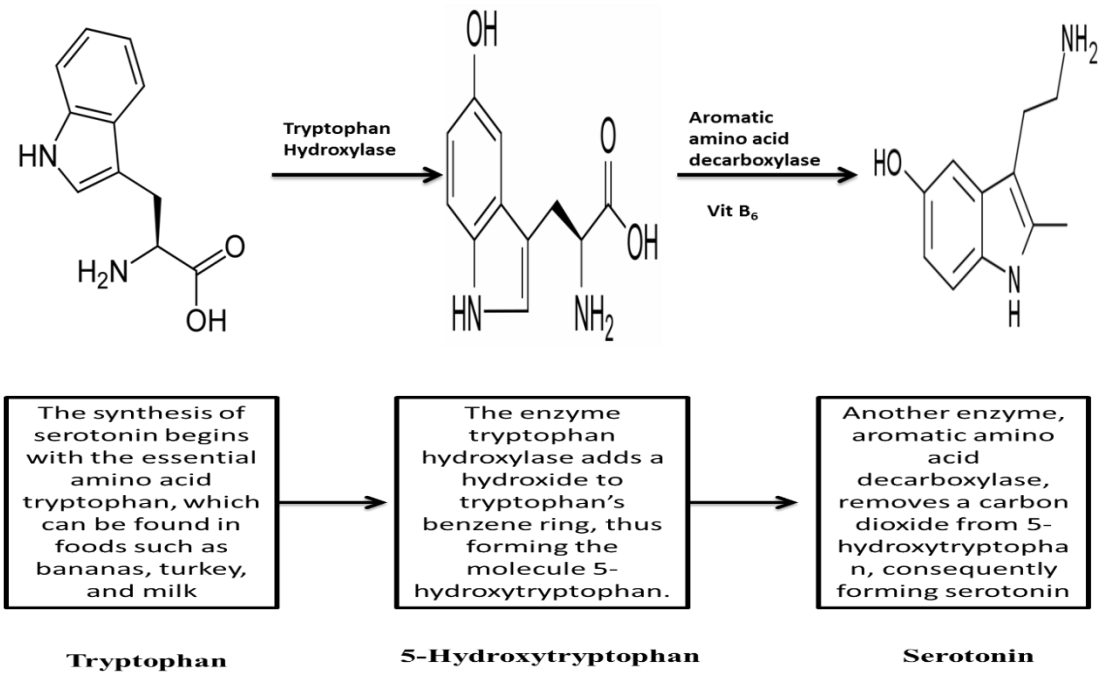


Figure 6. Synthesis of serotonin

The initial hydroxylation of tryptophan, rather than the decarboxylation of 5-HTP, appears to be the rate-limiting step in serotonin synthesis. Evidence in support of this view includes the fact that 5-HTP is found only in trace amounts in brain, presumably because it is decarboxylated about as rapidly as it is formed. As might be expected if the hydroxylation reaction is rate-limiting, inhibition of this reaction results in a marked depletion of the content of 5-HT in brain. The enzyme inhibitor most widely used in experiments is parachlorophenylalanine (PCPA) by incorporating itself into the enzyme to produce an inactive protein. This results in a long-lasting reduction of 5-HT levels.[215]

Transformation of serotonin into melatonin, which should not be regarded as a degradation pathway because melatonin is also active, is carried out primarily in the pineal gland. It involves two steps:

1. Acetylation of the amine group by N-acetyl transferase leading to N-acetyl-serotonin.

2. Methylation of the OH group by 5- hydroxyindole-O-methyltransferase catalysing the transfer of a methyl group from S-adenosyl-methionine to obtain acetyl-5-methoxytryptamine or melatonin.

The concentration of melatonin in the pineal gland presents circadian variations: it follows the variations of N-acetyl transferase activity, increasing during the night and decreasing during the day, darkness and light playing a regulatory role via catecholamines. Light inhibits melatonin biosynthesis.

2.2.2 Distribution:

Serotonin is found in many tissues:

1. Digestive tract contains about 95% of the total amount of serotonin of the body, localized in enterochromaffin cells.
2. Platelets: practically all blood serotonin (concentration going from 100 to 200 micrograms per litre) is found in platelets which do not synthesize it, but take it from plasma where it is released by these enterochromaffin cells. The uptake of serotonin by platelets is very fast and Serotonin released from platelets in plasma has a relatively localized effect on the vessels where it is released, for example during migraine.
3. Central nervous system of all species: higher concentrations are found in brainstem than in cortex. Serotonin, released by presynaptic serotonergic neurons in synaptic clefts, activates specific receptors and is partly reuptaken by presynaptic neurons.

The neurons of the raphe nuclei are the principal source of 5-HT release in the brain.[216] The raphe nuclei are neurons grouped into about nine pairs and distributed along the entire length of the brainstem, centered around the reticular formation. Axons from the

neurons of the raphe nuclei form a neurotransmitter system, reaching almost every part of the central nervous system. Axons of neurons in the lower raphe nuclei terminate in the cerebellum and spinal cord while the axons of the higher nuclei spread out in the entire brain.

Serotonin is released into the space between neurons, and diffuses over a relatively wide gap (>20 μm) to activate 5-HT receptors located on the dendrites, cell bodies and presynaptic terminals of adjacent neurons.

5-HT receptors are the receptors for serotonin. They are located on the cell membrane of nerve cells and other cell types in animals and mediate the effects of serotonin as the endogenous ligand and of a broad range of pharmaceutical and hallucinogenic drugs. With the exception of the 5-HT_3 receptor, a ligand gated ion channel, all other 5-HT receptors are G protein coupled seven transmembrane (or *heptahelical*) receptors that activate an intracellular second messenger cascade cAMP.[217]

Serotonergic action is terminated primarily via uptake of 5-HT from the synapse. This is through the specific monoamine transporter for 5-HT, SERT, on the presynaptic neuron (Figure 7). Various agents can inhibit 5-HT reuptake including MDMA (ecstasy), amphetamine, cocaine, dextromethorphan (an antitussive), tricyclic antidepressants (TCAs) and selective serotonin reuptake inhibitors (SSRIs). 5-HT clearance may also be monoamine transporter, known as PMAT.[218]

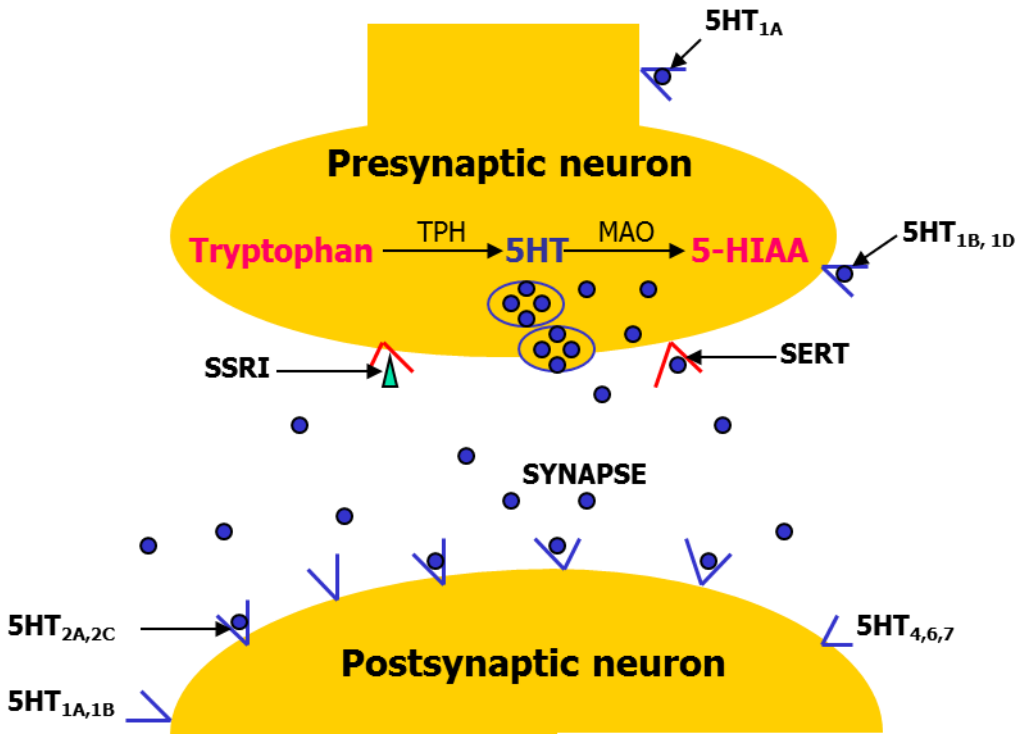


Figure 7. Schematic diagram of 5-HT metabolism

The half-life of serotonin is long in platelets and intestine, and very short, a few minutes, in the brain. The concentration of a compound in an organ, static view of a dynamic phenomenon, is not necessarily a good representation of metabolic activity and turn-over.

Melatonin found in plasma is released from the pineal gland. Its plasma concentration decreases during ageing.

2.2.3 Inactivation

Serotonin is converted into inactive molecules by biotransformation in the liver. Oxydative deamination of the lateral amino chain by monoamine oxidase, leads to 5-hydroxy-indol-acetaldehyde which is then oxidized into 5-hydroxy-indol-acetic acid (5-HIAA). 5-HIAA is excreted from the kidneys in urines in quantities normally lower than 10

mg/24 h (Figure 8). Low levels of 5-HIAA are found in the CSF of patients with personality disorders, anxiety, suicide and other psychiatric problems.[219;220]

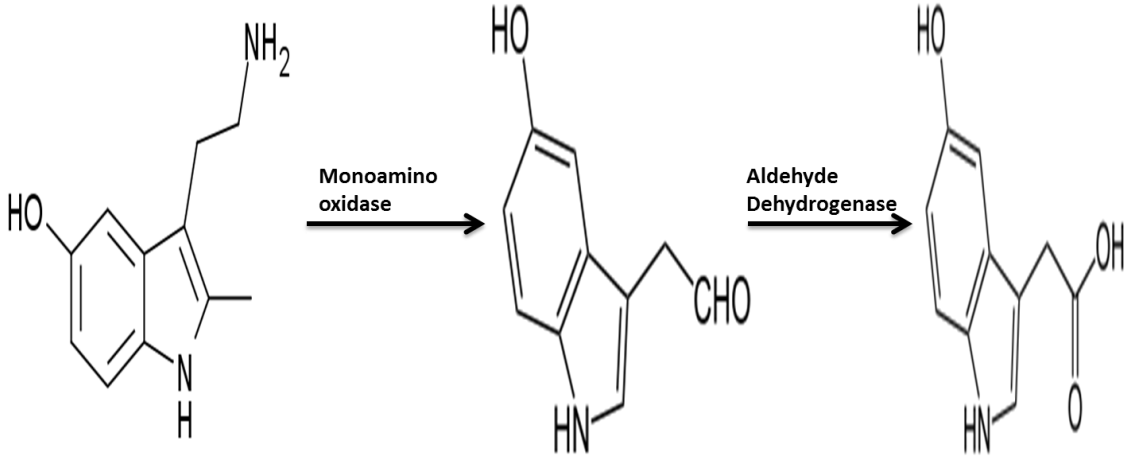


Figure 8. Serotonin Metabolism

2.3 OVARIAN STEROIDS AND SEROTONERGIC PATHWAY

Many studies have shown that Oestrogen and Progesterone are neuroprotective.[221;222] Gonadal steroids have shown to play a role in all stages of neural development including survival and death.[223]

The receptors for the gonadal steroids modulate the synthesis of the enzymes and receptor proteins for many neurotransmitters and neuropeptides.[224] Serotonin neurons contain oestrogen receptor-β[225] and progestin receptors. Oestrogen and progesterone regulate gene and protein expression in serotonin neurons in a manner that would increase serotonin production, increase serotonin turnover, increase serotonin neural

firing and decrease serotonin degradation.[226-228] The protein receptors for the 17β Estradiol and progesterone are located in the cell nucleus of the dorsal raphe, hypothalamus and limbic areas.[229;230] Hence these receptors function as the transcription factors and modulate gene expression related to serotonin and serotonin receptors.[227;231]

Recently a study on macaques showed the mRNA for tryptophan hydroxylase (TPH), the committal enzyme in serotonin synthesis, increased significantly with the Oestrogen treatment and remained elevated when the progesterone was added to the Oestrogen regimen. Serotonin reuptake transporter (SERT) mRNA decreased significantly with the oestrogen treatment and addition of progesterone had no further effect.[227]

There is enough evidence to show that oestrogen stimulates serotonin neurons and down-regulates the expression and activity of 5-HT1A autoreceptors.[227;228;232] There is a sex difference on the effect of steroids on the 5-HT1A receptor and hence more release of serotonin in females in response to some medications.[233]

Studies designed to identify the chemical messenger in the CNS that mediate Oestrogen control of the serotonin of pituitary gonadotrophin led to the discovery that Oestrogen increases the density in forebrain of the serotonin 2A receptor. The ERβ in the, hypothalamus, periventricular nuclei and supraoptic nucleus[234] have been shown to increase the density of 5HT2A receptors in this area involved in mood and reproductive status.[235] The density of these receptors has been shown to especially increase during the Oestrogen induced LH surge and this response has been shown to be absent following ovariectomy[236] and reversing with Oestrogen replacement.[237] This receptor has been implicated in mood disorders as well as in schizophrenia.

Together this data indicate that ovarian hormones and their synthetic analogues could modify cognitive and autonomic neural functions by acting on the serotonin neural pathway.

2.4 PMS/PMDD AND ABNORMALITIES OF SEROTONERGIC PATHWAY

Many studies have identified various abnormalities in the serotonin system in women with PMS and PMDD. These include abnormal levels of whole blood serotonin, serotonin platelet uptake and platelet tritiated imipramine binding; abnormal responses to serotonergic probes such as L-tryptophan, buspirone, metachlorophenylpiperazine and fenfluramine; and exacerbation of premenstrual symptoms after metergoline or tryptophan depletion.[25;55;238]

A review article by Halbreich U et.al (1993) the data presented suggested that postsynaptic serotnergic responsivity might be altered during the late-luteal-premenstrual phase of the menstrual cycle.[239] Some serotonergic functions of the women with PMS might be altered during the entire cycle and be associated with a vulnerability trait. It is shown that gonadal hormones might change the activity of 5-HT systems as part of a multidimensional interactive system.

Inoue Y et.al (2007) showed the serotonergic function in the follicular phase was higher in PMDD subjects but lower in luteal phase compared to women with PMS without PMDD and normal controls.[240] Hence suggesting that PMDD women have fluctuating serotonergic function across their menstrual cycles and that the pattern may be different from PMS without PMDD.

Several studies have also suggested that women with PMDD have decreased luteal-phase levels of GABA, abnormal ALLO levels and decreased luteal-phase sensitivity of the

$GABA_A$ receptor.[25;56;71;182] Imaging studies have reported altered serotonin function[241;242] and altered GABAergic function[243;244] in women with PMDD when compared with healthy control subjects. It is possible that the rapid efficacy of selective serotonin reuptake inhibitors (SSRIs) in PMDD may be due in part to their ability to increase ALLO levels in the brain and enhance $GABA_A$ receptor function.[245] Alternative hypotheses for the fast action of SSRIs in PMDD include enhanced function of 5-HT_{2C} receptors[246] and inhibition of the serotonin transporter with resulting decreased LH production.[247] It has also been hypothesized that the increase in ALLO after SSRI administration underlies the improvement in depressive symptoms of MDD in both sexes.[248-250] PMDD subjects showed higher serotonergic function in follicular phase but lower serotonergic function in luteal phase, compared with women with PMS without PMDD and normal controls. Thus we can conclude that women with PMDD have fluctuating serotonergic function across their menstrual cycles and their pattern may be different from PMS without PMDD. [240]

2.5 SEROTONIN RECEPTORS

The initial suggestion that there might be more than one type of receptor for serotonin came from experiments on the isolated guinea pig ileum. In the 1970s, the development of radioligand-binding assays furthered our understanding of subtypes of receptors for serotonin. Bradley and associates in 1986 proposed a classification scheme with three major types of receptors for serotonin, using pharmacological criteria and functional responses primarily in peripheral tissues. [207] The receptors were called "5-HT_1-like," 5-HT_2 and 5-HT_3.

Molecular biological techniques have led to the rapid discovery of additional serotonin-receptor subtypes and their properties. The current classification scheme is based on operational criteria, such as drug-related characteristics and intracellular signal-transduction mechanisms and amino acid sequence of the receptor protein. The study of these receptor protein have been carried out by localising the protein by the use of antibodies (immunocytochemistry), its mRNA could be detected (*in situ* hybridization), or, it could be localized by virtue of its binding by a radiolabeled ligand (receptor autoradiography). Each of these methods provides a distinct class of information.

Modern Serotonin Receptor Classification and Subtypes[251] show there are three serotonin receptor subfamilies, the 5-HT_1 family; the 5-HT_2 family; and the family that includes the 5-HT_4, 5-ht_6 and 5-HT_7 receptors, represent the three major classes of serotonin receptor that are members of the G protein-coupled receptor superfamily. Unlike the 5HT1 and 5HT2 families which inhibit the adenylate cyclase activity, the 5HT4, 5HT6 and 5HT7 stimulate adenylate cyclase. The 5-HT_3 receptor is a ligand-gated ion channel and is a separate subfamily. Although each serotonin receptor can be activated potently by serotonin, differences in signal-transduction mechanisms, neuroanatomical distribution and affinities for synthetic chemicals create opportunities for drug discovery and make each serotonin receptor subtype a potential therapeutic target. The most important and well-studied are 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2C} for mood regulation.[252;253]

2.5.1 The 5-HT_1 Receptor Family

The 5-HT_1 receptor family contains receptors that are negatively coupled to adenylyl cyclase and includes the 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-ht_{1E} and 5-ht_{1F} receptors.[251]

The 5-HT_{1A} receptor is coupled via G proteins to two distinct effector systems: (i) inhibition of adenylyl cyclase activity and (ii) the opening of K^+ channels, which results in neuronal hyperpolarization. In terminal field areas of serotonergic innervation, such as the hippocampus, 5-HT_{1A} receptors are coupled to both effector systems (Table 6). However, in the dorsal raphe nucleus, 5-HT_{1A} receptors are coupled only to the opening of potassium channels. 5-HT1A receptors are present in high density in the hippocampus, septum, amygdala, hypothalamus and neocortex (Table 6). Destruction of serotonergic neurons with the neurotoxin 5,7-dihydroxytryptamine (5,7-DHT) does not reduce 5-HT1A receptor number in forebrain areas, indicating that 5-HT1A receptors are located postsynaptically in these brain regions. Many of these serotonergic terminal field areas are components of the limbic system, the pathway thought to be involved in the modulation of emotion. This indicates that the reported effects of 5-HT or serotonergic drugs on emotional states could be mediated by 5-HT1A receptors.[254] The presence of 5-HT1A receptors in the neocortex suggests that this receptor also may be involved in cognitive or integrative functions of the cortex. 5-HT1A receptors are also present in high density in serotonergic cell body areas, in particular the dorsal and median raphe nuclei, where they function as somatodendritic auto receptors, modulating the activity of serotonergic neurons. Activation of these auto receptors causes a decrease in the rate of firing of serotonergic neurons and a reduction in the release of 5-HT from serotonergic terminals. Neurotoxin-induced destruction of serotonergic cell bodies dramatically reduces the number of 5-HT1A receptors in these areas, consistent with their location on serotonergic soma.

The 5-HT_{1B} and 5-HT_{1D} receptor subtypes are also linked to inhibition of adenyl cyclase activity (table 6). In the substantia nigra, where a high density of 5-HT_{1B} or 5-HT_{1D}

receptors has been demonstrated by radioligand-binding studies, these serotonin receptors are linked to the inhibition of adenylyl cyclase through a G protein.

Although biochemical, pharmacological and functional data suggest that the 5-HT_{1B} receptor found in rats and mice and the 5-HT_{1D} receptor found in other species, including humans, are functionally equivalent species homologs, the story has been complicated somewhat by the discovery of two genes encoding the human 5-HT_{1D} receptor, 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$.[251] Radioligand-binding studies currently do not allow the differentiation of 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors, and the binding profiles of these receptor subtypes match the previously described 5-HT_{1D}-binding site Because there are no compounds currently available to differentiate between the 5-$HT_{1D\alpha}$ and the 5-$HT_{1D\beta}$ receptors, they are referred to as 5-HT_{1D}.

The 5-HT1B receptor in rats and mice and the 5-HT1D receptor in bovine and human brain are located in high density in the basal ganglia, particularly in the globus pallidus and the substantia nigra (Table 6). Functional studies indicate that the 5-HT1B and 5-HT1D receptors are located on presynaptic terminals of serotonergic neurons and modulate the release of serotonin. Release of 5-HT from the dorsal raphe nucleus also appears to be under the control of 5-HT1B/1D receptors, although it is unclear whether these receptors are located on serotonergic terminals or cell bodies. The 5-HT1B and 5-HT1D receptors also are located postsynaptically, where they may modulate the release of other neurotransmitters, such as acetylcholine (ACh) in the hippocampus and DA in the prefrontal cortex. The presence of these receptors in high density in the basal ganglia raises the interesting possibility that they may play a role in diseases of the brain which involve the basal ganglia, such as Parkinson's disease.

The 5-ht_{1E} receptor originally was identified in homogenates of human frontal cortex by radioligand-binding studies with [^{3}H]5-HT in the presence of 5-carboxamidotryptamine (5-CT) to block 5-HT_{1A} and 5-HT_{1D} receptor sites. Because of the lack of specific radioligands for the 5-ht_{1E} receptor, the overall distribution in brain is unknown.

With the cloning of the various subtypes of receptors for serotonin, knowledge of receptor sequences has been used to generate radioactive probes for mRNAs encoding individual serotonin receptor subtypes. Using *in situ* hybridization histochemistry, the localization of these mRNAs and, thus, the distribution of cells expressing the mRNAs for serotonin receptors can be established in brain. 5-ht_{1E} receptor mRNA has been found in the caudate putamen, parietal cortex and olfactory tubercle.[255] The function of the 5-ht_{1E} receptor in intact tissue is not known due to the lack of selective agonists or antagonists. In transfected cells, the 5ht_{1E} receptor is coupled to the inhibition of adenylyl cyclase activity. The 5-ht_{1E} receptor displays a higher degree of homology with the 5-HT_{1D} receptor (64%) than any other 5-HT_1 receptors.[251]

The 5-HT_{1F} receptor was cloned and sequenced in 1993 and shares the greatest sequence homology with the 5-ht_{1E} receptor (61%). 5-ht_{1F} receptor mRNA is found in cortex, hippocampus, dentate gyrus, nucleus of the solitary tract, spinal cord, trigeminal ganglion neurons, uterus and mesentery. In transfected cells, the 5-ht_{1F} receptor is coupled to the inhibition of adenylyl cyclase.[251] Because selective agonists or antagonists for the 5-ht_{1F} receptor have not been available until very recently, little is known about the distribution or function of the 5-ht_{1F} receptor in brain. The selective agonist radioligand has been used to demonstrate the presence of 5-ht_{1F} receptor sites in cortex, striatum, hippocampus and olfactory bulb. Activation of 5-ht_{1F} receptors *in vivo* inhibits neurogenic dural inflammation and dural protein extravasation.

2.5.2 The 5-HT_2 Receptor Family

The 5-HT_2 receptor family includes the 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} (formerly the 5-HT_{1C}) receptor subtypes. 5-HT_{2A} receptor-mediated stimulation of phosphoinositide hydrolysis has been well characterized in cerebral cortex and the choroid plexus (table5). Activation of 5-HT_{2A} receptors also mediates neuronal depolarization, a result of the closing of potassium channels.

Cloning of the 5-HT_{2A} receptor has been used to gain insight into a controversy over the nature of agonist binding to the 5-HT_{2A} receptor. A single gene produces a protein with two separate binding sites, 5-HT_{2A} receptor antagonist [^{3}H]ketanserin and the 5-HT_2 receptor agonist [^{3}H]DOB substantiating the view that agonist and antagonist binding are to different states, rather than to two different subtypes, of the 5-HT_{2A} receptor.

A high density of 5-HT2A receptors is found in many cortical areas. These receptors are particularly concentrated in the frontal cortex. 5-HT2A receptors also are found in high density in the claustrum, a region which is connected to the visual cortex; in parts of the limbic system; and in the basal ganglia and the olfactory nuclei (Table 6).

FAMILY	MECHANISM	POTENTIAL	SUBTYPE	MAIN SIGNALLING PATHWAY	MAIN EXPRESSION SITES IN CNS	KNOCK OUT PHENOTYPE
5-HT$_1$	↓ cAMP	Inhibitory	5HT$_{1A}$	Gi/oAC i	Widespread in brain (mainly hippocampus,cortex, raphe nuclei)	Increased anxiety, Antidepressant-like phenotype Cognitive impairment
			5HT$_{1B}$	Gi/oAC i	Widespread in brain (mainly basal ganglia, cortex)	Increased aggression Decreased anxiety, Enhanced learning performance , Enhanced cocaine response
			5HT$_{1D}$	Gi/oAC i	Basal ganglia, hippocampus, cortex	
			5HT$_{1E}$	Gi/oAC i	Cortex, caudate putamen, claustrum	(not existing in mice)
			5HT$_{1F}$	Gi/oAC i	Dorsal raphe nucleus, hippocampus, cortex, claustrum, caudate nucleus, brainstem	
5-HT$_2$	↑ IP$_3$, ↑DAG	Excitatory	5HT$_{2A}$	Gq$_{/11}$ PLC s	Cortex, claustrum, hippocampus, hypothalamus, basal ganglia	Decreased anxiety
			5HT$_{2B}$	Gq$_{/11}$ PLC s	Cerebellum, septum, hypothalamus, amygdala	
			5HT$_{2C}$	Gq$_{/11}$ PLC s	Choroid plexus, cortex, hippocampus, amygdala, striatum, substantia nigra	Increased appetite, overweight Spontaneous convulsions Cognitive impairment Enhanced cocaine response
5-HT$_3$	Depolarising plasma membrane	Excitatory	5-HT3A with 5-HT3B,C,D, And E	Ligand gated Na+ and K+ channel	Widespread in brain (mainly area postrema, nucleus tractus solitarius, dorsal vagal complex, limbic structures)	Reduced pain perception (5-HT3A)
5-HT$_4$	↑cAMP	Excitatory	5HT$_4$	GsAC s	Basal ganglia, cortex, septum, hippocampus	Decreased stress response, Increased convulsive response
5-HT$_5$	↓ cAMP	Inhibitory	5HT$_{5A}$	Gi/oAC i	Hippocampus, hypothalamus, olfactory bulb, cortex, thalamus, striatum, pons	Increased exploratory activity, Altered LSD response
			5HT$_{5B}$	?	Habenula, raphe nuclei, hippocampus(in rodents); pseudogene (in humans)	
5-HT$_6$	↑cAMP	Excitatory	5HT$_6$	GsAC s	Widespread in brain (mainly striatum,amygdala, hippocampus, cortex)	Altered alcohol response
5-HT$_7$	↑cAMP	Excitatory	5HT$_7$	GsAC s	Thalamus, hippocampus, cortex, amygdala, suprachiasmatic nucleus	Antidepressant-like phenotype Disturbed circadian rhythms, Disturbed thermoregulation

Table 6: Serotonin receptors Classification and Subtypes

5-HT2A receptors in the cortex are thought to be located postsynaptically on intrinsic cortical neurons as destruction of projections to the cortex does not reduce 5-HT2A receptors. Because of the lack of selective agonists to differentiate between members of the 5-HT2 receptor family, many of the functional and clinical correlates of the 5-HT2A receptor may very well involve or be attributed to the 5-HT2C receptor.

Although the 5-HT_{2B} receptor is the most recently cloned of the 5-HT_2 receptor class, it was among the first of the serotonin receptors to be characterized using pharmacological criteria. The first report of the sensitivity of rat stomach fundus to serotonin was published by Vane in 1959. This receptor, whose activation results in the contraction of fundus smooth muscle, originally was placed in the 5-HT_1 receptor class by Bradley and associates[207] because of its sensitivity to serotonin and because responses mediated by it were not blocked by 5-HT_2 or 5-HT_3 receptor antagonists. It has been reclassified as a 5-HT_2 receptor because of its similar pharmacological profile to the 5-HT_{2C} receptor. The recombinant receptor expressed in clonal cells is coupled to the stimulation of inositol lipid hydrolysis. However, in rat stomach fundus, the 5-HT_{2B} appears not to be coupled to phosphoinositide hydrolysis. 5-HT_{2B} receptor-mediated contraction of rat stomach fundus is dependent on the influx of calcium through voltage-sensitive channels, intracellular calcium release and activation of PKC.[256] The effector system to which this receptor is coupled in the CNS remains to be established. In humans, 5-HT_{2B} receptor mRNA has been found peripherally and in cerebellum, cerebral cortex, amygdala, substantia nigra, caudate, thalamus, hypothalamus and retina. [251]

5-HT_{2C} receptors are present in high density in the choroid plexus. High-resolution autoradiography has shown that they are enriched on the epithelial cells of the choroid plexus. It has been proposed that 5-HT-induced activation of 5-HT_{2C} receptors could

regulate the composition and volume of the cerebrospinal fluid. 5-HT_{2C} receptors also are found throughout the brain, particularly in areas of the limbic system, including the hypothalamus, hippocampus, septum, neocortex and regions associated with motor behaviour, including the substantia nigra and globus pallidus. 5-HT_{2C} receptors are present in much lower concentrations in these areas than in the choroid plexus (Table 6). 5-HT_{2C} receptor gene are widely located in the brain and have been shown to be involved in the regulation of endocrine responses, including the production and secretion of adrenocorticotropic hormone (ACTH)[257], Oxytocin[258] and Prolactin.[259]

2.5.3 The 5-HT_3 Receptor

The 5-HT_3 receptor belongs to the ligand-gated ion channel superfamily. The 5-HT_3 receptor is a serotonin-gated cation channel that causes the rapid depolarization of neurons (Table 6). The depolarization mediated by 5-HT_3 receptors is caused by a transient inward current, specifically the opening of a channel for cations. A single subunit of the 5-HT_3 receptor, the 5-HT_3-A receptor subunit, has been cloned. An alternatively spliced variant, the 5-HT_3-As receptor subunit, has been identified in mouse, rat and human. The cloned receptor subunit exhibits sequence similarity to the α subunit of the nicotinic acetylcholine receptor and to the β_1 subunit of the $GABA_A$ receptor. It is not known whether the native 5-HT_3 receptor is composed of this single subunit or several different subunits. Although single subunits of members of the ligand-gated ion channel receptor family can form functional receptors, they generally lack some of the properties of the native, multisubunit receptor. The cloned subunit of the 5-HT_3 receptor has been studied in *Xenopus* oocytes injected with mRNA encoding this receptor. Although the expressed 5-HT_3-A and 5-HT_3-As receptors are functional, they do not display all of the

characteristics of native 5-HT_3 receptors. The 5-HT_3 receptor, like other members of the ligand-gated ion channel superfamily, appears to possess additional pharmacologically distinct recognition sites for alcohols and anesthetic agents, by which the function of this receptor can be allosterically modulated.[260]

5-HT_3 receptors initially appeared to be confined to peripheral neurons, where they mediate depolarizing actions of 5-HT and modulate neurotransmitter release. 5-HT_3 receptors are found in high density in peripheral ganglia and nerves, including the superior cervical ganglion and vagus nerve, as well as in the substantia gelatinosa of the spinal cord. Their localization in spinal cord and medulla suggests that 5-HT could modulate nociceptive mechanisms via the 5-HT_3 receptor. 5-HT_3 receptors facilitate the release of substance P in the spinal cord.[261] The localization of 5-HT_3 receptor-binding sites in cortical and limbic areas of the brain is consistent with behavioural studies in animals which suggest that 5-HT_3 receptor antagonists may have potential anxiolytic, antidepressant and cognitive effects. 5-HT_3 receptors are located postsynaptically, where they modulate the release of neurotransmitters such as ACh or DA. 5-HT_3 receptors modulate the activity of dopaminergic neurons in the ventral tegmental area. In the cortex and hippocampus, the majority of neurons expressing 5-HT_3 receptor mRNA are GABAergic. The highest density of 5-HT_3 receptor sites in the brain is in the area postrema, the site of the chemoreceptor trigger zone (Table 6).

2.5.4 The 5-HT4 , 5-ht6 and 5-HT7 receptors

5-HT_4 , 5-ht_6 and 5-HT_7 receptors are included in a family of serotonin receptors coupled to the stimulation of adenylyl cyclase. The 5-HT_4 receptor originally was described in cultured murine collicular neurons as a serotonin receptor coupled to the stimulation of

adenylyl cyclase activity, possessing pharmacological characteristics distinct from those of the 5-HT_1, 5-HT_2 or 5-HT_3 receptors. The 5-HT_4 receptor gene has been cloned from rat brain RNA by reverse transcriptase (RT)-PCR.[262] Two different cDNA clones, the long isoform, 5.5-kb 5-HT_{41}, and the short isoform, 4.5-kb 5-HT_{4s}, have been isolated and are most likely the result of alternative splicing of 5-HT_4 receptor mRNA.

Studies of the 5-HT_4 receptor, originally characterized by measuring cAMP production in cultured mouse collicular neurons, have been hampered by the absence of a high-affinity radioligand. The synthesis and development of specific radioligands, [^{3}H]GR 113808 and [^{125}I]SB 207710, have provided the necessary tools for the study and characterization of the 5-HT_4 receptor. 5-HT_4 receptor binding sites are localized with high densities in the striatum, substantia nigra and olfactory tubercle and have been reported in the hippocampus as well (Table 6). The 5-HT_4 receptor indirectly mediates the enhancement of striatal DA release by 5-HT, although 5-HT_4 receptors do not appear to be located on striatal DA terminals. In the alimentary tract, 5-HT_4 receptors are located on neurons, for example, the myenteric plexus of the ileum, smooth muscle cells and secretory cells, where they evoke secretions and the peristaltic reflex.

The 5-ht_6 receptor is approximately 30% homologous to other serotonin receptors. When expressed in transfected cells, it shows high affinity for [^{125}I]LSD and [^{3}H]5-HT. The pharmacology of this recombinant receptor is unique. Interestingly, this receptor has high affinity for various antipsychotic and antidepressant drugs, such as clozapine, amitriptyline, clomipramine, mianserin and ritanserin. The 5-ht_6 receptor stimulates adenylyl cyclase when expressed in some, but not all, cell systems. The function of the 5-ht_6 receptor in intact tissue has not been characterized due to the lack of selective

agonists or antagonists. Expression of 5-ht_6 receptor mRNA has been detected in the striatum, nucleus accumbens, olfactory tubercle, hippocampus and cerebral cortex.[251]

The 5-HT_7 receptors are widely expressed in central nervous system and peripheral tissues including human vascular smooth muscle cells.[263-265] There are at least four 5-HT7 receptor isoforms that are produced by alternative splicing[266], but the differences in their pharmacological profiles are not yet known. Studies have provided evidence that 5-HT_7 receptors may be involved in the regulation of emotions, thermoregulations, circadian rhythmicity, memory process and smooth muscle relaxation.[267;268]

5-HT_7 receptor-binding sites in the rat brain have been described using receptor autoradiography in layers 1–3 of the cortex, septum, thalamus, hypothalamus, amygdala and superior colliculus[269] (Table). The 5-HT7 receptor has been suggested as a target for treating depression since inactivation or blockade of the receptor has an antidepressant-like behavioural effect.[270] A putative neuroendocrine role for 5-HT7 receptors has also been suggested in immortalized hypothalamic neurones in culture (GT-1 cells), where they were directly involved in the 5-HT-induced release of luteinizing hormone-releasing hormone.[271] It was also shown that the effect of 5-HT on aldosterone secretion in rat adrenal glands is mediated by 5-HT7 receptors.[272]

2.5.5 The 5-ht5A and 5-HT5B receptors

The 5-ht_{5A} and 5-HT_{5B} receptors may constitute a new family of serotonin receptors mainly found in the central nervous system. The receptor proteins are 77% identical to each other, whereas the homology to other serotonin receptors is low. The 5-HT(5) receptors remain as two of the least studied and understood receptor subtypes.

5-ht_{5A} receptor mRNA transcripts have been detected by *in situ* hybridization in the cerebral cortex, hippocampus, granule cells of the cerebellum, medial habenula, amygdala, septum, several thalamic nuclei and olfactory bulb of the rat, mouse and human. The 5-ht5A gene was located on human chromosome 7 (position 7q36). 5-HT_{5B} mRNA has been detected by *in situ* hybridization in the hippocampus, habenula and the dorsal raphe nucleus of rat and is the first example of a brain-specific protein that is absent in human.[251]

The 5-ht_{5A} receptor has been demonstrated to couple to G proteins through Gi/o to inhibit adenylyl cyclase activity.[273] At the present time, the functional correlate and transductional properties are unknown for the 5-HT_{5B} receptor.

Few recent studies implicate 5-ht_{5A} receptor may control the circadian rhythms, mood and cognitive function from brain localisation and functional studies, whilst gene association studies implicate the receptor in the aetiology of schizophrenia. Much is still to be learnt about the function of the 5-ht5A receptor and selective ligands may show utility in psychiatric disorders

HYPOTHESIS:

Premenstrual Dysphoric Disorder like many other psychiatric and behavioural disorders is complex and multifactorial.[274] From our literature review we have gathered strong evidence supporting serotonin dysregulation in PMS[94;275-278] with ovulatory progesterone or progestogens providing the trigger.[227;279] This is further emphasised by the fact that serotonin selective reuptake inhibitor (SSRIs) are an effective and acceptable first-line treatment for severe PMS.[154;280-287] Evidence from family and twin studies suggests that PMDD is in total or in part genetically determined as well.[288-290] It may be associated with

a single or multiple genetic cause in combination with environmental and lifestyle factors.[290] Initial studies of key polymorphisms of genes regulating the synthesis (tryptophan hydroxylase 1), membrane reuptake (serotonin transporter), and catabolism (monoamineoxidase A) of serotonin failed to demonstrate a significant association between genotype and premenstrual dysphoria.[291;292]

With the convincing evidence that PMS is inheritable and that serotonin is important in the pathogenesis of PMS, we are convinced that one of the polymorphisms in the genes involved in the biosynthesis of Serotonin maybe related to PMDD. Furthermore, failed initial studies on the synthesis, reuptake and catabolism of serotonin, we can hypothesise that functional polymorphisms of the serotonin receptor genes are involved in the pathogenesis of Premenstrual Dysphoric Disorder.

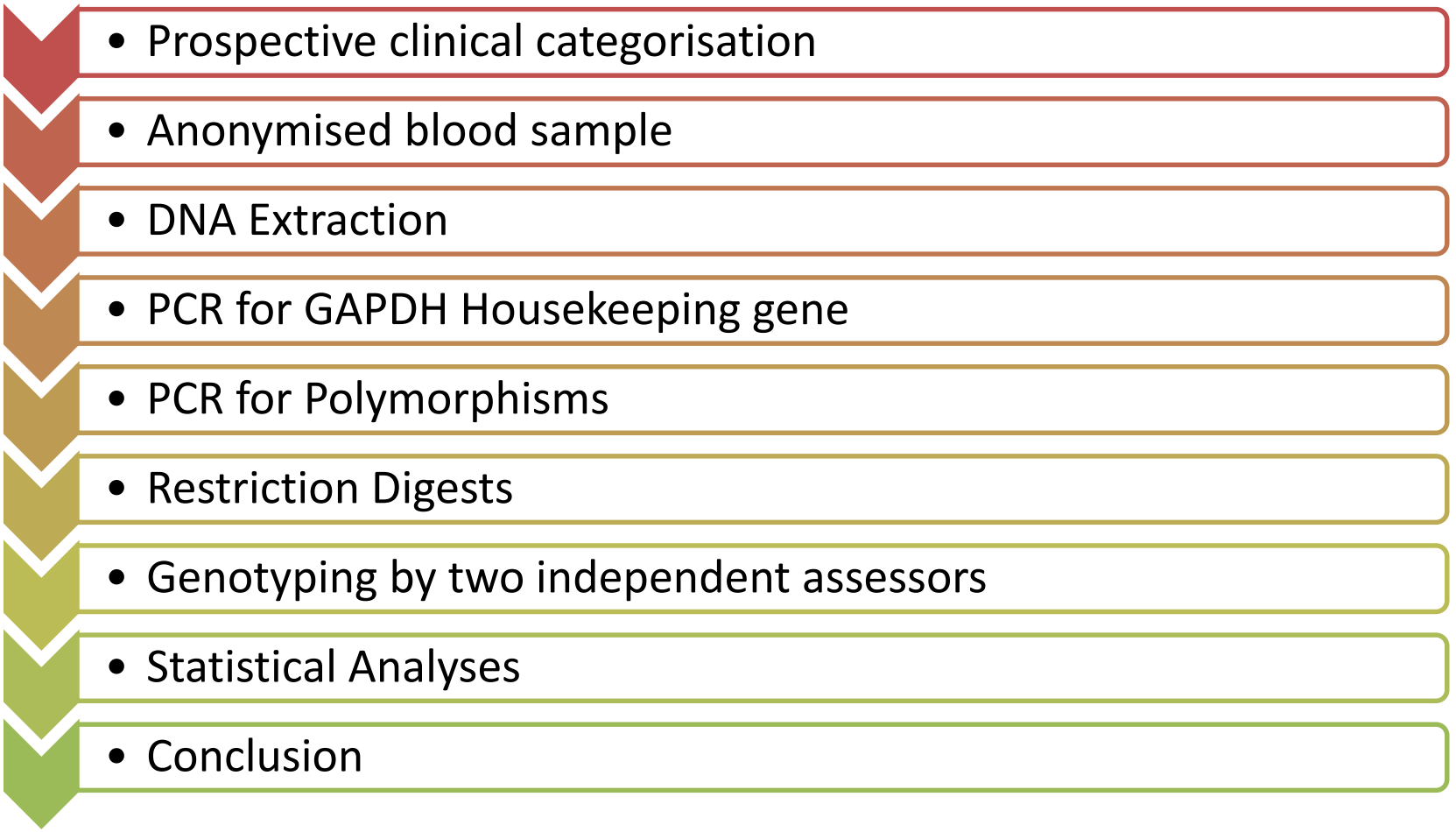
AIM:

The aim of this study falls under two broad categories:

A) Using Bioinformatics to identify the evolutionary relationship between the various serotonin receptors and their subtypes by drawing the phylogenetic tree and delineating the primary, secondary and tertiary structures of the receptors we aim to select the most likely candidate genes to regulate serotonergic receptors' function.

B) Apply genomic techniques to investigate any association between commonly known polymorphic genotypes in the identified candidate genes and the Premenstrual Dysphoric Disorder phenotype.

Summary Of Study Protocol



CHAPTER 3:

GENETICS OF PREMENSTRUAL SYNDROME: INVESTIGATION OF SPECIFIC SEROTONIN RECEPTORS POLYMORPHISM: BIOINFORMATICS

3.1 AIM

I am interested in learning about the evolutionary science and the structural relationship in between the Serotonin receptor proteins using Bioinformatics. Serotonin is a neurotransmitter which plays an important role in a number of physiological and pathological processes in many organs.[293] Serotonin functions through one of the seven different types of receptors 5-HT_{1-7}, grouped on the basis of sequence identity and on the nature of the second-messenger systems to which they are coupled.[294] Apart from $5HT_3$ receptor, which are inotropic all other receptors are G protein coupled receptors. In the brain the function of these 5-HT receptors can be associated with various overlapping functions.

My aim is firstly to understand the function of serotonin receptors and similarity if any between them. We are going to be investigating the human serotonin receptor phylogeny. Secondly, knowing a protein's 3-dimensional structure helps us to understand its functionality and provides means for planning experiments and drug design. To achieve this, first we will use phylogenetic analysis to examine the relationship between the sequences of various serotonin receptors including 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E}, 5-HT_{1F}, 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C}, 5-HT_{3A}, 5-HT_{3B}, 5-HT_{3C}, 5-HT_{3D}, 5-HT_{3E}, 5-HT_4, 5-HT_5, 5-HT_6, and 5-HT_7 using various bioinformatics tools. Here we assume the fact that homologous sequences are evolutionarily related.

Then we will predict and provide the transmembrane structure using the TMFMM method and the secondary and tertiary structure for the serotonin receptors.

3.2 METHODOLOGY and RESULTS

3.2.1 PHYLOGENETIC ANALYSIS: The evolutionary relationship between the serotonin receptors

Raw Sequence Data: The first step in constructing a tree is building the dataset, this means finding and retrieving sequences from the public domain.

Protein sequence for various serotonin receptors was retrieved using Protein database BLAST in NCBI (FATSA). http://www.ncbi.nlm.nih.gov/sites/entrez. It is the most commonly used database search tool. (Appendices 1)

Alignment

Alignment of serotonin receptor protein sequences was done using ClustalX (http://www.clustal.org/download/current/clustalx-2.0.12-win.msi). The default alignment parameters were applied. Clustal[295] X is a windows interface for the Clustal W multiple sequence alignment program and performs multiple sequence and profile alignments and analyses the results.[296] The sequence alignment is displayed in a window on the screen. Usually manual alignment is advocated.[297] Clustal X is formatted as PHYLIP tree file and can be imported into other tree drawing programs.

Annotation

The aligned sequence are annotated using CHROMA. The programme can be downloaded from (http://www.llew.org.uk/chroma/download/CHROMA.msi). Chromatic Representation of Multiple Alignments (CHROMA) is a tool for generating annotated multiple sequence alignments in a convenient format for publication. CHROMA runs only under Microsoft windows operating systems.

Alignment of serotonin receptor protein sequencing was done using ClustalX and the aligned sequences are annotated using CHROMA. (Appendices 2)

Generation of Phylogenetic Tree:

The alignment from ClustalX was viewed using Seaview (http://pbil.univ-lyon1.fr/software/seaview.html) here we aligned the sequence and then made the tree using Phyml (maximum length) by bootstapping 100 replicates, the file was saved as unrooted tree. This tree was opened using fig tree. The tree used to guide the final multiple alignment process are calculated from the distance matrix using the neighbour joining method.[298] This produces the unrooted trees with branch lengths proportionate to the estimated divergence along each branch. The root is placed by a pinpoint method.[299] Trees generated showing all the serotonin receptors is shown in (Figure 9).

Even though the phylogentetic tree for all serotonin receptors most likely shows a Common Evolutionary Ancestors 'Divergent Evolution', that is one serotonin receptor gene dividing to give many receptors types but all bind to one common serotonin ancestor. Sequences that are the most closely related are drawn as neighbouring branches on a tree. The phylogenetic analysis can help determine which genes are likely to have equivalent functions.

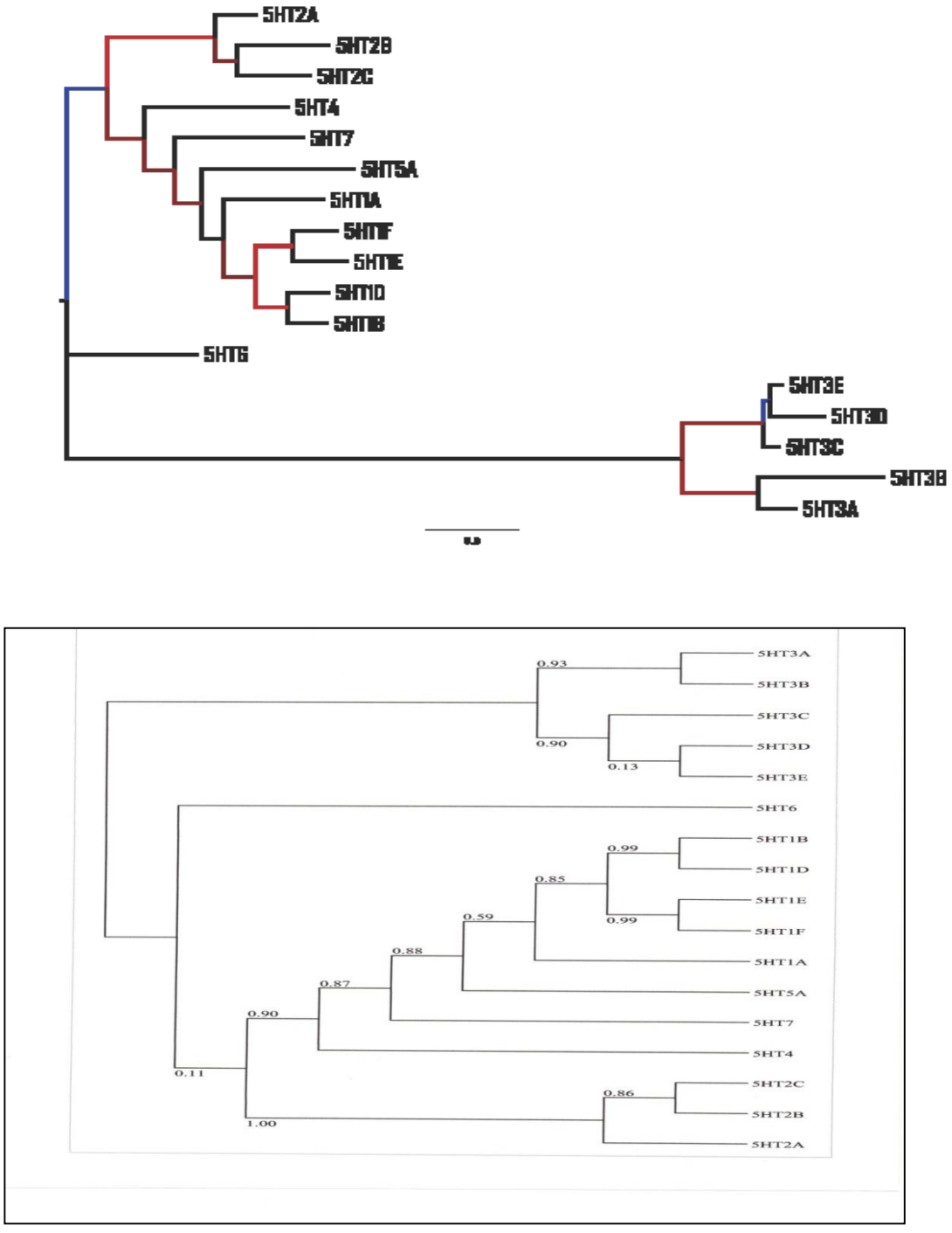


Figure 9. Phylogenetic tree for serotonin receptors

Phylogenetic Analysis of the Two Families of Serotonin Receptors done Separately :

It becomes increasingly difficult to find good alignments and to evaluate all the receptors simultaneously as more divergent sequences were included. Also in view of the large

distance in between 5-HT3 receptors and others, the phylogenetic trees for the two groups were dealt with separately.

Alignment of serotonin receptors 5-HT1,2,4,5,6,7 protein sequences was done using ClustalX and the aligned sequence are annotated using CHROMA (Appendices 3).

