VARIATION OF THE PSEUDOGENE FOR A SEROTONIN-7-LIKE RECEPTOR

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ABSTRACT

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Antipsychotic drugs are central to the treatment of schizophrenia and psychosis. The search for a selective mechanism shared by all antipsychotics has focused mainly on the antipsychotic effects mediated by dopamine D_2 receptor blockade. However, the risk of Parkinsonism is high when antipsychotics occupy dopamine D_2 receptors above 80%. Antipsychotics which are atypical have a low tendency to induce these extrapyramidal side effects in humans and require high doses to elicit catalepsy in animals. Blockade of serotonin receptors has been suggested as a basis for atypical antipsychotic drug action. The current evidence on dopamine/serotonin interactions, however, is inconclusive. Thus, speculation of other such receptor sites contributing to the absence or low Parkinsonism observed with atypical antipsychotics has here been the impetus for a novel receptor search. Using a strategy based on the strong homology between dopamine and serotonin receptors, a serotonin-7B receptor pseudogene was previously isolated. In an effort to identify the functional serotonin-7B receptor gene through homologous hybridization screening techniques, a serotonin-7B receptor pseudogene polymorphism was detected from human fetal brain cDNA. In comparison to the pseudogene, its polymorphism contains four nucleotide substitutions and one deletion creating four amino acid changes. The intronless coding sequence of 1325 base pairs produces a truncated protein of 84 amino acids, the function of which is not known. The continuing search for the functional serotonin-7B receptor gene may elucidate these and other issues pertaining to antipsychotic drug action.

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ABBREVIATIONS

amp	Ampicillin
bp	Base pair
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
cpm	Counts per minute
dATP	Dideoxyadenosine-5'-triphosphate
ddH_2O	Double-distilled water
dCTP	Dideoxycytidine-5'-triphosphate
dGTP	Dideoxyguanosine-5'-triphosphate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase I	Deoxyribonuclease I
dNTP	Deoxynucleoside-5'-triphosphate
DTT	Dithiothreitol
dTTP	Dideoxythymidine-5'-triphosphate
EDTA	Ethylenediaminetetraacetic acid
EtOH	Ethanol
G protein	Guanine nucleotide regulatory protein
IPTG	Isopropylthio-β-D-galactoside
kb	Kilobase pair
Kd	Dissociation constant
LB	Luria-Bertani medium containing bactotrypton, bactoyeast extract and sodium chloride
MOPS	3-[N-Morpholino]propanesulfonic acid

NZY	LB medium containing casein enzymatic hydrolysate (N-Z-Amine), bactoyeast extract, sodium chloride, magnesium sulfate
MES	2-(N-morpholino)ethanesulfonic acid
mRNA	Messenger ribonucleic acid
µCi	Microcurie
PCR	Polymerase chain reaction
RNase	Ribonuclease
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
SSC	Standard saline citrate buffer (pH 7.0) containing 150 mM NaCl, 15 mM sodium citrate
SM	Phage elution buffer containing NaCl, $MgSO_4$, Tris-HCl (pH 7.5), 2% gelatin
TBE	Tris buffer containing 45 mM Tris-borate, 2 mM EDTA (pH 8.0)
TE (pH 8.0)	Buffer containing 10 mM Tris (pH 8.0) 1 mM EDTA (pH 8.0)
TEMED	N,N,N',N',-Tetramethylethylenediamine
TM	Transmembrane
U	Unit
X-gal	5-Bromo-4-chloro-3-indolyl-β-D-galactoside

INTRODUCTION

1.0 Antipsychotic Therapy

Investigation into the therapeutic actions of antipsychotic drugs has led to an increased understanding of the biological basis for schizophrenia (Seeman, 1966; Seeman *et al*, 1974; Matthysse *et al*, 1973) and the underlying biochemical processes of other psychotic disorders. Since the first reported use of chlorpromazine (a phenothizaine) in 1952 to alleviate delusions and hallucinations (Delay *et al*, 1952), researchers have tried to isolate the target site(s) of antipsychotic drug action. An early link between psychosis and dopamine was detected (Van Rossum, 1967) by observing the side effects of antipsychotic drug use. Similar to the clinical signs of Parkinson's disease (akinesia, tremor, rigidity), these side effects suggested the involvement of brain dopamine which was known to be absent or markedly decreased in patients with Parkinson's disease.