Tree generated for the 5-HT1,2,4,5,6,7 receptors (Figure 10)

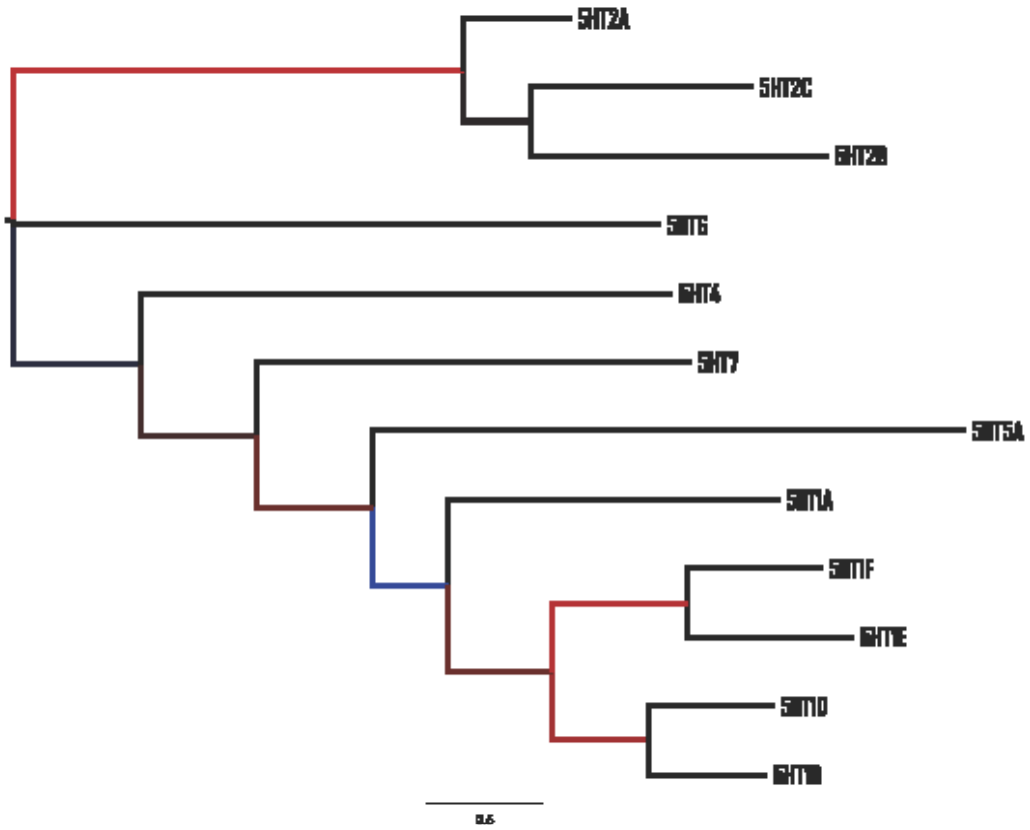


Figure 10: Phylogenetic tree for Serotonin Receptors

Alignment of serotonin receptors 5-HT3 protein sequences was done using ClustalX and the aligned sequence are annotated using CHROMA (Appendices 4) Tree generated for 5-HT3 receptor subtypes (Figure 11)

The alignment was impossible to improve by eye when 5HT3 receptor was considered separately to other receptors; hence a useful result was achieved.

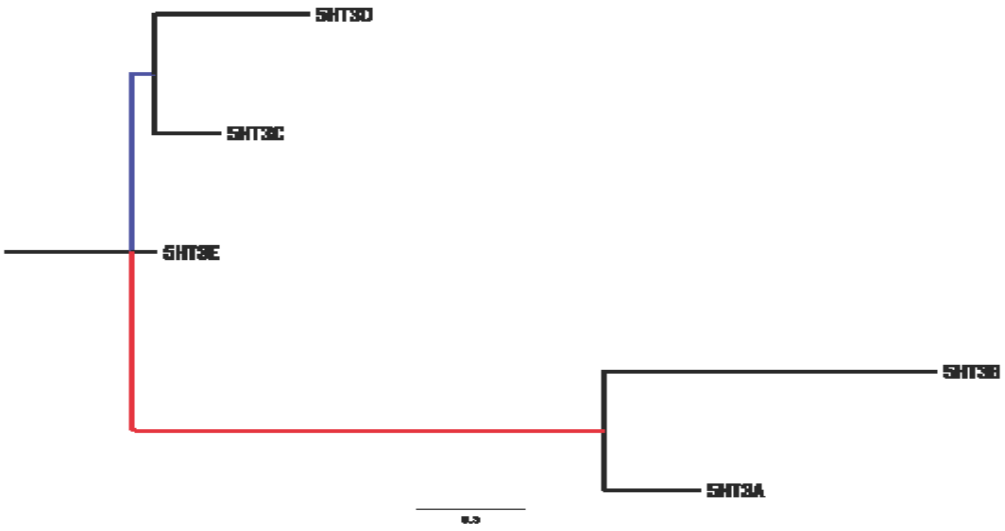


Figure 11: Phylogenetic tree for Serotonin Receptors

3.2.2 PROTEIN STRUCTURE: Prediction of the serotonin receptors structure

Structure of various receptors makes them capable of transducing an extracellular signal into the cell. The serotonin (5-hydroxytryptamine, or 5-HT) class of Gprotein-coupled receptors (GPCR) serve as neurotransmitters involved in many processes in the central nervous system, including the regulation of feeding, aggression, mood, perception, pain and anxiety.[293]

Trans-membrane Prediction (TMHMM model)

Trans**M**embrane prediction using **H**idden **M**arkov **Models** -- is a program for predicting transmembrane helices based on a hidden Markov model. TMFMM predicts the 7 transmembrane segments of GPCRs exactly.[300] The TMHMM was used to predict the structure of all 7 types of serotonin receptors including $5HT_{1A}$, $5HT_{1B}$, $5HT_{2A}$, $5HT_{2C}$, $5HT_{3A}$, $5HT_{4}$, $5HT_{5}$, $5HT_{6}$, $5HT_{7}$.

FASTA was used to format all the sequences by Search SMART (Simple Molecular Architectural Research Tool) database http://smart.embl-heidelberg.de/smart/ (Appendices 5). TMHMM was used to predict the trans-membrane structure

http://www.cbs.dtu.dk/services/TMHMM/ . The lengthy results include the graphical plot for the protein structure and the following information. (Table 7)

1. *Length:* the length of the protein sequence
2. *Number of predicted TMHs*: The number of predicted transmembrane helices.
3. *Exp number of AAs in TMHs*: The expected number of amino acids in transmembrane helices. If this number is larger than 18 it is very likely to be a transmembrane protein (OR have a signal peptide)
4. *Exp number, first 60 AAs*: The expected number of amino acids in transmembrane helices in the first 60 amino acids of the protein. If this expected number of amino acids is more than 60, one should be warned that a predicted transmembrane helix in the N-term could be a signal peptide.
5. *Total prob of N-in*: The total probability that the N-term is on the cytoplasmic side of the membrane.

RECEPTOR	LENGTH	Number of predicted TMHs	Exp number of AAs in TMHs	Exp number, first 60 AAs	Total prob of N-in
5-HT1A	422	7	158.05958	21.53471	0.00307
5-HT1B	390	7	155.62023	9.99705	0.00939
5-HT2A	471	7	159.64767	0.0018	0.00302
5-HT2C	458	7	179.77408	27.51182	0.97688
5-HT6	440	7	156.66362	23.05112	0.00002
5-HT7	479	7	154.44007	0.00361	0.00138
5-HT3	510	4	91.81364	0.4317	0.01408

Table 7: Results obtained with prediction of TMHMM structure of serotonin receptors

The results showed the prediction of the 7 transmembrane helices for all the G protein coupled receptors (5HT-1,2,4,5,6,7) (Table 8) (Figure 12) except 5HT-3 (Figure 13)

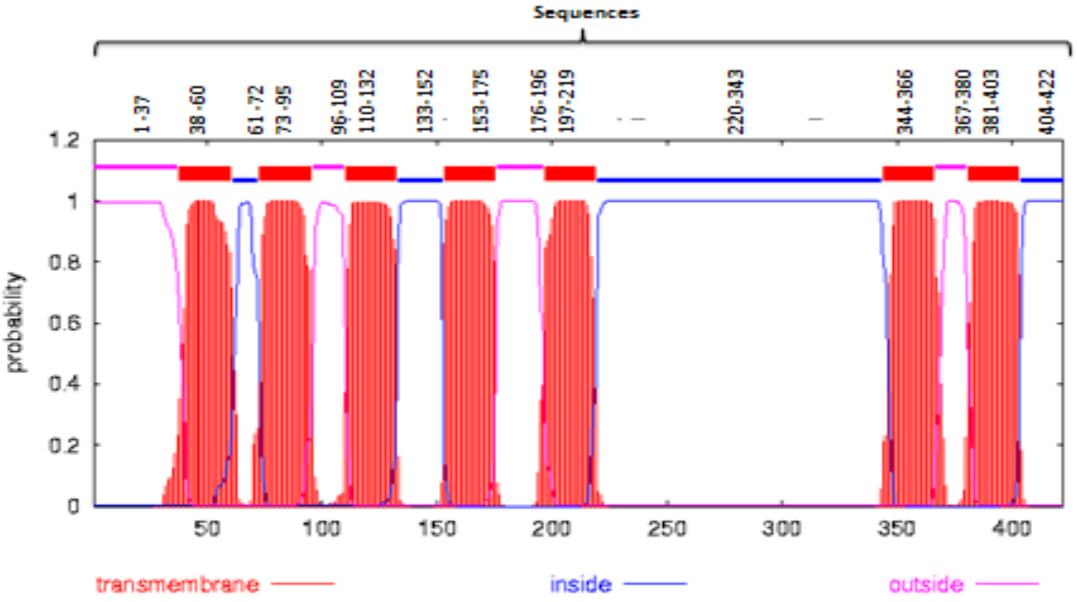
The results confirmed the GPCR protein super family shows definitive common features of:

1. Seven hydrophobic transmembrane α helices
2. Three extracellular loops and three or four intracellular loops in the cytoplasm
3. Extracellular N-terminus
4. Intracellular C-terminus
5. They are related to Class A GPCR's (rhodopsin and adrenergic receptors)

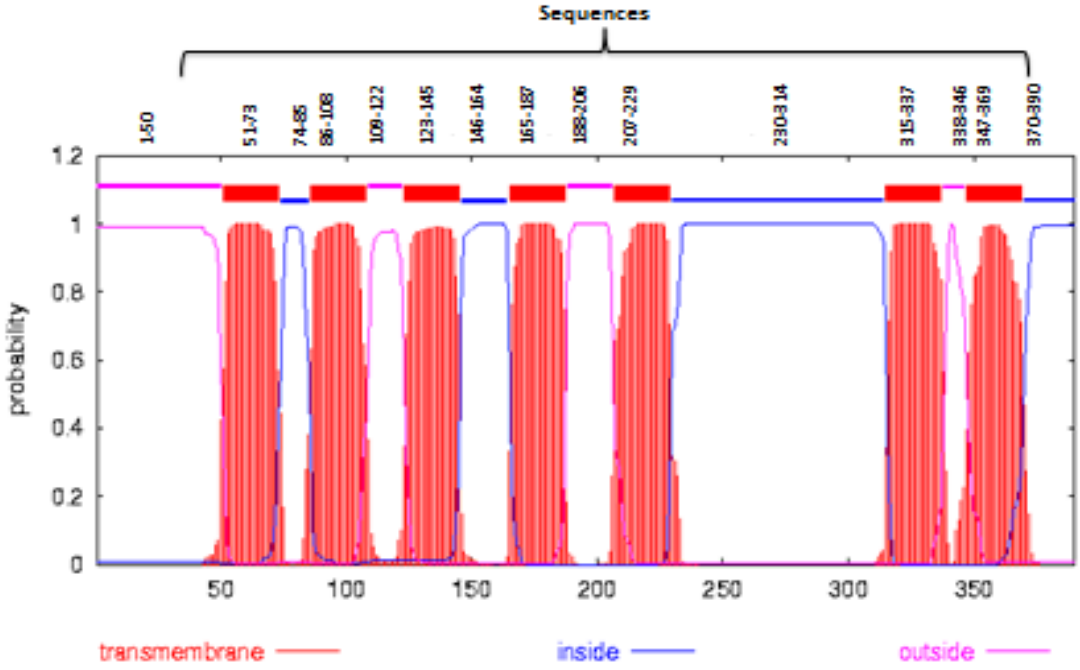
RECEPTOR	NUMBER OF TRANSMEMBRANE HELICES PREDICTED	PREDICTION TO HOMOLOGY TO GPCR's	E-value
5HT-1A	7	√	2.10e-05
5HT-1B	7	√	1.0e-02
5HT-2A	7	√	1.8e-03
5HT-2C	7	√	5.8e-03
5HT-4	7	√	
5HT-5	7	√	
5HT-6	7	√	2.4e-07
5HT-7	7	√	3.90e-02
5HT-3A	3	**X**	

Table 8: TMHMM Structure of Serotonin Receptor 5-HT 1A, 1B, 2A, 2C, 6, 7

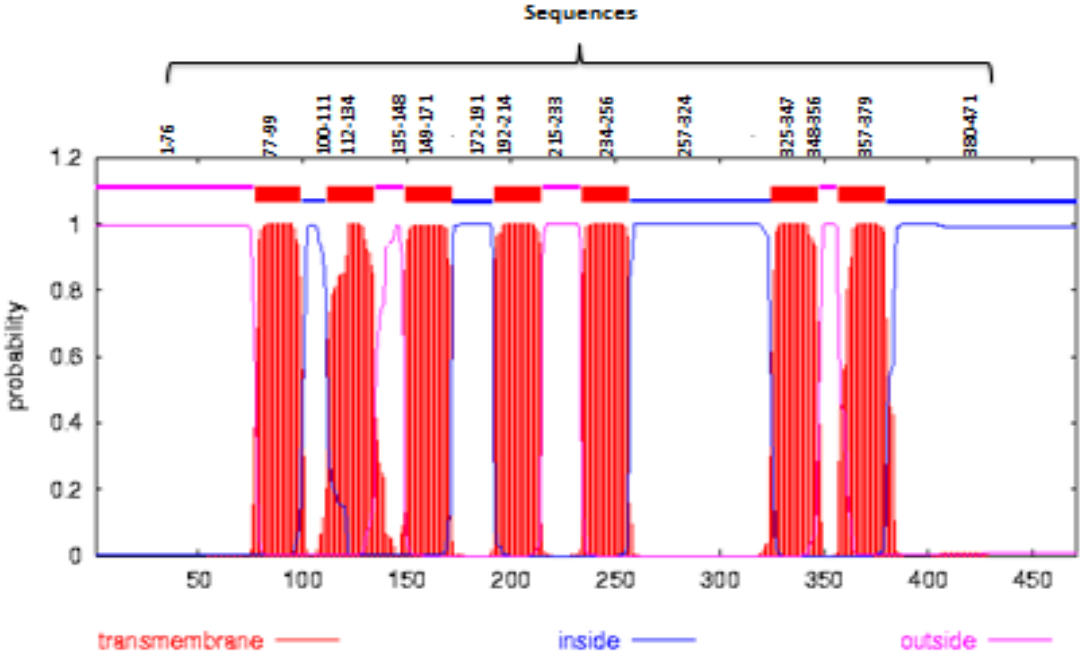
Figure 12: TMHMM Structure of Serotonin Receptor 5-HT 1A, 1B, 2A, 2C, 6, 7



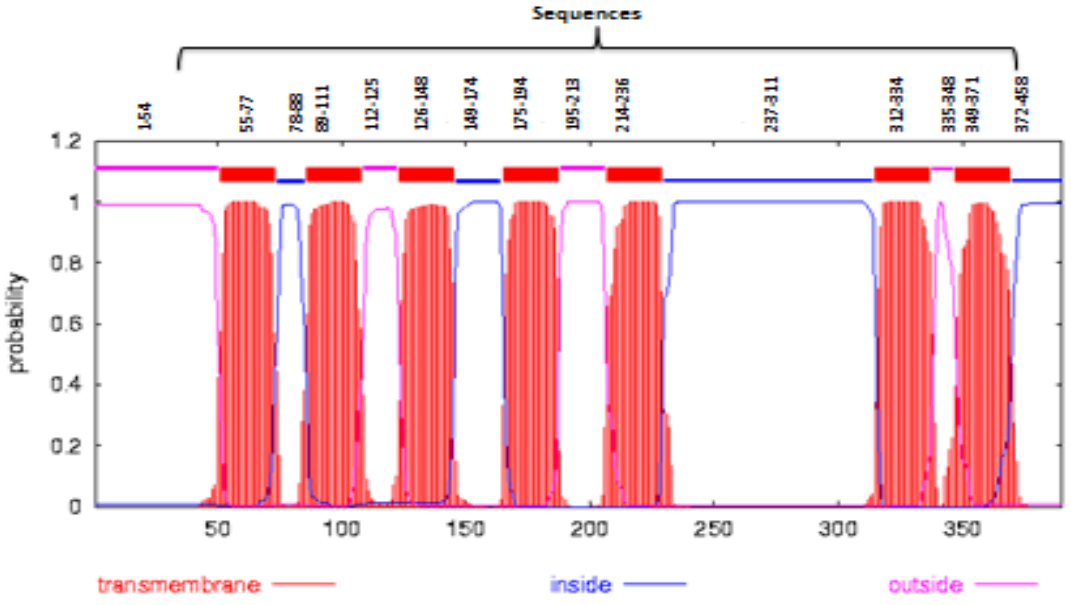
TMHMM posterior probabilities for sp_P08908_5HT1A_HUMAN



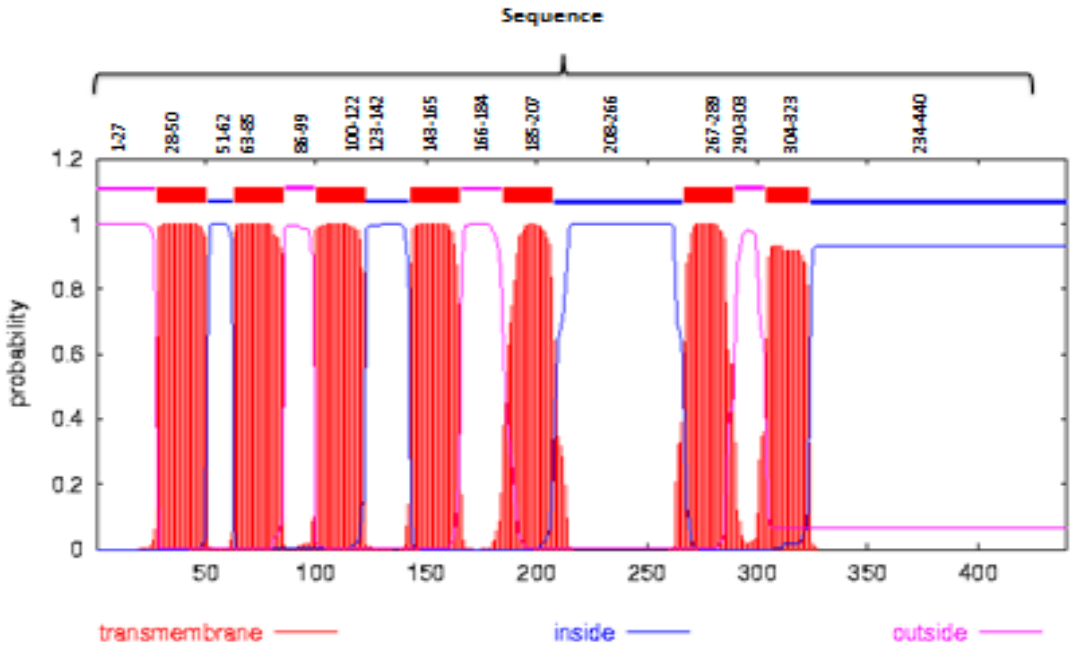
TMHMM posterior probabilities for sp_P28222_5HT1B_HUMAN



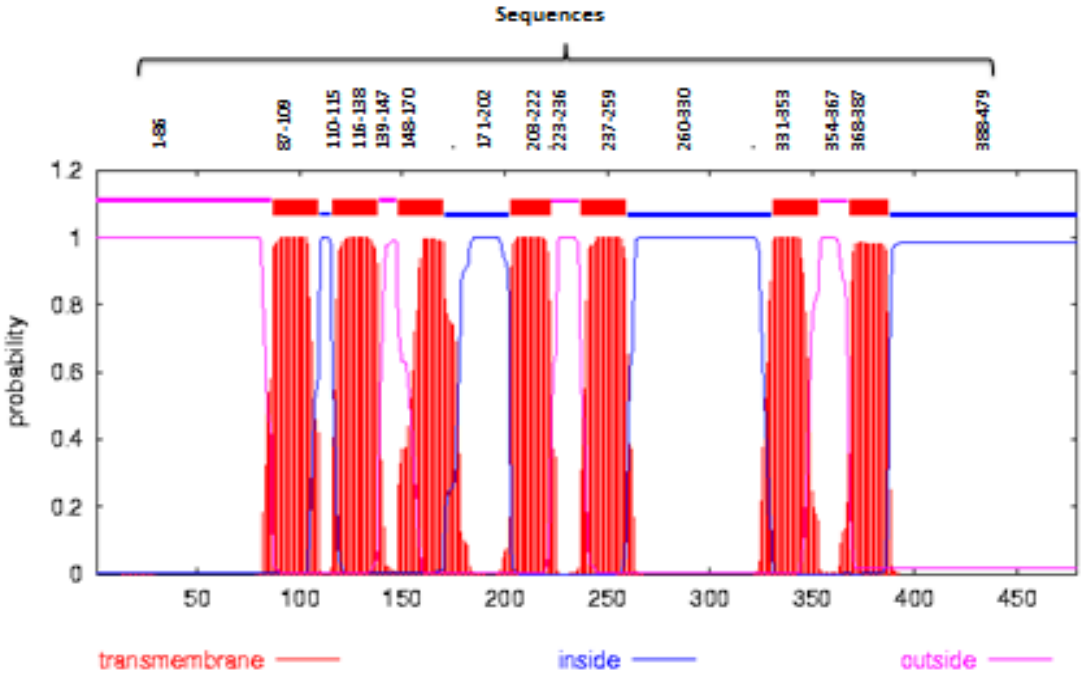
TMHMM posterior probabilities for sp_P28223_5HT2A_HUMAN



TMHMM posterior probabilities for sp_P28335_5HT2C_HUMAN



TMHMM posterior probabilities for sp_P50406_5HT6_HUMAN

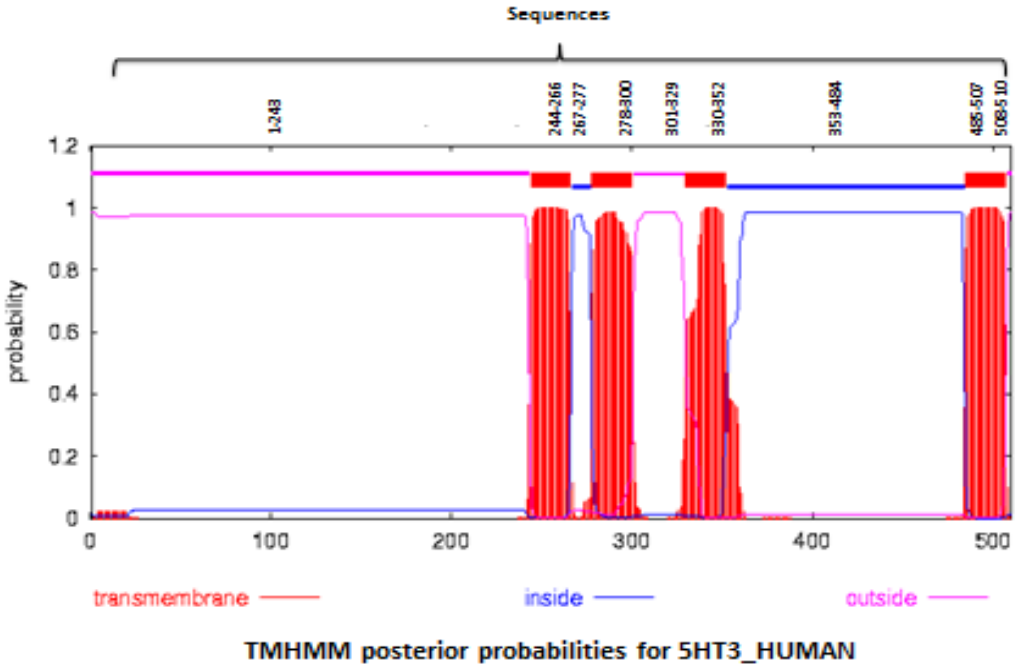


TMHMM posterior probabilities for sp_P34969_5HT7_HUMAN

In contrast to the other receptors 5HT-3 which is a ligand-gated ion channel shares features of the ion channel family members. It has:

1. A large extracellular cysteine loop
2. Four hydrophobic transmembrane segment
3. A large intracellular loop between the third and the fourth transmembrane region
4. Extracellular C-terminus

Figure 13: TMHMM Structure of Serotonin Receptor 5-HT3A



Secondary Protein Structure: We selected the representative 5-HT1A and the 5-HT3 receptors and predicted the secondary structure for the GPCR receptors (Figure 14) and the ionic channel receptors (Figure 15).

We used PSIPRED (Protien Sequence analysis workbench) - a highly accurate method for secondary structure prediction for serotonin receptors. http://bioinf.cs.ucl.ac.uk/psipred/.

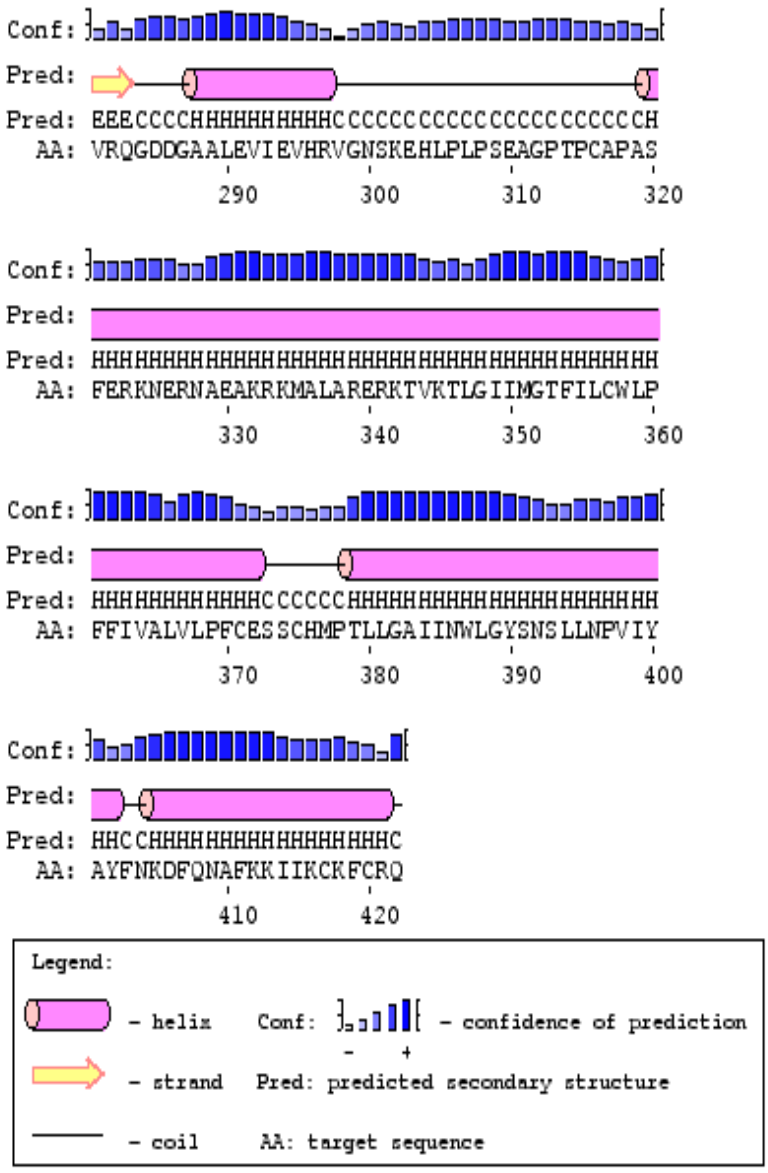


Figure 14: 5HT1A Secondary structure

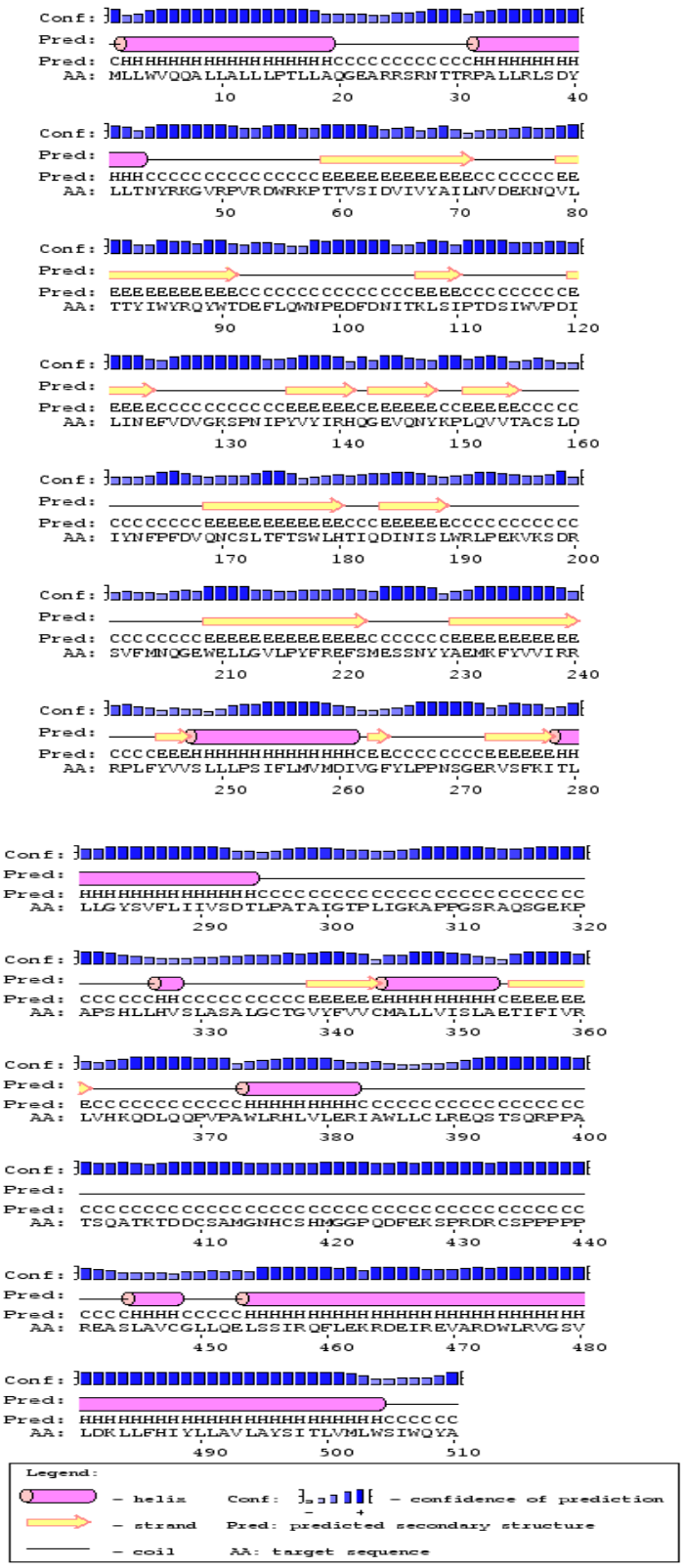


Figure 15: 5-HT3 Serotonin Receptor Secondary Structure

Tertiary Protein Structure: To crystallise serotonin, purification is a very slow and laborious procedure, hence currently there is no known serotonin structure. The prediction method that I have used is the Comparative modelling method, If the sequence to model has a homologue in the PDB (Protein Data Bank) which it is very similar to, the homologue may be used as the target and a structural model is built on the basis of this template.

To get the crystalloid structure of serotonin receptor structure, get the receptor sequence using The Basic Local Alignment Search Tool (BLAST) which finds regions of local similarity between sequences. The program compares nucleotide or protein sequences from the same or different organisms. Sequence similarity searching can be used to predict the location and function of protein-coding and transcription-regulation regions in genomic DNA.

I have compared this data sequence using BLAST to the Brookhaven Protein Data Bank (PDB), the repository for protein-structure obtained by X-ray crystallography and NMR. All sequences in the PDB with an E-value of Blast below a certain threshold of 10 are considered as candidates for the template. http://blast.ncbi.nlm.nih.gov/Blast.cgi. (Table 9) The PDB code is unique code for the crystal structure.(Table 9) This PDB code is used to view the crystal structure in jmol on the PDB website http://www.pdb.org.

The PDB file is downloaded and the structure is modified to give the crystal figure using Molosoft. To Get the results for two representative receptors 5-HT1A and 5-HT3 following steps have been followed:

1) www.ensembl.org
    a) Select human
    b) Search for serotonin receptor

c) By species- homosapeines

RECEPTOR		CCDS	ACCESSION No.	PDB code	DESCRIPTION	QUERY COVERAGE	E VALUE
5HT-1A	ENSG00000178394		NP000515.2	3D4S A	Chain A, Cholesterol Bound Form Of Human Beta2 Adrenergic Receptor	88%	5e-45
5HT-1B	ENSG00000148680	7408	NP062873.1	2R4R A	Chain A, Crystal Structure Of The Human Beta2 Adrenoceptor	66%	5.8e-03
5HT-2C	ENSG00000147246	14564	NP000859.1	2R4R A	Chain A, Crystal Structure Of The Human Beta2 Adrenoceptor	67%	3e-37
5HT-2A	ENSG00000102468	9405	NP000612.1	2R4R A	Chain A, Crystal Structure Of The Human Beta2 Adrenoceptor	67%	8e-36
5HT-4	ENSG00000164270	4291	NP955525.1	2R4R A	Chain A, Crystal Structure Of The Human Beta2 Adrenoceptor	88%	4e-53
5HT-5	ENSG00000157219	5936	NP076917.1	2R4R A	Chain A, Crystal Structure Of The Human Beta2 Adrenoceptor	94%	2e-33
5HT-6	ENSG00000158748	197	NP000862.1	2R4R A	Chain A, Crystal Structure Of The Human Beta2 Adrenoceptor	72%	2e-42
5HT-7	ENSG00000148680	7408	NP062873.1	2R4R A	Chain A, Crystal Structure Of The Human Beta2 Adrenoceptor	66%	8e-42
5HT-3	ENSG00000166736	8366	NP001155244	2BG9 B	*Chain B, Refined Structure Of The Nicotinic Acetylcholine Receptor*	71%	3e-37

Table 9: Results of Comparing this Data Sequence using BLAST to the Brookhaven Protein Data Bank (PDB)

2) Select the receptor required

   a) External references

   b) Ref sequence peptide

3) Use accession number and run BLAST in database select PDB proteins

4) Get sequence producing significant alignment

5) Click on protein 3Dstructure or

6) Put the PDB code on www.pdb.org

7) View image in Jmol

To get the crystalloid structure of serotonin receptor structure, 5-HT1A was compared to the data sequence using BLAST to the protein databank (PDB). The PDB code 3D4S is unique code for the crystal structure. This PDB code is used to view the crystal structure on the PDB website.(Figure 16) This showed the structure similarity to Chain A, Cholesterol Bound Form Of Human Beta2 Adrenergic Receptor.

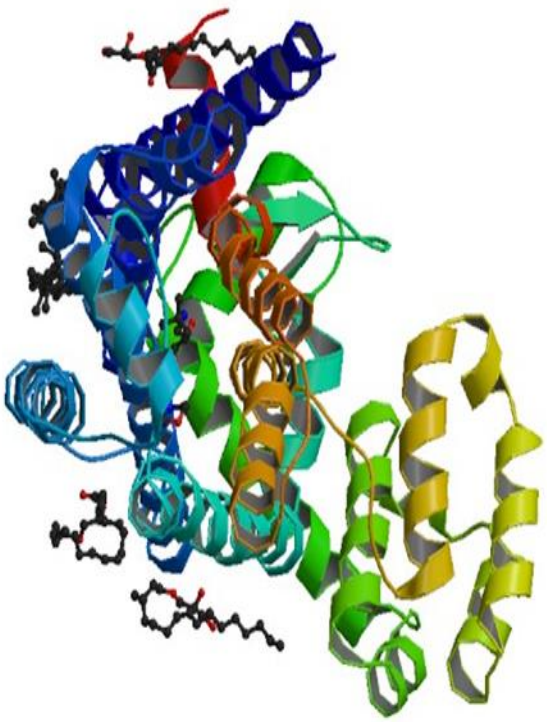


Figure 16: Assumed Biological Molecule for the 5HT-1A Serotonin Receptor Cholesterol bound form of human beta2 adrenergic receptor.[301]

Whereas the tertiary structure for the 5-HT3 serotonin receptor shows similarity to the Nicotine Acetylcholine Receptor. (Figure 17)

Figure 17: Prediction of Tertiary Structure for 5HT3 Serotonin Receptor. Similar to Nicotine Acetylcholine Receptor. (Refined structure of the nicotinic acetylcholine receptor at 4A resolution.)[302]

The PDB file is downloaded and the structure is modified to give the crystal figure using Molosoft. (Figure 18) showing the ligand binding sites. More the number of amino acids in the ligand site more the specificity of binding. Hence if there is a polymorphism in the this coding region it will change the function (a subtle effect of the signalling cascade)

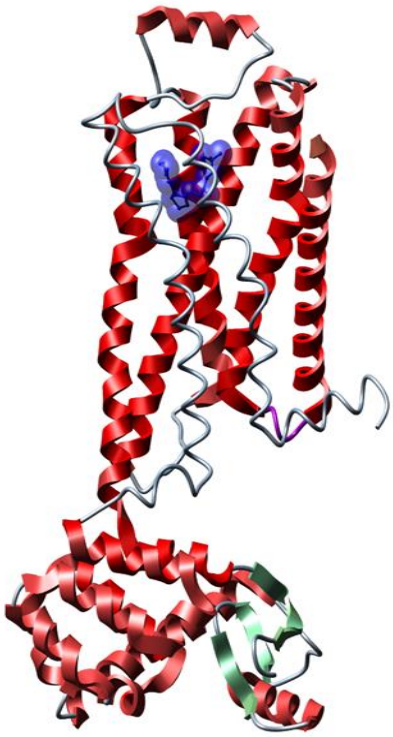


Figure 18: Modified Tertiary Structure of 5-HT1A to give the crystal figure using Molosoft showing the ligand binding sites

3.3 DISCUSSION:

Phylogenetic analysis gives insight into how a family of related sequences has been derived during evolution. Our results show, with high bootstrap support, the tree of 17 member serotonin receptor family can be divided into three main CLADS (Figure 9) comprising of 5-HT3 receptor subtypes, 5-HT6 and the third group of 5HT1,2,4,5,7. Each clad which is a group of receptors that include the most recent common ancestors of all of its members and descendants. Members of a clad share a common evolutionary history and are more related to each other. These clusters of homologues may be functionally related groups.

The evolutionary relationships among the sequences are shown as branches of a tree. The length and nesting of these branches reflects the degree of similarity or divergence between any two given sequences. Sequences that are the most closely related are drawn

as neighbouring branches on a tree, for example, our results show that 5-HT2B is more related to 5-HT2A than 5-HT2C to 5-HT2A (Figure 9). Similarly 5-HT7 is related more to 5-HT4 than 5-HT5 to 5-HT4. From the three major taxons, the branch length shows divergence between 5-HT1B and 5-HT1A. note that the 5-HT5 and 5-HT7 represent distinct classes of receptors but seems to be more closely related to the 5-HT1 receptor than 5-HT2. 5-ht6 also falls out of other serotonin receptor classes.

To further emphasise the significance of our tree findings, the members of the above three clads also diverse in their intracellular transduction mechanisms which defines the receptor superfamily. The most diverged group of 5-HT3 receptors are ligand gated ion channel whereas 5-HT1,2,4,5,6,7 receptors are G-protein coupled receptors. Further in the GPCR group of receptors 5-HT1 are negatively coupled to adenylyl cyclase (↓cAMP), 5-HT2 are coupled to protein kinase, while 5-HT4,5,6,7 are positively linked to adenylyl cyclase (↑cAMP).

It has been extensively shown in the case of bioamine G protein-coupled receptors, that the ligand binding site is embedded in the membrane, surrounded by the seven transmembrane α-helices.[303;304] The amino acids in receptor structures interact with the ligands, but change in receptor sequences during evolution to form paralogous genes has caused agonists or antagonists compounds to discriminate between different receptor class or subtypes of receptors.[305]

The overall structure of a receptor protein becomes conserved and adapted to a function, the structure is fixed by strong functional requirements. Hence the details of the receptor shapes can change only through millions of year down the revolution.[306] Within a gene family two main evolutionary mechanisms cause diversifications. Firstly, during a variety of duplication mechanism unrepaired point mutations cause a sequence drift leading to

two different but similar genes.[307;308] mostly these genes result in pseudogenes and are eliminated over time, rarely two receptors encoded by duplicated genes exhibit different biological characteristics.[309] These paralogous genes will form different receptor subtypes with their specific functions. The second type of diversification occurs between different species encoding the same receptor subtypes. These orthologous genes have the same function showing a much higher degree of same sequence identity.[310] Hence the cluster of the 5-HT3 receptor are paralogous genes with very different mechanism of action and function.

In view of the large distance in between 5-HT3 receptors and others, the phylogenetic trees for the two groups were dealt with separately. The tree generated for 5-HT1,2,4,5,6,7 confirmed the same findings as above.(Figure 10)

By drawing the 5-HT3 receptors separately we see that there are three major evolutionary branches (Figure 11) 5-HT3A and 5-HT3B map closely to each other suggesting that they have arisen by gene duplication.

To further analyse the putative similarities between the serotonin receptor family we extended the analysis by generating the TMHMM structure and produced a model tertiary structure. Important information regarding the intracellular transduction mechanism defines the receptor superfamily. From our results in predicting the TMHMM structures of the various serotonin receptors, the GPCR have a very similar structure (figure 12) compared to the ligand gated ion channel receptor 5HT3 (figure 13). Structurally all serotonin receptors except 5HT3, which forms a part of the cation channels belong to the G protein superfamily and contain the characteristic structure of 7 hydrophobic transmembrane segments with extracellular amino terminus and intracellular carboxyl terminal. (Figure 19)

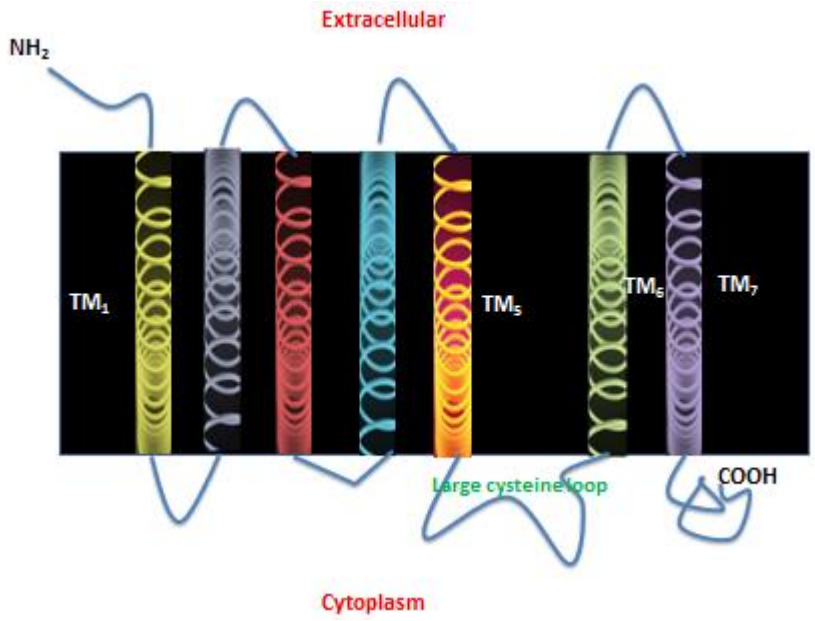


Figure 19: G protein superfamily structure containing the characteristic structure of 7 hydrophobic transmembrane segments with extracellular amino terminus and intracellular carboxyl terminal.

The seven sequence stretches of about 25-35 residues represents the α helices, they span the plasma membrane enabling an extracellular ligand to exert a specific effect into the cell. All of these receptors (5HT 1,2,4,5,6,7) transduce extracellular signals though interaction with guanine nucleotide binding (G) protein. However the 5HT3 receptor shares the characteristic feature of the other ion channel families.(Figure 20) It has a large extracellular domain containing the cysteine loop, four hydrophobic transmembrane segments. It has a large intracellular loop between the third and fourth transmembrane region and a extracellular Carboxyl terminus.

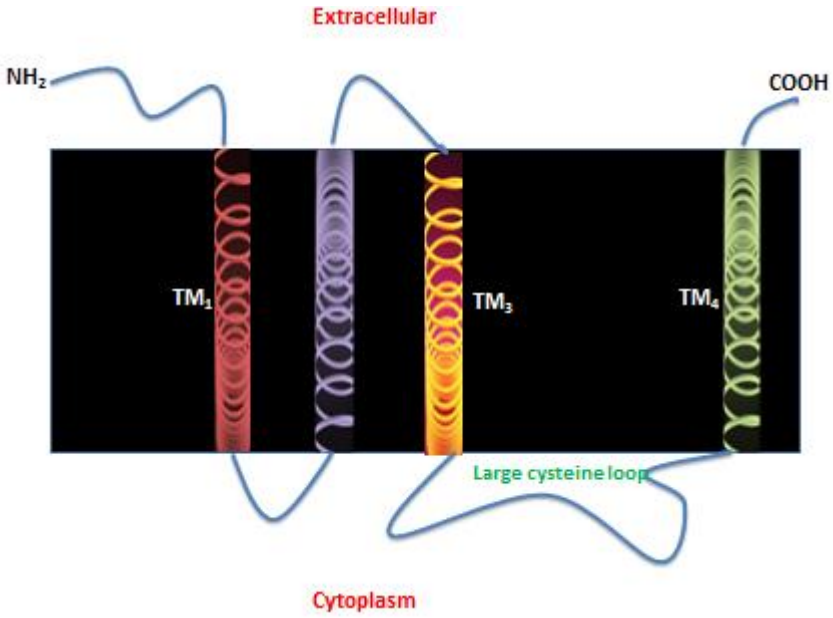


Figure 20: Ion Channel superfamily structure containing the characteristic structure of 4 hydrophobic transmembrane segments with extracellular amino and carboxyl terminal.

The secondary structures of the representative GPCR 5HT1A and the ionic channel 5-HT3 receptor confirms the structure differences (Figure 6&7).

The predicted tertiary structural model of 5-HT1A receptor has the same structure as the Cholesterol bound form of human beta2 adrenergic receptor (a GPCR). The 5-HT3 model has shown similarity to Nicotine Acetylcholine Receptor (an ion channel receptor). Great caution is needed when using the homology based models for detail functional and structural annotations since the loops and helix are often different in different receptors. But homology modelling of the receptors can be utilised in understanding of ligand-protein interaction and further identification of new and potent ligands.

Hence we have shown that serotonin receptors not only share the common ancestors but have several common structural features except 5HT3.

3.4 CONCLUSION

It is clear from the phylogenetic tree and prediction of the serotonin receptor protein structure, there are two representative groups. The Serotonin receptors 5HT-1,2,4,5,6,7 are G protein coupled receptors (GPCR's) and have a 7 α transmembrane helices (7TMR) and they mediate signal transduction through the G protein. They are key controllers of diverse physiological process (signal transduction) hence they are major drug targets. GPCR play a very important role however it is very difficult to resolve their tertiary structure by x-ray crystallography. Hence computational methods have been developed to predict their structure based on their sequences. These receptors have shown homology to the human β-adrenergic receptor, hence may carry out functional roles traditionally associated with adrenergic receptors like carbohydrate metabolism, modulation of sensory input, memory etc.

Whereas the 5HT-3 receptors, are far in the phylogenetic tree in spite of the common ancestor, hence have been dealt with separately. This fact is further emphasised by our protein structure predictions that unlike the others they are ligand- gated ion channel sharing the features of the ion channel family members. They show greater structural similarity to the Nicotine Acetylcholine Receptors. This is supported by the fact that they have a large intracellular loop between third and fourth transmembrane region. These receptors are channels for the conduction of both cations and anions which form the basis of the transfer of information at the neuronal synaptic junction.

This analysis allows us to conclude that although close to the rest of the serotonin receptors in the evolution tree, 5-HT3 receptors constitute a separate family of receptors. Hence we hypothesize that the clad containing the 5-HT1,2,4,5,7 group of receptors comprises a series of homologous genes arisen by gene duplication and share common

structural features. We will select representative receptor types, evenly distributed in this CLAD of receptors to see their contribution to the development of PMDD.

CHAPTER 4:

GENETICS OF PREMENSTRUAL SYNDROME: INVESTIGATION OF SPECIFIC SEROTONIN RECEPTORS POLYMORPHISM: RECRUITMENT OF STUDY PARTICIPANTS

The chapter is the continuation from the thesis "GENETICS OF THE SEROTONERGIC PATHWAY IN PREMENSTRUAL DYSPHORIC DISORDER" on permission of Prof KMK Ismail as the clinical categorisation of the recruited patients and controls is the same.

4.1 RECRUITMENT OF STUDY PARTICIPANTS:

The study was conducted in University Hospital North Staffordshire, Keele University Hospital. The unit has a specialised PMS clinic run by Prof Obrien, Specialist nurse, Health assistant and research fellow (clinical scientist). The department has had a longstanding interest in PMS and its treatments. Many research and review articles have been published making enormous input into the understanding, diagnosis and management of this condition. The study has been funded by departmental funds. Patient referrals have been via GP's directly, other units in the West Midlands and fellow consultant teams locally.

ETHICS

The study was approved by the Staffordshire and Shropshire Ethics Committee and informed written consent was obtained from each participant. Women were recruited from a specialised PMS clinic, general gynaecology clinics or following advertisement on the hospital intranet system.

First Contact

In order to achieve a successful first contact, without the need for additional attempts, and hence saving costs, ladies were seen by the Specialised Research Nurse on one to one basis for at-least 30mins consultation. Detail history and presenting symptoms were noted. The ladies were introduced to the study and invited to participate. An invitation

letter and information leaflet were given to the ladies along with the DRSP forms (complete daily diaries for 2 to 3 menstrual cycle) return addressed and stamped envelopes. The appointment could be followed by a phone call in order to confirm and schedule next appointment.

The Invitation Letter and the Information Leaflet (Appendices 6) were combined together. This was a personal invitation to participate in the study, and was short as other relevant information was given in an attached information leaflet. The leaflet contains key information on the study in a concise form, targeted at provisional persons. It was in simple words, easily understandable and informative. It typically addressed:

- WHAT ARE WE AIMING TO DO?
- WHY ARE WE ASKING YOU FOR YOUR HELP?
- WHO IS ORGANISING THE STUDY?
- WHAT WILL WE BE ASKING YOU TO DO?
- DO I HAVE TO TAKE PART?
- WHO WILL SEE MY RECORDS AND KNOW ABOUT ME TAKING PART?
- WILL MY EXPENSES BE PAID?
- WHOM DO I CONTACT WITH ANY CONCERNS?

Re-Contacts

If the lady did not respond or did not attend further appointments, she was contacted via telephone call 3 attempts and a written letter with reschedule appointment date. If the person was not contactable, moved residence or refused the recruitment ended at that point. If she agreed to participated she was enrolled either as a case or controls. The **GOPMS Recruitment Sheet** (Appendices 7) was completed which included information like Study number, name, contact address and telephone number and Date Enrolled. The

GOPMS Data Sheet (Appendices 8) was also completed at this visit which included relevant history, presenting complaints, medication, family history and measure used for diagnosis.

4.2 PARTICIPANTS: CASES/CONTROLS

The Inclusion and exclusion criteria were very robust and can be seen in our previous GOPMS studies. Women were considered to be potentially suitable for inclusion in that study if following criteria were met:

1. The ladies had to be white Europeans.
2. Age group between 18 – 48 years
3. Should have regular menstrual cycles (28 ± 4 days).
4. These women should not be on any form of hormonal therapy, contraception or HRT at least for 2 months before and during the 2 cycles of symptom rating.

Women were considered non-eligible or excluded from the above group for GOPMS study if:

1. She was pregnant
2. Planning a pregnancy
3. Any history of existing or past relevant psychiatric disorder or
4. If using any psychotropic medications.

104 white European women between the ages of 18-48 were enlisted from the local 6 population, and categorized into two groups; PMDD and controls. All subjects reported regular menstrual cycles (28 ± 4 days) and none was taking oral contraceptives, hormone replacement therapy or psychotropic drugs. Any woman known to have an existing or previous psychiatric disorder was excluded from the study.

4.3 CLINICAL CATEGORISATION: CASES/CONTROLS

Clinical diagnosis was determined by prospective symptom rating using the daily record of severity of problems (DRSP) scale[14], based on self-assessment reports spanning two consecutive menstrual cycles. Symptom ratings of menstrual cycle days 6-12 and the seven days immediately before the next menstrual period were used to calculate the mean follicular and mean luteal scores respectively, using the formula:

Equation 1: Symptom rating for clinical categorisation

$$\text{Severity rating (\%)} = \frac{\text{(mean luteal score – mean follicular score)}}{\text{mean follicular score}} \text{X100}$$

Women were diagnosed with PMDD if there was a ≥200% increase in severity of one or more, or a ≥100% increase of two or more of the DSM-IV PMDD-defining symptoms.[105;311] According to DSM-IV to diagnose PMDD, require the presence of five out of 11 possible symptoms limited to the late-luteal phase of the menstrual cycle, including at least one of the following PMDD defining symptoms: marked depression, anxiety/tension, affective lability and irritability (criterion A).[12] The severity of symptoms must significantly disrupt work performance or social functioning (criterion B). Furthermore, the disturbance must not be an exacerbation of symptoms of an existing psychiatric disorder (criterion C).

The control group comprised women who reported no significant premenstrual symptoms, and did not meet the above criteria.

4.4 RESULTS OF CLINICAL CATEGORISATION

One hundred and seven Caucasian women who fulfilled the inclusion /exclusion criteria for the study were recruited. Two study participants (numbers 33 and 92) failed to follow-up hence excluded from the final analysis. One study participant was excluded as the blood sample was not enough to perform the experiments and on re-contact we found out that she had moved out of the area. Data were complete for one hundred and four European Caucasian women who were categorised into two groups; controls (n=51) and PMDD (n=53). The mean age of the control group was 36.2 years (age range 22-48 years) and the PMDD group had a mean age of 37.7 years (age range 27-46 years). In line with the DSM-IV diagnostic criteria for PMDD, Women were diagnosed with PMDD if there was a ≥200% increase in severity of one or more, or a ≥100% increase of two or more of the symptoms during the luteal phase compared to the follicular phase – in both menstrual cycles using the formula presented in **equation 1**. Otherwise they were categorized as controls. The follicular, luteal and percentage of difference for each of these symptoms in both groups are presented in **Appendices 9** **and** **Appendices 10**.

4.5 SAMPLE COLLECTION AND STORAGE

After consent Five to ten ml of blood were taken by venupuncture and placed in ethylene diamine tetra-acetic acid (EDTA) tubes. These samples were collected by a research sister and were anonymised using the unique study number issued for each participant at time of recruitment. Samples were transferred to the laboratory where they were stored at -20^0C for further analysis. The two researchers (VD and JM) who independently genotyped the samples were blind to clinical categorisation till the end of the study.

CHAPTER 5:

GENETICS OF PREMENSTRUAL SYNDROME: INVESTIGATION OF SPECIFIC SEROTONIN RECEPTORS POLYMORPHISM: LABORATORY METHODOLOGY

5.1 BACKGROUND

5.1.1 Genetic Polymorphisms

The human genome is the sum total of DNA molecules found within every cell except Red Blood Cell. Every cell in a person's body has the same DNA. Most DNA is located in the cell nucleus, but a small amount of DNA can also be found in the mitochondria. The information in DNA is stored as a code made up of four chemical bases: adenine (A), guanine (G), cytosine (C), and thymine (T). Human DNA consists of about 3000 million bases, and more than 99% of those bases are the same in all people. Sequence, of these bases determines the information available and required for building and maintaining an organism. The information is in the form of genes.

A gene is the basic physical and functional unit of heredity, acting as instructions to make molecules called proteins. Genes form only 3% of the total genome and rest of the DNA is non-coding but have a functional role in regulating and promoting gene expression. They also have a structural role in chromosome integrity segregation. The function of large fraction of genome is yet not known and does not depend on the sequence of the bases. Genes are made up of exons (coding sequence) and introns (non-coding sequence). Every person has two copies of each gene, most genes are the same in all people, but a small number of genes (less than 1% of the total) are slightly different between people. *Alleles* are forms of the same gene with small differences in their sequence of DNA bases and contribute to each person's unique physical features. In some cases, different alleles may produce different phenotypes, as in mutations responsible for monogenic disorders; in others they may not have any effect.

Mutation is the process whereby genes change from one allelic form to another and may form an entirely new allele. The wildtype allele dictates the most common phenotype in a natural population. Genes can mutate randomly, at any time and in any cell. Mutations occur during normal replication, due to mutagens or due to erroneous repair following a exposure to a mutagen, radiation, or infection by viruses. Mutant alleles may be recessive or dominant. Many common mutations at the DNA level have no consequences for the individual's phenotype. Mutations are revealed in an individual's phenotype if the function of genes and their products are affected. Different types of mutations at the DNA level are:

a) Missense mutation: This type of mutation is a change in one DNA base pair that results in the substitution of one amino acid for another in the protein. The amino acid substitution may have no affect or may render the protein non-functional

b) Nonsense mutation: nonsense mutation is also a change in one DNA base pair forming a STOP codon instead of a codon specifying an amino acid. This type of mutation results in a shortened protein that may function improperly or not at all.

c) Insertion: An insertion changes the number of DNA bases in a gene by adding a single base or a piece of DNA.

d) Deletion: A deletion changes the number of DNA bases by removing a single base pair or a piece of DNA.

e) Duplication: A duplication consists of a piece of DNA that is abnormally copied one or more times.

f) Frameshift mutation: This type of mutation occurs when the addition or loss of DNA bases (number of base pairs are not divisible by three) changes a gene's reading frame. A

frameshift mutation shifts the grouping of these bases and changes the code for amino acids.

g) Repeat expansion: Nucleotide repeats are short DNA sequences that are repeated a number of times in a row, hence altering the function of a protein. For example, a trinucleotide repeat is made up of 3-base-pair sequences, and a tetranucleotide repeat is made up of 4-base-pair sequences.

Genetic polymorphism is the presence of two or more allelic forms in a species ("many morphs") when the alternative forms are common, i.e. wildtype alleles. Polymorphism may occur at the phenotypic level (Mendel's study) or at the protein level.

5.1.2 Types of Genetic Polymorphisms

There are various kinds of polymorphisms: (appendices 11)

a) Single Nucleotide Polymorphisms (SNPs): alteration in a single nucleotide in the DNA sequence. It may have the following function:
   i. Majority are "silent" with no known functional change. A SNP in which both alleles produce the same amino acid sequence are called synonymous polymorphism.
   ii. Alter gene expression or regulation by acting at the promoter region and enhancing or silencing the function. It may affect the mRNA stability or gene splicing.
   iii. Alter function of gene product by changing the sequence of protein, due to missense, nonsense polymorphism.

b) Restriction Fragment Length Polymorphisms (RFLPs)

Because polymorphisms in a restriction sites translates into variability in the length of fragments after digestion of DNA with that restriction enzyme, these DNA markers are called RFLPs

c) Minisatellites or VNTRs (Variable Numbers of Tandem Repeats): short repeated segments of identical DNA at a particular locus in the genome.

d) Microsatellites or SSRs (Simple Sequence Repeats)

5.1.3 Methods of detection of Genetic Polymorphism

Up to date the most frequent used procedure to diagnose polymorphism is Gel Electrophoresis. Many new techniques are emerging that rapidly screen large number of samples at any one time, like the capillary array electrophoresis.[312] It is sensitive and has the ability for automating the rapid electrophoretic separation of a number of low-volume samples with relatively short analysis times.[313;314] Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) is the newest procedure in use to detect microsatellite polymorphisms in couple of seconds.[315]

In Gel electrophoresis the samples are loaded into a gel medium which is either agarose or polyacrylamide. Due to electric current the negatively charged DNA molecules migrate towards the positive pole at different rates depending on the size of the molecules and the concentration of the gel matrices. Once the molecules have been separated the polymorphism can be viewed either by using ethidium bromide in the agarose gel or silver nitrate staining in polyacrylamide gel. The other methods which are used to view these molecules are to use radiolabelled nucleotide during the PCR or use Laser technology where the primers are labelled with fluorescent dye. Computer programmes are used to analyse the output results. RFLF's may be detected by using southern hybridization

procedure where the molecules separated are transferred to a nylon membrane, which is hybridised to a probe with the sequence of interest.

Polymorphic markers of known location in the genome are used for:

a) Gene mapping

b) Association of genes to phenotypes

c) Genetic Identity

d) Population Genetics

5.2 Laboratory Methodology

All procedures were performed using strict laboratory protocols which adhered to the *Control of Substances Hazardous to Health (COSHH) guidelines*.

5.2.1 Extraction of DNA from Whole Blood (Laboratory Protocol)

Introduction:

Isolation of DNA from whole Blood can be difficult. Erythrocytes constitute more than 99% of blood cells; however, they lack nuclei and therefore possess no DNA. Nucleated blood cells are used to prepare genomic DNA. Only leukocytes (0.3% of total blood cells) contain nuclei and DNA. In the DNA extraction protocol described below the cells are first washed, then lysed and the nuclei (left intact) are pelleted in a low speed centrifuge run. The nuclei are re-suspended in a small volume, and lysed with SDS and proteinase K. Ethanol and chloroform extractions follow to remove most of the non-nucleic acid organic molecules. The remaining DNA is extracted into chloroform and is subsequently recovered *via* ethanol precipitation. The DNA which is precipitated is collected on a glass hook, and re-suspended.

Equipment:

1. Greiner v-bottomed 50 ml tubes.
2. Centrifuge capable of accommodating 50 ml Greiner v-bottomed tubes.
3. Micro-centrifuge.
4. Vortex mixer.
5. Incubator oven set at 65^{o}C.
6. Horizontal spiral mixer.
7. Sterile syringe needles.
8. Gilson P200, P1000 and P5000 pipettes.
9. Sterile yellow, blue and large white (5 ml) Gilson pipette tips.
10. Sterile 1.5 ml Eppendorf tubes.
11. Sterile 5 ml Bijoux bottles.
12. Ice bucket.
13. Ice.

Reagents: (appendices 12)

1. Reagent A

   (Contains 10 mM Tris HCL, 320 mM sucrose, 1% v/v Triton X-100.)

2. Reagent B

   (Contains 400mM of Tris HCL pH 8.0, 60 mM of EDTA, 150 mM of NaCl.)

3. 5M Sodium perchlorate
4. Chloroform [Sigma C-2432]. Store at -20^{o}C.
5. Ethanol 95% or 100%. Store at -20^{o}C.
6. Ethanol 70%. Store at 4^{o}C.

Procedure

1. Pipette two to four ml of EDTA blood were pipetted into 45 ml of Reagent A in a 50 ml Greiner tube.
2. Mix the solution was mixed on a horizontal spiral mixer for 10 minutes at room temperature.
3. Centrifuge at 2600 rpm for 4 minutes.
4. Carefully decant off the lysed red cells, leaving the nuclear pellet behind. If the pellet is difficult to visualize, do not decant every last drop of lysed cell. The white cells will be in the small fraction left behind.
5. The nuclear pellets are thoroughly re-suspended in 1.0 ml of Reagent B by carefully aspirating the reagent/pellet mixture up and down using a P1000 Gilson pipette (40 times).
6. The solution was then vortexed.
7. Add 250 μL of 5M sodium perchlorate
8. Vortex for 40secs
9. spiral mixed for 15 minutes at room temperature.
10. Incubate at 65^0C for 25 minutes with occasional mixing.
11. Cool the tubes ice and add 2.0 ml of chloroform at -20^{o}C, using a P5000 Gilson pipette.
12. The solution was then vortexed and then spiral-mixed for 10 minutes at room temperature.
13. Now centrifuge at 2600 rpm for 4mins.
14. Carefully remove the upper layers into a 1.5 ml Eppendorf tubes with each containing 200μl aliquots.

15. Micro-centrifuge at 13000 rpm for 10 minutes.
16. Carefully remove the upper layer (~1.0 ml in 200 μL aliquots) into Bijoux tube
17. Add 2.0 ml of 95% ethanol at -20^{o}C
18. mixed well by inversion to precipitate the DNA.
19. DNA was hooked out using a sterile syringe needle, and transfered to a sterile 1.5 ml Eppendorf tube containing 1 ml of 70% ethanol.
20. The DNA was washed by inverting the tube a few times.
21. Micro-centrifuge at 13000 rpm for 5 minutes to pellet the DNA, then the ethanol decanted off.
22. The tube was then dried and the DNA was dissolved in 0.25 - 1.0 ml water (depending on the pellet size) and stored at 4^{o}C.

5.2.2 Polymerase Chain Reaction (PCR)

Introduction

The polymerase chain reaction (PCR) is a relatively simple technique that amplifies a DNA template to produce specific DNA fragments in vitro.[316] The efficiency of amplification or the sequence of the amplified material can then be examined for any of many purposes, including genotyping or the characterization of new genes, gene expression patterns, mutations or polymorphisms. PCR consists of repeated cycles of copying of DNA or cDNA templates between two oligonucleotide primers of known sequence that promote synthesis towards each other.

A typical amplification reaction includes target DNA, two oligonucleotide primers, deoxynucleotide triphosphates (dNTPs), a thermo stable DNA polymerase, reaction buffer and magnesium. Once prepared, the reaction is placed in a thermal cycler, an instrument

that subjects the reaction to a series of different temperatures for set amounts of time. This series of temperature and time adjustments is referred to as one cycle of amplification. Each PCR cycle theoretically doubles the amount of targeted sequence (amplicon) exponentially in the reaction.

Each cycle is composed of the following steps (Figure 21):

a) Heat Lid: if the reaction is heated to temperatures >60°C before polymerization begins, synthesis of undesired PCR products and primer-dimer is avoided or reduced.
b) Pause
c) Denaturation: The initial step denatures the target DNA (single stranded DNA) by heating it to 95°C or higher for 3 minutes. This produces the necessary single-stranded DNA template for replication by the thermo stable DNA polymerase.
d) Annealing: at 50–70°C the oligonucleotide primers can form stable associations (anneal) with the denatured target DNA and serve as primers for the DNA polymerase.
e) Extension: the synthesis of new DNA begins as the reaction temperature is raised to 70–74°C. Taq acts in and dNTP'S line up.
f) The next cycle begins with a return to 94°C for denaturation.
g) After 20–40 cycles, the amplified product may be analyzed for size, quantity, sequence, etc., or used in further experimental procedures.
h) Final Extension: 72°C for 5 mins following annealing
i) Hold: after cooling down at 26°C room temperature.

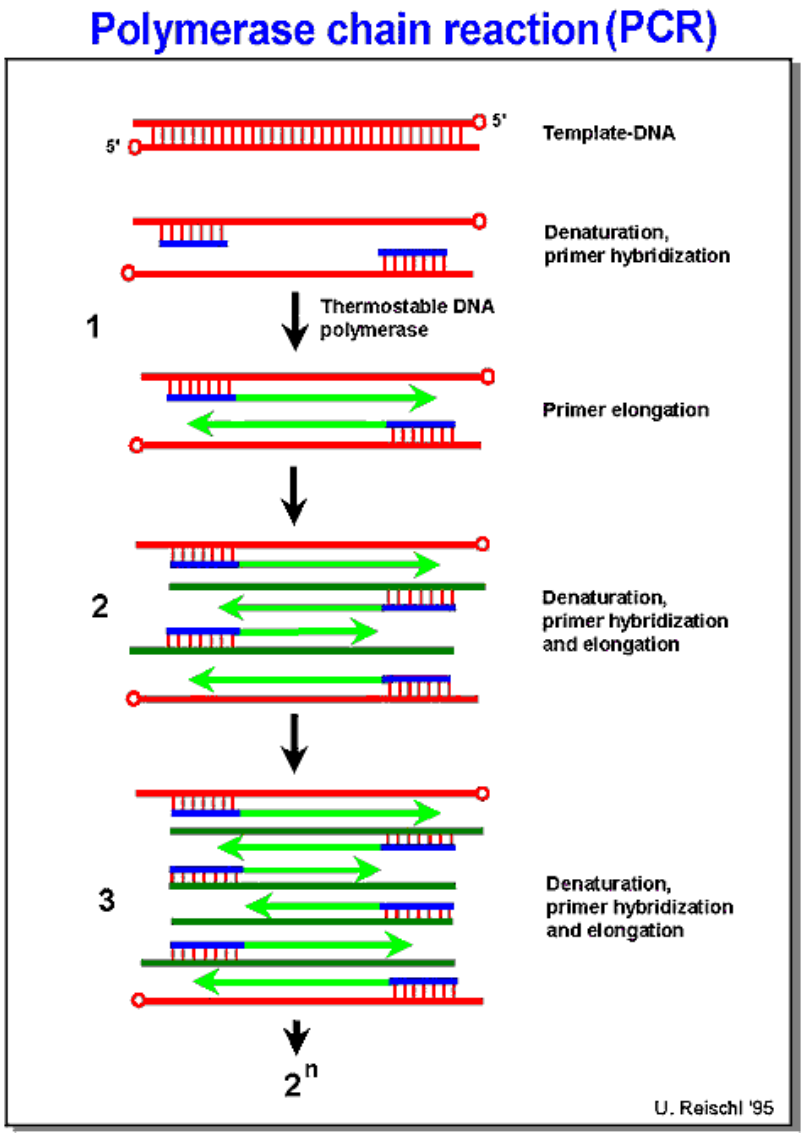


Figure 21: Schematic Representation Of PCR (Institute of Medical Microbiology and Hygiene, University of Regensburg, Franz-Josef-Strauß-Allee 11, 93053 Regensburg, Germany)[317]

j) Amplification efficiency is decreased because of competitive reactions, substrate exhaustion, polymerase inactivation and target reannealing. As the number of cycles increases, the amplification efficiency decreases, eventually resulting in a plateau effect. (Figure 22)

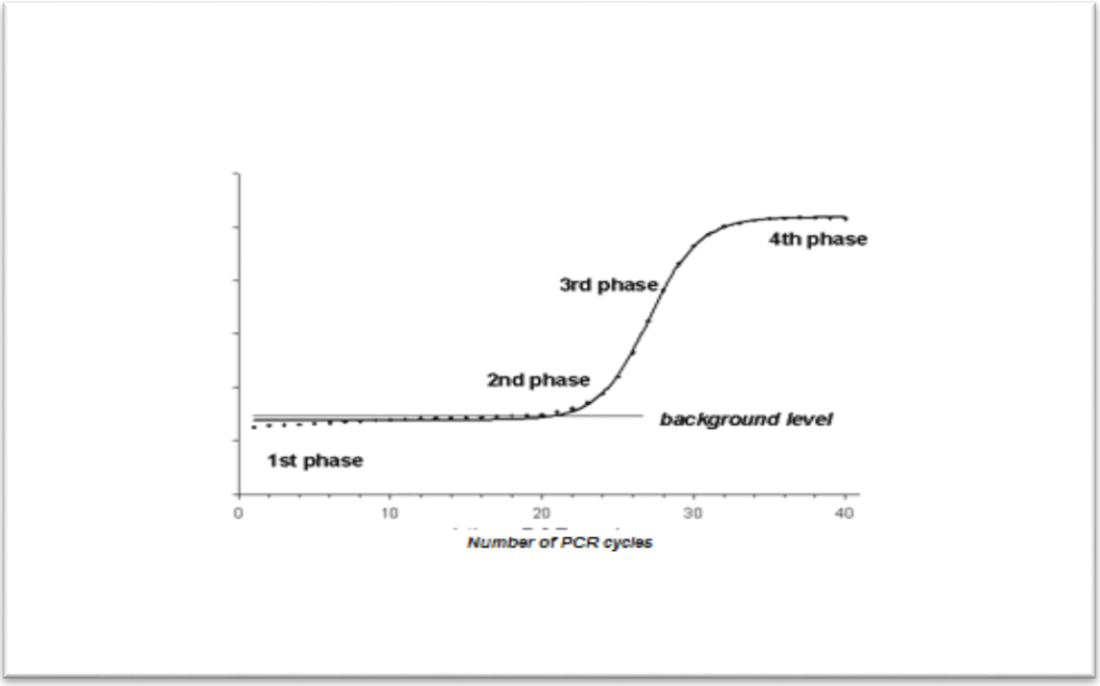


Figure 22: Amplification Efficiency Curve. The Amplification Efficiency decreases eventually resulting in a plateau effect as the number of cycles increases.

Equipment

1. Automated thermal cycler
2. Vortex mixer
3. Centrifuge
4. 0.5 mL Eppendorf PCR tubes
5. Gilson pipettes: 20 μL, 200 μL and 500 μL
6. Sterile yellow and blue Gilson pipette tips
7.

Reagents (for standard PCR procedure) (appendices 13)

a) GoTaq DNA Polymerase: Taq DNA polymerase is isolated from *Thermus aquaticus* and catalyses the primer-dependent incorporation of nucleotides into duplex DNA in the 5'→3' direction in the presence of Mg^{2+}. used to amplify PCR products of 5kb or less.

The fidelity of Taq DNA polymerase is slightly higher at lower pH, lower magnesium concentration and relatively low dNTP concentration.[318;319]

Most reaction buffers consist of a buffering agent, most often a Tris-based buffer, and salt, commonly KCl. The buffer regulates the pH of the reaction, which affects DNA polymerase activity and fidelity.[319] Some of the GoTaq comes colourless or coloured which does not require the loading dye. They may come supplied at pH 8.5 and contain MgCl2 at a concentration of 7.5mM for a final concentration of 1.5mM. Many DNA polymerases are supplied with a magnesium-free reaction buffer and a tube of 25mM MgCl2 so that you can adjust the Mg2+ concentration to the optimal level for each reaction.

b) Primers: go to www.ensemble.org follow homosapiens and search for 5HT. A complete genomic sequence with highlighted exon and small areas of introns given. Define the target region to be amplified and generally PCR primers range in length from 15–30 bases. Select GC content of40-60% and avoid 3 G or C residues near the 3' end. the melting temperature (Tm) of the two primers should be within 5°C so that the primers anneal efficiently at the same temperature.

c) $MgCl_2$: Magnesium is a required cofactor for thermo stable DNA polymerases, and magnesium concentration is a crucial factor that can affect amplification success. Empirically determine the optimal magnesium concentration for each target because in the absence of adequate free magnesium, TaqDNA polymerase is inactive, whereas excess free magnesium reduces enzyme activity[318] and may increase the level of nonspecific amplification.[320;321]

d) dNTP's: Contains 100mM solutions of dATP, dCTP, dGTP and dTTP.

Procedure:

Prepare master mix of 24µl for each sample using the following (table 10):

a) Add 1µl DNA sample into 24µl master mix in a pre-labelled eppendorf PCR tubes

b) Vortex

c) Known positive controls (from other studies) were used for reference comparison on gel electrophoresis.

d) Negative control samples contained sterile water with no DNA to confirm the integrity of reagent components.

e) PCRs were performed in duplicates using both Phoenix thermal cyclers under strictly controlled conditions.

Component	1 Test Sample (µl)	5 Test Sample (µl)	10 Test Sample (µl)	15 Test Sample (µl)	20 Test Sample (µl)
Water	13.9	69.5	139	208.5	278
Forward Primer	2.5	12.5	25	37.5	50
Reverse Primer	2.5	12.5	25	37.5	50
10X Buffer	2.5	12.5	25	37.5	50
dNTP's (5Mm)	1	5	10	15	20
$MgCl_2$	1.5	7.5	15	22.5	30
Taq DNA	0.1	0.5	1	1.5	2

Table 10: Master Mix Depending on the Number of Samples

Procedure for Phoenix Thermal Cycler (Figure 24)**:**

The two most commonly altered cycling parameters are annealing temperature and extension time. Primer sequence is a major factor that determines the optimal annealing temperature, which is often within 5°C of the melting temperature of the primers. Using an annealing temperature lower than the primer Tm (Oligonucleotide synthesis facilities will often provide an estimate of a primer's Tm) can result in higher yields, as the primers anneal more efficiently at the lower temperature. One way to calculate the annealing temperature is to use the formula

T_M = {2 x (total number of A & T)} + {4 x (total number of C & G)}

For example (Figure 23):

R Primer: 5'-CAAGTGACATCAGGAAATAG-3'

F Primer: 5'-AGCAGAAACTATAACCTGTT-3'

T_M (R Primer) = {2 x 12} + {4 x 8} = 56

T_M (F Primer) = {2 x 13} + {4 x 7} = 54

Use the lower T_M . Hence annealing temperature for this PCR would be 54°C

Figure 23: Example of Calculating the Annealing Temperature

The length of the extension cycle, which may need to be optimized, depends on PCR product size and the DNA polymerase being used and is usually between 1-2mins. The risk of undesirable PCR products appearing in the reaction increases as the cycle number increases hence PCR typically involves 25–35 cycles of amplification.

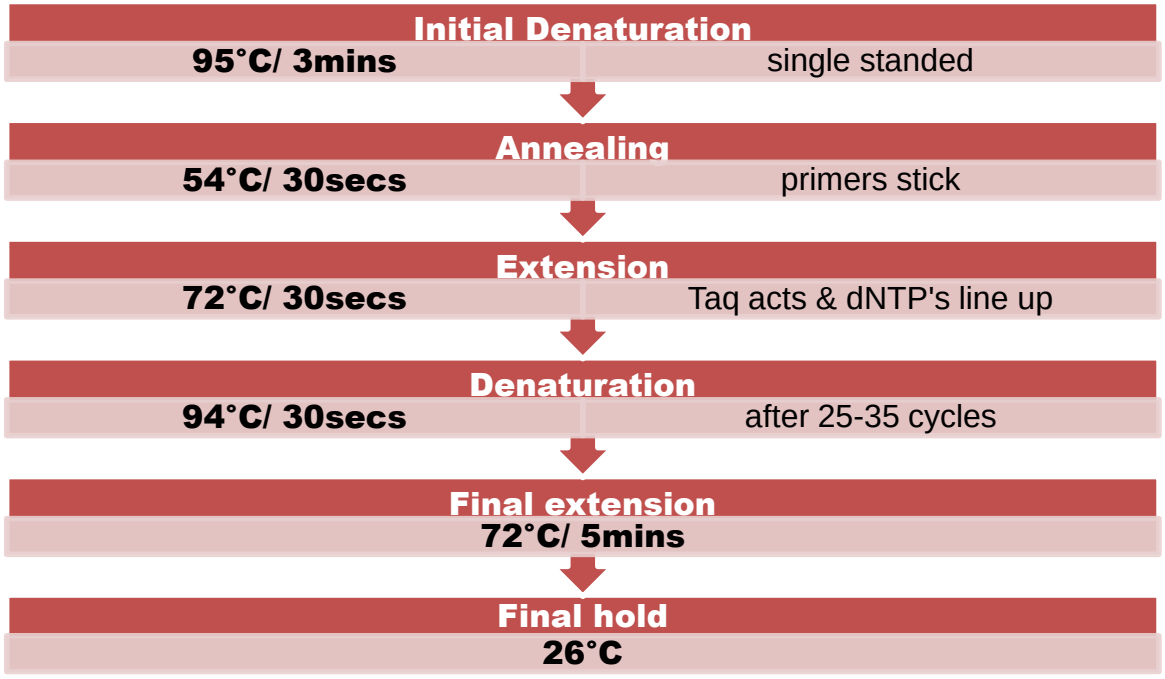


Figure 24: Procedure for Phoenix Thermal Cycler

Analyse 5µl of the PCR products by agarose gel electrophoresis. The products should be readily visible by UV transillumination of the ethidium bromide-stained gel. Store reaction products at –20°C until needed.

Troubleshooting:

- Thaw the magnesium solution completely prior to use and vortex the magnesium solution for several seconds before pipetting.
- Cross-contamination between samples can occur and should be minimised to prevent carryover of RNA and DNA from one experiment to the next. Use separate work areas and pipettes for pre- and post-amplification steps. To reduce cross-contamination during pipetting use positive displacement pipettes or aerosol-resistant tips. Wear gloves, and change them often between different experiments and steps.

- Making up a master-mix of PCR reagents reduces the number of pipetting steps and the likelihood of contamination.

5.2.3 Electrophoresis of PCR products

Electrophoresis is a technique to purify or to separate macromolecules especially proteins and nucleic acids. The negatively charged nucleic acids (due to their phosphate back bone) when placed in an electric field migrate towards the positive pole (anode).

Proteins and nucleic acids are electrophoresed within a "gel", which is cast in the shape of a thin slab, with wells for loading the sample. The gel is immersed within an electrophoresis buffer that provides ions to carry a current and some type of buffer to maintain the pH at a relatively constant value. The gel itself is composed of either agarose or polyacrylamide, each of which is suitable to do a particular task.

Detection nucleic acids in agarose or acrylamide gels can be performed in several ways:

1. The method of choice is staining with the intercalating dye, ethidiurn bromide, either after electrophoresis or during the run. Visualization of the DNA-dye complex by UV illumination[322;323]
2. Staining of the gel with acridine orange and visualization of the nucleic acid-dye complex by UV illumination.
3. UV shadowing
4. Silver staining. This is the most sensitive method and provides a permanent record of the actual separation. A destructive method molecules can not be recovered from gel after staining.
5. The gel can be blotted onto a membrane filter and the blot either exposed to X- Ray film or hybridized.

Agarose Gel Electrophoresis of PCR products

Agarose is a polysaccharide extracted from seaweed. It is widely used for the fractionation of DNA by varying the concentration between 0.5 to 2%.

Agarose gels have a large range of separation, but relatively low resolving power. By varying the concentration of agarose, fragments of DNA from about 200 to 50,000 bp can be separated using standard electrophoretic techniques. (Figure 25)

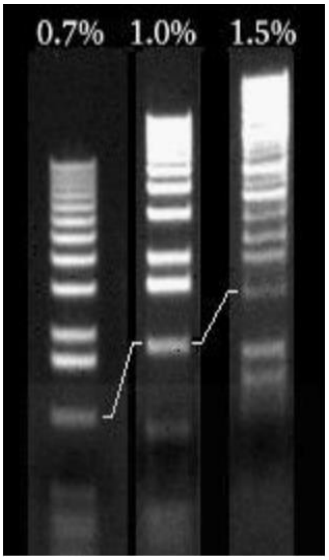


Figure 25: Migration of a set of DNA fragments in three concentrations of agarose, The larger fragments are much better resolved in the 0.7% gel, while the small fragments separated best in 1.5% agarose. The 1000 bp fragment is indicated in each lane.

Agarose gels have the added advantage, they are extremely easy to prepare and perform: you simply mix agarose powder with buffer solution, melt it by heating, and pour the gel. It is also non-toxic.

Reagents (Appendices 14)

1. Tris-Borate-EDTA buffer [TBE 10X]
2. Molecular Biology Certified Agarose 500 g [Bio-Rad 137632D]

3. Ethidium Bromide* 10 mg/mL

   Prepare a stock solution in deionised water (1 g/100mL). Aliquot into 2 ml volumes for use. Store the stock solution at 4^{o}C protected from light. Store the working aliquot at room temperature protected from light

4. Gel Loading Buffer:

   0.25% w/v bromophenol blue

   0.25% w/v xylene cyanol

   40% w/v sucrose in deionised water

Equipment:

1. Anachem Scotlab Horizontal electrophoresis units
2. Power supply unit
3. Microwave oven
4. 250ml conical flask
5. 100ml measuring cylinder
6. Gilson P20 pipette
7. Sterile yellow Gilson pipette tips

Procedure:

Before starting it is necessary to have some idea of the sizes of DNA fragments to be resolved. Generally, a 1.0% or a 2.0% gel is used for the separation of fragments between 100 and 1000 base pairs.

1. For 100ml 2.0% agarose weigh out 2.0gm agarose into a clean flask and add 100ml 1X TBE buffer

2. Mix and dissolve by heating in a microwave oven at maximum power (~600 watts) for approximately 1min, (watch to avoid boiling over). The agarose solution may need reheating to ensure all agarose has dissolved.
3. Allow to cool to approximately 50^0C and add 5µl ethidium bromide (10mg/ml) solution.
4. Use autoclave tape to seal the ends of a clean dry casting tray. Position a comb at one end and pour the agarose solution into the mould. Leave at room temperature.
5. When the gel has set remove the tape and comb. Place the gel and casting tray into the electrophoresis tank. Add sufficient 1X TBE buffer to cover the surface of the gel. (Figyre 26)
6. To each 5µl DNA sample add 2µl sucrose loading buffer. Choose an appropriate size DNA size marker and add sufficient sucrose loading buffer. Load each sample to the well in the gel. (Figure 27)

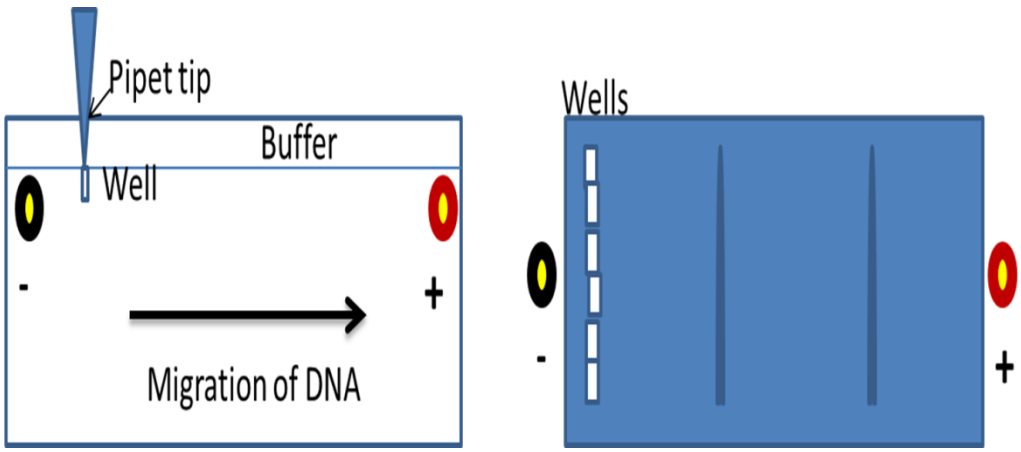


Figure 26: Agarose Gel Electrophoresis; loading sample into the well

7. Run the gel at a suitable voltage, until separation of the products is achieved. As a rough guide, 150v for about 10minutes will let you see whether the PCR has worked and then it is down to the individual as to how much longer the gel should be run.

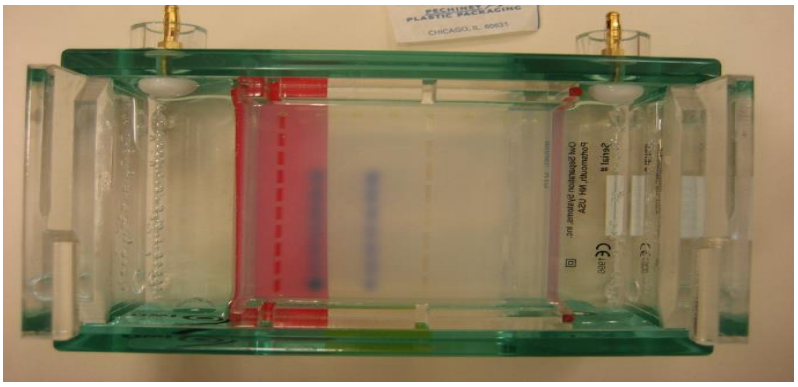


Figure 27: Electrophoresis Tank with Gel and Casting Tray in 1XTBE Buffer

8. After electrophoresis the gel is removed and examined on the ultraviolet trans illuminator. (Figure 28) The ethidium bromide intercalates with DNA allowing PCR products to be visualised when exposed to ultraviolet light. Take care to position a UV protective shield between yourself and the trans illuminator as UV light damages eyesight.

Figure 28: System for visualizing, analyzing and documenting ethidium bromide stained agarose and acrylamide gels

9. Take a photo of your notebook and interpret the digest and PCR reactions.

Troubleshooting:

- Agarose gel may be remelted and reused for subsequent electrophoresis of PCR products. Gels should be used a maximum of five times and then disposed of by incineration.
- To avoid wasting agarose the gel casting tray should be measured and the approximate volume of agarose gel required calculated.

Polyacrylamide Gel Electrophoresis of PCR products

Polyacrylamide gel electrophoresis (PAGE) provides a versatile, gentle, high resolution method for fractionation and physical-chemical characterization of molecules on the basis of size, conformation, and net charge.[324]

Polyacrylamide is a cross-linked polymer of acrylamide monomers. The length of the polymer chains is dictated by the concentration of acrylamide used, which is typically between 3.5 and 20%. When this polymer is formed, it turns into a gel and electricity is used to pull the proteins through the gel so the entire process is called polyacrylamide gel electrophoresis (PAGE).

Polyacrylamide gels have a rather small range of separation, but very high resolving power. In the case of DNA, polyacrylamide is used for separating fragments of less than about 500 bp. However, under appropriate conditions, fragments of DNA differing is length by a single base pair are easily resolved. In contrast to agarose, polyacrylamide gels are used extensively for separating and characterizing mixtures of proteins.

Reagents: (Appendices 15)

1. Stock Acrylamide/Bis 29:1 solution. (Store at 4°C).
2. 10% Ammonium persulfate APS (10gms + 100 ml H_2O)

3. Fixative: 10% methanol + 0.5% acetic acid (mix 100ml methanol with 5ml acetic acid and make upto 1lt with deionised water and storeat room temperature).
4. Silver stain: Silver nitrate 0.1%(1gm dissolve in 1lt H_2O in a dark bottle at room temperature)
5. Developer: 1.5% Sodium Hydroxide (dissolve 15gms of NaOH with 1lt deionised water and store room temperature)
6. formaldehyde
7. 1X TBE
8. TEMED

Equipment:

1. Large plate
2. Small plate
3. 2 plate clamps
4. Plate stand
5. Gel comb
6. 20ml syringe
7. Biorad Protean II Rig
8. Electrophoresis tank (Figure 29)
9. Gilson P20 pipette
10. Sterile yellow Gilson pipette tips
11. 100ml cylinder
12. clean 250ml beaker
13. Glass or plastic dishes, at least 20 cm square and capable of holding 100 mL of solution.

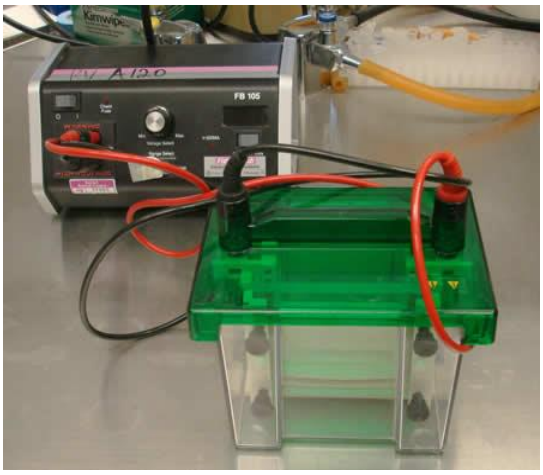


Figure 29: PAGE the Polyacrylamide Electrophoresis Tank

Procedure:

a) preparing gel plates

i. rinse the two plates and spacers and dry them thoroughly.

ii. Place large plate flat on surface, position spacers at the side, then position the small plate on top. Square up and put on plate clamps. tighten slightly and place vertically in plate stand. Slip card in between plates and make sure it moves freely up and down. Tighten or loosen clamps accordingly

iii. Check the bottom of the plates to ensure alignment is square, make sure the bottom of both plates and spacers are flush with one another to prevent leaking. Now move to clamping section of plate stand and secure

iv. Make sure the squaretooth comb fits between the plates. Finally, make sure the gel will not leak by filling the area between the plates with deionized water. If the water leaks, check to see that the bottom of the plates and the spacers align and that the area between the plates is sealed at the bottom when placed in the

loading part of the apparatus. When the gel does not leak, drain the water and dry the area between the plates with a paper towel.

b) preparing the polyacrylamide gel

i. To prepare 8% gel, to 30ml 1X TBE add 10ml of acrylamide. (40%, 29:1) in a 100ml cylinder then poured into a clean 250ml beaker. Then add 40 µl of TEMED (starts acting as catalyst). Then add 400µl of 10% APS (initiation of catalyst process).

ii. Using a sterile large syringe carefully pour the gel between the glass plates. Pour against the large back plate one side at an angle of 45° with a constant flow to prevent bubble formation. If any bubbles are noticeable, gently tap the glass plate to remove the bubble. Pour until the gel is about a centimetre away from the top and then stop. Put any remaining gel solution into a carefully labelled container and use it as a polymerization control.

iii. Insert the squaretooth comb with 15, 20 or 25 teeth into the gel so that the wells are submerged in the gel solution, but leaving a bit of air in the top to prevent bubble formation and to allow for the addition of extra gel as the result of leakage prior to polymerization.

iv. Allow the gel to set for at least an hour, using the polymerization control to verify that the gel polymerized between the plates.

v. Flush out the wells using syringe containing 1X TBE although water is as good alternative if 1X TBE is not available.

vi. Once the gel has set place the completed gel in the electrophoresis rig. Place the rig holding the gels in the electrophoresis tank and fill the top of the rig with 1XTBE buffer.

c) sample preparation

2 µl of loading dye were added to 5 µL PCR product (briefly spin to mix) and the samples loaded into the wells under the buffer, using a Gilson P10 pipette.

d) sample loading and electrophoresis

i. Assemble the entire electrophoresis apparatus. Fill both the top and bottom chambers of the apparatus with ~300 mL of 1X TBE buffer, ensuring the platinum wires of the apparatus are submerged at both ends.

ii. The samples are loaded into the wells under the buffer.

iii. The central cooling core was then lowered into the lower buffer chamber.

iv. The hose tubing attached to the core cooling ports and water supply switched on.

v. Connect the leads to a power supply. Turn on the power supply, ensuring that DC power is on. Press start on the power supply. Adjust the settings to approximately ~25 - 30milliamps and ~800 volts for 1- 11/2 hrs.

e) **Visualisation of PCR Products using Silver Nitrate Staining** (Table 11)

i. Once the gel has run to experimental specifications, cut off the power source and disconnect the leads.

ii. Empty the buffer chambers of the apparatus. Take unit out of the tank and unclip the plates. Remove the clamps and gently remove the spacers and the top plate off. Cut off the top of the gel with the spacer.

iii. To remove the gel from the plate turn upside down over the tray and loosen corner of the gel with spacer gel should pop off the plate. Do not use fingers.

iv. Place acrylamide gel into the container with 100ml of fixative. Incubate at room temperature for 6mins on the shaker.

v. Remove and wash the fixative

vi. Overlay gel with 100ml of Silver Nitrate staining solution. Incubate at room temperature for 20mins on the shaker.

vii. Remove stain and wash.

viii. To 100mls of developer add 250µls formaldehyde, and place on the gel. Incubate at the room temperature on the mixture until the bands are visualised.

ix. Wash and then dry the backside of the plate with a paper towel and doc the gel on it. Use UV-Transilluminator to print an image of the electrophoresis patterns.

Step	*Solution*	*Duration*
A	Fixative	6 min
B	deionized water (repeat twice)	2 x 2 min
C	staining solution with $AgNO_3$	20 min
D	deionized water	10 sec
E	developer solution (4-10˚C)	45-60 min (until bands are visible)
F	deionized water	2 min

Table 11: Silver Nitrate Staining of the PCR Product

Troubleshooting:

- if the gel begins to leak from the bottom of the plates, use the syringe to take up whatever leaks and return it to the rest of the gel at the top. Keep repeating the process until the gel has set and hence stops leaking.
- When adding the comb often it is best to place it on one side then the other as this will stop the air bubbles being trapped into the gel.
- Wet rubber seal on electrophoresis rig to give a better seal between the gel plates and rig.
- When handling acrylamide wear gloves as it is a neurotoxin.

5.3 Polymorphism Studied

Five Key Polymorphisms were studied in $5HT_{1A}$ (C-1019G), $5HT_{1B}$ (A-161T), $5HT_{2A}$ (102T/C), $5HT_{2C}$ (Cys23-Ser23) and $5HT_{7}$ (Pro-257-Leu) receptors. These receptors and genes were selected according to the following criteria: involvement of the receptors in the function of serotonin; representative of the common receptor protein structure and equally spread around the evolutionary tree; they have been studied and shown to be linked to various other psychiatric diseases in a variety of different population groups with significant results.

5.4 Polymerase Chain Reaction

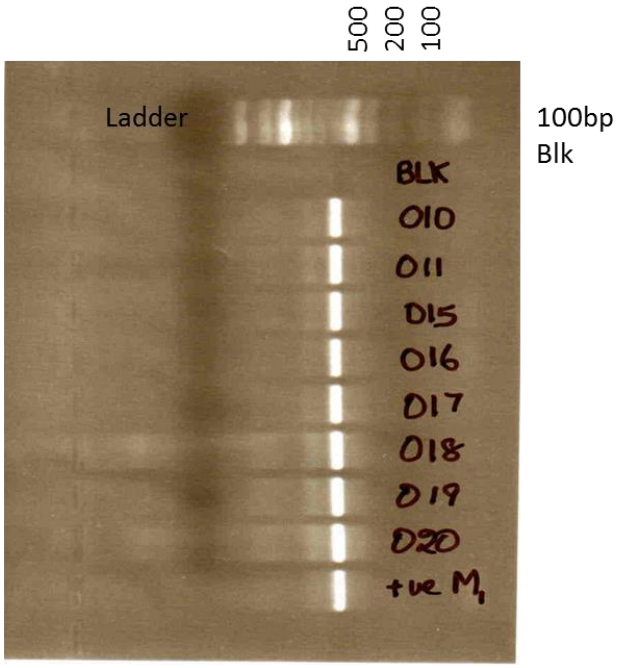
Genomic DNA was isolated from peripheral blood leucocytes using the technique mentioned in 5.2.1, once again as the samples from our old studies in the laboratory were contaminated. Once DNA was extracted GAPDH was performed to all the samples.

5.4.1 Glyceraldehyde 3-phosphate dehydrogenase gene (GAPDH):

The GAPDH gene, also known as "housekeeping gene"[325;326] is often stably and constitutively expressed at high levels in most tissues and cells and hence used as a control. GAPDH has been proven time and time to be the best housekeeping genes for study.[327;328] When performing GAPDH strict conditions (drugs and chemicals) has to be maintained as many factors can influence the housekeeping gene expression.[329;330]

GAPDH reaction condition included initial denaturation at 95°C for 3mins, then primer annealing/ primer extension/ denaturation at 58/74/94°C, 30secs/cycle for 30 cycles. Final extension was set at 74°C for 5mins. PCR product was run in 1% agarose gel and the electrophoresis conditions included standard 110volts for 1hr30mins.

This test presented data describing the expression of DNA for GAPDH in all the samples extracted from whole blood of both controls and cases (Figure 30).



GAPDH PCR product electrophoresis on 1% agarose gel

Figure 30: Glyceraldehyde 3-phosphate dehydrogenase gene (GAPDH)

5.4.2 Selection of primers:

To Select primers we obtained the protein and nucleotide sequence from the site www.ensembl.org. and then Primer picked using PRIMER 3 software from the website http://www.frodo.wi.mit.edu and the general rules to design these primers applied. These primers include the target region to be amplified and the general rules to design these primers are as following:

a. Length should be between 15-30bp.

b. GC content should be between 40-60%

c. Avoid three G or C residues near the 3' primer end to avoid nonspecific primer

annealing.

d. The melting temperature (T_M) should be between 52 -58°C

e. The difference in the melting temperature (T_M) of the two primers should not be more than 5°C

Oligonucleotides (Primers) are received in dry ice from Operon Biotechnologies. To Make 100 µM Spin the primers at 100rpm for 5mins before opening and then re-suspend in a sterile buffered solution i.e TE at pH 8.0, vortex and then spiral mix. To make working primer concentration of 5µM, dilute stock primers in the ratio of 1:20 (5µl stock +95µl of water). Store at -20°C and to use again thaw at room temperature on ice for some time and then spiral mix before use.

The primers for 5-HT1A (appendices 16) penultimate nucleotide at the 3' end of the reverse primer was deliberately altered from a thymine (T) to an adenine (A, underlined below), to introduce BseGI restriction site in the G allele. The forward and reverse primer sequences were 5'-GTAAGGCTGGACTGTTAGATG-3' and 5'-GGAAGAAGACCGAGTGTGTC**A**T-3' respectively.

Since the polymorphism is not detectable as a conventional RFLP[331] a PCR forward primer for 5-HT2C (appendices 19) that introduces a base substitution close to the codon of interest was used to create an artificial restriction site with two allelic form. The 3rd last nucleotide at the 3' end of the forward primer has been changed from Adenine (A) to Cytosine (C). The forward and reverse primer sequences were 5'-GGCCTATTGGTTTGGC**C**AT-3' and 5'-GCCATGATCACAAGGATG-3' respectively.