With a focus on dopamine receptors, other antipsychotic drugs were developed. In particular, a new chemical class, namely butyrophenones such as haloperidol, was discovered (Janssen, 1976). In the 1970s with the synthesis of selective radiolabelled ligands and the radioligand binding assay, a strong correlation emerged between the affinity of neuroleptics for dopamine D_2 receptors and their clinical therapeutic doses (Seeman *et al*, 1975; 1976). The search to isolate dopamine receptors accelerated using molecular biological techniques in the 1980s and early 1990s. Currently, five different dopamine receptors of the G protein-coupled class have been cloned (Table 1): D_1 (Sunahara *et al*, 1990), D_2 (Grandy *et al*, 1989), D_3 (Sokoloff *et al*, 1990), D_4 (Van Tol *et al*, 1991) and D_5 (Sunahara *et al*, 1991). These fall into two dopamine receptor subtypes, termed D1 (D_1, D_5) and D2 (D_2, D_3, D_4), and are grouped for their abilities to stimulate or inhibit adenylyl cyclase, respectively (Spano *et al*, 1976; Cools and Van Rossum, 1976; Kebabian and

Table 1: Summary of Dopamine Receptors

	D1 GROUP		D2 GROUP		
Receptor type	D1	D5	D2	D3	D4
Found alone in	Parathyroid	?	Anterior pituitary	?	?
High density in	Putamen, caudate, accumbens	?	Putamen, caudate, accumbens	Accumbens, Calleja isles	Accumbens, hippocampus
Adenylate cyclase	Stimulates	Stimulates	Inhibits	Inhibits	Inhibits
Amino acids	446	475-477	414-444	400-446	387-515
Human variants	None	None	Five	Three	>25
Introns in gene	None	None	Six	Five	Three
On chromosome	Five	Four	Eleven	Three	Eleven
Pseudogenes	?	Yes	?	?	?
Blocked by	Schering no. 23390	Schering no. 23390	Antipsychotics, raclopride	Antipsychotics	Clozapine, olanzapine, risperidone
Stimulated by	Dopamine, fenoldopam	Dopamine, fenoldopam	Dopamine, bromocriptine, naxagolide	Dopamine, 7-OH-DPAT	Dopamine
Electrical response	Excitation	Excitation	Inhibition	Inhibition?	Inhibition?

[Reproduced from Seeman, 1995 with permission from author.]

Calne, 1979). Cloning of the various dopamine receptors has provided new insights into the structure and function of these proteins. The affinity of the various neuroleptics for the different dopamine receptors has led to the conclusion that after 30 years, the dopamine D_2 receptor remains as the primary site of action for antipsychotic drugs to mediate antipsychosis (Farde *et al*, 1992; 1994; Nordström *et al*, 1994).

1.1 Neuroleptic Parkinsonism

The hypothesis of dopamine overactivity in schizophrenia is primarily based on the evidence that neuroleptics block dopamine receptors *in vivo* and *in vitro* (Andén *et al*, 1970; Seeman *et al*, 1974;1975; Creese *et al*,1976). Positron emission tomography (PET) studies (Figure 1) have established that patients with schizophrenia responding to conventional doses of classical or typical neuroleptics have consistently high dopamine D_2 receptor occupancy between 70-89% (Farde *et al*, 1988). However, there is an increased risk of neuroleptic Parkinsonism [also referred to as extrapyramidal side effects (EPS) and will be used interchangeably] at dopamine D_2 receptor occupancies above 80% (Figure 2). Therefore, these findings suggest that the optimal range for dopamine D_2 receptor occupancy is between 70-80% (Farde *et al*, 1994) to alleviate the symptoms of psychosis without the risk of EPS (Seeman, 1992; 1995).