The primers for 5-HT1B (appendices 17), 5-HT2A (appendices 18), 5HT7 (appendices 20) receptors containing the polymorphisms were designed and general rules applied accordingly. (Table 12)

Receptor Polymorphisms	Oligonucleotide Primer Sequences	Length	T_m	GC%
5-HT1A (C-1019G)	**Forward** 5'-GAGTAAGGCTGGACTGTTAGATG-3'	23	57.2	47.8
	Reverse 5'-GGAAGAAGACCGAGTGTGTCAT-3'	22	59.7	50.0
5-HT1B (A161T)	**Forward** 5'- CTCAAGCAGTACTTCACCCTCC-3'	22	60.3	54.5
	Reverse 5'- GTTCCTCCATGGCTCTCCTCG-3'	21	65.5	61.9
5-HT2A (102T/C)	**Forward** 5'-GAGCAGAAACTATAACCTGTT-3'	21	54.6	38
	Reverse 5'-CAAGTGACATCAGGAAATAG-3'	20	56	45
5-HT2C(Cys23-Ser23)	**Forward** 5'-GGCCTATTGGTTTGGCCAT-3'	19	59.7	47
	Reverse 5'-GCCATGATCACAAGGATG-3'	18	55.5	50
5-HT7 (Pro-279-Leu)	**Forward** 5'-CCTCCATCACCTTACCTCCA-3'	20	62.4	55
	Reverse 5'-GGTGGCTGCTTTCTGTTCTC-3'	20	62.4	55

Table 12: Oligonucleotide primer sequences for the chosen receptor polymorphisms.

5.4.3 Amplification

Genomic regions encompassing each of the six polymorphic sites were amplified in duplicate by Polymerase Chain Reaction. Water, PCR buffer, $MgCl_2$, TaqDNA polymerase was mixed as required by the protocol for PCR (Table 10). PCR conditions were optimised accordingly for each polymorphism (Table13).

Each 25µl reaction mixture contained approximately 100ng DNA, 1x Taq DNA polymerase buffer containing1.5mmol/L $MgCl_2$, 12.5pmol of each forward and reverse primer, 5nmol of dNTP's, and 0.5 units of Taq DNA polymerase. PCR cycling parameters are listed in table 13 and the PCR product sequence for each polymorphisms site of interest is included in Table 14.

Each set of amplification was done in duplicate and was accompanied by a negative and positive control. The PCR reaction mixture was then incubated in a thermocycler.

Receptor Polymorphism	PCR Conditions				PCR Product Size
	Step	**Temp**	**Time**	**No of Cycles**	
5-HT1A (C-1019G)	1.Initial denaturation	95^0C	3mins	35	169
	2.Denaturation	94^0C	30secs		
	Primer annealing	59.5^0C	1min 30sec		
	Primer extension	72^0C	30secs		
5-HT1B (A161T)	1.Initial denaturation	95^0C	3mins	35	360
	2.Denaturation	94^0C	30secs		
	Primer annealing	63^0C	30secs		
	Primer extension	72^0C	30secs		
5-HT2A (102T/C)	1.Initial denaturation	95^0C	3mins	36	412
	2.Denaturation	94^0C	30sec		
	Primer annealing	54^{0-C}	30sec		
	Primer extension	72^0C	30sec		
5-HT2C (Cys23-Ser23)	1.Initial denaturation	96^0C	5min	36	182
	2.Denaturation	94^0C	30sec		
	Primer annealing	50^0C	1min30sec		
	Primer extension	72^0C	1min		
5-HT7 (Pro-279-Leu)	1.Initial denaturation	96^0C	5min	35	350
	2.Denaturation	94^0C	30sec		
	Primer annealing	60^0C	1min		
	Primer extension	72^0C	1min		

Table 13: Amplification of the genotypic fragments containing the polymorphic sites was studied using optimised conditions and the standard methods

5.4.4 Detection:

5µl of each amplified product will then be analysed by gel ectrophoresis using 1-2% agarose in the presence of Ethidium Bromide by UV illumination. The amplified product gave the required sizes (table 13)

Which was compared using a molecular weight marker (Generuler 50/100bp DNA Ladder). If positive signal is obtained the samples were treated with endonucleases as required. (Figures 31-35)

Receptor	Product Sequence
5-HT1A	GAGTAAGGCTGGACTGTTAGATGATAACGGAGGTACCGTTTTGTTGT TGTTGTCGTCGTTGTTCGTTTGTTTTTGGAGACGGAGTCTCGCTCTG TCGCCCAGGCTGGAGTGCAATGGCGCGAGAACGGAGGTAGCTTTTT AAAAAGGAAGAC▼↕ACACTCGGTCTTCTTCC T
5-HT1B	CTCAAGCAGTACTTCACCCTCCTGCACTAGACGCCTCCAGGGAGCT GGAGCGGAGCAGGGCTCGGTGGGCCAGCTCTTAGCAACCCAGGTC TAAGACCCGGTGTGGAGAGGAACAACCACAGACGCGGCGGCTTAGC TAGGCGCTCTGGAAGTGCAGGGGAGGCGCCCGCCTGCCTTGGCTG CCGCACCCATG▼ACCTCTAGTTTCAGCTGTGAACCTGGGCGGAGGA ATAATTGAGGAACTCACGGAACTATCAACTGGGGACAAACCTGCGAT CGCCACGGTCCTTCCGCCCTCTCCTTCGTCCGCTCCATGCCCAAGA GCTGCGCTCCGGAGCTGGGGCGAGGAGAGCCATGGAGGAAC
5-HT2A	GAGCAGAAACTATAACCTGTTAGTCCTTCTACACCTCATCTGCTACAAGT TCTGGCTTAGACATGGATATTCTTTGTGAAGAAAATACTTCTTTGAGCTC AACTACGAACTCCCTAATGCAATTAAATGATGACACCAGGCTCTACAGTA ATGACTTTAACTCC▼GGAGAAGCTAACACTTCTGATGCATTTAACTGGA CAGTCGACTCTGAAAATCGAACCAACCTTTCCTGTGAAGGGTGCCTCTC ACCGTCGTGTCTCTCCTTACTTCATCTCCAGGAAAAAAACTGGTCTGCTT TACTGACAGCCGTAGTGATTATTCTAACTATTGCTGGAAACATACTCGTC ATCATGGCAGTGTCCCTAGAGAAAAAGCTGCAGAATGCCACCAACTATT TCCTGATGTCACTTG
5-HT2C	C GGCCTATTGGTTTGGCAATG▼TGATATTTCTGTGAGCCCAGTAGCAGC TATAGTAACTGACATTTTCAATACCTCCGATGGTGGACGCTTCAAATTCC CAGACGGGGTACAAAACTGGCCAGCACTTTCAATCGTCATCATAATAAT CATG▼ACAATAGGTGGCAACATCCTTGTGATCATG▼GC
5-HT7	CCTCCATCACCTTACCTCCACTCTTTGGATGGGCTCAGAATGTAAAT GATGATAAGGTGTGCTTGATCAGCCAGGACTTTGGCTATACGATTTA CTCTACCGCAGTGGCATTTTATATCCCCATGTCCGTCATGCTTTTCAT GTACTACCAGATTTACAAGGCTGCCAGGAAGAGTGCTGCCAAACACA AGTTTCCTGGCTTCCC▼TCGAGTGGAGCCAGACAGCGTCATCGCCC TGAATGGCATAGTGAAGCTCCAGAAGGAGGTGGAAGAGTGTGCAAA CCTTTCGAGACTCCTCAAGCATGAAAGGAAAAACATCTCCATCTTTAA GCGAGAACAGAAAGCAGCCACC

Table 14: PCR Product Sequence for each Polymorphism of interest.

5.5 Restriction Enzyme Digest

The PCR products were digested with the restriction endonucleases to selectively cleave the DNA strands at various restriction sites, also known as DNA fragmentation to obtain and note the required polymorphisms. All restriction digests were incubated in duplicates, in a total reaction volume of 20 µl, containing 3-5µl of PCR product, 1X reaction buffer and one or two units of the respective restriction endonuclease. Wherever possible, pairs of complementary restriction enzymes were used in separate digests reactions, each of which targeted a different allele. The cleaved variants have been shown in the table after each enzyme. The conditions were optimized accordingly depending on the enzyme properties. The products were analysed on 2-4% agarose gel or 8-10% polyacrylamide gels for electrophoresis and compared with a molecular weight marker (Generuler of 100bp/50bp/25bp DNA ladder) (Table 15)

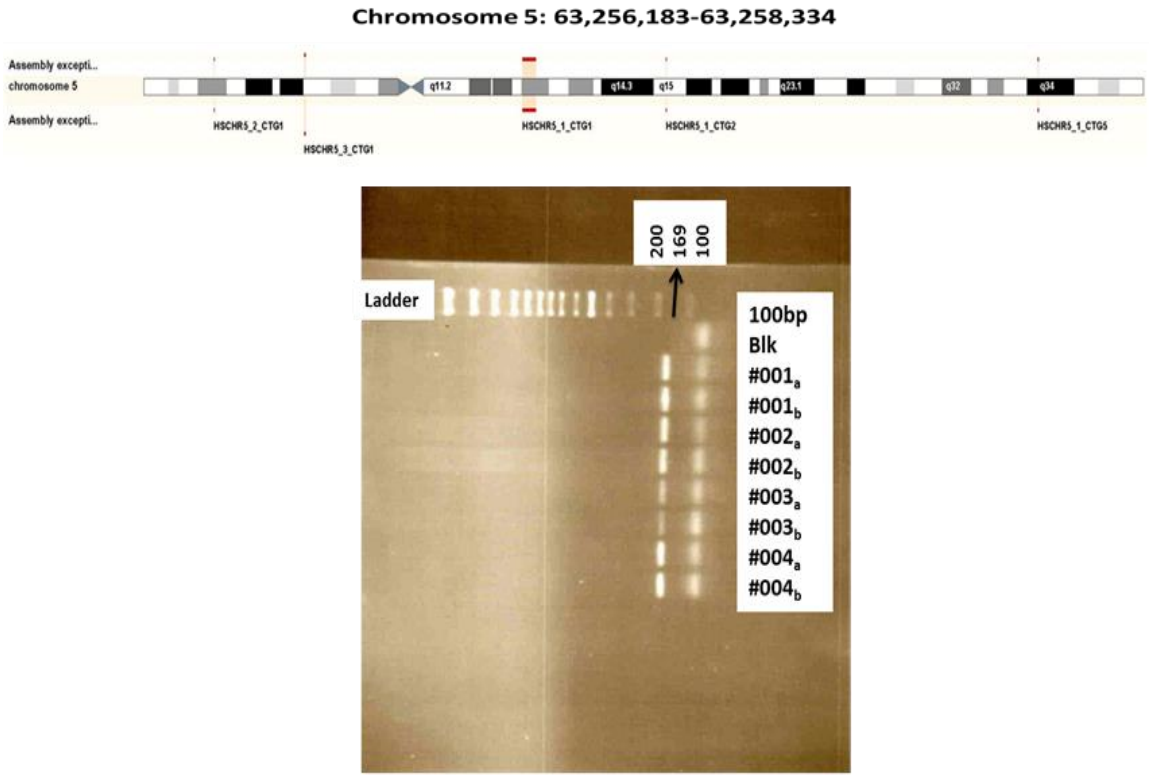
Endonucleases were ordered from FERMENTAS Life Sciences, they arrived with the buffers, enzyme information including recommended conditions for use and storage requirements. 5-HT_{1A}(C-1019G) PCR products was incubated at 55°C overnight with BseGI with the cleavage site 5'-GGATGNN↓-3' and 3'-CCTAC↑NN-5' were analysed on 4% agarose gel at 120volts for 2hrs.(Figure 31) 5-HT_{1B}(A-161T) Amplicons were digested at 37°C for 4 hours using NIaIII (cleavage sites 5'---CATG↓---3' and 3'---↑GTAC---5') and analysed on 2% agarose gel.(Figure 32) 5-HT_{2A}(102T/C) products were digested with MspI at 37°C for 4 hours and analysed on 2% agarose gel on 110volts for 2 hours.(Figure 33) For 5-HT_{2C}(CYS23-Ser23) 10 units of NIaIII enzyme with 5µl of sample was left overnight in the water bath at 37°C and then analysed by using 8% polyacrylamide gel electrophoresis at 30mA, 210 volts for 2 hours. Silver Nitrate staining was performed as explained in section

5.2.3 and (table 11). Polyarylamide gel was used for 5-HT_{2C}(CYS23-Ser23) due to small product basepair size for both wild and polymorphic samples.(Figure 34) 5-HT_7(Pro-279-Leu) PCR products of 35obp were digested with the XhoI in various repeatd different conditions to confirm the results and there was uniformly no difference in the digested product results, but we were sure that the enzyme was definitely working. After standardising the conditions all samples were digested at 37°C under water bath for 3 hours and were analysed on 3% agarose gel.(Figure 35). Genotypes were independently determined by two researchers (VD and JM), without prior knowledge of clinical group status.

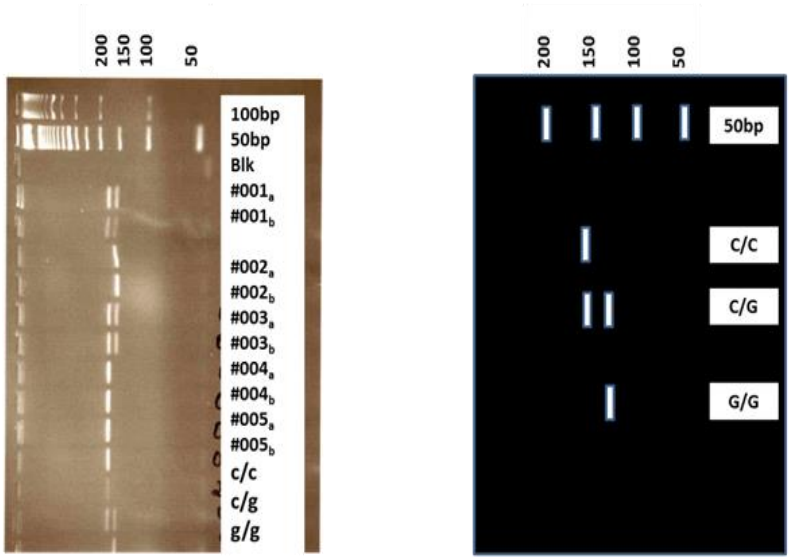
Receptor Polymorphism	Enzyme	Cleavage Site	Electrophoresis			Product
			Gel	Temp	Time	
5-HT1A (C-1019G)	$BseG_1$	5'-GGATGNN↓-3' 3'-CCTAC↑NN-5'	4% Agarose	55°C	4hrs	Wild Type: 169 Polymorphism: 152+17
5-HT1B (A161T)	NlaIII	5'---CATG↓---3' 3'---↑GTAC---5'	2% Agarose	37°C	4hrs	Wild Type: 312+41+7 Polymorphism: 192+120+41+7
5-HT2A (102T/C)	Msp I	5'---C↓CGG---3' 3'---GGC↑C---5'	2% Agarose	37°C	4hrs	Wild Type: 410 Polymorphism: 163+247
5-HT2C(Cys23-Ser23)	NlaIII	5'---CATG↓---3' 3'---↑GTAC---5'	8% PAGE	37°C	Overnight	Wild Type: (150+146)+30+(2+6) Polymorphism: (20+16)+130+30+(2+6)
5-HT7(Pro-279-Leu)	Xho I	5'--C↓TCGAG--3' 5'--GAGCT↑C--3'	3% Agarose	37°C	3hrs	Wild Type: 145+205 Polymorphism: 350

Table 15: Restriction endonucleases to fragment DNA and expected products to recognize the polymorphisms

Figure 31: Schematic presentation of the 5-HT1A receptor, PCR product and BseG$_1$ Enzyme digest to demonstrate Genotypes

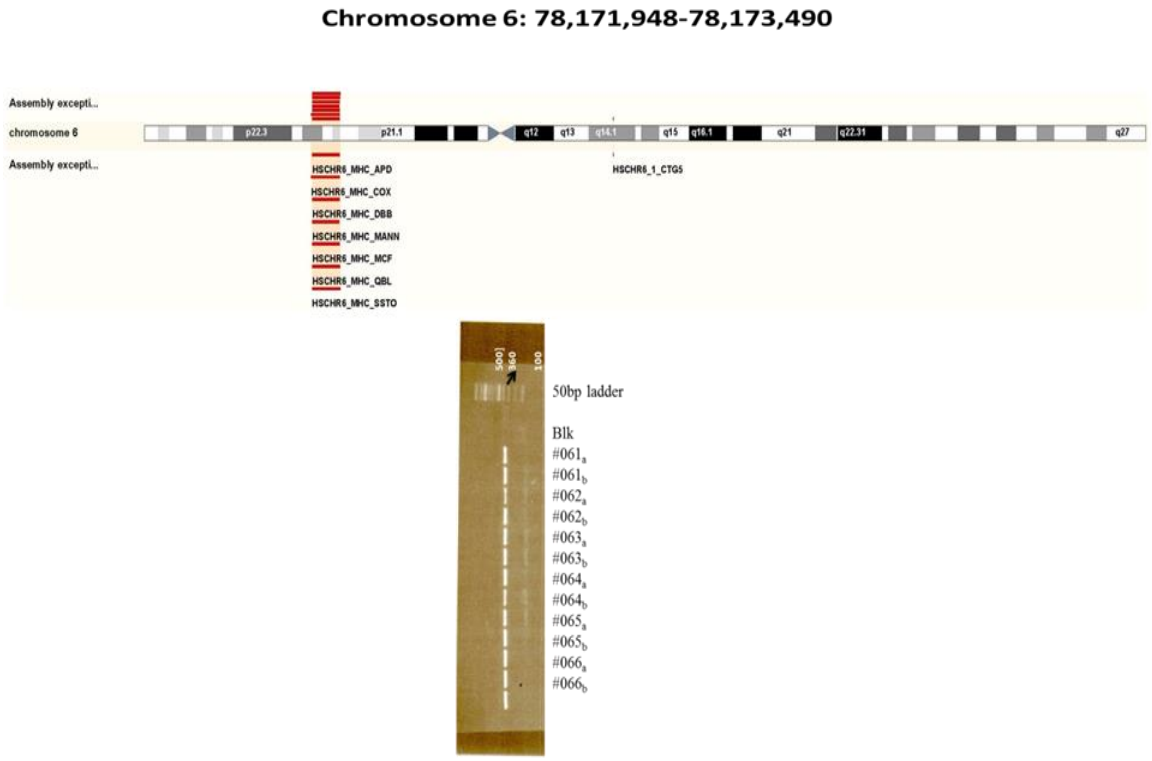


5-HT1A: PCR Product electrophoresis on 1% agarose gel

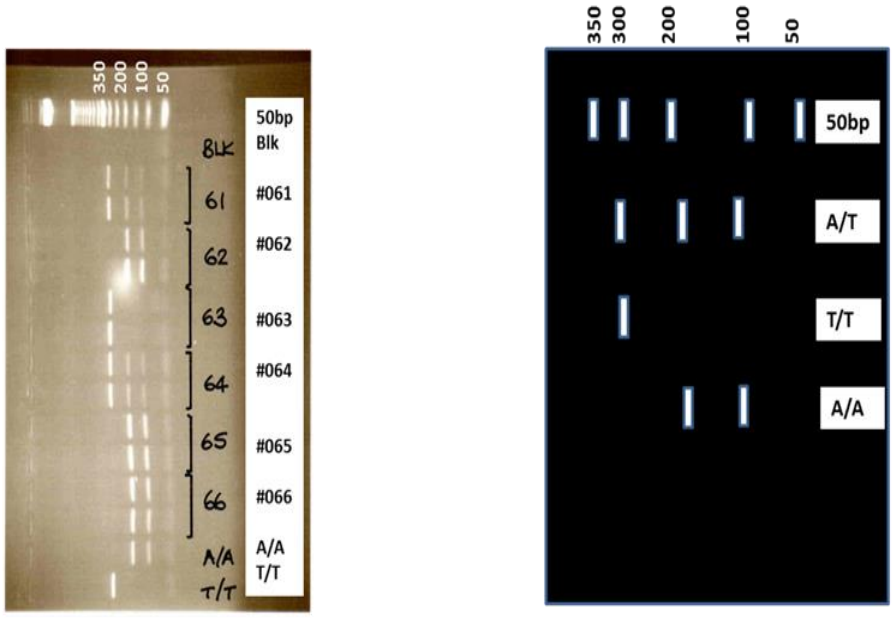


4% Agarose Gel Electrophoresis for 5-HT1A (C-1019G) After Restriction Digest with BSeG$_1$ Enzyme

Figure 32: Schematic presentation of the 5-HT1B receptor, PCR product and NlaIII Enzyme digest to demonstrate Genotypes

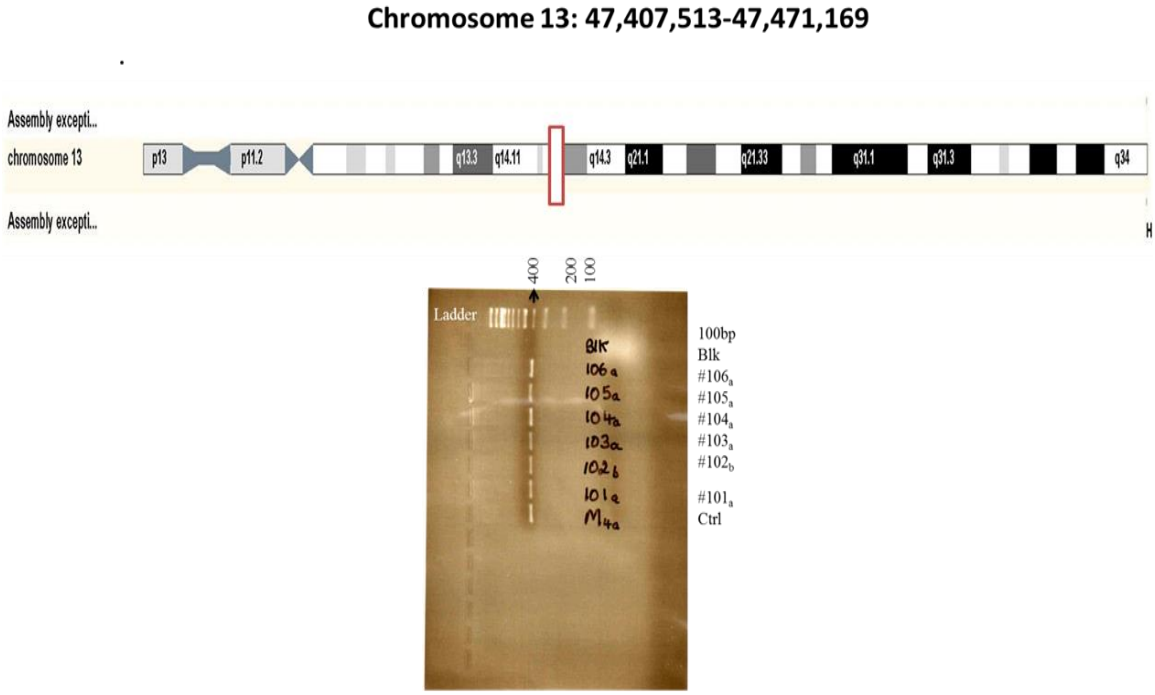


5-HT1B: PCR Product electrophoresis on 1% agarose gel

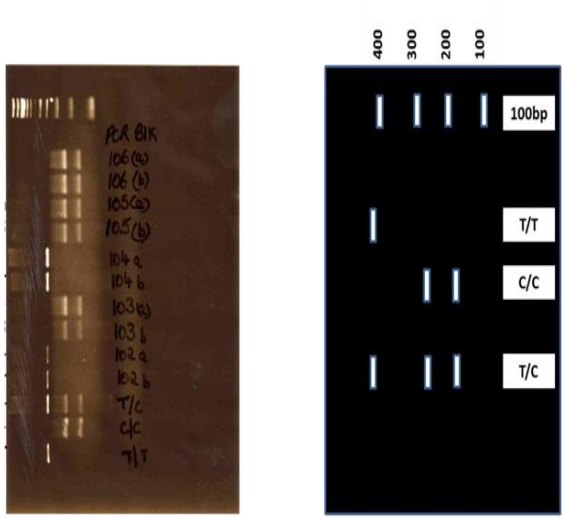


2% Agarose Gel Electrophoresis for 5-HT1B (A-161T) After Restriction Digest with NlaIII Enzyme

Figure 33: Schematic presentation of the 5-HT2A receptor, PCR product and MspI Enzyme digest to demonstrate Genotypes

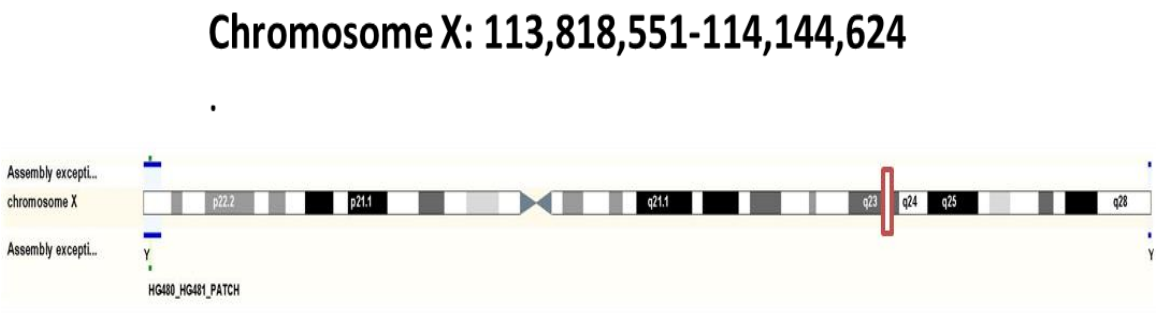


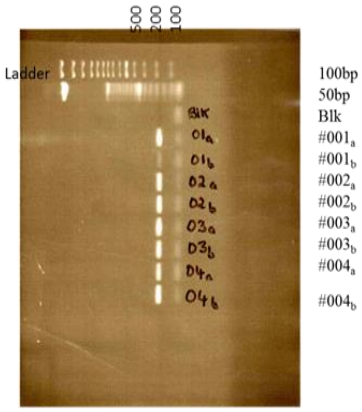
5-HT2A: PCR Product electrophoresis on 1% agarose gel



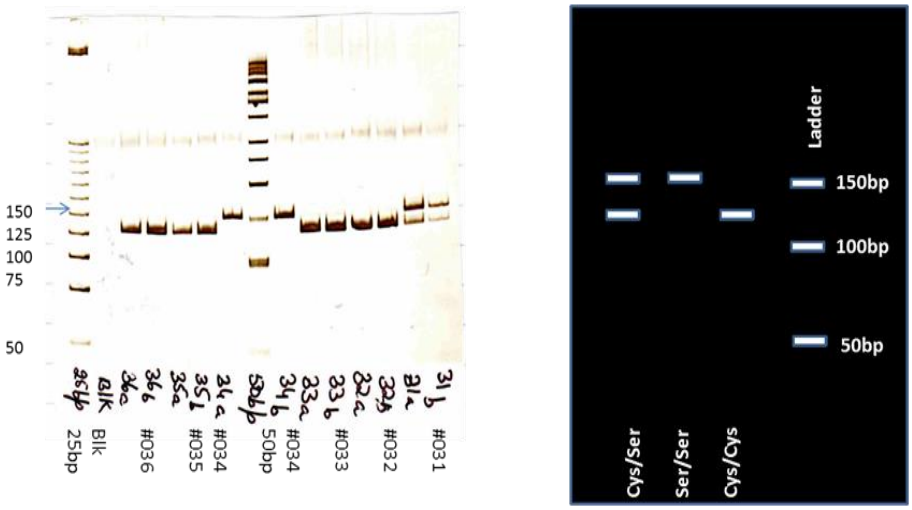
2% Agarose Gel Electrophoresis for 5-HT2A (102T/C) After Restriction Digest with MspI Enzyme

Figure 34: Schematic presentation of the 5-HT2C receptor, PCR product and NIaIII Enzyme digest to demonstrate Genotypes



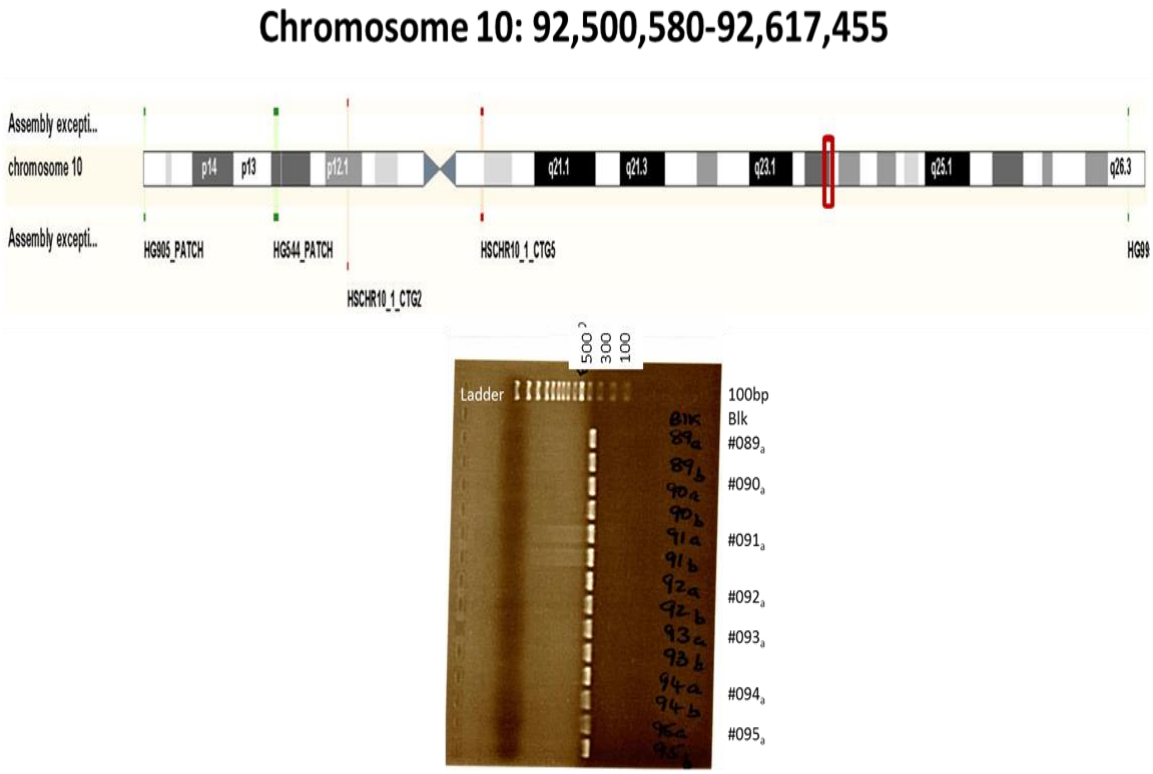


5-HT2C: PCR Product electrophoresis on 1% agarose gel

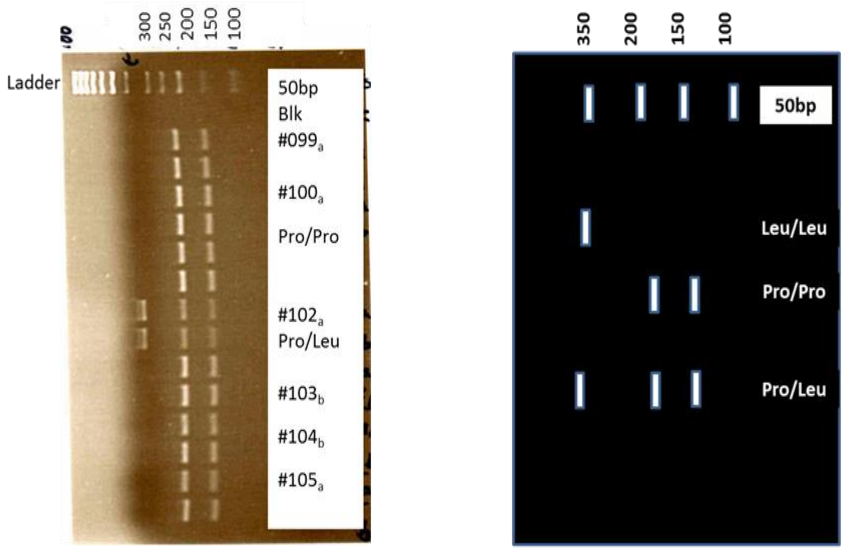


Polyacrylamide Gel Electrophoresis for 5-HT2C (Cys23-Ser23) After Restriction Digest with NIaIII Enzyme

Figure 35: Schematic presentation of the 5-HT7 receptor, PCR product and XhoI Enzyme digest to demonstrate Genotypes



5-HT7: PCR Product electrophoresis on 1% agarose gel



3% Agarose Gel Electrophoresis for 5-HT7 (Pro-279-Leu) After Restriction Digest with XhoI Enzyme

5.6 Sequencing

To check and confirm our results of PCR and endonuclease function, selected samples of each receptor (5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A}, 5-HT_{2C}, and 5-HT_{7}) forward and reverse strands of wild, polymorphic and heterozygous genotype were verified by direct DNA sequencing to Cambridge geneservice. The data files sent to us were in the ABI format. These were opened and reviewed using a freely downloadable software called " Finch TV". This software can be accessed by clicking on the following link. http://www.geospiza.com/Products/finchtv.shtml . The sequencing confirmed our results and hence the polymorphisms for each receptor. (Figures 36-40)

From the sequencing results we can confirm the working primer for 5-HT1A where the penultimate nucleotide at the 3' ends of the reverse primer was deliberately altered from a thymine (T) to an adenine (A). Similarly in 5-HT2C the 3rd last nucleotide at the 3' end of the forward primer has been changed from Adenine (A) to Cytosine (C) to create a restriction site for the action of the endonuclease.

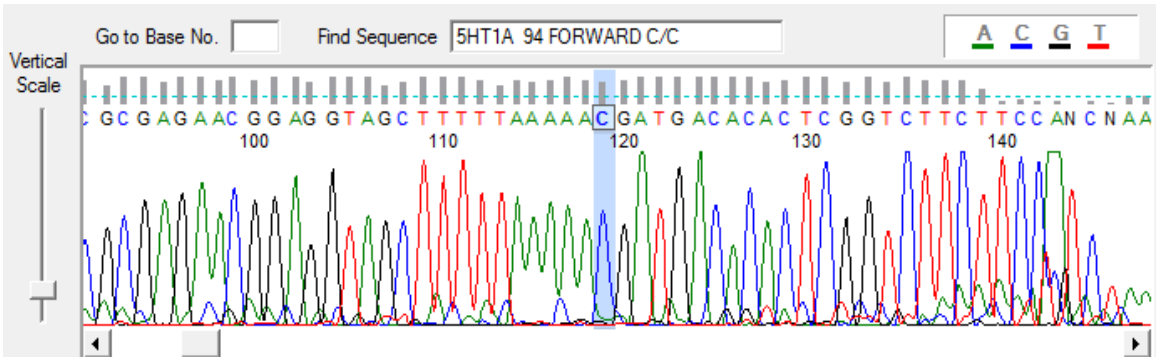
Figure 36: Sequencing snapshots for 5HT1A showing and confirming 3 different genotypes in both forward and reverse complements

5HT 1A (Ensembl sequence)

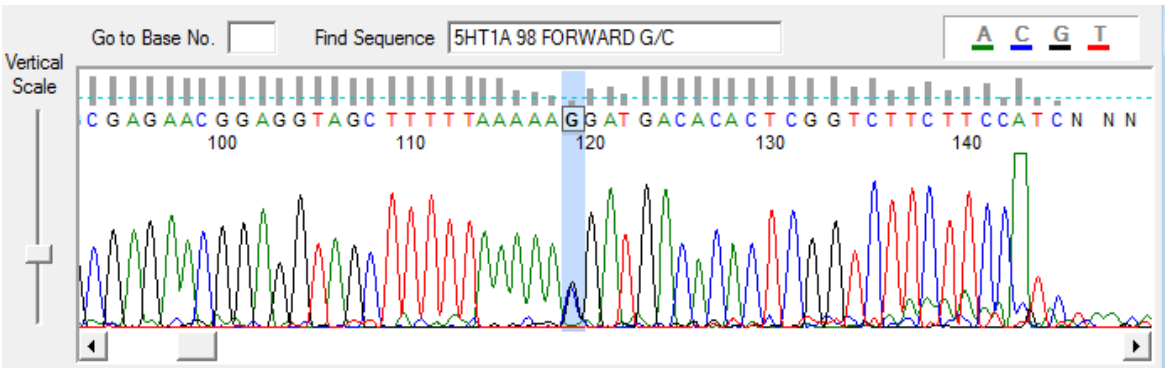
GAGTAAGGCTGGACTGTTAGATCATAACGGAGGTACCGTTTTGTTGTTGTTGTCGTCGTTGTTCGTTTGTTTTTGG
AGACGGAGTCTCGCTCTGTCGCCCAGGCTGGAGTGCAATGGCGCGAGAACGGAGGTAGCTTTTTAAAAACGATGACA

CACTCGGTCTTCTTCC

5HT 1A (94) Forward & Reverse C/C



5HT1A *(98) Forward & REVERSE : G/C*



5HT1A (99) Forward & Reverse : G/G

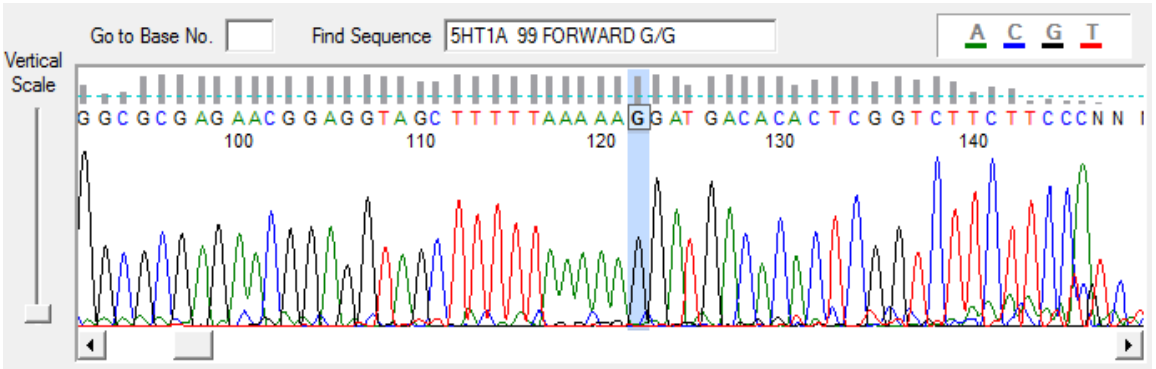
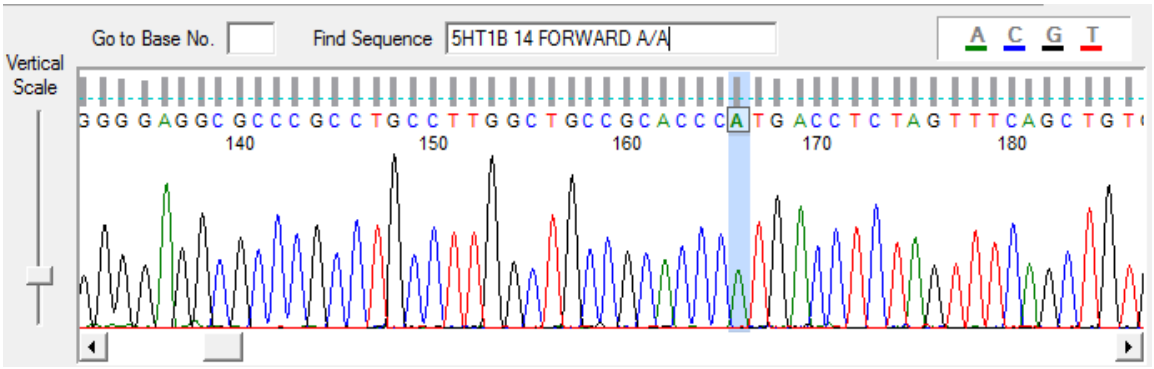


Figure 37: Sequencing snapshots for 5HT1B showing and confirming 3 different genotypes in both forward and reverse complements

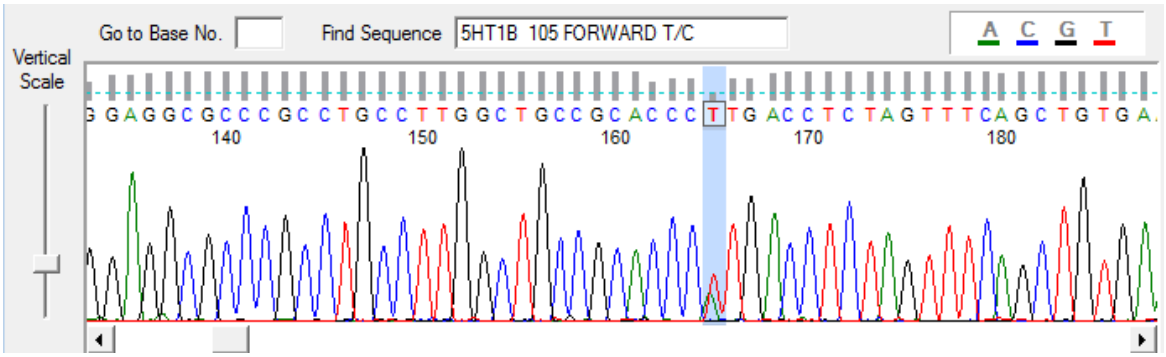
5HT 1B (Ensembl sequence)

CTCAAGCAGTACTTCACCCTCCTGCACTAGACGCCTCCAGGGAGCTGGAGCGGAGCAGGGCTCGGTGGGCCAGCTCT
TAGCAACCCAGGTCTAAGACCCGGTGTGGAGAGGAACAACCACAGACGCGGCGGCTTAGCTAGGCGCTCTGGAAGTG
CAGGGGAGGCGCCCGCCTGCCTTGGCTGCCGCACCCCATGACCTCTAGTTTCAGCTGTGAACCTGGGCGGAGGAATAA
TTGAGGAACTCACGGAACTATCAACTGGGGACAAACCTGCGATCGCCACGGTCCTTCCGCCCTCTCCTTCGTCCGCT
CCATGCCCAAGAGCTGCGCTCCGGAGCTGGGGCGAGGAGAGCCATGGAGGAAC

5HT 1B (14) Forward & Reverse A/A



5HT1B *(105) Forward & REVERSE : T/C*



5HT1B (98) Forward & Reverse : T/T

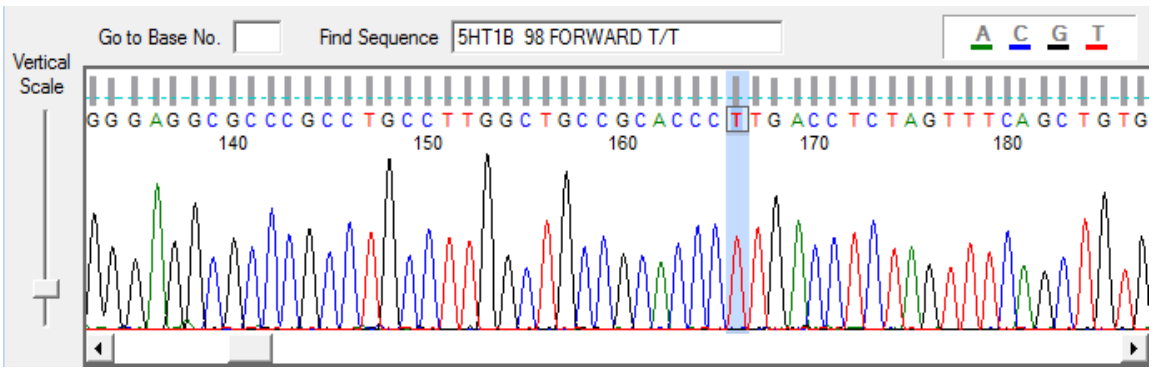


Figure 38: Sequencing snapshots for 5HT2A showing and confirming 3 different genotypes in both forward and reverse complements

5HT 2A (Ensembl sequence)

GAGCAGAAACTATAACCTGTTAGTCCTTCTACACCTCATCTGCTAC*AAGT*TCTGGCTTAGACATGGATATTCTTTGTGAAG

AAAATACTTCTTTGAGCTCAACTACGAACTCCCTAATGCAATTAAATGATGACACCAGGCTCTACAGTAATGACTTTAACT

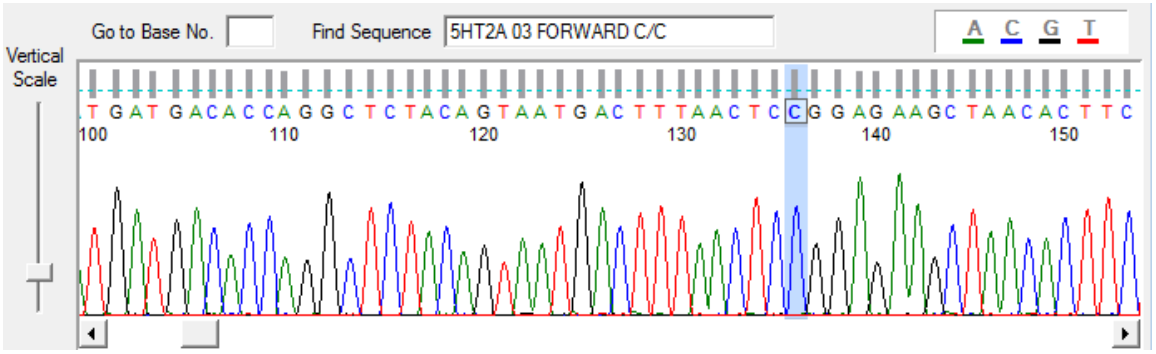
CCGGAGAAGCTAACACTTCTGATGCATTTAACTGGACAGTCGACTCTGAAAATCGAACCAACCTTTCCTGTGAAGGGTGCC

TCTCACCGTCGTGTCTCTCCTTACTTCATCTCCAGGAAAAAAACTGGTCTGCTTTACTGACAGCCGTAGTGATTATTCTAA

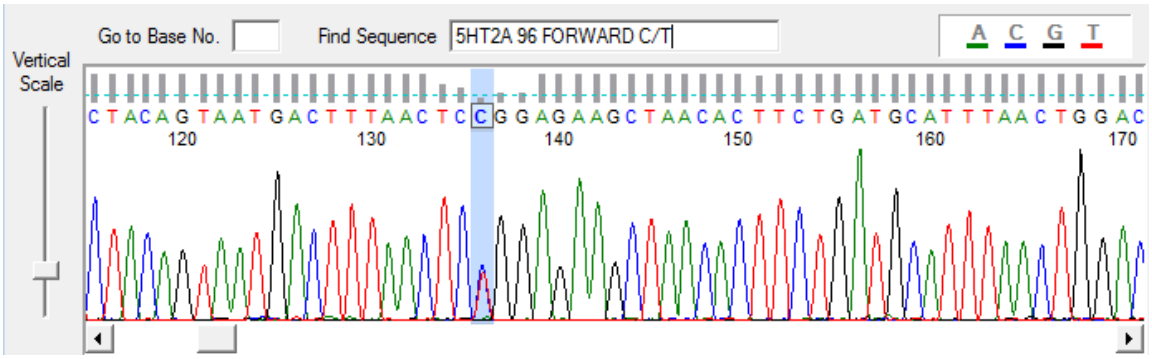
CTATTGCTGGAAACATACTCGTCATCATGGCAGTGTCCCTAGAGAAAAAGCTGCAGAATGCCACCAACCTATTTCCTGATGT

CACTTG

5HT 2A (03) Forward & Reverse C/C



5HT2A *(96) Forward & REVERSE : C/T*



5HT2A (92) Forward & Reverse : T/T

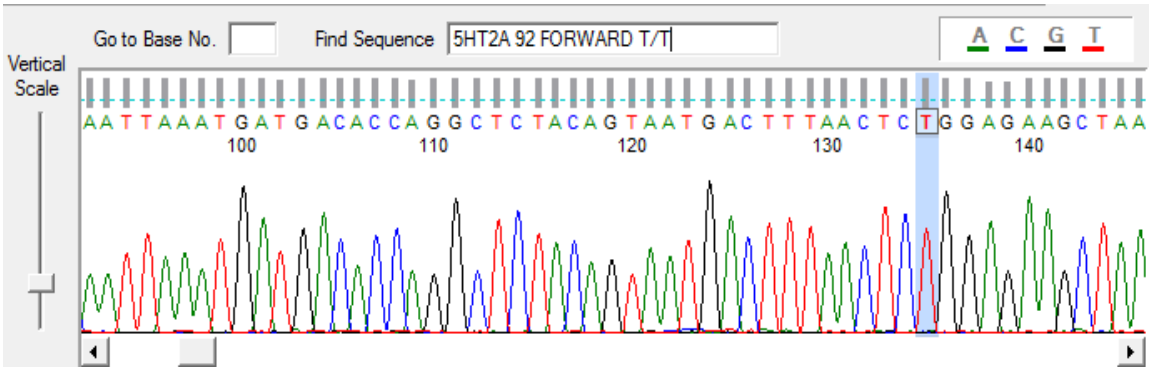


Figure 39: Sequencing snapshots for 5HT2C showing and confirming 3 different genotypes in both forward and reverse complements

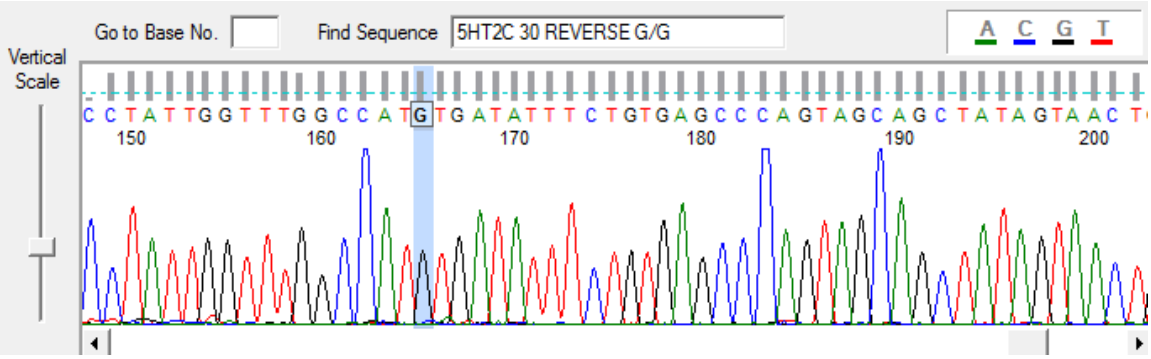
5HT 2C (Ensembl sequence)

GGCCTATTGGTTTGGCCATGTGATATTTCTGTGAGCCCAGTAGCAGCTATAGTAACTGACATTTTCAATACCT

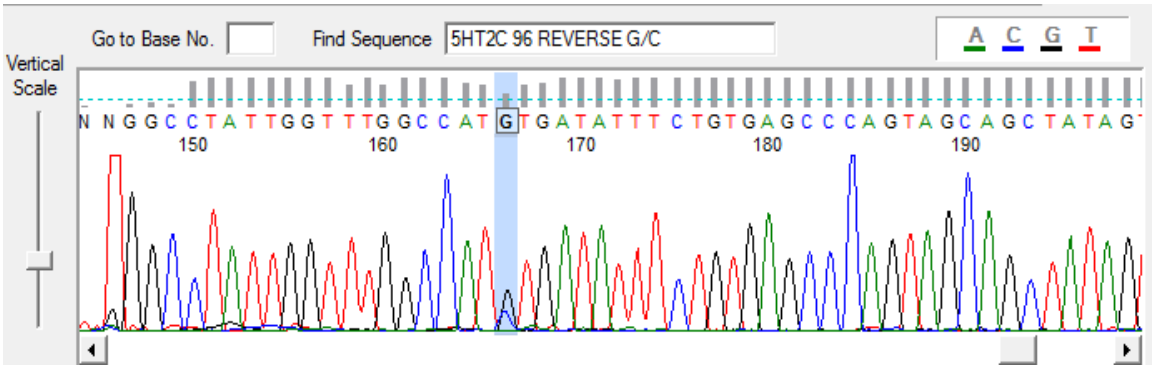
CCGATGGTGGACGCTTCAAATTCCCAGACGGGGTACAAAACTGGCCAGCACTTTCAATCGTCATCATAATAAT

CATGACAATAGGTGGCAACATCCTTGTGATCATGGC

5HT 2C (30) Reverse: G/G



5HT2C *(96) Forward & Reverse: G/C*



5HT2C (33) Forward & Reverse : G/G

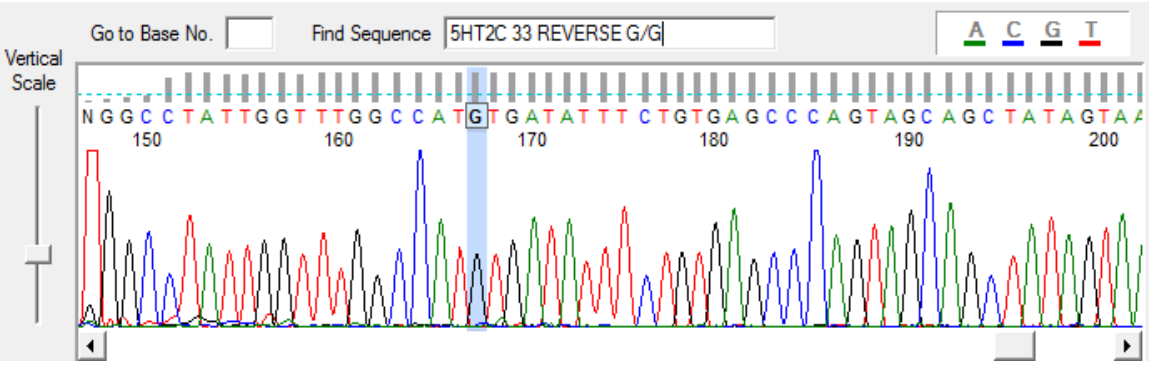


Figure 40: Sequencing snapshots for 5HT7 showing and confirming 3 different genotypes in both forward and reverse complements

5HT 7 (Ensembl sequence)

CCTCCATCACCTTACCTCCACTCTTTGGATGGGCTCAGAATGTAAATGATGATAAGGTGTGCTTGATCAGCCAGGACTTTGGC

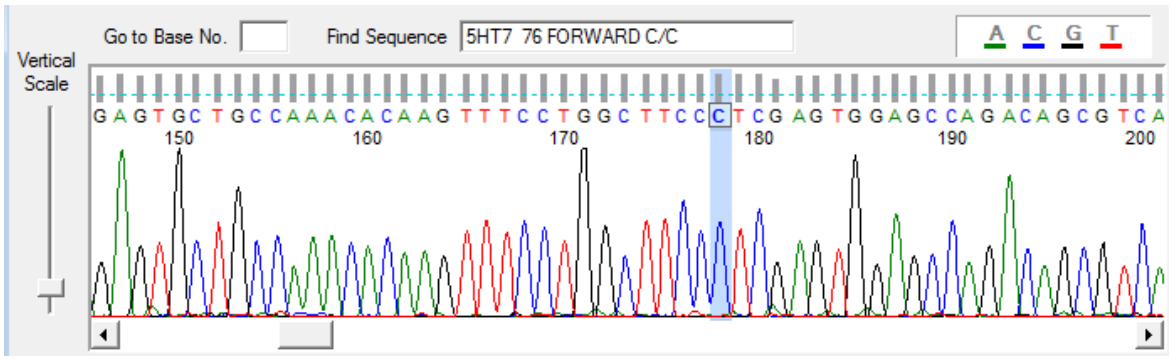
TATACGATTTACTCTACCGCAGTGGCATTTTATATCCCCATGTCCGTCATGCTTTTCATGTACTACCAGATTTACAAGGCTGCCA

GGAAGAGTGCTGCCAAACACAAGTTTCCTGGCTTCCCTCGAGTGGAGCCAGACAGCGTCATCGCCCTGAATGGCATAGTGAA

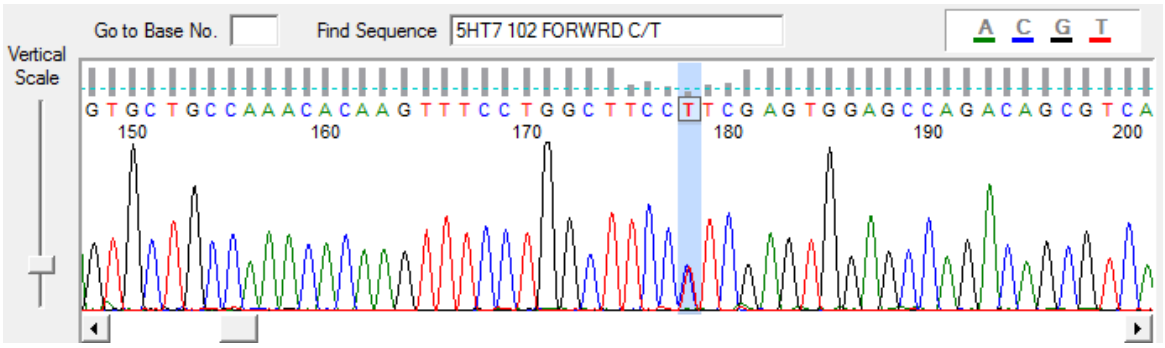
GCTCCAGAAGGAGGTGGAAGAGTGTGCAAACCTTTCGAGACTCCTCAAGCATGAAAGGAAAAACATCTCCATCTTTAAGCGA

GAACAGAAAGCAGCCACC

5HT 7 (76) Reverse: C/C



5HT7 *(102) Forward & Reverse: C/T*



5.7 Statistical Analysis

The frequency of the mutant alleles responsible for a monogenic disorder in a population is specified by the Hardy-Weinbuerg Distribution, which states that :

$p^2 + 2pq + q^2 = 1$

where p is the allele frequency of the more common allele and q the frequency of the less common allele. The equation is only true if certain conditions are met , such as mating is random and there is no migration into or from the population.