1.2 Antipsychotic drugs: Typicals and atypicals

Antipsychotic drugs through dopamine D_2 receptor antagonism not only produce antipsychotic effects but are also accompanied by Parkinson's-like side effects (akinesia, tremor at rest, rigidity). Classical or typical neuroleptics, such as haloperidol and chlorpromazine, refer to compounds that produce antipsychotic effects at 70-80% (high affinity) dopamine D_2 receptor occupancy and elicit Parkinsonism in man and catalepsy in

COURTESY OF SHITIJ KAPUR AND SYLVAIN HOULE THE CLARKE INSTITUTE OF PSYCHIATRY AND THE UNIVERSITY OF TORONTO

Figure 1 Positron Emission Tomography shows radiolabelled raclopride binding to dopamine D_2 receptors in the caudate nucleus and putamen in an untreated patient's brain (left). After treatment with a daily dose of an antipsychotic drug, risperidone for one week (right), the amount of radioactive raclopride was reduced by 75%. Courtesy of Drs. Kapur and Seeman, The Clarke Institute of Psychiatry and the University of Toronto.

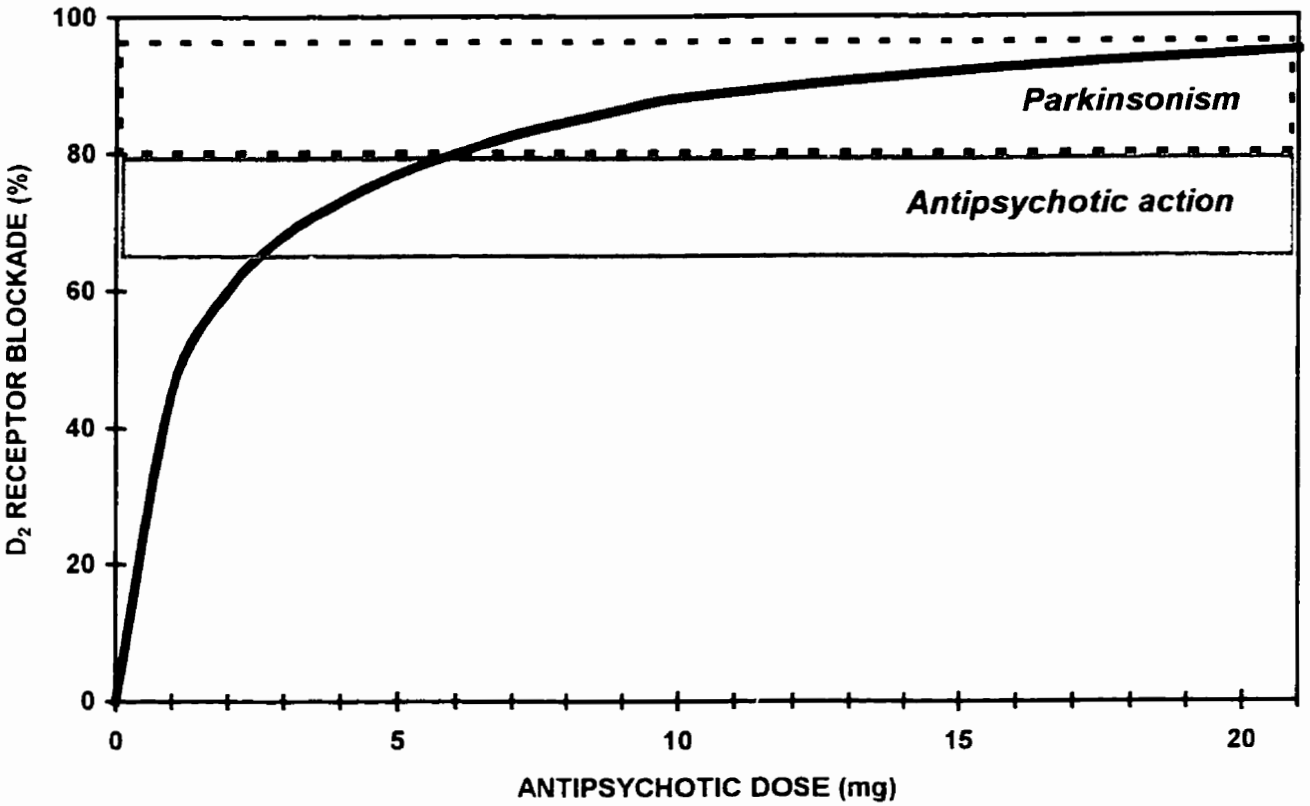


Figure 2 **The relationship between antipsychotic dose and dopamine D_2 receptor blockade in patients.** Antipsychosis is in the range of ~75%. Overdosing will exceed D_2 occupation over 80% causing signs of Parkinsonism. [Adapted from Seeman *et al*, 1995 with author's permission]

animals (Delay *et al*, 1957; Farde *et al*, 1992). As well, plasma prolactin levels are elevated. In contrast, neuroleptics, such as clozapine and olanzapine, that provide efficacy at 40-50% (low affinity) dopamine D_2 receptor occupancy and have little or no incidence of Parkinsonism, are considered to be atypicals. These produce no changes in plasma prolactin levels (Meltzer *et al*, 1979; Kane *et al*, 1981; Casey, 1989). Thus, clarification of clozapine's atypical effects may provide new strategies for drug development as well as provide new insights into the pathophysiologic mechanisms of schizophrenia. It is possible that by examining atypicals, such as clozapine, the atypical effects observed clinically may also be unique with respect to biochemical factors in the human brain. Moreover, the important role of central dopamine D_2 receptors in antipsychotic efficacy has not precluded the involvement of other neurotransmitter systems in a modulatory capacity to alleviate neuroleptic-induced side effects. Currently, many of these neurotransmitter systems are being studied to investigate the receptor basis for atypical neuroleptics.