Hardy-Weinberg equilibrium for genotype frequencies was tested in both patients and controls using chi-square tests (χ^2). Simple chi-square tests of independence were also performed to confirm the presence or absence of allele or genotype associations. Odds ratios (OR) with 95% confidence intervals (CI) were estimated for the effects of high-risk genotypes and alleles.

To account for low frequencies in some groups, Fisher exact p tests were used to compare genotype and allelic distribution between the PMDD and control group. Stat Xact -4 software was used for initial analysis of 5-HT1A results. This was further confirmed and for rest of the receptors on line calculator was used Kirkman, T.W. (1996) “Statistics to Use” http://www.physics.csbsju.edu/stats/

Statistical significance was considered at exact p values of <0.05. Odds ratio were calculated to determine whether a particular genotype or allele was associated with an increased risk of PMDD.

In the end a stepwise logistic analysis was performed to see any evidence of a specific association between any genotype and clinical category.

CHAPTER 6:

GENETICS OF PREMENSTRUAL SYNDROME: INVESTIGATION OF SPECIFIC SEROTONIN RECEPTORS POLYMORPHISM: RESULT

6.1 CLINICAL CATEGORISATION

One hundred and four white European women from Stoke on Trent in the United Kingdom were identified and categorized into two groups. The group diagnosed with Premenstrual Dysphoric Disorder if there was a ≥200% increase in severity of one or more, or a ≥100% increase of two or more of the symptoms during the luteal phase compared to the follicular phase during the two menstrual cycles, this group included fifty three cases, PMDD (n=53), with mean age 37.7 years (range 27-46 years). The other arm included fifty one ladies in the control group (n=51), with mean age 36.2 years (range 22-48 years).

6.2 POLYMORPHISM STUDIED

Five Key Polymorphisms were studied in $5HT_{1A}$ (C-1019G), $5HT_{1B}$ (A-161T), $5HT_{2A}$ (102T/C), $5HT_{2C}$ (Cys23-Ser23) and $5HT_{7}$ (Pro-257-Leu) receptors. These receptors and genes were selected according to the following criteria: involvement of the receptors in the function of serotonin; representative of the common receptor protein structure and equally spread around the evolutionary tree; they have been studied and shown to be linked to various other psychiatric diseases in a variety of different population groups with significant results.

6.2.1 Receptor 5-HT 1A (C-1019G):

C-1019G (identity number: rs6295 G/C) is the most investigated gene variation (SNP) on 5-HT1A receptor which is present on chromosome 5q11.2-q13.[332] A functional variant in the promoter region of the gene consists of a C to G substitution located at nucleotide position

92,928 (GDB: AC008965), and is 1019 base pairs (bp) downstream of the transcription initiation site.[333]

YEAR	AUTHER	DISORDER	Yes/No	OUTCOME
2003	Lemonde et al[334]	Major depression & suicide	Yes	G (-1019) allele depresses 5-HT1A autoreceptor expression predisposing to suicide & depression.
2003	Strobe et al	Anxiety- and depression	Yes	Does play a role in the development and modulation of anxiety- and depression-related personality traits.
2004	Chen TJ et al[335]	Major Depression	Yes	findings suggest that this 5-HT1A polymorphism may affect AEP P2 latency in a gender-dependent manner
2004	Serretti A et al[336;337]	Antidepressant response in depression	Yes	5HT1A- C/C variants influence the antidepressant efficacy in bipolar subjects.
2004	Huang et al[338]	Depression and substance abuse	Yes	An association seen between the polymorphism and schizophrenia, substance abuse and panic disorder
2004	Rothe C et al[339]	Panic disorder and Agrophobia	Yes	Significant association of the G allele in patients with panic disorder with agrophobia.
2004	Lemonde et al[340]	Antidepressant response	Yes	C-1019G 5-HT1A gene polymorphism is a potential marker for antidepressant response.
2006	Yu YW et al[341]	Fluoxetine response in major depressive disorder	Yes	Female patients with -1019c/c showed a better response to fluoxetine.
2008	Wu et al[342]	Major depressive disorder	yes	5-HTR1A C (-1019) G polymorphism is probably associated with MDD and it is likely to be the susceptible gene locus for the female and late-onset MDD.
2008	Le François B et al[343]	Mental Illness	Yes	There is an association of the G(-1019) allele with increased raphe 5-HT1A binding potential, increased amygdala reactivity to emotional stimuli, and reduced amygdala volume, particularly in disease states, suggests a functional role for the C(-1019)G site in 5-HT1A receptor dys-regulation and predisposition to mental illness.

Table 16: Studies Reporting Association Between 5-HT$_{1A}$ (C-1019G) Polymorphism and Several Diseases

This C(-1019)G polymorphism sits within a 26 bp palindromic region, which in humans binds two repressors known as Deaf-1 and He5.[334] The polymorphism has been reported to be involved in modulating the rate of transcription of the 5-HT1A gene. The G allele fails to bind to these repressors, with a consequent upregulation of 5-HT1A autoreceptor expression and a reduction of serotonergic neurotransmission.[344] Several studies have reported an association between the (-1019)G allele and major depression, panic disorder and suicide, and a decreased response to SSRIs in both Caucasian and Chinese subjects. (Table 16) Since low serotonin has also been implicated in the pathogenesis of PMDD, we postulated that women carrying at least one G allele would be at increased risk of premenstrual dysphoria.

6.2.2 Receptor 5-HT 1B (A-161T)

HTR1B is a presynaptic and postsynaptic receptor; it is located within the region of 6q13-26. 5-HT1B is a single exon gene (does not have introns). A-161T polymorphism (rs130058 SNP) located on the Chromosome 6:78173281 (forward strand) has been described in the 5'UTR.[345] It is a regulatory region and is on the binding site for the transcription factor AP-1, hence affecting the AP-1 binding activity and gene expression.

A-161T has been shown to modify transcriptional activity in choriocarcinoma and colon adenocarcinoma cell lines.[346] A-161T has been associated in a population-based study with alcohol dependence in Chinese Han,[346] and A-161 has been associated significantly in a family-based study of schizophrenia.[345] Since HTR1B is involved in modulating the effects of serotonin (and indirectly other neurotransmitters) and A-161T has been shown to be functional,[347] it may affect the brain function in PMDD and response to drugs. (Table 17)

YEAR	AUTHER	DISORDER	Yes/No	OUTCOME
2002	Sun et al[346]	Alcohol dependence	Yes	The HTR1B A-161T polymorphism may be valuable both as a functional and as an anonymous genetic marker for HTR1B.
2003	Duan et al[347]	Function of the polymorphism	Yes	T-261G and A-161T exhibit opposing effect on gene expression
2004	Duan et al[348]	schizophrenia	No	HTR1B gene does not plays a major role in the etiopathogenesis of schizophrenia in Chinese Han population
2004	Hong et al[349]	Attempted suicide, aggressive behaviour and schizophrenia	No	This genetic polymorphism does not play a major role in the pathogenesis of schizophrenic disorders
2004	Tsai[350]	MDD	No	It does not play a major role in the susceptibility to MDD, nor related to suicidal attempt or the therapeutic response to fluoxetine in MDD.
2005	Li et al[351]	Attention Deficit Hyperactivity Disorder	Yes	Found a trend towards excess transmission of the 861G allele and the 861G/161A haplotype in ADHD-I.
2009	Lee et al[352]	Anxiety/depressio n alcoholism	yes	5HT1B gene A-161T polymorphisms might be one of the common genetic factors between the ANX/DEP ALC and antisocial ALC subgroups.
2010	Jin-Xia Caoa et al[353]	Alcohol dependence	Yes	A161Tpolymorphism was associated with alcohol dependence

Table 17: Studies Reporting Association Between 5-HT$_{1B}$ (A-161T) Polymorphism and Several Diseases

6.2.3 Receptor 5-HT 2A (102T/C)

The human 5-HT2A receptor gene consists of three exons spanning more than 20 kb[354] and is located on chromosome 13q14-21.[355] The silent C/T(102) polymorphism on exon 1 does not alter the 34TH amino acid (serine) sequence of the 5-HT2A receptor but the T and C allele quantitatively differ in their expression and function.[356]

YEAR	AUTHER	DISORDER	Yes/No	OUTCOME
1996	Ozaki et al[357]	Seasonal affective disorder	Yes	There was significant linkage disequilibrium bet 102-T/C and 516-T/C both in SAD patients and controls
1996	Erdmann et al[358]	Schizophrenia	Yes	There is an association of the non-coding polymorphism 102T/C with the development of schizophrenia.
1997	Zhang HY et al[359]	Mood disorder	Yes	The C allele frequency was higher in depressed patient
1998	Holmes et al[360]	Psychosis in Alzheimer's disease	Yes	It is associated with psychotic symptoms in AD but are clinically silent until the onset of the neurodegenerative process.
2001	Du. L et al[361]	Depression & Suicide	Yes	Showed this polymorphism was significantly associated with suicide ideation in depressed patient
2001	Arias B et al[362]	Seasonal pattern to major depression	Yes	Variation may play a role in the development of major depression with seasonal pattern
2001	Arias B et al[363]	Suicidal behaviour in Depressed	Yes	I increased risk of suicidality conferred by 5-HT(2A)-C allele.
2003	Robertson et al[364]	Bipolar affective puerperal psychosis	No	It does not appear to play a role in the development of bipolar affective puerperal psychosis.
2003	Rocchi et al[365]	Psychosis in Alzheimer disease	Yes	A significant association between C/C genotype and psychotic symptoms in AD.
2003	Holmes C et al[366]	Depression in Alzheimer's	Yes	Homozygous carriers of 5-HT2A C102allele were5 times more likely to have major depressive illness.
2003	Inada et al[367]	Panic disorder	Yes	HTR2A plays an important role in the pathogenesis of panic disorder
2004	Lam LC et al[368]	Alzheimer's disease	Yes	Certain symptoms in AD are associated with receptor polymorphism.
2005	Maron E et al[369]	Major Depression & Panic disorder	Yes	Pure PD was associated with the 5HT2A polymorphism
2005	Zalsman G[370]	Family based association study of suicide in adolescents	No	It is unlikely to be associated with suicidal behaviour in adolescent suicide behaviour.

Table 18: Studies Reporting Association Between 5-HT$_{2A}$ (102T/C) Polymorphism and Several Diseases

Over representation of the 102C allele has been shown to cause lower expression of the 5-HT2A receptor than the T allele,[356] and has been linked to many psychiatric disorders including schizophrenia.[371;372] (Table 18) The silent polymorphism contributing to the genetic disposition of disorder suggests that a functional mutation in a regulatory region of the gene is in linkage disequilibrium with the 102T/C variant.[253;358]

6.2.4 Receptor 5-HT 2C (Cys23-Ser23)

5-HT2C receptor is on the long arm of the X chromosomw. 5-HT2C is widely spread in the brain and is involved in the regulation, production and secretion of hormones like ACTH,[257] Oxytocin[373] and Prolactin.[374] A structural variant of the serotonin 2C (5-HT2C) on exon 2, receptor gene (68G/C), gives rise to a cysteine to serine substitution in the N terminal extracellular domain of the receptor protein (cys23ser). The site of Cys23-Ser23 substitution is within the first hydrophobic region of the human 5-HT2C receptor.[331] This substitution may alter the protien folding by altering the disulphide bonds.[331] Ser23 appears to be an abundant candidate allele capable of directly influencing inter-individual variation in behaviour and susceptibility to mental disorder.[375] (Table 19)

6.2.5 Receptor 5-HT 7(Pro-279-Leu)

The 5-HT7 gene (HTR7), cloned in 1993[376;377], locates in human chromosome 10q21-24[378] and contains three exons and one alternative splice exon. A Cytosine to Thymidine polymorphism leading to Proline to leucine amino acid substitution on 279 position in the third intracellular loop of the receptor protein has been studied in various disorders.[379;380] (Table 20) This point substitution changes the protein and affects the G-protein coupling sufficient to modify the function of the receptor.

YEAR	AUTHER	DISORDER	Yes/No	OUTCOME
1996	Gutierrez et al[381]	Bipolar affective disorder	Yes	The Ser23 allele may increase the susceptibility to bipolar affective disorder in women.
1997	Oruc et al[382]	Bipolar disorder	Yes	Variations in these genes may be responsible for a minor increase in susceptibility for bipolar disorder in women.
1997	Ebstein et al[383]	Human personality trait of reward dependence	Yes	It has been shown to be related to the personality trait of reward dependence
1998	Holmes et al[360]	Psychosis in Alzheimer's disease	Yes	It is associated with psychotic symptoms in AD but are clinically silent until the onset of the neurodegenerative process.
2000	Evans et al[384]	Impulsivity	Yes	Cys23Ser is related to the impulsiveness and deliberate self-harm.
2000	Segman et al[385]	Tardive dyskinesia in Chronic schizophrenia	Yes	Significant excess of Ser23 allele in females with TD in schizophrenia
2001	Lerer B et al[386]	Affective disorder	Yes	This variation does have a role in major affective disorder
2001	Gutierrez et al[387]	Bipolar affective disorder	Yes	Only a slight increase of s-Ser23 haplotype was found in the subgroup of bipolar women with family history of psychiatric illness
2003	Holmes C et al[366]	Depression in Alzheimer's disease	Yes	Homozygous or hemizygous carrier of 5-HT2C Ser were 12 times more likely to have major depressive illness.
2003	Johann et al[388]	Alcohol dependence with attention Deficit disorder.	No	This polymorphism does not contribute to the supposed common genetic predisposition of ADHD and alcohol dependence

Table 19: Studies Reporting Association Between 5-HT_{2c} (Cys23-Ser23) Polymorphism and Several Diseases

YEAR	AUTHER	DISORDER	Yes/No	OUTCOME
1995	Gelernter et al[378]	Tourette syndrome	No	Excluded genetic linkage between this locus and Tourette syndrome under a reasonable set of assumptions.
1996	Erdmann J et al[379]	Bipolar affective disorder & schizophrenia	No	Does not play a major role in the development of bipolar affective disorder and schizophrenia
1998	Pesonen et al[380]	Alcoholics	Yes	The 5-HT7Leu279 allele may be a predisposing allele in a subgroup of alcoholic offenders with multiple behavioral problems.
1999	Hinney et al[389]	Obesity, underweight or anorexia nervosa	No	None of the association tests revealed nominal P-values below 0.3.
2003	Kiel et al[390]	Psychiatric diseases	Yes	This may have relevance for the action of new drugs which affect psychiatric diseases like schizophrenia.

Table 20: Studies Reporting Association Between 5-HT$_7$ (Pro-297-Leu) Polymorphism and Several Diseases

6.3 GENOTYPES

DNA was extracted successfully from whole blood in all 104 samples. The GAPDH or housekeeping genes were identified in all above samples.

All experiments were done in duplicates and each genotyping was individually analysed by two researchers and assessors with aim to achieve 100% agreement. If there was any discrepancy with the result the experiment was performed again. We were blinded to the clinical categorisation during the course of the experiments until the time for analysing the data.

We achieved 100% agreement between the two assessors. The genotype distribution of the tested polymorphisms is shown in (table 21) for the controls and (table 22) for the PMDD group.

Patient ID Code	5HT 7	5-HT 1A	5-HT 1B	5-HT 2A	5-HT 2C
1	C/C	G/C	A/T	T/C	C/C
2	C/C	G/G	A/T	C/C	C/C
6	C/C	C/C	T/T	C/C	C/C
9	C/C	G/C	A/T	T/C	C/C
12	C/C	G/C	A/T	C/C	C/C
18	C/C	G/C	A/T	C/C	C/C
23	C/C	G/G	A/T	T/C	C/S
24	C/C	G/G	A/T	T/T	C/S
26	C/C	G/C	A/T	T/C	C/C
27	C/C	C/C	A/A	T/T	C/C
30	C/C	G/C	A/A	T/C	C/C
32	C/C	G/G	A/A	T/C	C/C
35	C/C	G/C	A/T	T/T	C/C
37	C/C	G/G	A/A	C/C	C/C
38	C/C	G/C	A/A	T/C	C/S
39	C/C	G/C	A/A	T/T	C/C
41	C/C	C/C	A/A	T/T	C/C
43	C/C	G/C	A/T	C/C	C/C
46	C/C	G/G	A/T	T/T	C/C
49	C/C	C/C	A/T	T/C	C/C
50	C/C	G/G	A/A	T/C	C/S
51	C/C	G/G	A/A	T/C	C/C
54	C/C	G/G	A/T	T/C	C/S
55	C/C	G/G	A/T	T/C	C/C

Table 21: Control group – genotypes

Patient ID Code	5-HT 7	5-HT 1A	5-HT 1B	5-HT 2A	5-HT 2C
56	C/C	G/C	A/A	T/C	C/C
57	C/C	G/C	A/T	T/C	C/C
58	C/C	G/G	A/T	C/C	C/C
59	C/C	G/G	A/A	C/C	C/C
61	C/C	G/G	A/T	C/C	C/C
62	C/C	G/G	A/A	C/C	C/C
63	C/C	G/C	T/T	C/C	C/C
65	C/C	G/G	A/A	T/C	C/C
66	C/C	C/C	A/A	T/C	C/C
73	C/C	C/C	A/A	T/C	C/S
74	C/C	G/G	A/T	C/C	C/C
86	C/C	G/G	T/T	T/C	C/C
88	C/C	G/C	A/A	T/C	C/S
89	C/C	G/C	A/T	T/T	C/C
90	C/C	G/C	A/A	C/C	C/C
94	C/C	C/C	A/A	T/T	C/C
95	C/C	G/G	A/A	T/C	C/C
96	C/C	C/C	A/A	T/C	S/S
97	C/C	G/C	A/T	T/C	C/C
98	C/C	G/C	T/T	T/C	C/C
99	C/C	G/G	A/T	T/C	C/S
100	C/C	G/C	A/T	C/C	C/C
102	T/C	G/C	A/A	T/C	C/C
103	C/C	G/C	A/A	C/C	C/S
104	C/C	G/G	T/T	T/T	C/S
105	C/C	G/C	A/T	C/C	C/S
106	C/C	C/C	A/A	C/C	C/S

Table 21: Control group – genotypes (Continued)

Patient ID Code	5HT 7	5-HT 1A	5-HT 1B	5-HT 2A	5-HT 2C
3	C/C	G/C	A/A	C/C	C/S
4	C/C	C/C	A/T	C/C	C/C
5	C/C	C/C	A/T	C/C	C/C
7	C/C	C/C	A/T	C/C	C/C
8	C/C	C/C	A/A	C/C	C/C
10	C/C	G/G	A/T	C/C	C/C
11	C/C	G/C	A/T	T/C	C/C
13	C/C	C/C	A/T	C/C	C/C
14	C/C	G/C	A/A	T/T	C/C
15	C/C	G/C	A/T	T/T	C/S
16	C/C	G/G	A/A	T/C	C/C
17	C/C	G/C	A/T	T/C	C/S
19	C/C	C/C	A/T	C/C	C/S
20	C/C	C/C	A/A	T/T	C/S
21	C/C	G/G	A/T	C/C	C/C
25	C/C	G/C	T/T	T/T	C/C
28	C/C	C/C	A/A	T/C	C/C
29	C/C	G/C	A/T	T/T	C/C
31	C/C	G/G	A/T	T/C	C/S
34	C/C	C/C	A/T	T/C	S/S
36	C/C	G/C	A/T	T/T	C/C
40	C/C	G/C	T/T	T/C	C/S
42	C/C	G/C	A/A	C/C	C/C
44	C/C	G/G	A/A	C/C	C/C
45	C/C	G/C	A/T	T/C	C/C
47	C/C	G/C	A/A	T/C	C/S
48	C/C	G/C	A/A	T/C	C/C

Table 22: PMDD group – genotypes

Patient ID Code	5-HT 7	5-HT 1A	5-HT 1B	5-HT 2A	5-HT 2C
52	C/C	G/C	A/T	T/C	C/C
53	C/C	G/C	A/A	T/C	C/C
60	C/C	G/G	A/A	T/C	C/C
64	C/C	C/C	A/T	C/C	C/S
67	C/C	G/C	A/A	T/C	C/S
68	C/C	C/C	T/T	T/T	C/C
69	C/C	G/G	T/T	C/C	C/C
70	C/C	G/C	A/T	C/C	C/C
71	C/C	G/G	A/A	T/C	C/C
72	C/C	G/C	A/A	T/C	C/S
75	C/C	C/C	A/T	T/C	C/C
76	C/C	G/G	A/A	T/T	C/S
77	C/C	C/C	A/A	T/C	C/C
78	C/C	G/C	A/A	C/C	C/S
79	C/C	G/C	A/A	C/C	C/S
80	C/C	C/C	A/A	T/C	C/C
81	C/C	C/C	A/T	C/C	C/C
82	C/C	C/C	A/A	T/C	C/C
83	C/C	G/C	A/A	T/C	C/C
84	C/C	G/C	A/A	T/T	C/C
85	C/C	G/G	A/A	T/C	C/C
87	C/C	C/C	A/A	T/C	C/C
(91	C/C	G/C	A/A	T/T	C/C
(93	C/C	G/C	A/A	T/T	C/C
101	C/C	C/C	A/T	T/C	C/C
107	C/C	G/G	A/T	T/C	C/C

Table 22: PMDD group – genotypes (continued)

Our results of PCR and endonuclease function, were checked and confirmed on selected samples of each receptor (5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A}, 5-HT_{2C}, and 5-HT_7). Both forward and reverse strands of wild, polymorphic and heterozygous genotype were verified by direct DNA sequencing. The genotype distribution and allelic frequencies in the PMDD and control groups is shown in (table 22) and (table 23) respectively.

The frequency of the mutant alleles responsible for a monogenic disorder in a population is specified by the Hardy-Weinbuerg Distribution. The equation is only true if certain conditions are met. Such as mating is random and there is no migration into or from the population. Hence it follows the principle stating that in an infinitely large, randomly mating population in which selection, migration, and mutation do not occur, the frequencies of alleles and genotypes do not change from generation to generation and this should equate to 1.

All genotype distributions conformed to the Hardy-Weinberg equilibrium, except for 5-HT7 in the Control cohort (χ^2 = 1.05,1 df). This deviation was attributed to the complete absence of the T/T genotype in women diagnosed with premenstrual dysphoria or Control. In 5-HT7 the amino acid substitution proline^{279}- leucine is due to a cytosine(836) to thymidine DNA base substitution. The allele frequency of this polymorphism is low in all women genotyped and the only subject with the Leu^{279} variant is heterozygous. The detailed characteristics of the individuals with Leu^{279}, was a healthy control in the control group. Hence Leu279 allele did not associate significantly to PMDD (P=.306).

All 5-HT1A genotypes conformed to the Hardy-Weinberg equilibrium (Controls χ2 1=0.46, PMDD χ2 1=0.32). Compared to the postulated 'high risk' G/G genotype, there was a marked overrepresentation of the C/C genotype in the PMDD group (odds ratio [OR] 3.63, 95% confidence intervals [CI] 1.22-10.78; χ2 1=5.61, exact p=0.034). Dichotomising

genotypes into presence or absence of the C allele showed that presence of at least one C allele was associated with a 2.5-fold increased risk of PMDD (OR 2.46, 95% CI 1.03-5.88; χ2 1=4.23, exact p=0.053), when compared with the control group. Similarly, allelic distributions showed significant association of the C variant with PMDD (χ2 1=6.3, exact p=0.013).

A stepwise logistic regression analysis of all other receptor genotypes showed no statistically significant association between any genotype and clinical category, and there were no statistically significant allelic distribution profiles between the PMDD and control groups (Table 22) and (Table 23).

GENOTYPE	GENOTYPE CODE	CONTROLS (N = 51)	PMDD (N=53)	χ^2	DF	p
5HT-1A						
G/C	27	22(43%)	24(45%)			
G/G	28	20(39%)	11(21%)	5.66	2	0.059
C/C	29	9(18%)	18(34%)			
5HT-1B						
A/T	30	23(45%)	21(40%)			
T/T	31	5(10%)	4(8%)	0.512	2	0.774
A/A	32	23(45%)	27(52%)			
5HT-2A						
T/C	33	25(49%)	25(47%)			
C/C	34	17(33%)	17(32%)	0.162	2	0.992
T/T	35	9(18%)	11(21%)			
5-HT-7						
C/C	36	50(98%)	53(100%)			
T/C	37	1(2%)	0	1.05	1	0.306
T/T	38	0	0			
5-HT-2C						
G/G	39	38(75%)	38(72%)			
G/C	40	12(23%)	14(26%)	0.115	2	0.994
C/C	41	1(2%)	1(2%)			

Table 22: Genotypic frequencies, χ^2 and exact p values for each polymorphism

RECEPTOR	ALLELS	CONTROLS (N=51)	PMDD (N=53)	χ^2	DF	P	EXACT p
$5HT_{1A}$ C-1019G				6.3	1	0.12	0.13
	G	62(61%)	46(43%)				
	C	40(39%)	60(57%)				
$5HT_{1B}$ A-161T				0.489	1	0.485	0.544
	A	69(68%)	75(72%)				
	T	33(32%)	29(28%)				
$5HT_{2A}$ T102C				0.193	1	0.661	0.676
	T	59(58%)	57(55%)				
	C	43(42%)	47(45%)				
$5HT_{2C}$ Cys_{23}-Ser_{23}				0.789	1	0.779	0.845
	G	88(86%)	90(85%)				
	C	14(14%)	16(15%)				
$5HT_7$ Pro-279-Leu				1.041	1	0.307	-
	C	101(99%)	106(100%)				
	T	1(1%)	0(0%)				

Table 23: Allelic frequencies, χ^2 and exact p values for each polymorphism

CHAPTER 7:

GENETICS OF PREMENSTRUAL SYNDROME: INVESTIGATION OF SPECIFIC SEROTONIN RECEPTORS POLYMORPHISM: DISCUSSION

7.1 DISCUSSION

The genetic basis of susceptibility to PMDD has been widely studied through candidate gene approach. The success of this depends on the choice of a good candidate which may be hampered by incomplete knowledge of the role of individual gene products within a complex biological system. In this study we have tried to find a relationship between the various serotonin receptors and their subtypes by identifying their primary, secondary and tertiary structural similarity and done a Phylogenetic analysis to determine which genes are likely to have equivalent or diverse functions. We have also described five various nucleotide polymorphisms in candidate serotonin receptor genes involved in serotonin response. The genes analysed were $5HT_{1A}$, $5HT_{1B}$, $5HT_{2A}$, $5HT_{2C}$ and $5HT_{7}$. These genes were selected according to the following criteria: involvement of the genes in the function of serotonin; representative of the common receptor protein structure and equally spread around the evolutionary tree; they have been studied and shown to be linked to various other psychiatric diseases in a variety of different population groups with significant results. Several authors have reported on the similarities and associations between PMS / PMDD and affective disorders for example anxiety, panic disorder, major depression and the seasonal affective disorder.[391-396] Drugs active at these receptors are used in the treatment of many of the aforementioned mental disorders, depression and PMDD. These polymorphisms have been previously described and their suitability for genotyping assessed. The functional significance for some polymorphisms is not known even though some may be non-functional, others may be of unrecognised physiological importance. Even then they may serve as a useful indicator of the involvement of other unidentified liked allelic variants of the same gene in the disease pathogenesis.

This is the first reported genotypic analysis of $5HT_{1A}$ (C-1019G), $5HT_{1B}$ (A-161T), $5HT_{2A}$ (T102C), $5HT_{2C}$ (Cys23Ser) and $5HT_{7}$ (Pro279Leu)

The serotonin-1A (5-HT1A) receptor is expressed on serotonin neurons as an autoreceptor where it regulates the activity of the entire serotonin system. Over-expression of the 5-HT1A autoreceptor has been implicated in reducing serotonergic neurotransmission,[397-399] and is associated with major depression and suicide, whereas post synaptic 5-HT1A receptors are reduced in depression[400-403] and anxiety.[404-407] 5-HT1A is a receptor protein with seven hydrophobic transmembrane domains, typical of G-protein coupled receptors and inhibits adenylyl cyclase activity, inactivate calcium channels, and activate potassium channels.[408] The 5HT1A receptor is an intronless gene located on human chromosome 5q11.2-13C.

The 5-HT1A C(–1019)G polymorphism is located in a transcriptional regulatory region and the sequence is within a 26-bp palindrome, a possible site of protein-DNA interactions. We studied the C(-1019)G polymorphism in PMDD. In our study there was a significant excess of the C/C genotype and C allele in the PMDD group compared to controls. Our finding is in stark contrast to previous studies of this marker in psychiatric disorders, which showed the G/G genotype and the G allele to be significantly associated with major depression, suicide, panic disorders and agoraphobia.[409;410]

Given that mood disorders and PMDD share several key symptoms such as irritability, depression, anxiety and affect lability, and the fact that SSRIs comprise standard treatment for both pathologies, our results were unexpected. However, there are two crucial differences between the psychological symptoms of premenstrual dysphoria and those of other mood disorders. First, PMDD symptoms are cyclical, occurring exclusively during the luteal-phase of ovulatory cycles, then dissipating during menses; in contrast to

the chronicity of symptoms in major depression. Second, the therapeutic response to SSRIs is more immediate in PMDD; in most cases within one menstrual cycle after starting treatment [411]. This characteristic has led to targeted luteal-phase SSRI therapy, which has been shown to be as effective as continuous treatment throughout the menstrual cycle [412;413]. In contrast, a lag of 3-6 weeks is required before SSRIs achieve their maximum effect in mood disorders. Recent studies of the C(-1019)G polymorphism in major depression have reported that female patients with the C/C genotype showed a better response to SSRIs than those with the G variant[414;415]. This improved response was independent from clinical variables ($p=0.036$)[416]. Although the functional characteristics of the C/C genotype do not explain why it would increase susceptibility to PMDD, it may provide insight into the rapid and effective response to SSRIs. Our control group C(-1019)G data did not concur with that of any white Caucasian study reported to date. However, there does appear to be a wide variation in genotype and allelic frequencies for this marker between different white Caucasian control groups, even within the same country[409;417-419.] Thus, our C(-1019)G data may represent a local genotypic anomaly. Our original PMDD study [420] which genotyped eight polymorphic markers of serotonin metabolism used the same cohort of women as presented here. The allelic frequencies in the control group concurred with those in other European Caucasian studies for all polymorphisms except the serotonin transporter (5-HTT) VNTR-2, which showed an excess of the 12-repeat allele compared to other reports. Thirdly, the association between the C(-1019)G marker and premenstrual dysphoria may be affected by sample size. Our study population is relatively small, nevertheless the control group statistics from our previous study indicate that the numbers are sufficient to provide reliable initial data for the C(-1019)G polymorphism.

5-HT1B is a receptor found both presynaptic and postsynaptic regions. It is widely distributed in the basal ganglia, hippocampus, and cortex. Studies have shown marked behavioural changes interestingly in both overexpression and in knockout mice.[421;422] The 5-HT1B receptors localized presynaptically on serotonergic terminals inhibit the release of 5-HT hence inhibitory autoreceptors, and postsynaptically on other nerve endings, where they inhibit the release of various neurotransmitters.[423]

The human 5-HT1B gene is located on chromosome 6q14.1. The 5-HT1B does not have introns hence has a single exon, and is implicated to harbour a schizophrenia susceptibility gene (SCZD5).[424] We looked at the A-161T polymorphism which has been described in the 5'UTR of 5-HT1B gene as it is a good candidate variant for association studies due to being functional.[347] A-161T has been shown to be associated with alcohol dependence and schizophrenia.[345;346] Based on the above, we hypothesize that A-161T polymorphism in the HTR1B gene may relate to the aetiology of PMDD.

We found no significant differences in allele (χ^2=0.489; df=1; Exact P=0.544) and genotype (χ^2=0.512; df=2; p=0.774) frequencies between the patient or control groups, although at this polymorphic site the A allele frequency was seen more than the T allele both in controls (frequency 0.68 vs 0.32) and study types(frequency 0.72 vs 0.38).

The 5-HT1B receptor is involved in modulating the release of serotonin and other neurotransmitters, especially dopamine, so the altered expression of the 5-HT1B receptor may lead to a disturbance in the central nervous system, which may be a factor in the development of PMDD. Because PMDD is a disorder that affects neurobehavioral features, it is likely some other polymorphism in 5-HT1B may lead to dysregulation the receptor in turn leading to suboptimal regulation of more than one neurotransmitter; this

ultimately may manifest as symptoms of the PMDD. Hence further studies are needed to investigate the other polymorphisms.

A cDNA that encodes HTR2A has been isolated, and the gene is localized on chromosome 13 q14-q21.[425;426] The cDNA shows an MspI polymorphic site at position T102C.[427] 5HT2A receptor is of great interest as various neuroleptic agents and antidepressant bind with high affinity to this receptor.[428] 5HT2 receptors have seven transmembrane domains that share amino acid identity with each other and with other members of the G-protein coupled receptor superfamily.

Statistically significant association between the silent T102C polymorphism in the 5HT2A receptor gene and multiple psychiatric disorders has been reported suggesting genetic predisposition to these conditions may be affected by a functional 5HT2A variant that is in linkage disequilibrium with 102T/C.[358;429] Hence we wanted to look for similar association in our study group with PMDD

We found no significant differences in allele (χ^2=0.193; df=1; Exact P=0.0676) and genotype (χ^2=0.162; df=2; p=0.992) frequencies between the patient or control groups, although at this polymorphic site the TCTG allele frequency was seen more than the TCCG allele both in controls(frequency 0.58 vs 0.42) and study types(frequency 0.55 vs 0.45). Even though in other studies with white European population; the TCCG allele is more prevalent in the normal population (frequency 0.6 versus 0.4).[358;427;429;430] In spite of the fact that all patients in this study were white European, it is still possible that stratification occurred.

We have failed to replicate the association between T102C polymorphism in $5HT_{2A}$ and other psychiatric conditions.[371;429;431] Association studies in unrelated individuals are designed to detect the coincidence of a polymorphic marker and disease states, the

association exists either because the mutation studied is pathogenetic or because it is in linkage disequilibrium with the causative mutation- i.e. it has not been separated by recombination in the time since the mutation occurred.[432] The fact that the C to T mutation does not change the amino acid composition of the receptor suggests that the linkage disequilibrium is the more likely possibility in the significant studies, the marker being in close proximity to the causative mutation.[433] Failure to replicate significant results in small size samples also points to a minor gene effect.

The serotonin (5-HT)2C receptor is distributed widely throughout the brain, but is densely expressed in regions implicated in anxiety, mood, drug-induced hallucinogenesis, reward, neuroendocrine regulation, and appetite.[434] The HTR2C gene is located on chromosome Xq24. A Cys–Ser polymorphism at amino acid 23 in the first hydrophobic region of HTR2C has been identified.[435] Allele frequencies in unrelated Caucasians in other studies were 0.13 and 0.87 for $5HT_{2C}$Ser and $5HT_{2C}$Cys respectively.[435;436] This coding SNP was choosen to study because an in vitro study showed that the Ser23 is more active than the Cys23.[436] We found no significant differences in allele (χ^2=0.789; df=1; Exact P=0.845) and genotype (χ^2=0.115; df=2; p=0.994) frequencies between the patient or control groups, although at this polymorphic site the Cys23 allele frequency was seen more than the Ser23 allele both in controls(frequency 0.88 vs 0.14) and study types(frequency 0.85 vs 0.15). This finding is consistent the other studies which have shown that the frequencies of the Cys23 and Ser23 alleles in unrelated Caucasians are 0.87 and 0.13, respectively.[435;436] We also noticed that the homozygous for Ser23/Ser23 in both controls and PMDD group was just 2% compared to the wild type Cys23/Cys23 (controls 75%, PMDD 72%) and heterozygous form Cyst23/Ser23 (controls 23%, PMDD 26%). Hence there is more transmission of Cys23 allele and Cys23/Ser23 haplotype in our female population.

In our study, no association between the Cys23Ser polymorphism and PMDD was found. This is in contrast to Arzu Gunes et.al.[437], who reported that the Ser23 allele was more frequent among male schizophrenic patients with Antipsychotic-induced extrapyramidal side effects (EPS). Segman et al. has also reported a similar significant association between the Cys23Ser polymorphism and tardive dyskinesia, with patients with dyskinesia having a higher frequency of the 23Ser allele.[385]

Since the HTR2C gene is located on the X chromosome, the gender distribution may have influenced the allele frequency calculations in previous studies showing positive association between Cys23Ser polymorphism and various mood disorders.

Taking the advantage of the X chromosome and PMDD in females it may be significant to look into the relationship between other polymorphism on 5-HT2C, or may be functional studies looking into interaction between various 5-HT2C and MAOA genes.

The gene encoding the 5HT7 receptor has been localised to human chromosome 10q.[438] Based on its sequence it has a structure of seven hydrophobic transmembrane helices separated by three extracellular and three intracellular loops.[269;376;379] Studies have evaluated the contribution of the genetic variation of the serotonin 5HT7 receptor to the development of schizophrenia and bipolar affective disorders.[268;379;439]

A cytosine to thymidine (C → T) polymorphism, leading to a proline to leucine amino acid substitution at position 279 (pro279leu) in the third intracellular loop of the receptor protein was genotyped in our study.[379] It has been postulate that the most likely consequence of the Pro279Leu point substitution is a change in local protein structure, which would affect G-protein coupling. Such an alteration at the molecular level could be sufficient to modify the function of the 5-HT7 receptor. In various studies the Leu279 variant has been found in similar frequencies in all groups,[379] whereas our study has

shown that not only the Pro279 genotype (frequency of 0.98 in controls and 1.00 in study group) but the C allele frequency is much more prevalent compared to the T allele (frequency 0.99 vs 0.01 in controls and 1.0 vs 0.0 in study group) which was seen in only one control candidate in the hetrozygous form (this has been confirmed by sequencing). Hence the allele frequency of this polymorphism is very low and the subject with the leucine variant is heterozygous who was in the control group. A similar finding was seen in a study by Pesonen et. al 1998[440] where The allele frequency of this polymorphism is low (0.004 in all Finns genotyped) and all the subjects with the Leu279 variant are heterozygous that is 6 out of the 752 Finns and 0 from other Caucasians. Another study with Han Chinese population showed the complete absence of the Pro279Leu polymorphism in their study.[441] The frequencies of this polymorphism in Western individuals are also rather low (around or smaller than 1%)[379;442]

The expression of mRNA for the 5-HT7 receptor in the midline hypothalmus, thalamic and limbic structures indicates a role in affective behaviour and receptor might be a target of antipsychotic agents.[390] Further the functional studies of Pro279Leu variation are of interest because the third intracellular loop of the receptor in which it is located plays an important role in G protein coupling hence strongly influences cAMP formation and may modify signal transduction.[390] The low leu279 allele frequency in our study limits the statistical power of the association analysis of this receptor, indicating that presence of this variant is not causally related to the development PMDD. Even the ambitious conscious effort to prove that PMDD is different to other psychiatric disorder may be impossible with this variant because of almost absence of the homozygous form in our study group. As our study group was only females and relatively small, there may be a link

between the presence of this allele and gender which may require further studies in males and females in larger population.

One after another monogenic diseases are being successfully mapped within the human genome, however in diseases with a complex mode of transmission like PMDD and other Psychiatric disorders linkage studies with DNA Markers have been far less conclusive than hoped. Rational strategies for the advancement of psychopharmacology are dependent on furthering our currently sparse knowledge of the patho-physiologic basis of PMDD. To this end, human genetic approaches offer a promising alternative to traditional biochemical and neurophysiologic investigations as twin, family, and adoption studies all support the heritability of many psychiatric syndromes. Unfortunately, attempts to first map (i.e., localize a unique region of DNA shared by patients with a particular disorder) and then identify genes predisposing to PMDD have been frustrated by the complexity of the genetic mechanisms underlying behavioural phenotypes. With single gene disorders (also referred to as mendelian disorders) there is a simple, direct relationship between variation in a single gene and the phenotype that results. In contrast, the relationship between phenotype and genotype is not straightforward for complex genetic traits. In this setting, multiple different susceptibility genes and environmental factors interact in varying combinations within individuals who appear to have clinically indistinguishable phenotypes. This means that in any given sample of patients diagnosed with premenstrual dysphoric disorder, the number of individuals actually sharing a disease gene or genes in common might be very small such that the ‘‘effective’’ sample size does not provide enough power to detect the responsible genes.

We have tried to reduce genetic heterogeneity in the patient sample by studying genetically isolated populations and by narrowing the affected phenotype under study

based on criteria of severity and robust Another approach is to greatly expand the sample size and number of DNA markers used in genetic association studies to increase the power to detect multiple possible genes contributing to disease in subsets of the sample.

More often than not, results are not reproducible from study to study, in large part because of the heterogeneous nature of psychiatric diseases, the absence of a specific diagnostic laboratory test, and the modest numbers of patients in many studies.

Some commonly studied polymorphisms, such as the C103T variant in the 5-HT2A receptor, are silent (i.e., do not change the genetic code), whereas other polymorphisms, such as the 5-HT2C receptor Cys23Ser allele, produce mutant proteins with no apparent alterations in functional properties. The clinical importance of such a subtle genetic variant may require analysis of other related genes in tandem.

Methods for detecting genetic polymorphisms are advancing rapidly and now allow simultaneous genotyping of several nucleotide polymorphisms.

7.2 SUMMARY

PMS/PMDD remains a matter of concern due to controversies in its definition, diagnostic criteria, aetiology, pathophysiology and effective treatment modalities. According to the American Psychiatric Association (APA) Diagnostic and Statistical manual (DSM-IV) fourth edition the prevalence of PMDD is 3-8%. Recently International Society for Premenstrual Disorders (ISPMD) group has developed an international universally acceptable multidisciplinary agreement regarding definition, quantification and clinical trial design of premenstrual disorders (PMD). The impact of the disease on quality of life, marital and parental social adjustment is similar to the impact of major depressive disorder (MDD).

The global burden of the disease can be calculated by using the number of years of healthy lost to disability. The most elaborate diagnostic criteria for PMDD is APA DSM-IV criteria but this has some unsolved issues too. There is currently no accepted objective means of assessing PMS and clinical diagnosis relies predominantly on the subjective self-reporting of symptomatology. There is very little doubt as to the direct relationship between the trigger caused by the normal ovarian cycle and CNS sensitivity to these hormonal changes.

Genetic factors are also pertinent to the aetiology of PMDD as shown by family and twin studies. A wide range of therapeutic pharmacological and nonpharmacological interventions have been tested in the treatment of premenstrual symptoms.

Continuous research is being conducted worldwide to find the aetiology of PMDD. Many studies have identified various abnormalities in the serotonin system in women with PMS and PMDD. These include abnormal levels of whole blood serotonin, serotonin platelet uptake, abnormal responses to serotonergic probes and exacerbation of premenstrual symptoms after tryptophan depletion.

This is the first study linking the 5HT1A C(-1019) allele and PMDD. There was a marked over-representation of the C/C genotype of 5-HT1A C(-1019)G polymorphism in the PMDD group. The presence of at least one C allele was associated with a 2.5-fold increased risk of PMDD. There were no significant associations between the other tested genotypes, allelic distribution and clinical category. These findings do not support a major role for common polymorphisms in contributing to susceptibility to PMDD.

A combination of genetics, neurotransmitter's pathology, endocrinology and imaging may help and lead us to the cause, diagnosis and management of this disorder. The Serotonin receptor gene polymorphisms not studied as yet are the potential areas for continued

investigation. However, preliminary data from our study and previously investigated polymorphisms by our group do not indicate that the genetic approach provides a robust diagnostic tool for PMDD.

Until an objective means (molecular and / or imaging) of diagnosing for PMS is agreed, diagnosis is likely to relay on daily questionnaires such as the DRSP which most closely relates to the symptom factors within DSM IV PMDD.