1.3 Receptor basis for low level of Parkinsonism by atypical neuroleptics

As mentioned previously, dopamine D_2 receptor blockade alleviates psychosis but produces Parkinsonism. Those neuroleptics which cause less Parkinsonism are commonly referred to as atypical antipsychotics (see section 1.2). There are several current views on the receptor basis for this atypical action of these particular neuroleptics:

1. Atypical neuroleptics may have low affinity for D_2 and are, therefore, readily displaced by high endogenous concentrations of dopamine in the human striatum.
2. Atypical neuroleptics may have both anti-D_2 and anticholinergic action.
3. Atypical neuroleptics may have a selective blockade of dopamine D_4 receptors.
4. Atypical neuroleptics may block both D_2 and serotonin 2A (5-HT_{2A}) receptors.

[References: 1. Seeman *et al*, 1996. 2. Seeman, 1990. 3. Van Tol *et al*, 1991; Seeman *et al*, 1996. 4. Malmberg *et al*, 1993; Leysen *et al*, 1994, Huttunen, 1995; Meltzer *et al*, 1995]

The different receptor hypotheses, mentioned above, for the clinically atypical action of the atypical neuroleptics may be examined using the dissociation constant (K_d) values.

(a) "Loose" neuroleptics displaceable by endogenous dopamine:

The first group are those atypical neuroleptics which have low affinity at D_2 and thus may be readily displaced by high endogenous concentrations of dopamine in the caudate/putamen. This group includes remoxipride, clozapine, perlapine, seroquel and melperone, all of which have K_d values in the range of 30 nM to 88 nM (Table 2). This is in contrast to most typical neuroleptics which have lower K_d values in the range of 0.3 to 6 nM (Table 2). Molindone is borderline, having a K_d value of 6 nM (Table 2). This was confirmed by Roth *et al* (1994) where K_d values of atypicals (using [^{3}H]spiperone) were between 45 nM and 1584 nM, suggesting that these compounds would be readily displaced at the D_2 receptor by high local concentrations of endogenous dopamine in the striatum. Of the typical neuroleptics tested by Roth *et al* (1994), these had K_d values (using [^{3}H]spiperone) which were between 0.06 nM and 8 nM, suggesting that these compounds would be less readily displaced at the D_2 receptor by high local concentrations of endogenous dopamine in the striatum.