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Appendices 1: Protein sequences for Serotonin Receptors (FASTA format)

Protein sequences for Serotonin Receptors (FASTA format)

1. 5-hydroxytryptamine receptor 1A; Short=5-HT1A

>gi|231454|sp|P08908.3|5HT1A_HUMAN
422 aa
MDVLSPGQGNNTTSPPAPFETGGNTTGISDVTVSYQVITSLLLGTLIFCAVLGNACVVAAIALERSLQNV
ANYLIGSLAVTDLMVSVLVLPMAALYQVLNKWTLGQVTCDLFIALDVLCCTSSILHLCAIALDRYWAITD
PIDYVNKRTPRRAAALISLTWLIGFLISIPPMLGWRTPEDRSDPDACTISKDHGYTIYSTFGAFYIPLLL
MLVLYGRIFRAARFRIRKTVKKVEKTGADTRHGASPAPQPKKSVNGESGSRNWRLGVESKAGGALCANGA
VRQGDDGAALEVIEVHRVGNSKEHLPLPSEAGPTPCAPASFERKNERNAEAKRKMALARERKTVKTLGII
MGTFILCWLPFFIVALVLPFCESSCHMPTLLGAIINWLGYSNSLLNPVIYAYFNKDFQNAFKKIIKCKFC
RQ

2. 5-hydroxytryptamine receptor 1B; Short=5-HT1B

>gi|112821|sp|P28222.1|5HT1B_HUMAN
390 aa
MEEPGAQCAPPPPAGSETWVPQANLSSAPSQNCSAKDYIYQDSISLPWKVLLVMLLALITLATTLSNAFV
IATVYRTRKLHTPANYLIASLAVTDLLVSILVMPISTMYTVTGRWTLGQVVCDFWLSSDITCCTASILHL
CVIALDRYWAITDAVEYSAKRTPKRAAVMIALVWVFSISISLPPFFWRQAKAEEEVSECVVNTDHILYTV
YSTVGAFYFPTLLLIALYGRIYVEARSRILKQTPNRTGKRLTRAQLITDSPGSTSSVTSINSRVPDVPSE
SGSPVYVNQVKVRVSDALLEKKKLMAARERKATKTLGIILGAFIVCWLPFFIISLVMPICKDACWFHLAI
FDFFTWLGYLNSLINPIIYTMSNEDFKQAFHKLIRFKCTS

3. 5-hydroxytryptamine receptor 1D; Short=5-HT1D

>gi|112819|sp|P28221.1|5HT1D_HUMAN
377aa
MSPLNQSAEGLPQEASNRSLNATETSEAWDPRTLQALKISLAVVLSVITLATVLSNAFVLTTILLTRKLH
TPANYLIGSLATTDLLVSILVMPISIAYTITHTWNFGQILCDIWLSSDITCCTASILHLCVIALDRYWAI
TDALEYSKRRTAGHAATMIAIVWAISICISIPPLFWRQAKAQEEMSDCLVNTSQISYTIYSTCGAFYIPS
VLLIILYGRIYRAARNRILNPPSLYGKRFTTAHLITGSAGSSLCSLNSSLHEGHSHSAGSPLFFNHVKIK
LADSALERKRISAARERKATKILGIILGAFIICWLPFFVVSLVLPICRDSCWIHPALFDFFTWLGYLNSL
INPIIYTVFNEEFRQAFQKIVPFRKAS

4. 5-hydroxytryptamine receptor 1E; Short=5-HT1E

>gi|112822|sp|P28566.1|5HT1E_HUMAN
365aa
MNITNCTTEASMAIRPKTITEKMLICMTLVVITTLTTLLNLAVIMAIGTTKKLHQPANYLICSLAVTDLL
VAVLVMPLSIIYIVMDRWKLGYFLCEVWLSVDMTCCTCSILHLCVIALDRYWAITNAIEYARKRTAKRAA
LMILTVWTISIFISMPPLFWRSHRRLSPPPSQCTIQHDHVIYTIYSTLGAFYIPLTLILILYYRIYHAAK
SLYQKRGSSRHLSNRSTDSQNSFASCKLTQTFCVSDFSTSDPTTEFEKFHASIRIPPFDNDLDHPGERQQ
ISSTRERKAARILGLILGAFILSWLPFFIKELIVGLSIYTVSSEVADFLTWLGYVNSLINPLLYTSFNED
FKLAFKKLIRCREHT

5. 5-hydroxytryptamine receptor 1F; Short=5-HT1F

>gi|398967|sp|P30939.1|5HT1F_HUMAN
366aa
MDFLNSSDQNLTSEELLNRMPSKILVSLTLSGLALMTTTINSLVIAAIIVTRKLHHPANYLICSLAVTDF
LVAVLVMPFSIVYIVRESWIMGQVVCDIWLSVDITCCTCSILHLSAIALDRYRAITDAVEYARKRTPKHA
GIMITIVWIISVFISMPPLFWRHQGTSRDDECIIKHDHIVSTIYSTFGAFYIPLALILILYYKIYRAAKT
LYHKRQASRIAKEEVNGQVLLESGEKSTKSVSTSYVLEKSLSDPSTDFDKIHSTVRSLRSEFKHEKSWRR
QKISGTRERKAATTLGLILGAFVICWLPFFVKELVVNVCDKCKISEEMSNFLAWLGYLNSLINPLIYTIF
NEDFKKAFQKLVRCRC

6. 5-hydroxytryptamine receptor 2A; Short=5-HT-2

>gi|543727|sp|P28223.2|5HT2A_HUMAN
471aa
MDILCEENTSLSSTTNSLMQLNDDTRLYSNDFNSGEANTSDAFNWTVDSENRTNLSCEGCLSPSCLSLLH
LQEKNWSALLTAVVIILTIAGNILVIMAVSLEKKLQNATNYFLMSLAIADMLLGFLVMPVSMLTILYGYR
WPLPSKLCAVWIYLDVLFSTASIMHLCAISLDRYVAIQNPIHHSRFNSRTKAFLKIIAVWTISVGISMPI

PVFGLQDDSKVFKEGSCLLADDNFVLIGSFVSFFIPLTIMVITYFLTIKSLQKEATLCVSDLGTRAKLAS
FSFLPQSSLSSEKLFQRSIHREPGSYTGRRTMQSISNEQKACKVLGIVFFLFVVMWCPFFITNIMAVICK
ESCNEDVIGALLNVFVWIGYLSSAVNPLVYTLFNKTYRSAFSRYIQCQYKENKKPLQLILVNTIPALAYK
SSQLQMGQKKNSKQDAKTTDNDCSMVALGKQHSEEASKDNSDGVNEKVSCV

7. 5-hydroxytryptamine receptor 2B; Short=5-HT2B

>gi|1168220|sp|P41595.1|5HT2B_HUMAN
481aa
MALSYRVSELQSTIPEHILQSTFVHVISSNWSGLQTESIPEEMKQIVEEQGNKLHWAALLILMVIIPTIG
GNTLVILAVSLEKKLQYATNYFLMSLAVADLLVGLFVMPIALLTIMFEAMWPLPLVLCPAWLFLDVLFST
ASIMHLCAISVDRYIAIKKPIQANQYNSRATAFIKITVVWLISIGIAIPVPIKGIETDVDNPNNITCVLT
KERFGDFMLFGSLAAFFTPLAIMIVTYFLTIHALQKKAYLVKNKPPQRLTWLTVSTVFQRDETPCSSPEK
VAMLDGSRKDKALPNSGDETLMRRTSTIGKKSVQTISNEQRASKVLGIVFFLFLLMWCPFFITNITLVLC
DSCNQTTLQMLLEIFVWIGYVSSGVNPLVYTLFNKTFRDAFGRYITCNYRATKSVKTLRKRSSKIYFRNP
MAENSKFFKKHGIRNGINPAMYQSPMRLRSSTIQSSSIILLDTLLLTENEGDKTEEQVSYV

8. 5-hydroxytryptamine receptor 2C; Short=5-HT-2C

>gi|112816|sp|P28335.1|5HT2C_HUMAN
458aa
MVNLRNAVHSFLVHLIGLLVWQCDISVSPVAAIVTDIFNTSDGGRFKFPDGVQNWPALSIVIIIIMTIGG
NILVIMAVSMEKKLHNATNYFLMSLAIADMLVGLLVMPLSLLAILYDYVWPLPRYLCPVWISLDVLFSTA
SIMHLCAISLDRYVAIRNPIEHSRFNSRTKAIMKIAIVWAISIGVSVPIPVIGLRDEEKVFVNNTTCVLN
DPNFVLIGSFVAFFIPLTIMVITYCLTIYVLRRQALMLLHGHTEEPPGLSLDFLKCCKRNTAEEENSANP
NQDQNARRRKKKERRPRGTMQAINNERKASKVLGIVFFVFLIMWCPFFITNILSVLCEKSCNQKLMEKLL
NVFVWIGYVCSGINPLVYTLFNKIYRRAFSNYLRCNYKVEKKPPVRQIPRVAATALSGRELNVNIYRHTN
EPVIEKASDNEPGIEMQVENLELPVNPSSVVSERISSV

9. 5-hydroxytryptamine receptor 3A; Short=5-HT3A

>gi|1168222|sp|P46098.1|5HT3A_HUMAN
Full=Serotonin-gated ion channel receptor
478aa
MLLWVQQALLALLLPTLLAQGEARRSRNTTRPALLRLSDYLLTNYRKGVRPVRDWRKPTTVSIDVIVYAI
LNVDEKNQVLTTYIWYRQYWTDEFLQWNPEDFDNITKLSIPTDSIWVPDILINEFVDVGKSPNIPYVYIR
HQGEVQNYKPLQVVTACSLDIYNFPFDVQNCSLTFTSWLHTIQDINISLWRLPEKVKSDRSVFMNQGEWE
LLGVLPYFREFSMESSNYYAEMKFYVVIRRRPLFYVVSLLLPSIFLMVMDIVGFYLPPNSGERVSFKITL
LLGYSVFLIIVSDTLPATAIGTPLIGVYFVVCMALLVISLAETIFIVRLVHKQDLQQPVPAWLRHLVLER
IAWLLCLREQSTSQRPPATSQATKTDDCSAMGNHCSHMGGPQDFEKSPRDRCSPPPPPREASLAVCGLLQ
ELSSIRQFLEKRDEIREVARDWLRVGSVLDKLLFHIYLLAVLAYSITLVMLWSIWQYA

10. 5-hydroxytryptamine receptor 3B; Short=5-HT3B

>gi|74705987|sp|O95264.1|5HT3B_HUMAN
451aa
MLSSVMAPLWACILVAAGILATDTHHPQDSALYHLSKQLLQKYHKEVRPVYNWTKATTVYLDLFVHAILD
VDAENQILKTSVWYQEVWNDEFLSWNSSMFDEIREISLPLSAIWAPDIIINEFVDIERYPDLPYVYVNSS
GTIENYKPIQVVSACSLETYAFPFDVQNCSLTFKSILHTVEDVDLAFLRSPEDIQHDKKAFLNDSEWELL
SVSSTYSILQSSAGGFAQIQFNVVMRRHPLVYVVSLLIPSIFLMLVDLGSFYLPPNCRARIVFKTSVLVG
YTVFRVNMSNQVPRSVGSTPLIGHFFTICMAFLVLSLAKSIVLVKFLHDEQRGGQEQPFLCLRGDTDADR
PRVEPRAQRAVVTESSLYGEHLAQPGTLKEVWSQLQSISNYLQTQDQTDQQEAEWLVLLSRFDRLLFQSY
LFMLGIYTITLCSLWALWGGV

11. RecName: Full=5-hydroxytryptamine receptor 3C; Short=5-HT3C

>gi|166198366|sp|Q8WXA8.2|5HT3C_HUMAN
447aa
MEGGWPARQSALLCLTVSLLLQGRGDAFTINCSGFDQHGVDPAVFQAVFDRKAFRPFTNYSIPTRVNISF
TLSAILGVDAQLQLLTSFLWMDLVWDNPFINWNPKECVGINKLTVLAENLWLPDIFIVESMDVDQTPSGL
TAYISSEGRIKYDKPMRVTSICNLDIFYFPFDQQNCTFTFSSFLYTVDSMLLGMDKEVWEITDTSRKVIQ
TQGEWELLGINKATPKMSMGNNLYDQIMFYVAIRRRPSLYIINLLVPSSFLVAIDALSFYLPAESENRAP
FKITLLLGYNVFLLMMNDLLPASGTPLISVYFALCLSLMVVSLLETVFITYLLHVATTQPPPMPRWLHSL
LLHCTSPGRCCPTAPQKGNKGLGLTLTHLPGPKEPGELAGKKLGPRETEPDGGSGWTKTQLMELWVQFSH
AMDTLLFRLYLLFMASSILTVIVLWNT

12. RecName: Full=5-hydroxytryptamine receptor 3D; Short=5-HT3D

>gi|74712683|sp|Q70Z44.1|5HT3D_HUMAN
454aa

MQKHSPGPPALALLSQSLLTTGNGDTLIINCPGFGQHRVDPAAFQAVFDRKAIGPVTNYSVATHVNISFT
LSAIWNCYSRIHTFNCHHARPWHNQFVQWNPDECGGIKKSGMATENLWLSDVFIEESVDQTPAGLMASMS
IVKATSNTISQCGWSASANWTPSISPSMDRARAWRRMSRSFQIHHRTSFRTRREWVLLGIQKRTIKVTVA
TNQYEQAIFHVAIRRRCRPSPYVVNFLVPSGILIAIDALSFYLPLESGNCAPFKMTVLLGYSVFLLMMND
LLPATSTSSHASLVAPLALMQTPLPAGVYFALCLSLMVGSLLETIFITHLLHVATTQPLPLPRWLHSLLL
HCTGQGRCCPTAPQKGNKGPGLTPTHLPGVKEPEVSAGQMPGPGEAELTGGSEWTRAQREHEAQKQHSVE
LWVQFSHAMDALLFRLYLLFMASSIITVICLWNT

13. RecName: Full=5-hydroxytryptamine receptor 3E; Short=5-HT3-E

>gi|162416113|sp|A5X5Y0.1|5HT3E_HUMAN
456aa
MEGSWFHRKRFSFYLLLGFLLQGRGVTFTINCSGFGQHGADPTALNSVFNRKPFRPVTNISVPTQVNISF
AMSAILDVNEQLHLLSSFLWLEMVWDNPFISWNPEECEGITKMSMAAKNLWLPDIFIIELMDVDKTPKGL
TAYVSNEGRIRYKKPMKVDSICNLDIFYFPFDQQNCTLTFSSFLYTVDSMLLDMEKEVWEITDASRNILQ
THGEWELLGLSKATAKLSRGGNLYDQIVFYVAIRRRPSLYVINLLVPSGFLVAIDALSFYLPVKSGNRVP
FKITLLLGYNVFLLMMSDLLPTSGTPLIGVYFALCLSLMVGSLLETIFITHLLHVATTQPPPLPRWLHSL
LLHCNSPGRCCPTAPQKENKGPGLTPTHLPGVKEPEVSAGQMPGPAEAELTGGSEWTRAQREHEAQKQHS
VELWLQFSHAMDAMLFRLYLLFMASSIITVICLWNT

14. 5-hydroxytryptamine receptor 4; Short=5-HT4

>gi|12644029|sp|Q13639.2|5HT4R_HUMAN

MDKLDANVSSEEGFGSVEKVVLLTFLSTVILMAILGNLLVMVAVCWDRQLRKIKTNYFIVSLAFADLLVS
VLVMPFGAIELVQDIWIYGEVFCLVRTSLDVLLTTASIFHLCCISLDRYYAICCQPLVYRNKMTPLRIAL
MLGGCWVIPTFISFLPIMQGWNNIGIIDLIEKRKFNQNSNSTYCVFMVNKPYAITCSVVAFYIPFLLMVL
AYYRIYVTAKEHAHQIQMLQRAGASSESRPQSADQHSTHRMRTETKAAKTLCIIMGCFCLCWAPFFVTNI
VDPFIDYTVPGQVWTAFLWLGYINSGLNPFLYAFLNKSFRRAFLIILCCDDERYRRPSILGQTVPCSTTT
INGSTHVLRDAVECGGQWESQCHPPATSPLVAAQPSDT

15. 5-hydroxytryptamine receptor 5A; Short=5-HT5A

>gi|1345607|sp|P47898.1|5HT5A_HUMAN
357aa
MDLPVNLTSFSLSTPSPLETNHSLGKDDLRPSSPLLSVFGVLILTLLGFLVAATFAWNLLVLATILRVRT
FHRVPHNLVASMAVSDVLVAALVMPLSLVHELSGRRWQLGRRLCQLWIACDVLCCTASIWNVTAIALDRY
WSITRHMEYTLRTRKCVSNVMIALTWALSAVISLAPLLFGWGETYSEGSEECQVSREPSYAVFSTVGAFY
LPLCVVLFVYWKIYKAAKFRVGSRKTNSVSPISEAVEVKDSAKQPQMVFTVRHATVTFQPEGDTWREQKE
QRAALMVGILIGVFVLCWIPFFLTELISPLCSCDIPAIWKSIFLWLGYSNSFFNPLIYTAFNKNYNSAFK
NFFSRQH

16. 5-hydroxytryptamine receptor 6; Short=5-HT6

>gi|1703010|sp|P50406.1|5HT6R_HUMAN
440aa
MVPEPGPTANSTPAWGAGPPSAPGGSGWVAAALCVVIALTAAANSLLIALICTQPALRNTSNFFLVSLFT
SDLMVGLVVMPPAMLNALYGRWVLARGLCLLWTAFDVMCCSASILNLCLISLDRYLLILSPLRYKLRMTP
LRALALVLGAWSLAALASFLPLLLGWHELGHARPPVPGQCRLLASLPFVLVASGLTFFLPSGAICFTYCR
ILLAARKQAVQVASLTTGMASQASETLQVPRTPRPGVESADSRRLATKHSRKALKASLTLGILLGMFFVT
WLPFFVANIVQAVCDCISPGLFDVLTWLGYCNSTMNPIIYPLFMRDFKRALGRFLPCPRCPRERQASLAS
PSLRTSHSGPRPGLSLQQVLPLPLPPDSDSDSDAGSGGSSGLRLTAQLLLPGEATQDPPLPTRAAAAVNF
FNIDPAEPELRPHPLGIPTN

17. 5-hydroxytryptamine receptor 7; Short=5-HT7

>gi|8488960|sp|P34969.2|5HT7R_HUMAN
479aa
MMDVNSSGRPDLYGHLRSFLLPEVGRGLPDLSPDGGADPVAGSWAPHLLSEVTASPAPTWDAPPDNASGC
GEQINYGRVEKVVIGSILTLITLLTIAGNCLVVISVCFVKKLRQPSNYLIVSLALADLSVAVAVMPFVSV
TDLIGGKWIFGHFFCNVFIAMDVMCCTASIMTLCVISIDRYLGITRPLTYPVRQNGKCMAKMILSVWLLS
ASITLPPLFGWAQNVNDDKVCLISQDFGYTIYSTAVAFYIPMSVMLFMYYQIYKAARKSAAKHKFPGFPR
VEPDSVIALNGIVKLQKEVEECANLSRLLKHERKNISIFKREQKAATTLGIIVGAFTVCWLPFFLLSTAR
PFICGTSCSCIPLWVERTFLWLGYANSLINPFIYAFFNRDLRTTYRSLLQCQYRNINRKLSAAGMHEALK
LAERPERPEFVLRACTRRVLLRPEKRPPVSVWVLQSPDHHNWLADKMLTTVEKKVMIHD

Appendices 2: Anotated Aligned Receptor sequences using CHROMA

Anotated Aligned Serotonin Receptor sequences using CHROMA

```
5HT2A
MDILCEENTSLSSTTNSLMQLNDDTRLYSNDFN(21)LSCEGCLSPSCLSLLH----LQE
5HT2C            MVNLRNAVHSFLVHLIGLLVWQCDISVSPVAAI----
VTDIFNTSDGGRFKFP----DGV
5HT2B            -MALSYRVSELQSTIPEHILQSTFVHVISSNWS----
GLQTESIPEEMKQIVE----EQG
5HT1B            MEEPGAQCAPPPPAGSETWVPQANLSSAPSQNC----SAKDYIYQDSISLPW-
-------
5HT1D            ----------MSPLNQSAEGLPQEASNRSLNAT-----ETSEAWDPRTLQAL-
-------
5HT1E            ------------------------MNITNCT----TEASMAIRPKTITE--
-------
5HT1F            --------------------MDFLNSSDQNLT----SEELLNRMPS------
-------
5HT1A            ----------------MDVLSPGQGNNTTSPPA----
PFETGGNTTGISDVTV----SY-
5HT5A            ---------MDLPVNLTSFSLSTPSPLETNHSL----GKDDLRPSSPLLSVF-
-------
5HT7R
MMDVNSSGRPDLYGHLRSFLLPEVGRGLPDLSPDGGADPVAGSWAPHLLSEVT(22)YGR
5HT3A            -MLLWVQQALLAL-LLPTLLAQG-EARRSRNTT----RPALLRLSDYLLTNY-
-------
5HT3B            -----MLSSVMAPLWACILVAAGILATDTHHPQ----DSALYHLSKQLLQKY-
-------
5HT3C            MEGGWPARQSALLCLTVSLLLQGRGDAFTINCS----
GFDQHGVDPAVFQAVF----D--
5HT3E            MEGSWFHRKRFSFYLLLGFLLQGRGVTFTINCS----
GFGQHGADPTALNSVF----N--
5HT3D            MQKHSPGPPALAL-LSQSLLTTGNGDTLIINCP----
GFGQHRVDPAAFQAVF----D--
5HT4R            ----------------------MDKLDANVS----SEEGFGSVE-------
-------
5HT6             ------------------MVPEPGPTANSTPA----WGAGPPSAPGG-----
-------
Consensus/80%
.......................h...s.pss........h..ss.h............


5HT2A            --KNWSALLTAVVIILTIAGNILVIMAVSLEKKLQNA-
TNYFLMSLAIADMLLGF---LV
5HT2C            --QNWPALSIVIIIIMTIGGNILVIMAVSMEKKLHNA-
TNYFLMSLAIADMLVGL---LV
5HT2B            NKLHWAALLILMVIIPTIGGNTLVILAVSLEKKLQYA-
TNYFLMSLAVADLLVGL---FV
5HT1B            --KVLLVMLLALITLATTLSNAFVIATVYRTRKLHTP-
ANYLIASLAVTDLLVSI---LV
5HT1D            --KISLAVVLSVITLATVLSNAFVLTTILLTRKLHTP-
ANYLIGSLATTDLLVSI---LV
5HT1E            --KMLICMTLVVITTLTTLLNLAVIMAIGTTKKLHQP-
ANYLICSLAVTDLLVAV---LV
5HT1F            --KILVSLTLSGLALMTTTINSLVIAAIIVTRKLHHP-
ANYLICSLAVTDFLVAV---LV
5HT1A            --QVITSLLLGTLIFCAVLGNACVVAAIALERSLQNV-
```

```
ANYLIGSLAVTDLMVSV---LV
5HT5A             --GVLILTLLGFLVAATFAWNLLVLATILRVRTFHRV-
PHNLVASMAVSDVLVAA---LV
5HT7R             VEKVVIGSILTLITLLTIAGNCLVVISVCFVKKLRQP-
SNYLIVSLALADLSVAV---AV
5HT3A             --RKGVRPVRDWRKPTTVSIDVIVYAILNVDEKNQVL-
TTYIWYRQYWTDEFLQWNPEDF
5HT3B             --HKEVRPVYNWTKATTVYLDLFVHAILDVDAENQIL-
KTSVWYQEVWNDEFLSWNSSMF
5HT3C             --RKAFRPFTNYSIPTRVNISFTLSAILGVDAQLQLL-
TSFLWMDLVWDNPFINWNPKEC
5HT3E             --RKPFRPVTNISVPTQVNISFAMSAILDVNEQLHLL-
SSFLWLEMVWDNPFISWNPEEC
5HT3D             --RKAIGPVTNYSVATHVNISFTLSAIWNCYSRIHTF-NCHH-
ARPWHNQFVQWNPDEC
5HT4R             --
KVVLLTFLSTVILMAILGNLLVMVAVCWDRQLRKIKTNYFIVSLAFADLLVSV---LV
5HT6              --SGWVAAALCVVIALTAAANSLLIALICTQPALRNT-
SNFFLVSLFTSDLMVGL---VV
Consensus/80%
..p.hh..hhsh.hhhsh.hshhlhhhl.hppplp...ssabbhsbshsDbblth...bs


5HT2A             MPVSMLTILYGYRWPLPSKLCAVWIYLD----------------------
VLFSTASI
5HT2C             MPLSLLAILYDYVWPLPRYLCPVWISLD----------------------
VLFSTASI
5HT2B             MPIALLTIMFEAMWPLPLVLCPAWLFLD----------------------
VLFSTASI
5HT1B             MPISTMYTVTG-RWTLGQVVCDFWLSSD----------------------
ITCCTASI
5HT1D             MPISIAYTITH-TWNFGQILCDIWLSSD----------------------
ITCCTASI
5HT1E             MPLSIIYIVMD-RWKLGYFLCEVWLSVD----------------------
MTCCTCSI
5HT1F             MPFSIVYIVRE-SWIMGQVVCDIWLSVD----------------------
ITCCTCSI
5HT1A             LPMAALYQVLN-KWTLGQVTCDLFIALD----------------------
VLCCTSSI
5HT5A             MPLSLVHELSGRRWQLGRRLCQLWIACD----------------------
VLCCTASI
5HT7R             MPFVSVTDLIGGKWIFGHFFCNVFIAMD----------------------
VMCCTASI
5HT3A             DNITKLSIPTDSIW-
VPDILINEFVDVGKSPNIPYVYIRHQGEVQNYKPLQVVTACSLDI
5HT3B             DEIREISLPLSAIW-
APDIIINEFVDIERYPDLPYVYVNSSGTIENYKPIQVVSACSLET
5HT3C             VGINKLTVLAENLW-
LPDIFIVESMDVDQTPSGLTAYISSEGRIKYDKPMRVTSICNLDI
5HT3E             EGITKMSMAAKNLW-
LPDIFIIELMDVDKTPKGLTAYVSNEGRIRYKKPMKVDSICNLDI
5HT3D             GGIKKSGMATENLW-LSDVFIEES--VDQTPAGLMA------------
SMSIVKATSNTI
5HT4R             MPFGAIELVQD-IWIYGEVFCLVRTSLD----------------------
VLLTTASI
5HT6              MPPAMLNALYG-RWVLARGLCLLWTAFD----------------------
VMCCSASI
Consensus/80%
hsbs.h.hhh..bW.bsphbh..bhshD.....................l.hs*hsI
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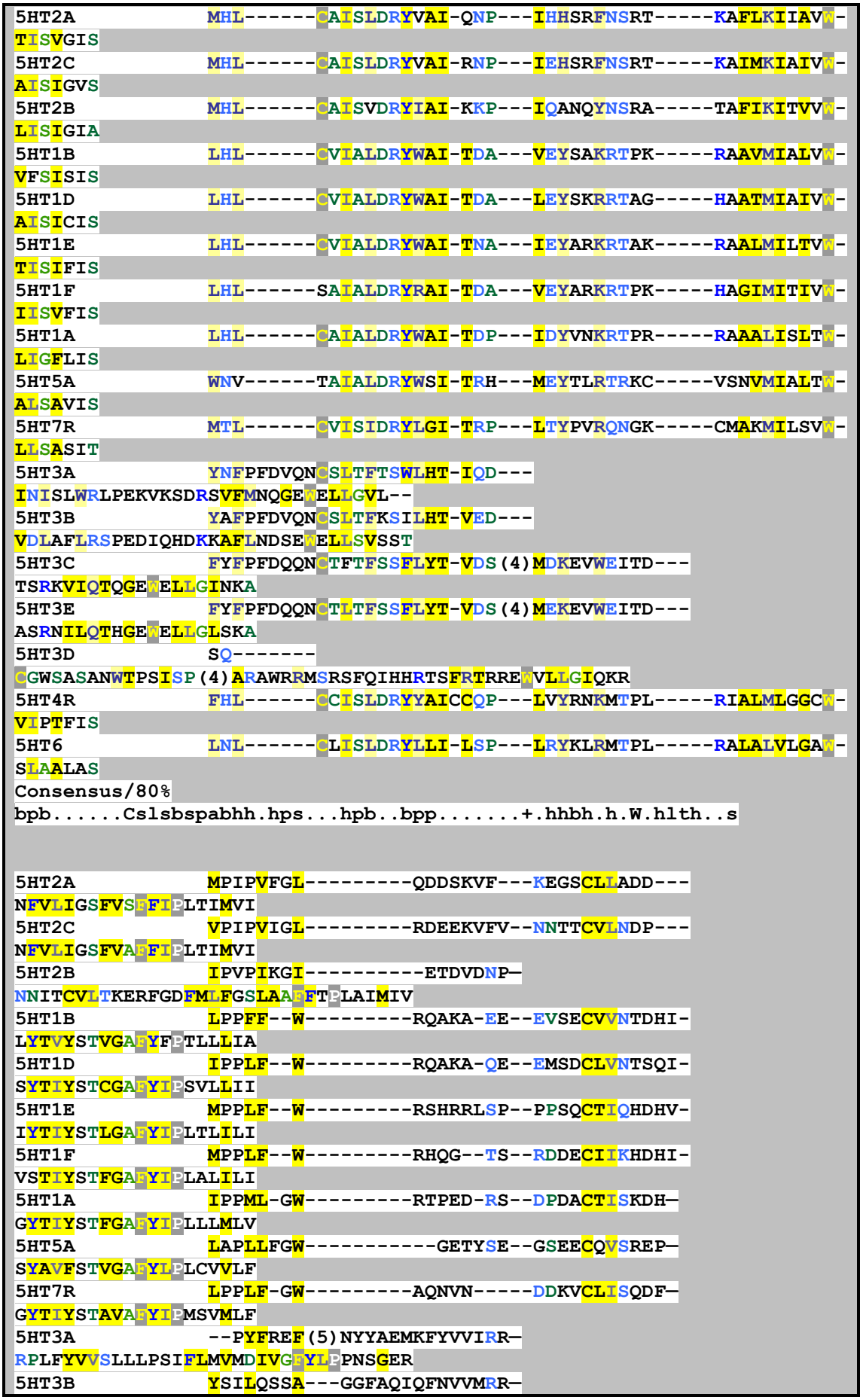
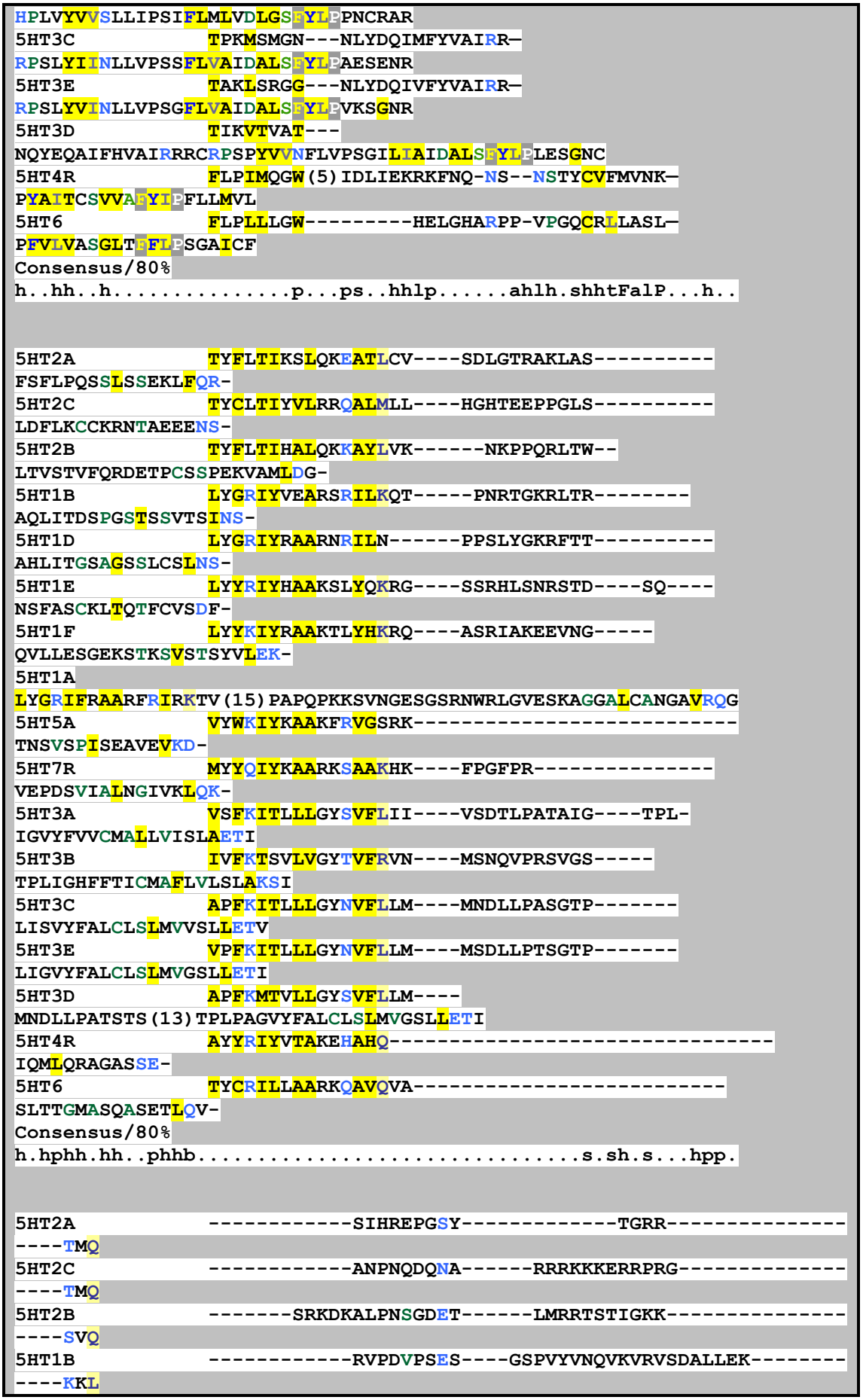
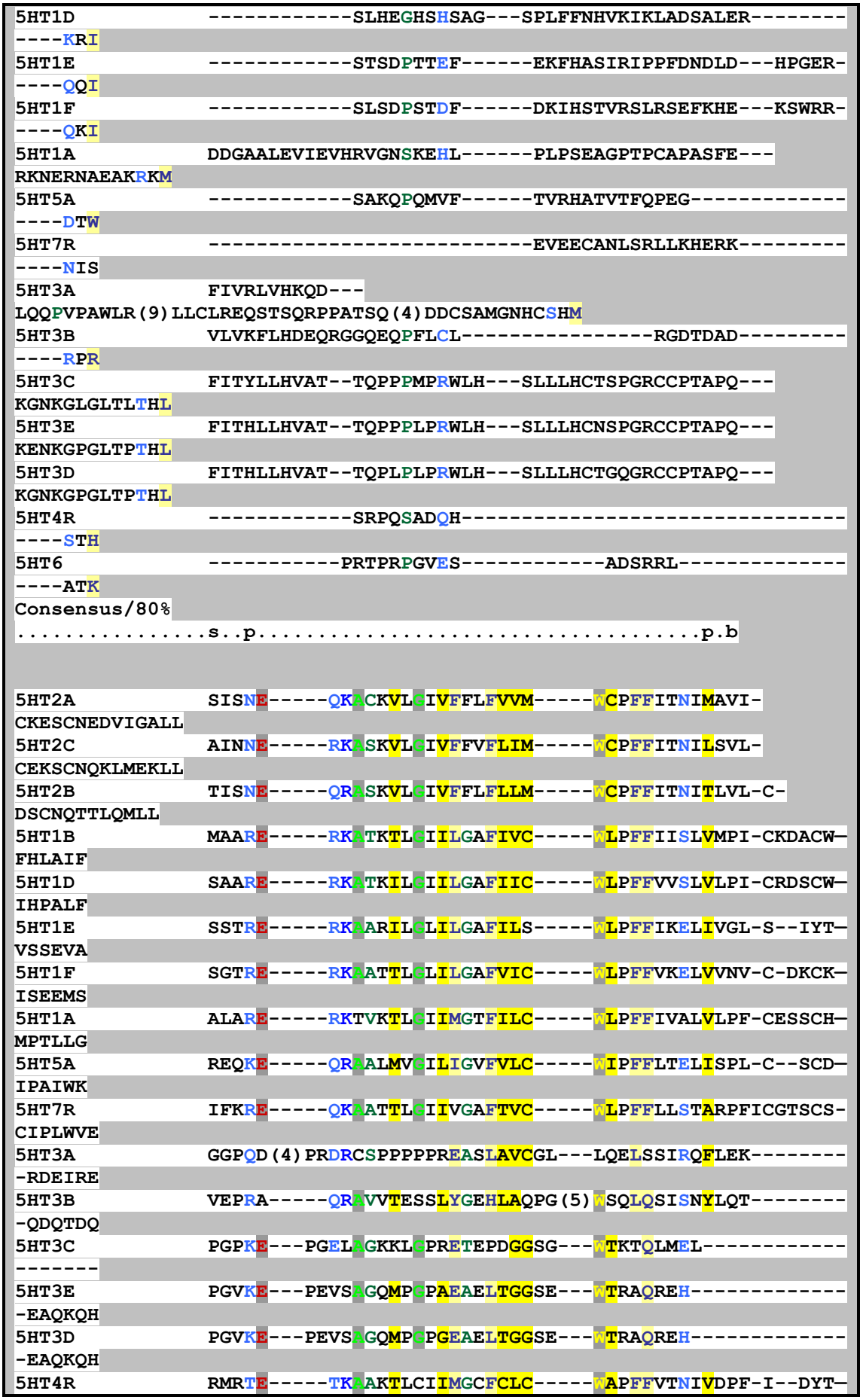

5HT2A MHL------CAISLDRYVAI-QNP---IHHSRFNSRT-----KAFLKIIAVW-
TISVGIS
5HT2C MHL------CAISLDRYVAI-RNP---IEHSRFNSRT-----KAIMKIAIVW-
AISIGVS
5HT2B MHL------CAISVDRYIAI-KKP---IQANQYNSRA-----TAFIKITVVW-
LISIGIA
5HT1B LHL------CVIALDRYWAI-TDA---VEYSAKRTPK-----RAAVMIALVW-
VFSISIS
5HT1D LHL------CVIALDRYWAI-TDA---LEYSKRRTAG-----HAATMIAIVW-
AISICIS
5HT1E LHL------CVIALDRYWAI-TNA---IEYARKRTAK-----RAALMILTVW-
TISIFIS
5HT1F LHL------SAIALDRYRAI-TDA---VEYARKRTPK-----HAGIMITIVW-
IISVFIS
5HT1A LHL------CAIALDRYWAI-TDP---IDYVNKRTPR-----RAAALISLTW-
LIGFLIS
5HT5A WNV------TAIALDRYWSI-TRH---MEYTLRTRKC-----VSNVMIALTW-
ALSAVIS
5HT7R MTL------CVISIDRYLGI-TRP---LTYPVRQNGK-----CMAKMILSVW-
LLSASIT
5HT3A YNFPFDVQNCSLTFTSWLHT-IQD---
INISLWRLPEKVKSDRSVFMNQGEWELLGVL--
5HT3B YAFPFDVQNCSLTFKSILHT-VED---
VDLAFLRSPEDIQHDKKAFLNDSEWELLSVSST
5HT3C FYFPFDQQNCTFTFSSFLYT-VDS(4)MDKEVWEITD---
TSRKVIQTQGEWELLGINKA
5HT3E FYFPFDQQNCTLTFSSFLYT-VDS(4)MEKEVWEITD---
ASRNILQTHGEWELLGLSKA
5HT3D SQ-------
CGWSASANWTPSISP(4)ARAWRRMSRSFQIHHRTSFRTRREWVLLGIQKR
5HT4R FHL------CCISLDRYYAICCQP---LVYRNKMTPL-----RIALMLGGCW-
VIPTFIS
5HT6 LNL------CLISLDRYLLI-LSP---LRYKLRMTPL-----RALALVLGAW-
SLAALAS
Consensus/80%
bpb......Cslsbspabhh.hps...hpb..bpp.......+.hhbh.h.W.hlth..s
5HT2A MPIPVFGL---------QDDSKVF---KEGSCLLADD---
NFVLIGSFVSGFIPLTIMVI
5HT2C VPIPVIGL---------RDEEKVFV--NNTTCVLNDP---
NFVLIGSFVAGFIPLTIMVI
5HT2B IPVPIKGI----------ETDVDNP-
NNITCVLTKERFGDFMLFGSLAAGFTPLAIMIV
5HT1B LPPFF--W---------RQAKA-EE--EVSECVVNTDHI-
LYTVYSTVGAFYFPTLLLIA
5HT1D IPPLF--W---------RQAKA-QE--EMSDCLVNTSQI-
SYTIYSTCGAFYIPSVLLII
5HT1E MPPLF--W---------RSHRRLSP--PPSQCTIQHDHV-
IYTIYSTLGAFYIPLTLILI
5HT1F MPPLF--W---------RHQG--TS--RDDECIIKHDHI-
VSTIYSTFGAFYIPLALILI
5HT1A IPPML-GW---------RTPED-RS--DPDACTISKDH-
GYTIYSTFGAFYIPLLLMLV
5HT5A LAPLLFGW----------GETYSE--GSEECQVSREP-
SYAVFSTVGAFYLPLCVVLF
5HT7R LPPLF-GW---------AQNVN-----DDKVCLISQDF-
GYTIYSTAVAFYIPMSVMLF
5HT3A --PYFREF(5)NYYAEMKFYVVIRR-
RPLFYVVSLLLPSIFLMVMDIVGFYLPPNSGER
5HT3B YSILQSSA---GGFAQIQFNVVMRR-


HPLVYVVSLLIPSIFLMLVDLGSFYLPPNCRAR
5HT3C TPKMSMGN---NLYDQIMFYVAIRR—
RPSLYIINLLVPSSFLVAIDALSFYLPAESENR
5HT3E TAKLSRGG---NLYDQIVFYVAIRR—
RPSLYVINLLVPSGFLVAIDALSFYLPVKSGNR
5HT3D TIKVTVAT---
NQYEQAIFHVAIRRRCRPSPYVVNFLVPSGILIAIDALSFYLPLESGNC
5HT4R FLPIMQGW(5)IDLIEKRKFNQ-NS--NSTYCVFMVNK—
PYAITCSVVAFYIPFLLMVL
5HT6 FLPLLLGW---------HELGHARPP-VPGQCRLLASL—
PFVLVASGLTFFLPSGAICF
Consensus/80%
h..hh..h.............p...ps..hhlp......ahlh.shhtFalP...h..
5HT2A TYFLTIKSLQKEATLCV----SDLGTRAKLAS----------
FSFLPQSSLSSEKLFQR-
5HT2C TYCLTIYVLRRQALMLL----HGHTEEPPGLS----------
LDFLKCCKRNTAEEENS-
5HT2B TYFLTIHALQKKAYLVK------NKPPQRLTW--
LTVSTVFQRDETPCSSPEKVAMLDG-
5HT1B LYGRIYVEARSRILKQT-----PNRTGKRLTR--------
AQLITDSPGSTSSVTSINS-
5HT1D LYGRIYRAARNRILN------PPSLYGKRFTT----------
AHLITGSAGSSLCSLNS-
5HT1E LYYRIYHAAKSLYQKRG----SSRHLSNRSTD----SQ----
NSFASCKLTQTFCVSDF-
5HT1F LYYKIYRAAKTLYHKRQ----ASRIAKEEVNG-----
QVLLESGEKSTKSVSTSYVLEK-
5HT1A
LYGRIFRAARFRIRKTV(15)PAPQPKKSVNGESGSRNWRLGVESKAGGALCANGAVRQG
5HT5A VYWKIYKAAKFRVGSRK--------------------------
TNSVSPISEAVEVKD-
5HT7R MYYQIYKAARKSAAKHK----FPGFPR---------------
VEPDSVIALNGIVKLQK-
5HT3A VSFKITLLLGYSVFLII----VSDTLPATAIG----TPL-
IGVYFVVCMALLVISLAETI
5HT3B IVFKTSVLVGYTVFRVN----MSNQVPRSVGS-----
TPLIGHFFTICMAFLVLSLAKSI
5HT3C APFKITLLLGYNVFLLM----MNDLLPASGTP-------
LISVYFALCLSLMVVSLLETV
5HT3E VPFKITLLLGYNVFLLM----MSDLLPTSGTP-------
LIGVYFALCLSLMVGSLLETI
5HT3D APFKMTVLLGYSVFLLM----
MNDLLPATSTS(13)TPLPAGVYFALCLSLMVGSLLETI
5HT4R AYYRIYVTAKEHAHQ--------------------------------
IQMLQRAGASSE-
5HT6 TYCRILLAARKQAVQVA--------------------------
SLTTGMASQASETLQV-
Consensus/80%
h.hphh.hh..phhb.............................s.sh.s...hpp.
5HT2A ------------SIHREPGSY-------------TGRR---------------
----TMQ
5HT2C ------------ANPNQDQNA------RRRKKKERRPRG--------------
----TMQ
5HT2B -------SRKDKALPNSGDET------LMRRTSTIGKK---------------
----SVQ
5HT1B ------------RVPDVPSES----GSPVYVNQVKVRVSDALLEK--------
----KKL


5HT1D ----------SLHEGHSHSAG---SPLFFNHVKIKLADSALER--------
----KRI
5HT1E ----------STSDPTTEF------EKFHASIRIPPFDNDLD---HPGER-
----QQI
5HT1F ----------SLSDPSTDF------DKIHSTVRSLRSEFKHE---KSWRR-
----QKI
5HT1A DDGAALEVIEVHRVGNSKEHL------PLPSEAGPTPCAPASFE---
RKNERNAEAKRKM
5HT5A ----------SAKQPQMVF------TVRHATVTFQPEG----------
----DTW
5HT7R ----------EVEECANLSRLLKHERK---------
----NIS
5HT3A FIVRLVHKQD---
LQQPVPAWLR(9)LLCLREQSTSQRPPATSQ(4)DDCSAMGNHCSHM
5HT3B VLVKFLHDEQRGGQEQPFLCL----------RGDTDAD---------
----RPR
5HT3C FITYLLHVAT--TQPPPMPRWLH---SLLLHCTSPGRCCPTAPQ---
KGNKGLGLTLTHL
5HT3E FITHLLHVAT--TQPPPLPRWLH---SLLLHCNSPGRCCPTAPQ---
KENKGPGLTPTHL
5HT3D FITHLLHVAT--TQPLPLPRWLH---SLLLHCTGQGRCCPTAPQ---
KGNKGPGLTPTHL
5HT4R ----------SRPQSADQH----------
----STH
5HT6 ----------PRTPRPGVES----------ADSRRL----------
----ATK
Consensus/80%
.............s..p.....................p.b
5HT2A SISNE-----QKACKVLGIVFFLFVVM-----WCPFFITNIMAVI-
CKESCNEDVIGALL
5HT2C AINNE-----RKASKVLGIVFFVFLIM-----WCPFFITNILSVL-
CEKSCNQKLMEKLL
5HT2B TISNE-----QRASKVLGIVFFLFLLM-----WCPFFITNITLVL-C-
DSCNQTTLQMLL
5HT1B MAARE-----RKATKTLGIILGAFIVC-----WLPFFIISLVMPI-CKDACW-
FHLAIF
5HT1D SAARE-----RKATKILGIILGAFIIC-----WLPFFVVSLVLPI-CRDSCW-
IHPALF
5HT1E SSTRE-----RKAARILGLILGAFILS-----WLPFFIKELIVGL-S--IYT-
VSSEVA
5HT1F SGTRE-----RKAATTLGLILGAFVIC-----WLPFFVKELVVNV-C-DKCK-
ISEEMS
5HT1A ALARE-----RKTVKTLGIIMGTFILC-----WLPFFIVALVLPF-CESSCH-
MPTLLG
5HT5A REQKE-----QRAALMVGILIGVFVLC-----WIPFFLTELISPL-C--SCD-
IPAIWK
5HT7R IFKRE-----QKAATTLGIIVGAFTVC-----WLPFFLLSTARPFICGTSCS-
CIPLWVE
5HT3A GGPQD(4)PRDRCSPPPPPREASLAVCGL---LQELSSIRQFLEK--------
-RDEIRE
5HT3B VEPRA-----QRAVVTESSLYGEHLAQPG(5)WSQLQSISNYLQT--------
-QDQTDQ
5HT3C PGPKE---PGELAGKKLGPRETEPDGGSG---WTKTQLMEL-----------

5HT3E PGVKE---PEVSAGQMPGPAEAELTGGSE---WTRAQREH-----------
-EAQKQH
5HT3D PGVKE---PEVSAGQMPGPGEAELTGGSE---WTRAQREH-----------
-EAQKQH
5HT4R RMRTE-----TKAAKTLCIIMGCFCLC-----WAPFFVTNIVDPF-I--DYT-

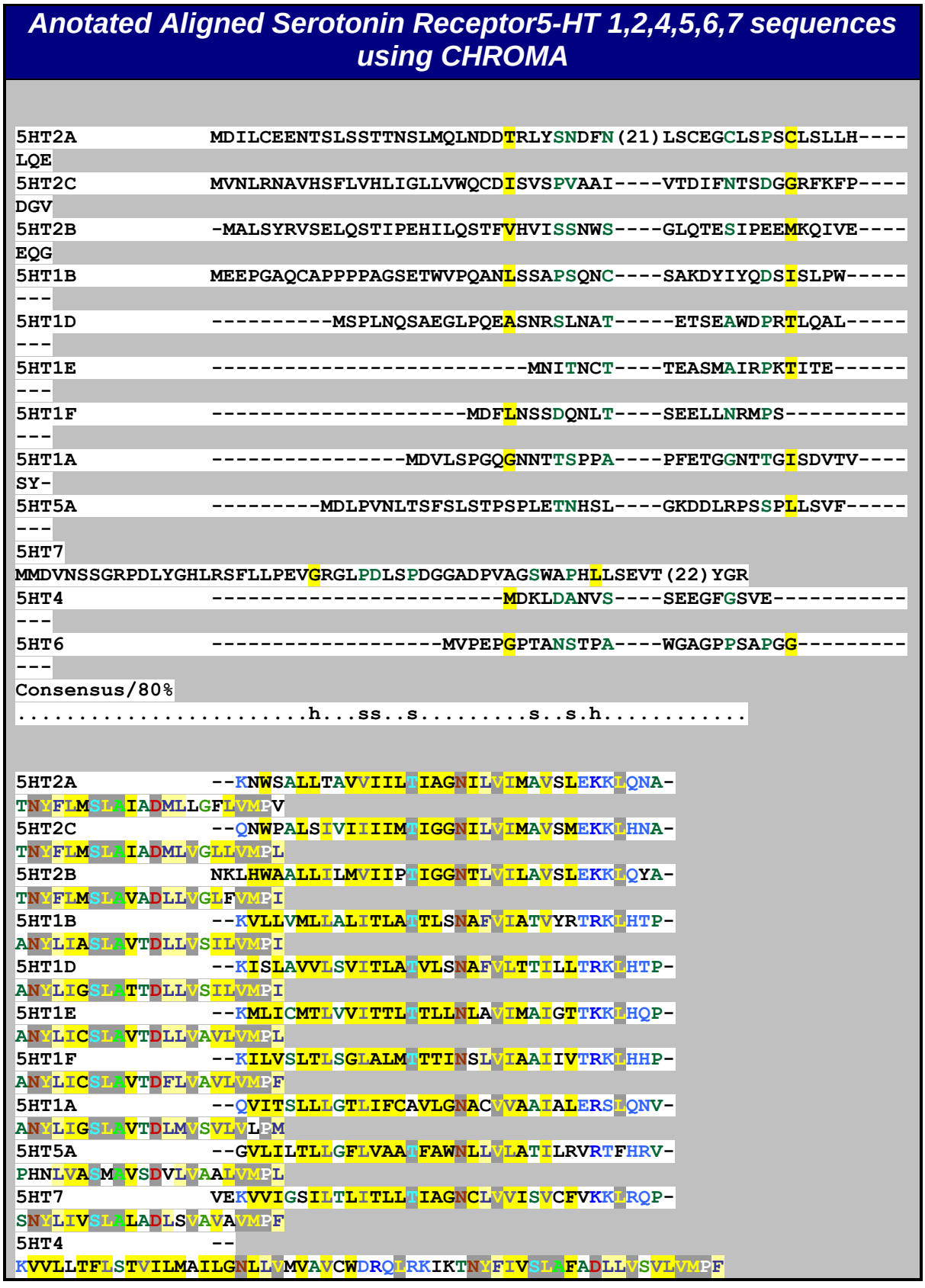
```
VPGQVW
5HT6              HSRKA-----LKASLTLGILLGMFFVT-----WLPFFVANIVQAV-C--DC--
-ISPGLF
Consensus/80%
...pE.....p+As.h.G.hbs.bhhh.....Wh.bb..p.h..................


5HT2A
NVFVWIGYLSSAVNPLVYTLFNKTYRSAFSRYIQCQYKENKKPLQLILVNTIPALAYKSS
5HT2C
NVFVWIGYVCSGINPLVYTLFNKIYRRAFSNYLRCNYKVEKKPPVRQIPRVAATALSGRE
5HT2B
EIFVWIGYVSSGVNPLVYTLFNKTFRDAFGRYITCNYRATKSVKTLRKRSSKIYFRNPMA
5HT1B              DFFTWLGYLNSLINPIIYTMSNEDFKQAFHKLIRFKCTS--------------
-------
5HT1D              DFFTWLGYLNSLINPIIYTVFNEEFRQAFQKIVPFRKAS--------------
-------
5HT1E              DFLTWLGYVNSLINPLLYTSFNEDFKLAFKKLIRCREHT--------------
-------
5HT1F              NFLAWLGYLNSLINPLIYTIFNEDFKKAFQKLVRCRC----------------
-------
5HT1A              AIINWLGYSNSLLNPVIYAYFNKDFQNAFKKIIKCKFCRQ-------------
-------
5HT5A              SIFLWLGYSNSFFNPLIYTAFNKNYNSAFKNFFSRQH----------------
-------
5HT7R
RTFLWLGYANSLINPFIYAFFNRDLRTTYRSLLQCQYRNINRKLSAAGMHEALKLAERPE
5HT3A              VARDWLRV-GSVLDKLLFHIYL-LAVLAYSITLVMLWSIWQYA----------
-------
5HT3B              QEAEWLVL-LSRFDRLLFQSYL-FMLGIYTITLCSLWALWGGV----------
-------
5HT3C              ----WVQF-SHAMDTLLFRLYL-LFMASSILTVIVLWNT--------------
-------
5HT3E              SVELWLQF-SHAMDAMLFRLYL-LFMASSIITVICLWNT--------------
-------
5HT3D              SVELWVQF-SHAMDALLFRLYL-LFMASSIITVICLWNT--------------
-------
5HT4R
TAFLWLGYINSGLNPFLYAFLNKSFRRAFLIILCCDDERYRRPSILGQTVPCSTTTINGS
5HT6
DVLTWLGYCNSTMNPIIYPLFMRDFKRALGRFLPCPRCPRERQASLASPSLRTSHSGPRP
Consensus/80%
phb.Wl.a.sShbsslla.ha...ab.tb.bhl.h.b.....................


5HT2A              QLQMGQKKNSKQDAKTTDNDCSMVALGKQHSEEASKDNSDGVNEKVSCV----
5HT2C              LNVNIYRHTNEPVIEKASDNEPGIEMQVENLELPVNPSSVVSERISSV-----
5HT2B              ENSKFFKKHGIRNGINPAMYQSPMRLRSSTIQSSSIILLDTLLLTENEG(10)
5HT1B              ----------------------------------------------------
5HT1D              ----------------------------------------------------
5HT1E              ----------------------------------------------------
5HT1F              ----------------------------------------------------
5HT1A              ----------------------------------------------------
5HT5A              ----------------------------------------------------
5HT7R              RPEFVLRACTRRVLLRPEKRPPVSVWVLQSPDHHNWLADKMLTTVEKKVMIHD
5HT3A              ----------------------------------------------------
5HT3B              ----------------------------------------------------
5HT3C              ----------------------------------------------------
5HT3E              ----------------------------------------------------
5HT3D              ----------------------------------------------------
5HT4R              THVLRDAVECGGQWESQCHPPATSPLVAAQPSDT-------------------
```

5HT6	GLSLQQVLPLPLPPDSDSDSDAGSGGSSGLRLTAQLLLPGEATQDPPLP(29)
Consensus/80%	.....

Appendices 3: Anotated Aligned Serotonin Receptor5-HT 1,2,4,5,6,7 sequences using CHROMA



```
5HT6            --SGWVAAALCVVIALTAAANSLLIALICTQPALRNT-
SNFFLVSLFTSDLMVGLVVMPP
Consensus/80%
..phhhshhlshlhhhThhhNhbVlhsl.hp+pLpps.sNYblhSLAhsDbbVtllVMPb

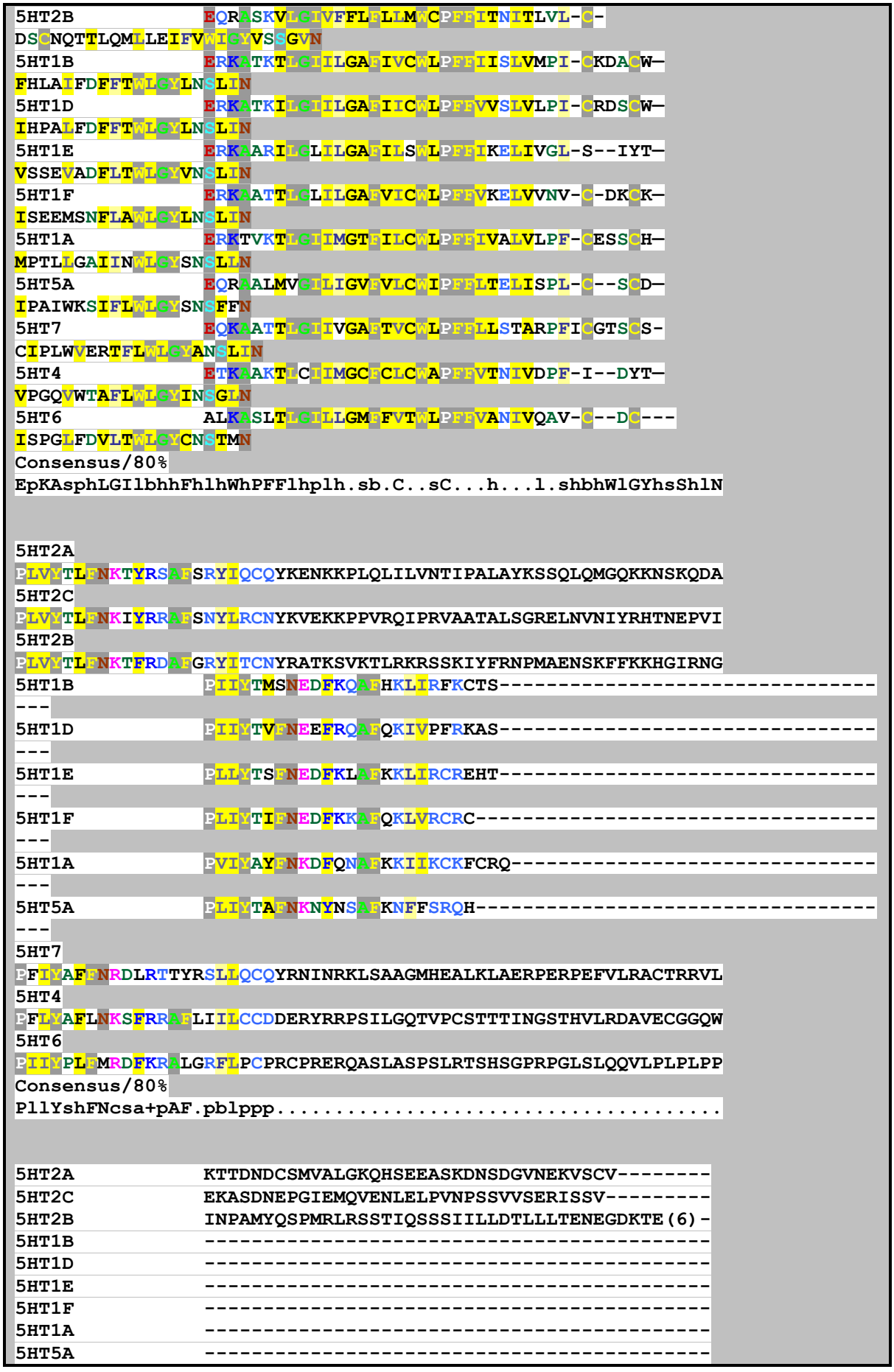

5HT2A           SMLTILYGYRWPLPSKLCAVWIYLDVLFSTASIMHLCAISLDRYVAI-
QNPIHHSRFNSR
5HT2C           SLLAILYDYVWPLPRYLCPVWISLDVLFSTASIMHLCAISLDRYVAI-
RNPIEHSRFNSR
5HT2B           ALLTIMFEAMWPLPLVLCPAWLFLDVLFSTASIMHLCAISVDRYIAI-
KKPIQANQYNSR
5HT1B           STMYTVTG-RWTLGQVVCDFWLSSDITCCTASILHLCVIALDRYWAI-
TDAVEYSAKRTP
5HT1D           SIAYTITH-TWNFGQILCDIWLSSDITCCTASILHLCVIALDRYWAI-
TDALEYSKRRTA
5HT1E           SIIYIVMD-RWKLGYFLCEVWLSVDMTCCTCSILHLCVIALDRYWAI-
TNAIEYARKRTA
5HT1F           SIVYIVRE-SWIMGQVVCDIWLSVDITCCTCSILHLSAIALDRYRAI-
TDAVEYARKRTP
5HT1A           AALYQVLN-KWTLGQVTCDLFIALDVLCCTSSILHLCAIALDRYWAI-
TDPIDYVNKRTP
5HT5A           SLVHELSGRRWQLGRRLCQLWIACDVLCCTASIWNVTAIALDRYWSI-
TRHMEYTLRTRK
5HT7            VSVTDLIGGKWIFGHFFCNVFIAMDVMCCTASIMTLCVISIDRYLGI-
TRPLTYPVRQNG
5HT4            GAIELVQD-
IWIYGEVFCLVRTSLDVLLTIASIFHLCCISLDRYYAICCQPLVYRNKMTP
5HT6            AMLNALYG-RWVLARGLCLLWTAFDVMCCSASILNLCLISLDRYLLI-
LSPLRYKLRMTP
Consensus/80%
thlh.l....W.bsphhC.lalthDlhhsTtSIbpLCsItLDRYbtI.ppslpas.bp*.


5HT2A           TKAFLKIIAVWTISVGISMPIPVFGL----QDDSKVF--KEGSCLLADD---
NFVLIGSF
5HT2C           TKAIMKIAIVWAISIGVSVPIPVIGL----RDEEKVFV-NNTTCVLNDP---
NFVLIGSF
5HT2B           ATAFIKITVVWLISIGIAIPVPIKGI-----ETDVDNP-
NNITCVLTKERFGDFMLFGSL
5HT1B           KRAAVMIALVWVFSISISLPPFF--W----RQAKA-EE-EVSECVVNTDHI-
LYTVYSTV
5HT1D           GHAATMIAIVWAISICISIPPLF--W----RQAKA-QE-EMSDCLVNTSQI-
SYTIYSTC
5HT1E           KRAALMILTVWTISIFISMPPLF--W----RSHRRLSP-PPSQCTIQHDHV-
IYTIYSTL
5HT1F           KHAGIMITIVWIISVFISMPPLF--W----RHQG--TS-RDDECIIKHDHI-
VSTIYSTF
5HT1A           RRAAALISLTWLIGFLISIPPML-GW----RTPED-RS-DPDACTISKDH-
GYTIYSTF
5HT5A           CVSNVMIALTWALSAVISLAPLLFGW------GETYSE-GSEECQVSREP-
SYAVFSTV
5HT7            KCMAKMILSVWLLSASITLPPLF-GW----AQNVN----DDKVCLISQDF-
GYTIYSTA
5HT4            LRIALMLGGCWVIPTFISFLPIMQGW(11)RKFNQ-NS-NSTYCVFMVNK-
PYAITCSV
5HT6            LRALALVLGAWSLAALASFLPLLLGW----HELGHARPPVPGQCRLLASL-
PFVLVASG
Consensus/80%
.pthhbIhhsWhlthhISbss.b..b.....p........s..Chl.ps...saslbt*h
```

```
5HT2A              VSFFIPLTIMVITYFLTIKSLQKEATLCV----SDLGTRAKLAS---------
FSFLPQS
5HT2C              VAFFIPLTIMVITYCLTIYVLRRQALMLL----HGHTEEPPGLS---------
LDFLKCC
5HT2B              AAFFTPLAIMIVTYFLTIHALQKKAYLVK------NKPPQRLTW-
LTVSTVFQRDETPCS
5HT1B              GAFYFPTLLLIALYGRIYVEARSRILKQT-----PNRTGKRLTR-------
AQLITDSPG
5HT1D              GAFYIPSVLLIILYGRIYRAARNRILN------PPSLYGKRFTT---------
AHLITGS
5HT1E              GAFYIPLTLILILYYRIYHAAKSLYQKRG----SSRHLSNRSTD---SQ----
NSFASCK
5HT1F              GAFYIPLALILILYYKIYRAAKTLYHKRQ----ASRIAKEEVNG----
QVLLESGEKSTK
5HT1A
GAFYIPLLLMLVLYGRIFRAARFRIRKTV(15)PAPQPKKSVNG(4)RNWRLGVESKAGG
5HT5A              GAFYLPLCVVLFVYWKIYKAAKFRVGSRK-------------------------
TNSVS
5HT7               VAFYIPMSVMLFMYYQIYKAARKSAAKHK----FPGFPR--------------
VEPDSVI
5HT4               VAFYIPFLLMVLAYYRIYVTAKEHAHQ-----------------------------
-IQ
5HT6               LTFFLPSGAICFTYCRILLAARKQAVQVA-----------------------
SLTTGM
Consensus/80%
sAFalPhhlblhhYhbhbbsh+pphhb.............................ss.


5HT2A              SLSSEKLFQR-----SIHREPGSY----------TGRR---------------
TMQSISN
5HT2C              KRNTAEEENS-----ANPNQDQNA---RRRKKKERRPRG--------------
TMQAINN
5HT2B              SPEKVAMLDGSRKDKALPNSGDET---LMRRTSTIGKK---------------
SVQTISN
5HT1B              STSSVTSINS-----RVPDVPSES-GSPVYVNQVKVRVSDALLEK--------
KKLMAAR
5HT1D              AGSSLCSLNS-----SLHEGHSHSAGSPLFFNHVKIKLADSALER--------
KRISAAR
5HT1E              LTQTFCVSDF-----STSDPTTEF---EKFHASIRIPPFDNDLD-HPGER---
QQISSTR
5HT1F              SVSTSYVLEK-----SLSDPSTDF---DKIHSTVRSLRSEFKHE-KSWRR---
QKISGTR
5HT1A              ALCANGAVRQ(12)HRVGNSKEHL---PLPSEAGPTPCAPASFE-
RKNER(5)RKMALAR
5HT5A              PISEAVEVKD-----SAKQPQMVF---TVRHATVTFQPEG-------------
DTWREQK
5HT7               ALNGIVKLQK-----------------EVEECANLSRLLKHERK---------
NISIFKR
5HT4               MLQRAGASSE-----SRPQSADQH-----------------------------
STHRMRT
5HT6               ASQASETLQV----PRTPRPGVES---------ADSRRL--------------
ATKHSRK
Consensus/80%
..p.....p.........p...p.............................p.b...p


5HT2A              EQKACKVLGIVFFLFVVMVCPFFITNIMAVI-
CKESCNEDVIGALLNVFVWIGYLSSAVN
5HT2C              ERKASKVLGIVFFVFLIMVCPFFITNILSVL-
CEKSCNQKLMEKLLNVFVWIGYVCSGIN
```


5HT2B EQRASKVLGIVFFLFLLMWCPFFITNITLVL-C-
DSCNQTTLQMLLEIFVWIGYVSSGVN
5HT1B ERKATKTLGIILGAFIVCWLPFFIISLVMPI-CKDACW-
FHLAIFDFFTWLGYLNSLIN
5HT1D ERKATKILGIILGAFIICWLPFFVVSLVLPI-CRDSCW-
IHPALFDFFTWLGYLNSLIN
5HT1E ERKAARILGLILGAFILSWLPFFIKELIVGL-S--IYT-
VSSEVADFLTWLGYVNSLIN
5HT1F ERKAATTLGLILGAFVICWLPFFVKELVVNV-C-DKCK-
ISEEMSNFLAWLGYLNSLIN
5HT1A ERKTVKTLGIIMGTFILCWLPFFIVALVLPF-CESSCH-
MPTLLGAIINWLGYSNSLLN
5HT5A EQRAALMVGILIGVFVLCWIPFFLTELISPL-C--SCD-
IPAIWKSIFLWLGYSNSFFN
5HT7 EQKAATTLGIIVGAFTVCWLPFFLLSTARPFICGTSCS-
CIPLWVERTFLWLGYANSLIN
5HT4 ETKAAKTLCIIMGCFCLCWAPFFVTNIVDPF-I--DYT-
VPGQVWTAFLWLGYINSGLN
5HT6 ALKASLTLGIILLGMFFVTWLPFFVANIVQAV-C--DC---
ISPGLFDVLTWLGYCNSTMN
Consensus/80%
EpKAsphLGIlbhhFhlhWhPFFlhplh.sb.C..sC...h...l.shbhWlGYhsShlN
5HT2A
PLVYTLFNKTYRSAFSRYIQCQYKENKKPLQLILVNTIPALAYKSSQLQMGQKKNSKQDA
5HT2C
PLVYTLFNKIYRRAFSNYLRCNYKVEKKPPVRQIPRVAATALSGRELNVNIYRHTNEPVI
5HT2B
PLVYTLFNKTFRDAFGRYITCNYRATKSVKTLRKRSSKIYFRNPMAENSKFFKKHGIRNG
5HT1B PIIYTMSNEDFKQAFHKLIRFKCTS----------

5HT1D PIIYTVFNEEFRQAFQKIVPFRKAS----------

5HT1E PLIYTSFNEDFKLAFKKLIRCREHT----------

5HT1F PLIYTIFNEDFKKAFQKLVRCRC----------

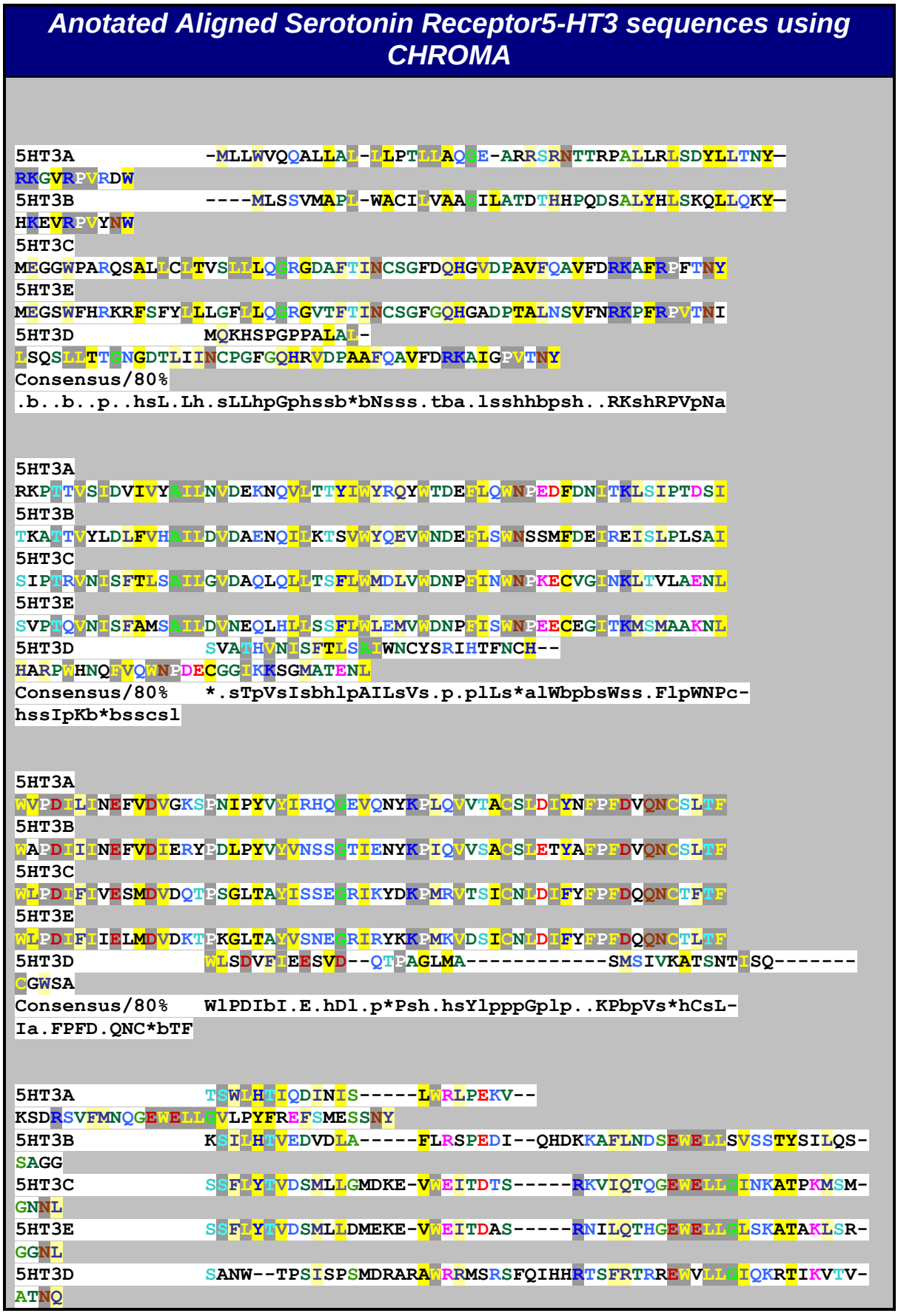
5HT1A PVIYAYFNKDFQNAFKKIIKCKFCRQ----------

5HT5A PLIYTAFNKNYNSAFKNFFSRQH----------

5HT7
PFIYAFFNRDLRTTYRSLLQCQYRNINRKLSAAGMHEALKLAERPERPEFVLRACTRRVL
5HT4
PFLYAFLNKSFRRAFLIILCCDDERYRRPSILGQTVPCSTTTINGSTHVLRDAVECGGQW
5HT6
PIIYPLFMRDFKRALGRFLPCPRCPRERQASLASPSLRTSHSGPRPGLSLQQVLPLPLPP
Consensus/80%
PllYshFNcsa+pAF.pblppp.....
5HT2A KTTDNDCSMVALGKQHSEEASKDNSDGVNEKVSCV--------
5HT2C EKASDNEPGIEMQVENLELPVNPSSVVSERISSV---------
5HT2B INPAMYQSPMRLRSSTIQSSSIILLDTLLLTENEGDKTE(6)-
5HT1B ----------
5HT1D ----------
5HT1E ----------
5HT1F ----------
5HT1A ----------
5HT5A ----------

5HT7	LRPEKRPPVSVWVLQSPDHHNWLADKMLTTVEKKVMIHD----
5HT4	ESQCHPPATSPLVAAQPSDT----------
5HT6	DSDSDSDAGSGGSSGLRLTAQLLLPGEATQDPPLPTRAA(25)
Consensus/80%	.....

Appendices 4: Anotated Aligned Serotonin Receptor5-HT3 sequences using CHROMA



```
Consensus/80%    *SbLaTlpsb.lt.....hWcbs-
ps.....RpsbbsptEWELLGlpphh.cb*..tsNb


5HT3A            YAEMKFYVVIRR--
RPLFYVVSLLLPSIFLMVMDIVGFYLPPNSGERVSFKITLLLGYSV
5HT3B            FAQIQFNVVMRR--
HPLVYVVSLLIPSIFLMLVDLGSFYLPPNCRARIVFKTSVLVGYTV
5HT3C            YDQIMFYVAIRR--
RPSLYIINLLVPSSFLVAIDALSFYLPAESENRAPFKITLLLGYNV
5HT3E            YDQIVFYVAIRR--
RPSLYVINLLVPSGFLVAIDALSFYLPVKSGNRVPFKITLLLGYNV
5HT3D
YEQAIFHVAIRRRCRPSPYVVNFLVPSGILIAIDALSFYLPLESGNCAPFKMTVLLGYSV
Consensus/80%
YsQbbFaVsIRR..RP.hYVlsLLlPShFLhslDhlSFYLPspS.sRssFKbTlLLGYsV


5HT3A            FLIIVSDTLPATAIG----TPL-IGVYFVVCMALLVISLAETIFIVRLVHKQDL--
QQ-P
5HT3B            FRVNMSNQVPRSVGS----TPL-
IGHFPTICMAFLVLSLAKSIVLVKFLHDEQRGGQEQP
5HT3C            FLLMMNDLLPASG------TPL-ISVYFALCLSLMVVSLLETVFITYLLHVATT-
QPPP
5HT3E            FLLMMSDLLPTSG------TPL-IGVYFALCLSLMVGSLLETIFITHLLHVATT-
QPPP
5HT3D            FLLMMNDLLPATSTS(13)TPLPAGVYFALCLSLMVGSLLETIFITHLLHVATT-
QPLP
Consensus/80%
FLlbMsDbLPs*t......TPL.IGVYFslCbtLbVhSLhETIFIs+LLHs.sp..Q..P


5HT3A            VPAWLR(9)LLCLREQSTSQRPPATSQATKT--
DDCSAMGNHCSHMGGPQD(4)PRDRCS
5HT3B            ---------FLCLRGDTDADRPRVEPRAQRAVVTESSLYGEHLAQPGTLKE------
---
5HT3C            MPRWLH---SLLLHCTSPGRCCPTAPQ------KGNKGLGLTLTHLPGPKE---
PGELAG
5HT3E            LPRWLH---SLLLHCNSPGRCCPTAPQ------KENKGPGLTPTHLPGVKE---
PEVSAG
5HT3D            LPRWLH---SLLLHCTGQGRCCPTAPQ------KGNKGPGLTPTHLPGVKE---
PEVSAG
Consensus/80%
hP.WL+....LhL+ss*stcpsPssPQ......c.spt.Gbp.*HbsGsKE...P...st


5HT3A
PPPPPRREASLAVCGLLQELSSIRQFLEKRDEIREVARDWLRVGSVLDKLLFHIYLLAVLA
5HT3B            ---------------
VWSQLQSISNYLQTQDQTDQQEAEWLVLLSRFDRLLFQSYLFMLGI
5HT3C            KKLGPRETEPDGGSGWTKTQ--------------
LMELWVQFSHAMDTLLFRLYLLFMAS
5HT3E            QMPGPAEAELTGGSEWTRAQR-----
EHEAQKQHSVELWLQFSHAMDAMLFRLYLLFMAS
5HT3D            QMPGPGEAELTGGSEWTRAQR-----
EHEAQKQHSVELWVQFSHAMDALLFRLYLLFMAS
Consensus/80%
...sP.Esp.ssst.W*chQp.....pppsp.pp.hcbWlpbtpsbD.LLF+lYLLbbtt


5HT3A            YSITLVMLWSIWQYA
```


5HT3B YTITLCSLWALWGGV
5HT3C SILTVIVLWNT----
5HT3E SIITVICLWNT----
5HT3D SIITVICLWNT----
Consensus/80% .hITllsLWsh....

Appendices 5: FASTA Results for Serotonin Receptor 5-HT 1A,1B,2A,2C,3A,4,5,6,7

FASTA Results for Serotonin Receptor 5-HT 1A,1B,2A,2C,3A,4,5,6,7

>sp|P08908|5HT1A_HUMAN 5-hydroxytryptamine receptor 1A OS=Homo sapiens GN=HTR1A PE=1 SV=3
MDVLSPGQGNNTTSPPAPFETGGNTTGISDVTVSYQVITSLLLGTLIFCAVLGNACVVAAIALERSLQNVANYLIGSLA
VTDLMVSVLVLPMAALYQVLNKWTLGQVTCDLFIALDVLCCTSSILHLCAIALDRYWAITDPIDYVNKRTPRRAAALISL
TWLIGFLISIPPMLGWRTPEDRSDPDACTISKDHGYTIYSTFGAFYIPLLLMLVLYGRIFRAARFRIRKTVKKVEKTGAD
TRHGASPAPQPKKSVNGESGSRNWRLGVESKAGGALCANGAVRQGDDGAALEVIEVHRVGNSKEHLPLPSEAGPT
PCAPASFERKNERNAEAKRKMALARERKTVKTLGIIMGTFILCWLPFFIVALVLPFCESSCHMPTLLGAIINWLGYSNS
LLNPVIYAYFNKDFQNAFKKIIKCKFCRQ

>sp|P28222|5HT1B_HUMAN 5-hydroxytryptamine receptor 1B OS=Homo sapiens GN=HTR1B PE=1 SV=1
MEEPGAQCAPPPPAGSETWVPQANLSSAPSQNCSAKDYIYQDSISLPWKVLLVMLLALITLATTLSNAFVIATVYRTR
KLHTPANYLIASLAVTDLLVSILVMPISTMYTVTGRWTLGQVVCDFWLSSDITCCTASILHLCVIALDRYWAITDAVEYS
AKRTPKRAAVMIALVWVFSISISLPPFFWRQAKAEEEVSECVVNTDHILYTVYSTVGAFYFPTLLLIALYGRIYVEARSRI
LKQTPNRTGKRLTRAQLITDSPGSTSSVTSINSRVPDVPSESGSPVYVNQVKVRVSDALLEKKKLMAARERKATKTL
GIILGAFIVCWLPFFIISLVMPICKDACWFHLAIFDFFTWLGYLNSLINPIIYTMSNEDFKQAFHKLIRFKCTS

>sp|P28223|5HT2A_HUMAN 5-hydroxytryptamine receptor 2A OS=Homo sapiens GN=HTR2A PE=1 SV=2
MDILCEENTSLSSTTNSLMQLNDDTRLYSNDFNSGEANTSDAFNWTVDSENRTNLSCEGCLSPSCLSLLHLQEKNW
SALLTAVVIILTIAGNILVIMAVSLEKKLQNATNYFLMSLAIADMLLGFLVMPVSMLTILYGYRWPLPSKLCAVWIYLDVLF
STASIMHLCAISLDRYVAIQNPIHHSRFNSRTKAFLKIIAVWTISVGISMPIPVFGLQDDSKVFKEGSCLLADDNFVLIGS
FVSFFIPLTIMVITYFLTIKSLQKEATLCVSDLGTRAKLASFSFLPQSSLSSEKLFQRSIHREPGSYTGRRTMQSISNEQ
KACKVLGIVFFLFVVMWCPFFITNIMAVICKESCNEDVIGALLNVFVWIGYLSSAVNPLVYTLFNKTYRSAFSRYIQCQY
KENKKPLQLILVNTIPALAYKSSQLQMGQKKNSKQDAKTTDNDCSMVALGKQHSEEASKDNSDGVNEKVSCV

>sp|P28335|5HT2C_HUMAN 5-hydroxytryptamine receptor 2C OS=Homo sapiens GN=HTR2C PE=1 SV=1
MVNLRNAVHSFLVHLIGLLVWQCDISVSPVAAIVTDIFNTSDGGRFKFPDGVQNWPALSIVIIIIMTIGGNILVIMAVSME
KKLHNATNYFLMSLAIADMLVGLLVMPLSLLAILYDYVWPLPRYLCPVWISLDVLFSTASIMHLCAISLDRYVAIRNPIEH
SRFNSRTKAIMKIAIVWAISIGVSVPIPVIGLRDEEKVFVNNTTCVLNDPNFVLIGSFVAFFIPLTIMVITYCLTIYVLRRQA
LMLLHGHTEEPPGLSLDFLKCCKRNTAEEENSANPNQDQNARRRKKKERRPRGTMQAINNERKASKVLGIVFFVFLI
MWCPFFITNILSVLCEKSCNQKLMEKLLNVFVWIGYVCSGINPLVYTLFNKIYRRAFSNYLRCNYKVEKKPPVRQIPRV
AATALSGRELNVNIYRHTNEPVIEKASDNEPGIEMQVENLELPVNPSSVVSERISSV

>sp|P50406|5HT6R_HUMAN 5-hydroxytryptamine receptor 6 OS=Homo sapiens GN=HTR6 PE=1 SV=1
MVPEPGPTANSTPAWGAGPPSAPGGSGWVAAALCVVIALTAAANSLLIALICTQPALRNTSNFFLVSLFTSDLMVGLV
VMPPAMLNALYGRWVLARGLCLLWTAFDVMCCSASILNLCLISLDRYLLILSPLRYKLRMTPLRALALVLGAWSLAAL
ASFLPLLLGWHELGHARPPVPGQCRLLASLPFVLVASGLTFFLPSGAICFTYCRILLAARKQAVQVASLTTGMASQAS
ETLQVPRTPRPGVESADSRRLATKHSRKALKASLTLGILLGMFFVTWLPFFVANIVQAVCDCISPGLFDVLTWLGYCN
STMNPIIYPLFMRDFKRALGRFLPCPRCPRERQASLASPSLRTSHSGPRPGLSLQQVLPLPLPPDSDSDSDAGSGGS
SGLRLTAQLLLPGEATQDPPLPTRAAAAVNFFNIDPAEPELRPHPLGIPTN

>sp|P34969|5HT7R_HUMAN 5-hydroxytryptamine receptor 7 OS=Homo sapiens GN=HTR7 PE=1 SV=2
MMDVNSSGRPDLYGHLRSFLLPEVGRGLPDLSPDGGADPVAGSWAPHLLSEVTASPAPTWDAPPDNASGCGEQIN
YGRVEKVVIGSILTLITLLTIAGNCLVVISVCFVKKLRQPSNYLIVSLALADLSVAVAVMPFVSVTDLIGGKWIFGHFFCN
VFIAMDVMCCTASIMTLCVISIDRYLGITRPLTYPVRQNGKCMAKMILSVWLLSASITLPPLFGWAQNVNDDKVCLISQ
DFGYTIYSTAVAFYIPMSVMLFMYYQIYKAARKSAAKHKFPGFPRVEPDSVIALNGIVKLQKEVEECANLSRLLKHERK
NISIFKREQKAATTLGIIVGAFTVCWLPFFLLSTARPFICGTSCSCIPLWVERTFLWLGYANSLINPFIYAFFNRDLRTTY
RSLLQCQYRNINRKLSAAGMHEALKLAERPERPEFVLRACTRRVLLRPEKRPPVSVWVLQSPDHHNWLADKMLTTV
EKKVMIHD

5HT3A
MLLWVQQALLALLLPTLLAQGEARRSRNTTRPALLRLSDYLLTNYRKGVRPVRDWRKPTTVSIDVIVYAILNVDEKNQ
VLTTYIWYRQYWTDEFLQWNPEDFDNITKLSIPTDSIWVPDILINEFVDVGKSPNIPYVYIRHQGEVQNYKPLQVVTAC
SLDIYNFPFDVQNCSLTFTSWLHTIQDINISLWRLPEKVKSDRSVFMNQGEWELLGVLPYFREFSMESSNYYAEMKF
YVVIRRRPLFYVVSLLLPSIFLMVMDIVGFYLPPNSGERVSFKITLLLGYSVFLIIVSDTLPATAIGTPLIGKAPPGSRAQS
GEKPAPSHLLHVSLASALGCTGVYFVVCMALLVISLAETIFIVRLVHKQDLQQPVPAWLRHLVLERIAWLLCLREQSTS
QRPPATSQATKTDDCSAMGNHCSHMGGPQDFEKSPRDRCSPPPPPREASLAVCGLLQELSSIRQFLEKRDEIREVA
RDWLRVGSVLDKLLFHIYLLAVLAYSITLVMLWSIWQYA

5HT4
MDKLDANVSSEEGFGSVEKVVLLTFLSTVILMAILGNLLVMVAVCWDRQLRKIKTNYFIVSLAFADLLVSVLVMPFGAIE
LVQDIWIYGEVFCLVRTSLDVLLTTASIFHLCCISLDRYYAICCQPLVYRNKMTPLRIALMLGGCWVIPTFISFLPIMQG
WNNIGIIDLIEKRKFNQNSNSTYCVFMVNKPYAITCSVVAFYIPFLLMVLAYYRIYVTAKEHAHQIQMLQRAGASSESRP
QSADQHSTHRMRTETKAAKTLCIIMGCFCLCWAPFFVTNIVDPFIDYTVPGQVWTAFLWLGYINSGLNPFLYAFLNKS
FRRAFLIILCCDDERYRRPSILGQTVPCSTTTINGSTHVLRDAVECGGQWESQCHPPATSPLVAAQPSDT

Appendices 6: GOPMS Information Leaflet

Women & Children's Division
Maternity Building
Newcastle Road
Stoke on Trent
ST4 6QG

Email: gail.chapman@uhns.nhs.uk

GENETICS OF PREMENSTRUAL SYNDROME

INVITATION TO TAKE PART IN RESEARCH INTO THE ROLE GENETICS PLAY IN PREMENSTRUAL SYNDROME AND PREMENSTRUAL DYSPHORIC DISORDER.

We would like to invite you take part in a research study. Before you decide whether or not you wish to take part it is important that you understand why we are doing the study and how you will be involved if you do take part. Please read the following information carefully. Discuss it with your partner and relatives if you wish. Ask if there is anything you do not understand or if you would like more information. You will be given as much time as want to make a decision and at any point you can change your mind.

WHAT ARE WE AIMING TO DO?

Premenstrual symptoms (PMS) affects 95% women during their reproductive years. Approximately 5% suffer from the more severe disorder, this is called premenstrual dysphoric disorder. In previous studies oestrogen and progesterone (hormones from the ovary) levels have been taken from women with and those without PMS, no differences have been shown. However, we do know that PMS occurs during the progesterone part of the cycle. In a research setting, serotonin (a brain hormone) levels have been shown to be lower during this part of the cycle in women with PMS and remain normal in women without PMS. By understanding which genes cause this, it may be possible to improve treatment, or even prevent the occurrence.

WHY ARE WE ASKING YOU FOR YOUR HELP?

PMS, as you are aware, is very disruptive to family life and relationships; this will be the main reason most of you have come to see us for advice on PMS. We are looking for women with and without the disorder so we can detect any difference in their genes.

We are seeking help from 100 women with varying degrees of the condition, so that we may understand why there are theses differences.

The white cells in your blood carry a complete set of your genes which we can study.

We would like to store some of these white blood cells in the laboratory because we believe it will take some years and experiments to fully understand the cause of premenstrual syndrome. For every new test or experiment on the white cells a new application will be made to the Ethic Committee for their approval before we can start. These tests will only be carried out for premenstrual syndrome and no other condition.

WHO IS ORGANISING THE STUDY?

Professor O'Brien is the Professor of Obstetrics and Gynaecology with Keele University and North Staffordshire Hospital (NHS) Trust. He has a longstanding interest in PMS and its treatments He will be leading a team consisting of research doctor, sister and clinical scientist. The study is being funded by departmental funds.

WHAT WILL WE BE ASKING YOU TO DO?

Some of you will have already been screened to define how severe you have the disease. For those of you who have not, we will ask you to complete daily diaries for 2 to 3 menstrual cycle, to measure the severity of your condition.

We will be asking you to donate a 30ml blood sample – one large syringe full, this will be taken through a needle from a vein in your arm. This usually causes only brief discomfort and occasionally a small bruise. We also need a 10ml sample of urine, this is like a specimen you would give for a pregnancy test. The sample will

be used to study the genes which we think differ in PMS and serotonin levels. We will ask some questions about your medical history, as well assess any other mood disorders you may have.

DO I HAVE TO TAKE PART?

Your taking part in this study is voluntary. If you would prefer not to take part you do not need to give a reason. You may also withdraw form the study at any time. This will not affect your medical treatment. We cannot guarantee to discover anything that will be of directly benefit you. Neither you nor the researchers will benefit financially from this research.

WHO WILL SEE MY RECORDS AND KNOW ABOUT ME TAKING PART?

The Research Doctors and Sister are the only ones who need to see your records and no other health professional needs to be made aware of your participation. The

information collected about you during the course of the study will be kept confidential. The result of the genetic testing on named individuals will not be available to any of the researchers, and the results of tests performed on your genes will not be divulged to you.

WILL MY EXPENSES BE PAID?

You will be offered payment for all reasonable additional expenses for taking part in the study, such as travelling expenses.

WHOM DO I CONTACT WITH ANY CONCERNS?

If you have any problems, concerns or other questions about this study, you should preferably first contact Gail Chapman, Research Sister on 01782 552449, where there is a “PMS Hotline” if you wish to leave a message or Professor O’Brien, via his secretary, on 01782 552472 at Academic Department of Obstetric and Gynaecology, Maternity Unit, City General Hospital, Newcastle Rd, Stoke on Trent. ST4 6QG.Alternatively you may contact Dave Crowley, Chief Executive, on 01782 715444, North Staffordshire Hospital (NHS) Trust, City General Hospital, Newcastle Rd, Stoke on Trent. ST4 6QG.

Appendices 7: GOPMS RECRUITMENT SHEET

GOPMS
Recruitment Sheet

Study No ……..…. **Subject Initials**

DOB / /

Name……..

Address…….

…….

…….

Post Code……..

Date Enrolled ……/……/……

Appendices 8: GOPMS DATA SHEET

GOPMS
Data Sheet

Study No ……..…. **Subject Initials**

DOB / /

Measure used to confirm diagnosis

DRSP SYMPTOMETRICS Device

COPE VAS

Other (Specify:……)

Diagnosis PMDD Control

Symptoms predominately Physical Psychological

Years of Problem ……..

Onset coincided with

Menarche Childbirth

Oral Contraceptive Sterilisation

Other (Describe ……..)

None

Family History of PMS/PMDD Yes No

Mother Daughter Sister Twin

Past medication
1 = not effective 2 = mild 3 = moderate 4 = effective 5 = very effective

Medication	**Effectiveness (1-5)**

Present medication
0 = no treatment 1 = not effective 2 = mild 3 = moderate 4 = effective 5 = very effective

Medication	**Effectiveness (1-5)**

Appendices 9: Control group – DRSP scores

Study Number	Depression Follicular	Depression Luteal	Depression Difference	Tension Follicular	Tension Luteal	Tension Difference	Lability Follicular	Lability Luteal	Lability Difference	Irritability Follicular	Irritability Luteal	Irritability Difference	Relationship Follicular	Relationship Luteal	Relationship Difference
1	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**
2	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**
6	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**
9	7	11	**57%**	7	7	**0%**	7	9	**29%**	7	16	**129%**	7	11	**57%**
12	9	12	**33%**	20	25	**25%**	12	18	**50%**	18	24	**33%**	19	18	**-5.26%**
18	18	19	**5%**	21	27	**33%**	16	22	**37%**	24	30	**25%**	21	21	**0%**
22	7	10	**42%**	7	7	**0%**	7	7	**0%**	7	10	**42%**	7	7	**0%**
23	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**
24	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**
26	25	28	**12%**	26	33	**26%**	24	27	**13%**	20	25	**25%**	29	26	**-10%**
27	20	25	**25%**	16	30	**88%**	17	30	**76%**	17	30	**76%**	14	27	**93%**
30	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**
32	7	9	**29%**	10	10	**0%**	9	8	**-11%**	10	12	**20%**	8	12	**50%**
35	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**
37	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**
38	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**
39	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**
41	7	8	**14%**	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**
43	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**

Appendices 9: Control group – DRSP scores (continued)

Study Number	Depression Follicular	Depression Luteal	Depression Difference	Tension Follicular	Tension Luteal	Tension Difference	Lability Follicular	Lability Luteal	Lability Difference	Irritability Follicular	Irritability Luteal	Irritability Difference	Relationship Follicular	Relationship Luteal	Relationship Difference
46	7	8	**14%**	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**
49	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**
50	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**
51	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**
54	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**
55	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**
56	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**
57	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**
58	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**
59	7	11	**57%**	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**
61	9	7	**-22%**	9	7	**-22%**	9	7	**-22%**	9	9	**0%**	7	7	**0%**
62	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**
63	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	9	**29%**
65	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**
66	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**
73	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**
74	7	7	**0%**	7	12	**71%**	7	9	**29%**	10	11	**10%**	9	9	**0%**
86	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**
88	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**

Appendices 9: Control group – DRSP scores (continued)

Study Number	Depression Follicular	Depression Luteal	Depression Difference	Tension Follicular	Tension Luteal	Tension Difference	Lability Follicular	Lability Luteal	Lability Difference	Irritability Follicular	Irritability Luteal	Irritability Difference	Relationship Follicular	Relationship Luteal	Relationship Difference
89	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**
90	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**
94	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**
95	7	9	**29%**	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**
96	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**
97	7	7	**0%**	7	7	**0%**	7	10	**42%**	7	7	**0%**	7	7	**0%**
98	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**
99	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**
100	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**
102	7	7	**0%**	7	8	**14%**	7	7	**0%**	7	7	**0%**	7	7	**0%**
103	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**
104	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	11	**57%**	7	7	**0%**
105	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**	7	7	**0%**
106	7	7	**0%**	7	10	**42%**	7	7	**0%**	7	7	**0%**	7	7	**0%**

Appendix 10: PMDD group – DRSP scores

Study Number	Depression Follicular	Depression Luteal	Depression Difference	Tension Follicular	Tension Luteal	Tension Difference	Lability Follicular	Lability Luteal	Lability Difference	Irritability Follicular	Irritability Luteal	Irritability Difference	Relationship Follicular	Relationship Luteal	Relationship Difference
3	11	25	**127%**	14	28	**100%**	7	7	**0%**	11	25	**127%**	8	34	**325%**
4	7	29	**314%**	7	41	**485%**	7	26	**271%**	8	40	**400%**	12	39	**225%**
5	9	25	**178%**	9	31	**244%**	10	29	**190%**	10	31	**210%**	7	24	**242%**
7	7	7	**0%**	7	42	**500%**	7	28	**300%**	7	27	**285%**	7	10	**42%**
8	9	18	**100%**	7	14	**100%**	8	17	**100%**	7	15	**100%**	7	14	**100%**
10	11	27	**145%**	15	15	**0%**	13	26	**100%**	16	12	**-25%**	12	28	**133%**
11	9	21	**133%**	10	28	**180%**	7	20	**185%**	7	21	**200%**	9	27	**200%**
13	9	19	**111%**	8	16	**100%**	7	14	**100%**	9	19	**100%**	9	20	**100%**
14	15	19	**27%**	17	29	**70%**	13	31	**138%**	19	31	**63%**	10	25	**150%**
15	9	11	**22%**	12	10	**-17%**	7	21	**200%**	12	18	**50%**	10	20	**100%**
16	7	22	**214%**	8	23	**187%**	7	23	**228%**	8	27	**237%**	7	23	**228%**
17	13	26	**100%**	19	34	**78%**	31	34	**10%**	14	34	**142%**	11	37	**236%**
19	14	34	**142%**	12	33	**175%**	21	30	**43%**	16	19	**19%**	14	33	**106%**
20	12	38	**216%**	13	41	**215%**	7	35	**400%**	12	36	**200%**	13	31	**138%**
21	8	9	**13%**	8	8	**0%**	7	25	**257%**	7	7	**0%**	7	10	**43%**
25	7	15	**114%**	8	18	**125%**	7	16	**129%**	9	21	**133%**	7	14	**100%**
28	8	24	**200%**	8	21	**163%**	7	22	**214%**	8	22	**175%**	7	25	**257%**

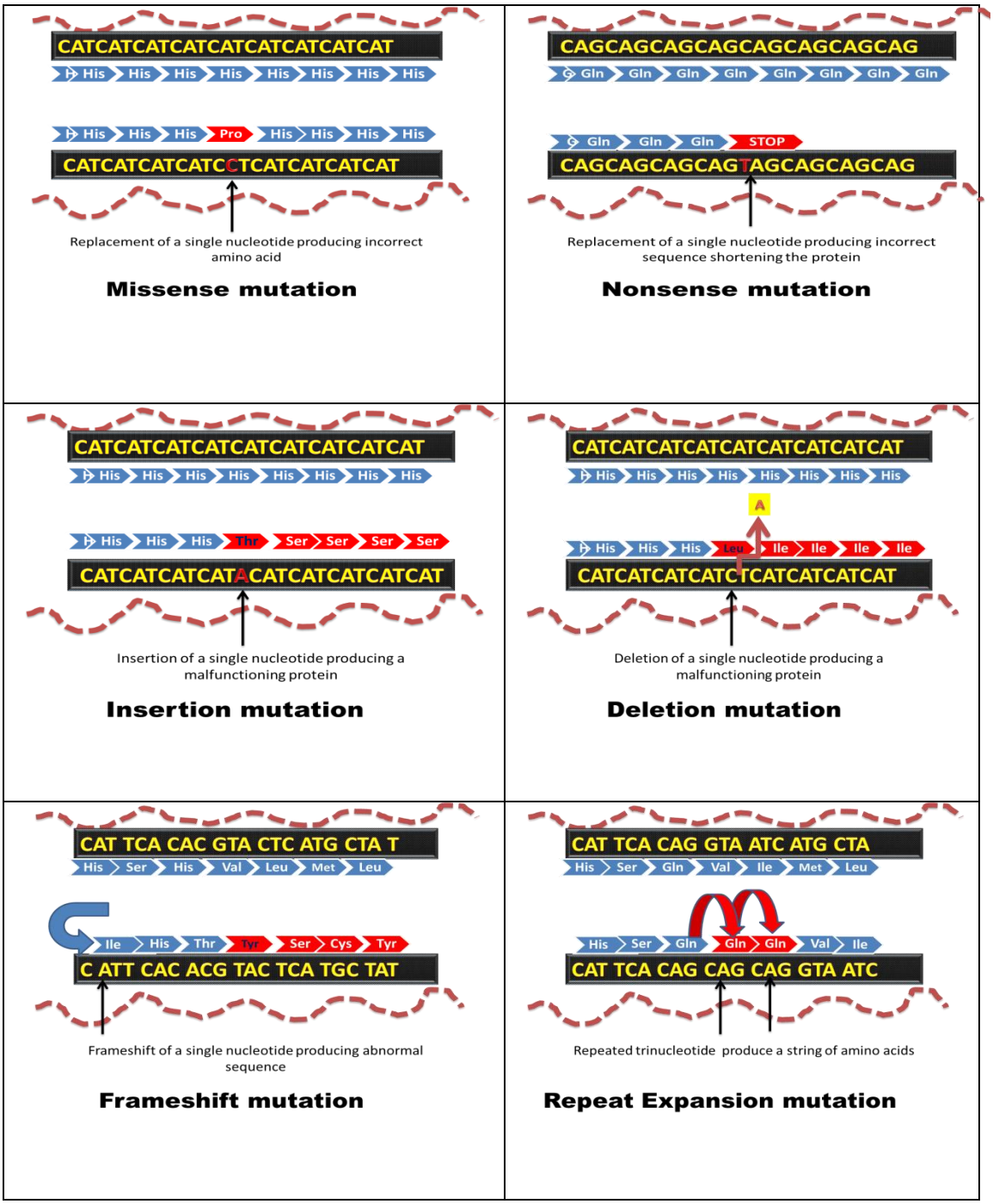
Appendices 10: PMDD group – DRSP scores (continued)

Study Number	Depression Follicular	Depression Luteal	Depression Difference	Tension Follicular	Tension Luteal	Tension Difference	Lability Follicular	Lability Luteal	Lability Difference	Irritability Follicular	Irritability Luteal	Irritability Difference	Relationship Follicular	Relationship Luteal	Relationship Difference
29	8	15	**100%**	8	21	**162%**	12	16	**33%**	13	26	**100%**	15	31	**100%**
31	8	34	**325%**	9	40	**344%**	8	28	**250%**	11	40	**263%**	7	28	**300%**
34	7	34	**385%**	9	32	**255%**	8	30	**275%**	8	35	**337%**	7	37	**428%**
36	7	22	**214%**	7	27	**285%**	7	31	**342%**	7	25	**257%**	7	23	**229%**
40	8	29	**262%**	7	35	**400%**	7	31	**342%**	7	34	**385%**	7	34	**385%**
42	7	10	**43%**	7	21	**200%**	8	7	**-14%**	7	21	**200%**	9	21	**133%**
44	9	31	**244%**	11	36	**227%**	14	31	**121%**	8	35	**325%**	8	34	**325%**
45	7	28	**300%**	7	28	**300%**	7	28	**300%**	7	28	**300%**	7	28	**300%**
47	9	36	**300%**	9	40	**344%**	9	39	**333%**	11	40	**263%**	10	39	**290%**
48	7	34	**385%**	7	36	**385%**	7	29	**314%**	7	35	**385%**	7	32	**320%**
52	7	31	**342%**	7	31	**342%**	7	35	**400%**	7	32	**357%**	7	24	**242%**
53	8	16	**100%**	10	25	**150%**	7	16	**129%**	7	17	**143%**	8	16	**100%**
60	7	21	**200%**	8	19	**138%**	7	16	**129%**	8	20	**150%**	9	19	**111%**
64	8	12	**50%**	10	22	**120%**	8	18	**125%**	9	15	**67%**	7	14	**100%**
67	7	31	**342%**	7	24	**242%**	7	29	**314%**	7	21	**200%**	7	19	**171%**
68	7	22	**214%**	10	38	**280%**	7	35	**400%**	11	37	**236%**	8	35	**337%**
69	7	19	**171%**	7	19	**171%**	7	7	**0%**	7	19	**171%**	7	16	**129%**
70	7	17	**143%**	8	11	**38%**	7	12	**71%**	8	19	**138%**	10	18	**80%**
71	7	23	**229%**	7	26	**271%**	7	27	**289%**	7	28	**300%**	7	24	**242%**
72	7	25	**257%**	7	28	**300%**	7	28	**300%**	7	27	**285%**	7	42	**500%**

Appendices 10: PMDD group – DRSP scores (continued)

Study Number	Depression Follicular	Depression Luteal	Depression Difference	Tension Follicular	Tension Luteal	Tension Difference	Lability Follicular	Lability Luteal	Lability Difference	Irritability Follicular	Irritability Luteal	Irritability Difference	Relationship Follicular	Relationship Luteal	Relationship Difference
75	7	32	**357%**	7	32	**357%**	7	32	**357%**	7	32	**357%**	7	32	**357%**
76	13	26	**100%**	10	28	**180%**	10	28	**180%**	9	29	**222%**	7	31	**342%**
77	20	37	**85%**	19	36	**89%**	17	35	**105%**	13	33	**154%**	16	34	**113%**
78	9	21	**133%**	8	22	**175%**	9	16	**77%**	9	14	**55%**	8	17	**113%**
79	7	18	**157%**	9	12	**33%**	7	18	**157%**	8	19	**138%**	7	22	**214%**
80	7	29	**314%**	7	41	**485%**	7	26	**271%**	8	38	**375%**	13	39	**200%**
81	15	21	**40%**	20	26	**30%**	15	22	**47%**	11	33	**200%**	7	22	**214%**
82	9	19	**111%**	7	21	**200%**	7	22	**214%**	14	24	**71%**	8	24	**200%**
83	10	20	**100%**	9	23	**156%**	8	17	**113%**	8	21	**163%**	9	20	**122%**
84	7	11	**57%**	7	20	**186%**	7	7	**0%**	7	20	**186%**	7	21	**200%**
85	12	26	**117%**	9	20	**122%**	7	19	**171%**	8	17	**113%**	7	14	**100%**
87	7	14	**100%**	8	19	**138%**	12	29	**142%**	10	21	**110%**	9	18	**100%**
91	9	12	**33%**	7	18	**157%**	8	26	**225%**	13	30	**131%**	7	20	**186%**
93	12	28	**133%**	9	19	**111%**	8	22	**175%**	9	21	**133%**	10	22	**120%**
101	11	21	**90%**	10	24	**124%**	8	24	**200%**	8	17	**113%**	9	22	**144%**
107	7	16	**129%**	8	19	**138%**	8	26	**225%**	9	19	**111%**	19	22	**120%**

Appendices 11: Types Of Genetic Mutations



Appendices 12: Reagents for the extraction of DNA from Whole blood:

- *Reagent A*

  10 ml of 1M Trizma-HCL [Sigma T-5941]

  109.5 g of sucrose [Sigma S-7903]

  1.02 g of $MgCl_2.6H_2O$ [Sigma M-2670]

  10 ml of Triton X-100

  Approximately 800 ml deionised water

  Adjust the pH to 8.0 with 2 M NaOH, and make up to 1 litre with deionised water.

  Autoclave before use.

  Store at room temperature.

- *Reagent B*

  100 ml of 1M Trizma-HCL pH 8.0

  30 ml of 0.5 M EDTA pH 8.0 [Sigma EDS]

  15 ml of 2.5 M NaCl [Sigma S-3014]

  Make up to 237.5 ml with deionised water.

  Autoclave at this stage.

  Before use, add 12.5 ml of 20% w/v SDS.

  Store at room temperature.

- *5M Sodium perchlorate*

  Dissolve 70.25 g of $NaHClO_3$ [Sigma S-1041] in 100 ml of deionised water (no autoclaving required).

  Store at room temperature.

Appendices 13: Reagents for PCR

- *Stock Primers*

  Thaw at room temperature

  Vortex

  Dilute 1:20 stock primers: sterile water

  25µL (5µl) stock primer + 475µl (95µl) sterile water

  Spiral mix

  Keep on ice

- *dNTP's*

  100 mM solutions of dATP, dCTP, dGTP and dTTP.

  Vortex

  10µL of each solution were pipetted into a single sterile 1.5mL

  Eppendorf tube

  160µL of sterile water added to give a working solution of 5 mM.

  Store @ -20°C Temperature

- *Taq DNA polymerase in storage buffer A(100 units)*

  Storage buffer A consists of 50 mM Tris-HCL (pH 8.0 at 250C),

  100 mM NaCl,

  0.1 mM EDTA,

  1 mM DTT,

  50 % glycerol and 1% Triton X-100.

  Store at -20°C

  Thaw and vortex before use

- *Taq DNA Polymerase 10X Buffer containing 15 mM MgCl2*

  50 KCl,

  10 mM Tris-HCL (pH 9.0 at 250C),

  1.5mM MgCl2 and 0.1 % Triton X-100 when diluted 1 in 10

  Stored at -20°C

  Thaw and vortex before use

Appendices 14: Solutions for agarose gel

- *Tris-Borate-EDTA buffer [TBE 10X]*

  107.8 g Tris base

  1.44g (40ml of .5M EDTA solution Ph 8.0

  55.0 g boric acid

  Dissolve in 800 mL deionized water, then bring total volume to 1 L with deionized water

  Store in room temperature

- *Agarose*

  100ml of 1X TBE

  1,2 or 3gms of Agarose to produce 1%,2% or 3% gel

- *Ethidium Bromide*

  10mg/ml ethidium bromide.

  Prepare stock in deionized water (1g/100ml)

  Store at 4°C in dark

- *Gel loading buffer*

  0.125gms of 0.25% bromophenol blue

  20gms of 40%sucrose in 50ml deionized water (heat)

  0.125gms of 0.25% xylene cyanol

  Store in room temperature

* Ethidium bromide is a powerful mutagen/carcinogen and is moderately toxic. Gloves should always be worn when handling solutions and gels containing ethidium bromide.

Appendices 15: Solutions for polyacrylamide gel

- *Fixative*

  5 mL acetic acid
  100 mL Methanol

  Dissolve in 1lt deionized water

  Store in room temperature

- *40% acrylamide:bis (29:1)*

  290 g acrylamide
  10 g bisacrylamide

  dissolve in 250 mL deionized water, then bring total volume to

  500mL with deionized water

  store at 4°C

- *10X TBE buffer*

  107.8 g Tris base
  1.45g EDTA

  55.0 g boric acid

  Dissolve in 800 mL deionized water, then bring total volume to 1 L

  with deionized water

  Store in room temperature

- *10% Ammonium persulphate APS*

  10dms APS
  100 mL deionized water

  Store in room temperature

- *staining solution*

  1 g silver nitrate
  1000 mL deionized water

  Store in dark bottle and room temperature

- *developer solution*

  15gms of sodium hydroxide
  1000 mL deionized water

  Store in room temperature

Appendices 16: 5-HT1A (-C1019G) Designing Primers

www.ensembl.org : To obtain the nucleotide sequence

CTAGCCACAAAGCTATGGGAAGTGGCAGTGTCACTGAAATTACAAGTGTAGTAGTGATGG
AAAAGTGTGTGTGTGTTTAGAATATATATCACACTGAGTTTTGTTCTTCATTTCGAGATG
CAGTTGTTTACCTCTCCTTGTCCTTTGACACGTCCTTTATAATTTCGTTCTCTCCCGGTT
CCCCAACGTTAAAAAAAAAGTCACAGGCAATATTCTCCCTGAGGGAGTAAGGCTGGACTG
TTAGATGATAACGGAGGTACCGTTTTGTTGTTGTTGTCGTCGTTGTTCGTTTGTTTTTGG
AGACGGAGTCTCGCTCTGTCGCCCAGGCTGGAGTGCAATGGCGCGAGAACGGAGGTAGCT
TTTTAAAAACGAAGACACACTCGGTCTTCTTCCATCAATTAGCAATAATTGGGAGACTGA
CCCAGGACTGTTCACCTTCCCATTCAGGCTCCCTATGCTTCCTTTTCTCATCTCCTATTG
CCACTCTGGGATGCTGACACGATTTAAGAATTTGGCAGATAATATGAGGCAAGGAGTAGT
TGGAATTCCCTCCCCCAAGTTTTTCCAACCCCAGTTTTGCTGGGTTGGAGGCGGAGTTTA
TTTGTTACAACCTTGGTCTGACCGGCAGGGATCTGGTGTGTGTAAGTGAGTTCTGAGTCT
CTGTTGACAAAAAGAGACTCGAATGCAAAGACGCTGAGCTAGAGGGAGAGGAGGGCGGGG
ACCCAGAGGAAAGAGGCACTCCTCGGGGTTGGGGAAGTATTAGGAGGGGAGGGTTAGAGT
GGGAGGGAAGGAGCCTGGCTTTCGAAGCGACTCACAGAGGGATAAATAAAGGGAAGTGAG
GAGGAAGAGGGAGACTGAAAGGGAAGGCAGGTGGGGAGAAGGGGGACGAAAGAGGCAGAA
GAGAGAGAAGAGAGGAGGAGAGAGGGGGAGAGAGGGAAGGAAGGAAATAGGGAGAGGAGG
GTCACAGAGTGACCGTGGAGGATGGGGCTTCTCGGTTCTAG**ATATTTCTGGGATTGGAGA**
CTGTTTGCTAGTGGGGAGACTCCAGCTCCGGCAGCCAGTTCGGGAGCGGCAAAGTAAAAT
GGACAGCGACAGACAGACGTTCCAGCCACCTCTCCGCCGCCGGGAGATCCTGGAGCTGCT
TTCAGGCCAACTCCAGTTTCCCAGCTGGAGCTTCTGAACGCGCTGGACTGCGAGAGCCCA
GGGAGCGCCTGAAAGCTGCTCCTCGGAGATACCCTTCGCCGAAGCAGTAAGAACTTCCTG
CTTGGGTCTCTGCATTCCCTTCCTCCGAAACTTCCCAGGAGAAGGGCGGAAGACCCCAGG
GGAAGGGGCGAGGCGAATCTTCGCGCTGCTTTTTCTTCCCTCCCCCTTCCCGCGCCGGGC
GCGCAGGCATGGATGTGCTCAGCCCTGGTCAGGGCAACAACACCACATCACCACCGGCTC
CCTTTGAGACCGGCGGCAACACTACTGGTATCTCCGACGTGACCGTCAGCTACCAAGTGA
TCACCTCTCTGCTGCTGGGCACGCTCATCTTCTGCGCGGTGCTGGGCAATGCGTGCGTGG
TGGCTGCCATCGCCTTGGAGCGCTCCCTGCAGAACGTGGCCAATTATCTTATTGGCTCTT
TGGCGGTCACCGACCTCATGGTGTCGGTGTTGGTGCTGCCCATGGCCGCGCTGTATCAGG
TGCTCAACAAGTGGACACTGGGCCAGGTAACCTGCGACCTGTTCATCGCCCTCGACGTGC
TGTGCTGCACCTCATCCATCTTGCACCTGTGCGCCATCGCGCTGGACAGGTACTGGGCCA
TCACGGACCCCATCGACTACGTGAACAAGAGGACGCCCCGGCGCGCCGCTGCGCTCATCT
CGCTCACTTGGCTTATTGGCTTCCTCATCTCTATCCCGCCCATGCTGGGCTGGCGCACCC
CGGAAGACCGCTCGGACCCCGACGCATGCACCATTAGCAAGGATCATGGCTACACTATCT
ATTCCACCTTTGGAGCTTTCTACATCCCGCTGCTGCTCATGCTGGTTCTCTATGGGCGCA
TATTCCGAGCTGCGCGCTTCCGCATCCGCAAGACGGTCAAAAAGGTGGAGAAGACCGGAG
CGGACACCCGCCATGGAGCATCTCCCGCCCCGCAGCCCAAGAAGAGTGTGAATGGAGAGT
CGGGGAGCAGGAACTGGAGGCTGGGCGTGGAGAGCAAGGCTGGGGGTGCTCTGTGCGCCA
ATGGCGCGGTGAGGCAAGGTGACGATGGCGCCGCCCTGGAGGTGATCGAGGTGCACCGAG
TGGGCAACTCCAAAGAGCACTTGCCTCTGCCCAGCGAGGCTGGTCCTACCCCTTGTGCCC
CCGCCTCTTTCGAGAGGAAAAATGAGCGCAACGCCGAGGCGAAGCGCAAGATGGCCCTGG
CCCGAGAGAGGAAGACAGTGAAGACGCTGGGCATCATCATGGGCACCTTCATCCTCTGCT
GGCTGCCCTTCTTCATCGTGGCTCTTGTTCTGCCCTTCTGCGAGAGCAGCTGCCACATGC
CCACCCTGTTGGGCGCCATAATCAATTGGCTGGGCTACTCCAACTCTCTGCTTAACCCCG
TCATTTACGCATACTTCAACAAGGACTTTCAAAACGCGTTTAAGAAGATCATTAAGTGTA
AGTTCTGCCGCCAGTGATGACGGAGGAGTAGCCGGCCAGTCGAGGCTACAGGATCCGTCC
CATTCACTATGCTTCCCCCAACCCTAGGGAATCAACACTTAAGATAATTCGCCACTTCTC
CTCTTTCTCTCTGCTCCGCTCACGGCTTGCAGACCTGGTCCCCTCCCCACTTCCTGCTCC
ACGGCAGGGCCCTTTGTGCAAAGGAGACCCAGCGGAGGAGCGTTGAGAGCCCAGGAAATT
CAGAGAGTTTGTGAGAAGCGACATTGGCTCAGACTTCGCCTGTATCATCAGTTTTGATCC
CAGTAATTGCCTCTTCTCTCTTCTATCTCCTAAATCTTTGCGGTGGATGTTTAATGCTTA
GTTCAAGGCAGAAAATCCAAAAAATAAATAAACTGTACACACAGCCGCCGCGCCCACTAA
GGGGCTCCACCCCATCACCCAACTTCTAGAACTTGGACTTGAGGTTTGGGAATTTGCTCC
ATCTCTCCCGCCCTGTGGCCCTTGAAATATTCACCTTTTTCTCCCCATTTTTATCTTTTC
TTTTTTTCATTGCTTCCCTCTCTGCCCTCGCCCATCCTTATGCACGAATCTGCTGGTCCG
GAGAGGCGGAAGAGGCTGCTTTTCCCCGTGCCAGCGGTGCCCCCGGGATTCCCCGTCTTC
TCGCAGACTTGAGATTAACTCTCTCGTGCAGTGCACAACCCCTATTTCCTTT

Primer picked using http://frodo.wi.mit.edu and the general rules to design these primers applied:

Primer3 Output