The higher range of K_d values of 30 nM to 88 nM for these atypical drugs indicates that they are loosely attached to the D_2 receptors and may, therefore, be readily displaced by endogenous dopamine. The principle of displacement of a neuroleptic by endogenous dopamine has been shown for [^{3}H]raclopride (Seeman *et al*, 1989; Young *et al*, 1991), [^{11}C]raclopride (Innis *et al*, 1992; Dewey *et al*, 1992; 1993; Wong *et al*, 1995), [^{3}H]spiperone and [^{3}H]methylspiperone (De Jesus *et al*, 1986; Seeman *et al*, 1989; Young

Table 2: Dissociation Constants, Kd (nM)

	D2	D4	5-HT2A	D2/5-HT2A	D2/D4
Chlorpromazine	0.66	1.15	3.5	0.19	0.58
Clozapine	44	1.6	11	4	28
Fluphenazine	0.32	50	80	0.004	0.0064
Haloperidol	0.35	0.84	25	0.014	0.42
Isoclozapine	5.8	5.8	1.45	4	1
Loxapine	5.2	7.8	10.2	0.51	0.67
Melperone	88	410	280	0.31	0.22
Molindone	6	2400	5800	0.001	0.0025
Olanzapine	3.7	2	5.8	0.64	1.85
Perlapine	60	30	30	2	2
Raclopride	0.64	620	5400	0.00012	0.001
Remoxipride	30	2800	3100	0.01	0.011
Risperidone	0.3	0.25	0.14	2.14	1.2
Seroquel	78	3000	2500	0.03	0.026
Sertindole	0.95	0.85	0.3	3.1	1.12
Thioridazine	0.4	1.5	60	0.007	0.27
Trifluperazine	0.96	44	135	0.007	0.022

[Adapted from Seeman *et al*, 1996]

et al, 1991), [^{18}F]N-methylspiperone (Dewey *et al*, 1991; Logan *et al*, 1991) and [^{123}I]iodobenzamide (Innis *et al*, 1992; Laruelle *et al*, 1995).

Hence, loosely bound neuroleptics (with high K_d) would occupy more dopamine receptors in brain regions having low dopamine output (limbic regions, hypothalamus and pre-frontal cortex), but would occupy fewer dopamine receptors in regions having high dopamine output (caudate/putamen) as a result of the neuroleptic competition with endogenous dopamine. Loose neuroleptics, therefore, would be expected to occupy a lower fraction of dopamine receptors in the caudate/putamen but a higher fraction in the nonstriatal regions, with corresponding fewer extrapyramidal signs compared to the typical neuroleptics with low K_d values.

(b) Combined block of D_2 and muscarinic receptors

A second small group of two atypical neuroleptics, clozapine and thioridazine, strongly block both D_2 and muscarinic receptors. Clozapine, for example, is of the order of 20- to 50-fold more potent in blocking muscarinic acetylcholine receptors than blocking dopamine D_2 receptors (Seeman, 1990). Clozapine blocks muscarinic receptors at about 15 nM. Because anticholinergic drugs have an anti-Parkinson action, it might appear that the low value of 15 nM may readily account for the atypical action of clozapine. However, isoclozapine is equally anticholinergic (K_d of 11 nM; Rupard *et al*, 1989), yet elicits catalepsy in animals at low doses, in contrast to clozapine. Moreover, it has been argued that the combination of antagonists for dopamine (i.e. neuroleptic) and acetylcholine (i.e. benztropine) is not as effective in minimizing Parkinsonism as clozapine itself (Kane *et al*, 1988).

Thioridazine also blocks muscarinic receptors at about the identical concentrations that it blocks D_2 receptors (Seeman, 1990). Thus, the relatively low level of Parkinsonism caused by thioridazine may stem from its anticholinergic action.