```
PRIMER PICKING RESULTS FOR 5HT1A

No mispriming library specified
Using 1-based sequence positions
WARNING: Right primer is unacceptable: High end self complementarity

OLIGO            start  len      tm      gc%   any   3'  seq
LEFT PRIMER        225   23   57.22    47.83  3.00  0.00 GAGTAAGGCTGGACTGTTAGATG
RIGHT PRIMER       393   22   59.78    50.00  7.00  7.00 GGAAGAAGACCGAGTGTGTCTT
SEQUENCE SIZE: 420
INCLUDED REGION SIZE: 420

PRODUCT SIZE: 169, PAIR ANY COMPL: 3.00, PAIR 3' COMPL: 1.00

    1 CTAGCCACAAAGCTATGGGAAGTGGCAGTGTCACTGAAATTACAAGTGTAGTAGTGATGG

   61 AAAAGTGTGTGTGTGTTTAGAATATATATCACACTGAGTTTTGTTCTTCATTTCGAGATG

  121 CAGTTGTTTACCTCTCCTTGTCCTTTGACACGTCCTTTATAATTTCGTTCTCTCCCGGTT

  181 CCCCAACGTTAAAAAAAAAGTCACAGGCAATATTCTCCCTGAGGGAGTAAGGCTGGACTG
                                                >>>>>>>>>>>>>>>>

  241 TTAGATGATAACGGAGGTACCGTTTTGTTGTTGTTGTCGTCGTTGTTCGTTTGTTTTTGG
      >>>>>>>

  301 AGACGGAGTCTCGCTCTGTCGCCCAGGCTGGAGTGCAATGGCGCGAGAACGGAGGTAGCT

  361 TTTTAAAAAGGAAGACACACTCGGTCTTCTTCCATCAATTAGCAATAATTGGGAGACTGA
                  <<<<<<<<<<<<<<<<<<<<<<

KEYS (in order of precedence):
>>>>>> left primer
<<<<<< right primer

Statistics
Pair Stats:
considered 1, ok 1
primer3 release 1.1.4
(primer3_results.cgi release 0.4.0)
```

Apendices 17: 5-HT1B (A-161T) Designing Primers

www.ensembl.org : To obtain the nucleotide sequence

GGCGTCCGCACCCGCCGCGCCGCACGAGTTGCACTGCTCTGGCGGACCGGACCTGGACTC
TATATAAAGAGCCCATCTGCTCCGTAGCTCGCACGCTTCTCCCGGGCTGGTGCACGCCGC
GTCCCTCCAGCTCCCCCAGACACCTGCCCCTTCCCAGTGTGCCGCGCCAGGTCCTCCAGA
CCCGCGCACCCAGTGGCATGGCTCCGAGTGGCTCCCGTGGGACCAGGGTGGCGGTGGCGG
CGGCGCGGCCCGAGCAGCCGCAACTCCAGCCCCCGTGTCCCCCCTTTTATGGCTCCGTCT
CCGCGGGGCAGCTCGTCCGAGTGGCCAGAGAGTGAAAAGAGAGGGAGGGCAGAGCTCCGG
CGCGAGGCGCGGCGCAGCGCTGCTCCTAGACTTCACCCCACCCAGCTCTGGCGGCCGCTG
CAGCCCCCCAAAAGTGCCCCAGCTTGGGGCGAGGGGTGGGAATGCAAGATCTCGGGACCT
CTCGCTGGCCTGCAAGCTTTGGTCTCTACACCTAGGAAACTCCTGTGGGCAAAGTCTGCA
GATCCAAAAGCGTCCAGGTTAGGAGACGCTCAGCCTCAAGCAACTGGGGTAAGAGATCCC
ATTTGGTCAAAGCCTTCTC CTCAAGCAGTACTTCACCCTCCTGCACTAGACGCCTCCAGG
GAGCTGGAGCGGAGCAGGGCTCGGTGGGCCAGCTCTTAGCAACCCAGGTCTAAGACCCGG
TGTGGAGAGGAACAACCACAGACGCGGCGGCTTAGCTAGGCGCTCTGGAAGTGCAGGGGA
GGCGCCCGCCTGCCTTGGCTGCCGCACCCATGACCTCTAGTTTCAGCTGTGAACCTGGGC
GGAGGAATAATTGAGGAACTCACGGAACTATCAACTGGGGACAAACCTGCGATCGCCACG
GTCCTTCCGCCCTCTCCTTCGTCCGCTCCATGCCCAAGAGCTGCGCTCCGGAGCTGGGGC
GAGGAGAGCCATGGAGGAAC CGGGTGCTCAGTGCGCTCCACCGCCGCCCGCGGGCTCCGA
GACCTGGGTTCCTCAAGCCAACTTATCCTCTGCTCCCTCCCAAAACTGCAGCGCCAAGGA
CTACATTTACCAGGACTCCATCTCCCTACCCTGGAAAGTACTGCTGGTTATGCTATTGGC
GCTCATCACCTTGGCCACCACGCTCTCCAATGCCTTTGTGATTGCCACAGTGTACCGGAC
CCGGAAACTGCACACCCCGGCTAACTACCTGATCGCCTCTCTGGCGGTCACCGACCTGCT
TGTGTCCATCCTGGTGATGCCCATCAGCACCATGTACACTGTCACCGGCCGCTGGACACT
GGGCCAGGTGGTCTGTGACTTCTGGCTGTCGTCGGACATCACTTGTTGCACTGCCTCCAT
CCTGCACCTCTGTGTCATCGCCCTGGACCGCTACTGGGCCATCACGGACGCCGTGGAGTA
CTCAGCTAAAAGGACTCCCAAGAGGGCGGCGGTCATGATCGCGCTGGTGTGGGTCTTCTC
CATCTCTATCTCGCTGCCGCCCTTCTTCTGGCGTCAGGCTAAGGCCGAAGAGGAGGTGTC
GGAATGCGTGGTGAACACCGACCACATCCTCTACACGGTCTACTCCACGGTGGGTGCTTT
CTACTTCCCCACCCTGCTCCTCATCGCCCTCTATGGCCGCATCTACGTAGAAGCCCGCTC
CCGGATTTTGAAACAGACGCCCAACAGGACCGGCAAGCGCTTGACCCGAGCCCAGCTGAT
AACCGACTCCCCCGGGTCCACGTCCTCGGTCACCTCTATTAACTCGCGGGTTCCCGACGT
GCCCAGCGAATCCGGATCTCCTGTGTATGTGAACCAAGTCAAAGTGCGAGTCTCCGACGC
CCTGCTGGAAAAGAAGAAACTCATGGCCGCTAGGGAGCGCAAAGCCACCAAGACCCTAGG
GATCATTTTGGGAGCCTTTATTGTGTGTTGGCTACCCTTCTTCATCATCTCCCTAGTGAT
GCCTATCTGCAAAGATGCCTGCTGGTTCCACCTAGCCATCTTTGACTTCTTCACATGGCT
GGGCTATCTCAACTCCCTCATCAACCCCATAATCTATACCATGTCCAATGAGGACTTTAA
ACAAGCATTCCATAAACTGATACGTTTTAAGTGCACAAGTTGACTTGCCGTTTGCAGTGG
GGTCGCCTAAGCGACCTTTGGGGACCAAGTTGTGTCTGGTTCCACAGGTAGGTCGAATCT
TCTTTCGCGGTTTCTGGGTCCCAGCGAGGCTCTCTCTCCTGGGCAAGGGCAATGGATCCT
GAGAAGCCAGAATAGTCCTGAGAGAGAGCTCTGAAAGGAGAAGTGTTGAAACTAAATGTA
GAGCTTCCCTGCCCAGGAGGAGGCTCACTTCCTCCCCTCAAGCCCCGGGCTCAGCACTGA
CCCTGCGGCAGCCAATCCCAAAGGGGGTTGCAACTTTTAAAAATTGATAATGGAAGGGAA
TCCCTGCCCTGCTTTGGTATCGTGGATAATGCCCACTAGAAGCAGTGTACTTGTAATTGT
TGTCTGAAGCCTGTCTGAGACAGATCTACATACAGCCTGGCAGTACTTGAACTAGACGCT
TAATGCCCTGTGTTTTTGGGGGGAGAACTTTGTGTTACAGCTTAATTTAAGAACAGTTAC
TTTGGCATCATTCAGTCTTCACTTTTTGTCTATTTAAACTTGGTTGGAGAAACTTGTGGA
TTTGGTGCTTCAAACCCTATGTGTGGCTTGGATGGCGCAGAGA

Primer picked using http://frodo.wi.mit.edu and the general rules to design these primers applied:

Primer3 Output

```
No mispriming library specified
Using 1-based sequence positions
WARNING: Left primer is unacceptable: High 3' stability; Right primer is
unacceptable: Tm too high/High 3' stability

OLIGO            start  len      tm     gc%   any   3' seq
LEFT PRIMER         20   22   60.30   54.55  6.00  0.00 CTCAAGCAGTACTTCACCCTCC
RIGHT PRIMER       380   21   65.61   61.90  6.00  2.00 GTTCCTCCATGGCTCTCCTCG
SEQUENCE SIZE: 540
INCLUDED REGION SIZE: 540

PRODUCT SIZE: 361, PAIR ANY COMPL: 3.00, PAIR 3' COMPL: 1.00

    1 ATTTGGTCAAAGCCTTCTCCTCAAGCAGTACTTCACCCTCCTGCACTAGACGCCTCCAGG
                         >>>>>>>>>>>>>>>>>>>>>>

   61 GAGCTGGAGCGGAGCAGGGCTCGGTGGGCCAGCTCTTAGCAACCCAGGTCTAAGACCCGG


  121 TGTGGAGAGGAACAACCACAGACGCGGCGGCTTAGCTAGGCGCTCTGGAAGTGCAGGGGA


  181 GGCGCCCGCCTGCCTTGGCTGCCGCACCCATGACCTCTAGTTTCAGCTGTGAACCTGGGC


  241 GGAGGAATAATTGAGGAACTCACGGAACTATCAACTGGGGACAAACCTGCGATCGCCACG


  301 GTCCTTCCGCCCTCTCCTTCGTCCGCTCCATGCCCAAGAGCTGCGCTCCGGAGCTGGGGC
                                                                  <

  361 GAGGAGAGCCATGGAGGAACCGGGTGCTCAGTGCGCTCCACCGCCGCCCGCGGGCTCCGA
      <<<<<<<<<<<<<<<<<<<

  421 GACCTGGGTTCCTCAAGCCAACTTATCCTCTGCTCCCTCCCAAAACTGCAGCGCCAAGGA


  481 CTACATTTACCAGGACTCCATCTCCCTACCCTGGAAAGTACTGCTGGTTATGCTATTGGC


KEYS (in order of precedence):
>>>>>> left primer
<<<<<< right primer


Statistics
Pair Stats:
considered 1, ok 1
primer3 release 1.1.4


(primer3_results.cgi release 0.4.0)
```

Appendices 18: 5-HT2A(102T/C) Designing Primers

www.ensembl.org: To obtain the protein and nucleotide sequence

5-HT2A Protein Sequence:

MDILCEENTSLSSTTNSLMQLNDDTRLYSNDFNSGEANTSDAFNWTVDSENRTNLSCEGC
LSPSCLSLLHLQEKNWSALLTAVVIILTIAGNILVIMAVSLEKKLQNATNYFLMSLAIAD
MLLGFLVMPVSMLTILYGYRWPLPSKLCAVWIYLDVLFSTASIMHLCAISLDRYVAIQNP
IHHSRFNSRTKAFLKIIAVWTISVGISMPIPVFGLQDDSKVFKEGSCLLADDNFVLIGSF
VSFFIPLTIMVITYFLTIKSLQKEATLCVSDLGTRAKLASFSFLPQSSLSSEKLFQRSIH
REPGSYTGRRTMQSISNEQKACKVLGIVFFLFVVMWCPFFITNIMAVICKESCNEDVIGA
LLNVFVWIGYLSSAVNPLVYTLFNKTYRSAFSRYIQCQYKENKKPLQLILVNTIPALAYK
SSQLQMGQKKNSKQDAKTTDNDCSMVALGKQHSEEASKDNSDGVNEKVSCV

5-HT2A Nucleotide Sequence (Exon1):

CCAGCCCCGGGAGAACAGCATGTACACCAGCCTCAGTGTTACAGAGTGTGGGTACATCAA
GGTGAATGGTGAGCAGAAACTATAACCTGTTAGTCCTTCTACACCTCATCTGCTACAAGT
TCTGGCTTAGACATGGATATTCTTTGTGAAGAAAATACTTCTTTGAGCTCAACTACGAAC
TCCCTAATGCAATTAAATGATGACACCAGGCTCTACAGTAATGACTTTAACTCGGAGAA
GCTAACACTTCTGATGCATTTAACTGGACAGTCGACTCTGAAAATCGAACCAACCTTTCC
TGTGAAGGGTGCCTCTCACCGTCGTGTCTCTCCTTACTTCATCTCCAGGAAAAAAACTGG
TCTGCTTTACTGACAGCCGTAGTGATTATTCTAACTATTGCTGGAAACATACTCGTCATC
ATGGCAGTGTCCCTAGAGAAAAAGCTGCAGAATGCCACCAACTATTTCCTGATGTCACTT
GCCATAGCTGATATGCTGCTGGGTTTCCTTGTCATGCCCGTGTCCATGTTAACCATCCTG
TATG

Primer picked using http://frodo.wi.mit.edu and the general rules to design these primers applied:

Primer3 Output

```
No mispriming library specified
Using 1-based sequence positions
WARNING: Left primer is unacceptable: Tm too low/High end self
complementarity

OLIGO            start  len      tm     gc%   any   3' seq
LEFT PRIMER         71   21   54.61   38.10  4.00  4.00 GAGCAGAAACTATAACCTGTT
RIGHT PRIMER       515   20   56.11   45.00  3.00  2.00 CAAGTGACATCAGGAAATAG
SEQUENCE SIZE: 544
INCLUDED REGION SIZE: 544

PRODUCT SIZE: 410, PAIR ANY COMPL: 3.00, PAIR 3' COMPL: 3.00

     1 CCAGCCCCGGGAGAACAGCATGTACACCAGCCTCAGTGTTACAGAGTGTGGGTACATCAA


    61 GGTGAATGGTGAGCAGAAACTATAACCTGTTAGTCCTTCTACACCTCATCTGCTACAAGT
                >>>>>>>>>>>>>>>>>>>>


   121 TCTGGCTTAGACATGGATATTCTTTGTGAAGAAAATACTTCTTTGAGCTCAACTACGAAC
```

```
  181 TCCCTAATGCAATTAAATGATGACACCAGGCTCTACAGTAATGACTTTAACTCCGGAGAA

  241 GCTAACACTTCTGATGCATTTAACTGGACAGTCGACTCTGAAAATCGAACCAACCTTTCC

  301 TGTGAAGGGTGCCTCTCACCGTCGTGTCTCTCCTTACTTCATCTCCAGGAAAAAAACTGG

  361 TCTGCTTTACTGACAGCCGTAGTGATTATTCTAACTATTGCTGGAAACATACTCGTCATC

  421 ATGGCAGTGTCCCTAGAGAAAAAGCTGCAGAATGCCACCAACTATTTCCTGATGTCACTT
                                                  <<<<<<<<<<<<<<<<<<

  481 GCCATAGCTGATATGCTGCTGGGTTTCCTTGTCATGCCCGTGTCCATGTTAACCATCCTG
      <

  541 TATG


KEYS (in order of precedence):
>>>>>> left primer
<<<<<< right primer

ADDITIONAL OLIGOS
                     start  len      tm     gc%   any    3' seq

 1 LEFT PRIMER         71   21   50.61   38.10  4.00  4.00 GAGCAGAAACTATAACCTGTT
   RIGHT PRIMER       484   20   60.24   45.00  4.00  0.00 TGGCAAGTGACATCAGGAAA
   PRODUCT SIZE: 414, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 3.00

 2 LEFT PRIMER         71   21   50.61   38.10  4.00  4.00 GAGCAGAAACTATAACCTGTT
   RIGHT PRIMER       509   20   59.15   50.00  4.00  2.00 AGGAAACCCAGCAGCATATC
   PRODUCT SIZE: 439, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 3.00

 3 LEFT PRIMER         71   21   50.61   38.10  4.00  4.00 GAGCAGAAACTATAACCTGTT
   RIGHT PRIMER       485   20   59.09   45.00  6.00  0.00 ATGGCAAGTGACATCAGGAA
   PRODUCT SIZE: 415, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 2.00

 4 LEFT PRIMER         71   21   50.61   38.10  4.00  4.00 GAGCAGAAACTATAACCTGTT
   RIGHT PRIMER       508   20   61.00   50.00  4.00  1.00 GGAAACCCAGCAGCATATCA
   PRODUCT SIZE: 438, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 3.00

Statistics
         con   too    in    in          no    tm    tm  high  high        high
         sid  many   tar  excl   bad    GC   too   too   any    3'  poly   end
        ered    Ns   get   reg   GC% clamp   low  high compl compl     X  stab
ok
Right   1267     0     0     0     0     0   403   486     0     2     0    27
349
Pair Stats:
considered 14, unacceptable product size 9, ok 5
primer3 release 1.1.4


(primer3_results.cgi release 0.4.0)
```

Appendices 19: 5-HT2C (Cys23Ser) Designing Primers

www.ensembl.org: To obtain the protein and nucleotide sequence

5-HT2A Protein Sequence:

MVNLRNAVHSFLVHLIGLLVWQCDISVSPVAAIVTDIFNTSDGGRFKFPDGVQNWPALSI
VIIIIMTIGGNILVIMAVSMEKKLHNATNYFLMSLAIADMLVGLLVMPLSLLAILYDYVW
PLPRYLCPVWISLDVLFSTASIMHLCAISLDRYVAIRNPIEHSRFNSRTKAIMKIAIVWA
ISIGVSVPIPVIGLRDEEKVFVNNTTCVLNDPNFVLIGSFVAFFIPLTIMVITYCLTIYV
LRRQALMLLHGHTEEPPGLSLDFLKCCKRNTAEEENSANPNQDQNARRRKKKERRPRGTM
QAINNERKASKVLGIVFFVFLIMWCPFFITNILSVLCEKSCNQKLMEKLLNVFVWIGYVC
SGINPLVYTLFNKIYRRAFSNYLRCNYKVEKKPPVRQIPRVAATALSGRELNVNIYRHTN
EPVIEKASDNEPGIEMQVENLELPVNPSSVVSERISSV

5-HT2C Nucleotide Sequence (Exons):

ACCCGCGCGAGGTAGGCGCTCTGGTGCTTGCGGAGGACGCTTCCTTCCTCAGATGCACCG
ATCTTCCCGATACTGCCTTTGGAGCGGCTAGATTGCTAGCCTTGGCTGCTCCATTGGCCT
GCCTTGCCCCTTACCTGCCGATTGCATATGAACTCTTCTTCTGTCTGTACATCGTTGTCG
TCGGAGTCGTCGCGATCGTCGTGGCGCTCGTGTGATGGCCTTCGTCCGTTTAGAGTAGTG
TAGTTAGTTAGGGGCCAACGAAGAAGAAAGAAGACGCGATTAGTGCAGAGATGCTGGAGG
TGGTCAGTTACTAAGCTAGAGTAAGATAGCGGAGCGAAAAGAGCCAAACCTAGCCGGGGG
GCGCACGGTCACCCAAAGGAGGTCGACTCGCCGGCGCTTCCTATCGCGCCGAGCTCCCTC
CATTCCTCTCCCTCCGCCGAGGCGCGAGGTTGCGGCGCGCAGCGCAGCGCAGCTCAGCGC
ACCGACTGCCGCGGGCTCCGCTGGGCGATTGCAGCCGAGTCCGTTTCTCGTCTAGCTGCC
GCCGCGGCGACCGCTGCCTGGTCTTCCTCCCGGACGCTAGTG
GGTTATCAGCTAACACCCGCGAGCATCTATAACATAGGCCAACTGACGCCATCCTTCAAA
AACAACT
GTCTGGGAAAAAAAGAATAAAAAGTAGTGTGAGAGCAGAAAACGTGATTGAAACACGACC
AATCTTTCTTCAGTGCCAAAGGGTGGAAAAG
AAAGGATGATATGATGAACCTAGCCTGTTAATTTCGTCTTCTCAATTTTAAACTTTGGTT
GCTTAAGACTGAAGCAATCATGGTGAACCTGAGGAATGCGGTGCATTCATTCCT
TGTGCACCTAATTGGCCTATTGGTTTGGCAATGTGATATTTCTGTGAGCCCAGTAGCAGC
TATAGTAACTGACATTTTCAATACCTCCGATGGTGGACGCTTCAAATTCCCAGACGGGGT
ACAAAACTGGCCAGCACTTTCAATCGTCATCATAATAATCATGACAATAGGTGGCAACAT
CCTTGTGATCATGGCAGTAAGCATGGAAAAGAAACTGCACAATGCCACCAATTACTTCTT
AATGTCCCTAGCCATTGCTGATATGCTAGTGGGACTACTTGTCATGCCCCTGTCTCTCCT
GGCAATCCTTTATG

Primer picked using http://frodo.wi.mit.edu and the general rules to design these primers applied:

Primer3 Output

```
PRIMER PICKING RESULTS FOR HTR2C

No mispriming library specified
Using 1-based sequence positions
WARNING: Right primer is unacceptable: Tm too low

OLIGO            start  len      tm     gc%   any   3' seq
LEFT PRIMER         14   19   59.78   47.37  5.00  3.00
GGCCTATTGGTTTGGCAAT
RIGHT PRIMER       195   18   55.58   50.00  6.00  0.00
GCCATGATCACAAGGATG
SEQUENCE SIZE: 314
INCLUDED REGION SIZE: 314

PRODUCT SIZE: 182, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 1.00

    1 TGTGCACCTAATTGGCCTATTGGTTTGGCAATGTGATATTTCTGTGAGCCCAGTAGCAGC
                  >>>>>>>>>>>>>>>>>>>

   61 TATAGTAACTGACATTTTCAATACCTCCGATGGTGGACGCTTCAAATTCCCAGACGGGGT


  121 ACAAAACTGGCCAGCACTTTCAATCGTCATCATAATAATCATGACAATAGGTGGCAACAT
                                                                <<<

  181 CCTTGTGATCATGGCAGTAAGCATGGAAAAGAAACTGCACAATGCCACCAATTACTTCTT
      <<<<<<<<<<<<<<<

  241 AATGTCCCTAGCCATTGCTGATATGCTAGTGGGACTACTTGTCATGCCCCTGTCTCTCCT


  301 GGCAATCCTTTATG


KEYS (in order of precedence):
>>>>>> left primer
<<<<<< right primer


Statistics
Pair Stats:
considered 1, ok 1
primer3 release 1.1.4


(primer3_results.cgi release 0.4.0)
```

Appendices 20: 5-HT7 (Pro-279-Leu) Designing Primers

www.ensembl.org: To obtain the protein and nucleotide sequence

5-HT7 Protein Sequence:

```
MMDVNSSGRPDLYGHLRSFLLPEVGRGLPDLSPDGGADPVAGSWAPHLLSEVTASPAPTW
DAPPDNASGCGEQINYGRVEKVVIGSILTLITLLTIAGNCLVVISVCFVKKLRQPSNYLI
VSLALADLSVAVAVMPFVSVTDLIGGKWIFGHFFCNVFIAMDVMCCTASIMTLCVISIDR
YLGITRPLTYPVRQNGKCMAKMILSVWLLSASITLPPLFGWAQNVNDDKVCLISQDFGYT
IYSTAVAFYIPMSVMLFMYYQIYKAARKSAAKHKFPGFPRVEPDSVIALNGIVKLQKEVE
ECANLSRLLKHERKNISIFKREQKAATTLGIIVGAFTVCWLPFFLLSTARPFICGTSCSC
IPLWVERTFLWLGYANSLINPFIYAFFNRDLRTTYRSLLQCQYRNINRKLSAAGMHEALK
LAERPERPEFVLRACTRRVLLRPEKRPPVSVWVLQSPDHHNWLADKMLTTVEKKVMIHD
```

5-HT7 Nucleotide Sequence:

5’ upstream sequence

```
ATGATGGACGTTAACAGCAGCGGCCGCCCGGACCTCTACGGGCACCTCCGCTCTTTCCTTCTGCCAGAAGTGGGGCG
CGGGCTGCCCGACTTGAGCCCCGACGGTGGCGCCGACCCGGTCGCGGGCTCCTGGGCGCCGCACCTGCTGAGCGAGG
TGACAGCCAGCCCGGCGCCCACCTGGGACGCGCCCCCGGACAATGCCTCCGGCTGTGGGGAACAGATCAACTACGGC
AGAGTCGAGAAAGTTGTGATCGGCTCCATCCTGACGCTCATCACGCTGCTGACGATCGCGGGCAACTGCCTGGTGGT
GATCTCCGTGTGCTTCGTCAAGAAGCTCCGCCAGCCCTCCAACTACCTGATCGTGTCCCTGGCGCTGGCCGACCTCT
CGGTGGCTGTGGCGGTCATGCCCTTCGTCAGCGTCACCGACCTCATCGGGGGCAAGTGGATCTTTGGACACTTTTTC
TGTAATGTCTTCATCGCCATGGACGTCATGTGCTGCACGGCCTCGATCATGACCCTGTGCGTGATCAGCATTGA
CAGGTACCTTGGGATCACAAGGCCCCTCACATACCCTGTGAGGCAGAATGGGAAATGCATGGCGAAGATGATTCTC
TCCGTCTGGCTTCTCTCCGCCTCCATCACCTTACCTCCACTCTTTGGATGGGCTCAGAATGTAAATGATGATAAGGT
GTGCTTGATCAGCCAGGACTTTGGCTATACGATTTACTCTACCGCAGTGGCATTTTATATCCCCATGTCCGTCATGC
TTTTCATGTACTACCAGATTTACAAGGCTGCCAGGAAGAGTGCTGCCAAACACAAGTTTCCTGGCTTCCCTCGAGTG
GAGCCAGACAGCGTCATCGCCCTGAATGGCATAGTGAAGCTCCAGAAGGAGGTGGAAGAGTGTGCAAACCTTTCGAG
ACTCCTCAAGCATGAAAGGAAAAACATCTCCATCTTTAAGCGAGAACAGAAAGCAGCCACCACCCTGGGGATCATCG
TCGGGGCCTTTACCGTGTGCTGGCTGCCATTTTTCCTCCTCTCGACAGCCAGACCCTTCATCTGTGGCACTTCCTGC
AGCTGCATCCCACTGTGGGTGGAGAGGACATTTCTGTGGCTAGGCTATGCAAACTCTCTCATTAACCCTTTTATATA
TGCCTTCTTCAACCGGGACCTGAGGACCACCTATCGCAGCCTGCTCCAGTGCCAGTACCGGAATATCAACCGGAAGC
TCTCAGCTGCAGGCATGCATGAAGCCCTGAA
GCTTGCTGAGAGGCCAGAGAGACCTGAGTTTGTGCT
```

Primer picked using http://frodo.wi.mit.edu and the general rules to design these primers applied:

Primer3 Output

```
PRIMER PICKING RESULTS FOR 5HT7

No mispriming library specified
Using 1-based sequence positions
OLIGO            start  len      tm     gc%   any   3'   seq
```

```
LEFT PRIMER         92   20   59.92   55.00  2.00
0.00CCTCCATCACCTTACCTCCA
RIGHT PRIMER       441   20   60.00   55.00  3.00
0.00GGTGGCTGCTTTCTGTTCTC
SEQUENCE SIZE: 755
INCLUDED REGION SIZE: 755

PRODUCT SIZE: 350, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 0.00
    1 TACCTTGGGATCACAAGGCCCCTCACATACCCTGTGAGGCAGAATGGGAAATGCATGGCG

   61 AAGATGATTCTCTCCGTCTGGCTTCTCTCCGCCTCCATCACCTTACCTCCACTCTTTGGA
                                       >>>>>>>>>>>>>>>>>>>>

  121 TGGGCTCAGAATGTAAATGATGATAAGGTGTGCTTGATCAGCCAGGACTTTGGCTATACG


  181 ATTTACTCTACCGCAGTGGCATTTTATATCCCCATGTCCGTCATGCTTTTCATGTACTAC


  241 CAGATTTACAAGGCTGCCAGGAAGAGTGCTGCCAAACACAAGTTTCCTGGCTTCCCTCGA


  301 GTGGAGCCAGACAGCGTCATCGCCCTGAATGGCATAGTGAAGCTCCAGAAGGAGGTGGAA


  361 GAGTGTGCAAACCTTTCGAGACTCCTCAAGCATGAAAGGAAAAACATCTCCATCTTTAAG


  421 CGAGAACAGAAAGCAGCCACCACCCTGGGGATCATCGTCGGGGCCTTTACCGTGTGCTGG
       <<<<<<<<<<<<<<<<<<<


  481 CTGCCATTTTTCCTCCTCTCGACAGCCAGACCCTTCATCTGTGGCACTTCCTGCAGCTGC


  541 ATCCCACTGTGGGTGGAGAGGACATTTCTGTGGCTAGGCTATGCAAACTCTCTCATTAAC


  601 CCTTTTATATATGCCTTCTTCAACCGGGACCTGAGGACCACCTATCGCAGCCTGCTCCAG


  661 TGCCAGTACCGGAATATCAACCGGAAGCTCTCAGCTGCAGGCATGCATGAAGCCCTGAAG


  721 CTTGCTGAGAGGCCAGAGAGACCTGAGTTTGTGCT
KEYS (in order of precedence):
>>>>>> left primer
<<<<<< right primer
Statistics
        con   too    in    in          no    tm    tm  high  high        high
        sid  many   tar  excl   bad    GC   too   too   any    3'  poly   end
       ered    Ns   get   reg   GC% clamp   low  high compl compl     X  stab    ok
Left    1916     0     0     0     0     0   481   839     0     1     0    49   546
Right   3525     0     0     0     1     0   657  2019     0     1     0    79   768
Pair Stats:
considered 756, unacceptable product size 741, high end compl 4, ok 11
primer3 release 1.1.4
(primer3_results.cgi release 0.4.0)
```

Appendices 21: Ethics Approval

NHS

North Staffordshire Local Research Ethics Committee
Heron House
120 Grove Road
Fenton
Stoke-on-Trent
Staffordshire
ST4 4LX

31 May 2005

Mrs G Chapman
Research Sister
Academic Department of Obstetrics and Gynaecology
Maternity Building
City General Hospital
Newcastle Road
Stoke-on-Trent
ST4 6QG

GOPMS NC04183/MT

Dear Mrs Chapman

Study title: **Genetics of premenstrual syndrome/premenstrual dysphoric disorder**
REC reference: **1361**

Thank you for the notice of a substantial amendment that was received on 5 May 2005 and was reviewed at the meeting of the Sub-Committee of the Research Ethics Committee held on 25 May 2005.

Ethical opinion

The members of the Committee present gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

- The protocol
- Patient information sheet version 3 April 2005
- Consent form version 3 April 2005

Membership of the Committee

The members of the Ethics Committee who were present at the meeting are listed on the attached sheet.

Management approval

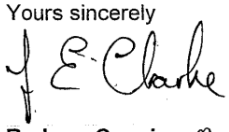
All investigators and research collaborators in the NHS should notify the R&D Department for the relevant NHS care organisation of this amendment and check whether it affects local management approval of the research.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

[REC reference number]: 1361	**Please quote this number on all correspondence**

Yours sincerely



Barbara Cannings
Committee Administrator

Enclosures *List of names and professions of members who were present at the meeting and those who submitted written comments*

SUB COMMITTEE COMPOSITION
NORTH STAFFORDSHIRE LOCAL RESEARCH ETHICS COMMITTEE

STATUS IN COMMITTEE	**DESIGNATION**	**OCCUPATION AND QUALIFICATION**	**MALE/FEMALE**
Chairman	Miss N. Brooks	Solicitor	Female
	Mr. P. Shott	Field Tutor of Philosophy	Male
	Dr. C.K. Tan	Pharmacy Representative	Male
	Dr E Thomas	Statistician	Female