(c) Selective block of dopamine D_4 receptors:

A third possible mechanism for atypical neuroleptic action may be the selective blockade of dopamine D_4 receptors. Studies from Seeman *et al* (1996) has shown a relation between the neuroleptic doses for rat catalepsy and the D_2/D_4 ratio of the K_d values (Table 2). Rat catalepsy decreased as selectivity at the D_4 receptor increased. Clozapine and isoclozapine are considerably different in their values for the D_2/D_4 ratio of K_d values, in relation to their different cataleptic potencies. Isoclozapine is equally selective at both the D_2 and D_4 receptors but elicits catalepsy. Clozapine which is 28 times more selective at the D_4 receptor does not elicit catalepsy. Roth *et al* (1995) have also found that the atypical drugs perlapine, olanzapine and clozapine were selective for the dopamine D_4 receptor, compared to the D_2 receptor.

Although sertindole is only now beginning to be tested in large numbers of patients, it has been reported that sertindole does not elicit Parkinsonism (Daniel, 1995). The low level or absence of EPS with sertindole appears related to its ability to block D_4 receptors more selectively than D_2 receptors (Table 2).

(d) Combined block of D_2 and 5-HT_{2A} receptors:

A fourth mechanism which may account for the clinically atypical action of atypical neuroleptics is that these drugs may simultaneously block both D_2 and serotonin 2A (5-HT_{2A}) receptors (Malmberg *et al*, 1993; Leysen *et al*, 1994; Huttunen, 1995; Meltzer *et al*, 1995).

The blockade of 5-HT receptors increases the release of dopamine, as measured indirectly by the fall in [^{11}C]raclopride binding to D_2 receptors (Smith *et al*, 1994; Pehek, 1995; Dewey *et al*, 1995). In turn, therefore, the increased release of endogenous dopamine displaces some of the neuroleptic from the D_2 receptors, thereby partly alleviating the Parkinsonism caused by D_2 blockade. This mechanism (of enhancing dopamine release) may explain the modest alleviation of neuroleptic-induced catalepsy (in rats) by ritanserin, a serotonin antagonist (Bligh-Glover *et al*, 1995). This alleviation only occurs, however, if the catalepsy is submaximum (Bligh-Glover *et al*, 1995), but not if the catalepsy is maximum as produced by a relatively high dose of haloperidol (Wadenberg, 1992; Jaskiw *et al*, 1994).

Ritanserin has been reported to alleviate neuroleptic-induced Parkinsonism and akathisia in patients (Bersani *et al*, 1990; Miller *et al*, 1992). However, ritanserin does not alleviate haloperidol-induced dystonia in monkeys, unlike clozapine, which is very effective in reversing this extrapyramidal syndrome (Casey, 1991; 1993; 1995). As well, other 5-HT receptor subtypes may also be involved. A more effective alleviation of neuroleptic-induced catalepsy is produced by 8-OH-DPAT, a 5-HT_{1A} agonist (Wadenberg, 1992; Invernizzi *et al*, 1988; Broekkamp *et al*, 1988; Hicks, 1990; Casey, 1992; Neal-Beliveau *et al*, 1993). There is mixed evidence, therefore, supporting the concept of a balanced block of D_2 and serotonin 5-HT_{2A} receptors to account for the low level or absence of Parkinsonism by clozapine and other atypical neuroleptics.

Thus, each of the four views purport a partial explanation for the atypical actions seen with atypical neuroleptics. Although further work is needed in each of these areas to study the receptor basis of atypical neuroleptics, the involvement of 5-HT receptors in antipsychotic therapy will be explored.

1.4 The role of 5-HT receptors

(a) 5-HT receptors in antipsychotic therapy:

Involvement of the serotonergic (5-hydroxytryptamine, 5-HT) system in the pathophysiology of schizophrenia was suggested in the early 1950s (Woolley and Shaw, 1954) from reports of lysergic acid diethylamide (LSD was already known to work through the 5-HT system) to induce schizophrenia-type symptoms. In the last two decades, support has grown gradually from reports of abnormal concentrations of 5-HT and 5-HT metabolites (5-hydroxyindoleacetic acid) in the blood and cerebral spinal fluid, respectively, of patients with schizophrenia (van Kammen and Gerlernter, 1987; Bleich, 1988) to preliminary clinical studies with 5-HT antagonists in conjunction with classical neuroleptics (Korsgaard, 1985; Hicks, 1990) to improve efficacy and/or decrease EPS. In animals, neuroleptic catalepsy was diminished by the destruction of the 5-HT receptors in the raphe nuclei (Kostowski *et al*, 1972; Costall *et al*, 1975). Whatever its role in antipsychosis, current studies implicating G protein-coupled 5-HT receptors (Table 3) to reduce the risk for neuroleptic-induced side effects in schizophrenic patients may have equally significant consequences (Nyberg *et al*, 1993).

(b) The role of 5-HT_{2A} receptors:

Support for the latter interest was initially observed from the varying affinities of typical and atypical neuroleptics to 5-HT receptors. It was shown that atypical neuroleptics (e.g. clozapine) blocked one 5-HT receptor in particular, 5-HT_{2A}, four times better than dopamine D_2 receptors (Corbett *et al*, 1995; Seeman *et al*, 1996). Typical neuroleptics (e.g. haloperidol) blocked dopamine D_2 receptors 20 times better than 5-HT_{2A} receptors (Corbett *et al*, 1995; Seeman *et al*, 1996). This difference in affinity of the two types of

Table 3: 5-HT Receptor Subtypes

G protein-coupled:		
	5-HT 1 A - F	- AC
	5-HT 2 A - C	- PI
	5-HT 4	+ AC
	5-HT 5 A, B	?
	5-HT 6	+ AC
	5-HT 7	+ AC
Ligand-gated ion channel:		
	5-HT 3	
Transporters:		
	5-HT uptake site	

[Adapted from Peroutka, 1994]

neuroleptics in addition to the other aspects of 5-HT involvement was the basis for further investigations (Meltzer *et al*, 1989; Meltzer and Nash, 1991; Farde *et al*, 1992; Nyberg *et al*, 1996). However, from the long list of existing 5-HT receptors and 5-HT receptor subtypes, only 5-HT_{2A} was shown to be implicated. However, there were some incongruencies. Clozapine, the most atypical neuroleptic with selectivity for 5-HT_{2A} receptors was also potent at many other receptors, including 5-HT_{1C} (Leysen *et al*, 1990; Kuoppamäki *et al*, 1993), 5-HT_5, 5-HT_6 (Kawagoe *et al*, 1992; Roth *et al*, 1994; Kohen *et al*, 1996) and the α1-adrenoreceptor (Lejeune *et al*, 1994) but not potent at 5-HT_3 receptors (Hoyer *et al*, 1989). As well, some neuroleptics (e.g. isoclozapine) with 5-HT_{2A} receptor selectivity equal to that of atypicals, such as clozapine, have shown to elicit catalepsy (Seeman *et al*, 1996). It was possible, therefore, that other such receptor sites contributed to the atypical action of clozapine. Hence, the question that arose was which other receptor, if any, could alleviate Parkinsonism?

1.5 Amino acid homology of dopamine and 5-HT receptors

The search for new receptor sites in the past has used homology screening to detect novel receptor sequences. From the strong homology of amino acid sequences between dopamine and 5-HT receptors (Figure 3), suitable degenerate primers could be created to screen for new receptors. Dopamine receptors are significant because of the key role they play in antipsychotic drug action; all antipsychotics target dopamine receptors. As well, a structural and evolutionary linkage between dopamine and 5-HT is also hinted by intron positions. For instance, the 5-HT_7 receptor intron positions coincide with D_2-like (D_2, D_3, D_4) intron positions, whereas comparsion of intron positions between 5-HT receptors (e.g. 5-HT_5, 5-HT_6) show completely dissimilar locations (Figure 3). Thus, if another receptor site exists, it is hypothesized that the receptor will have some dopamine characteristics.

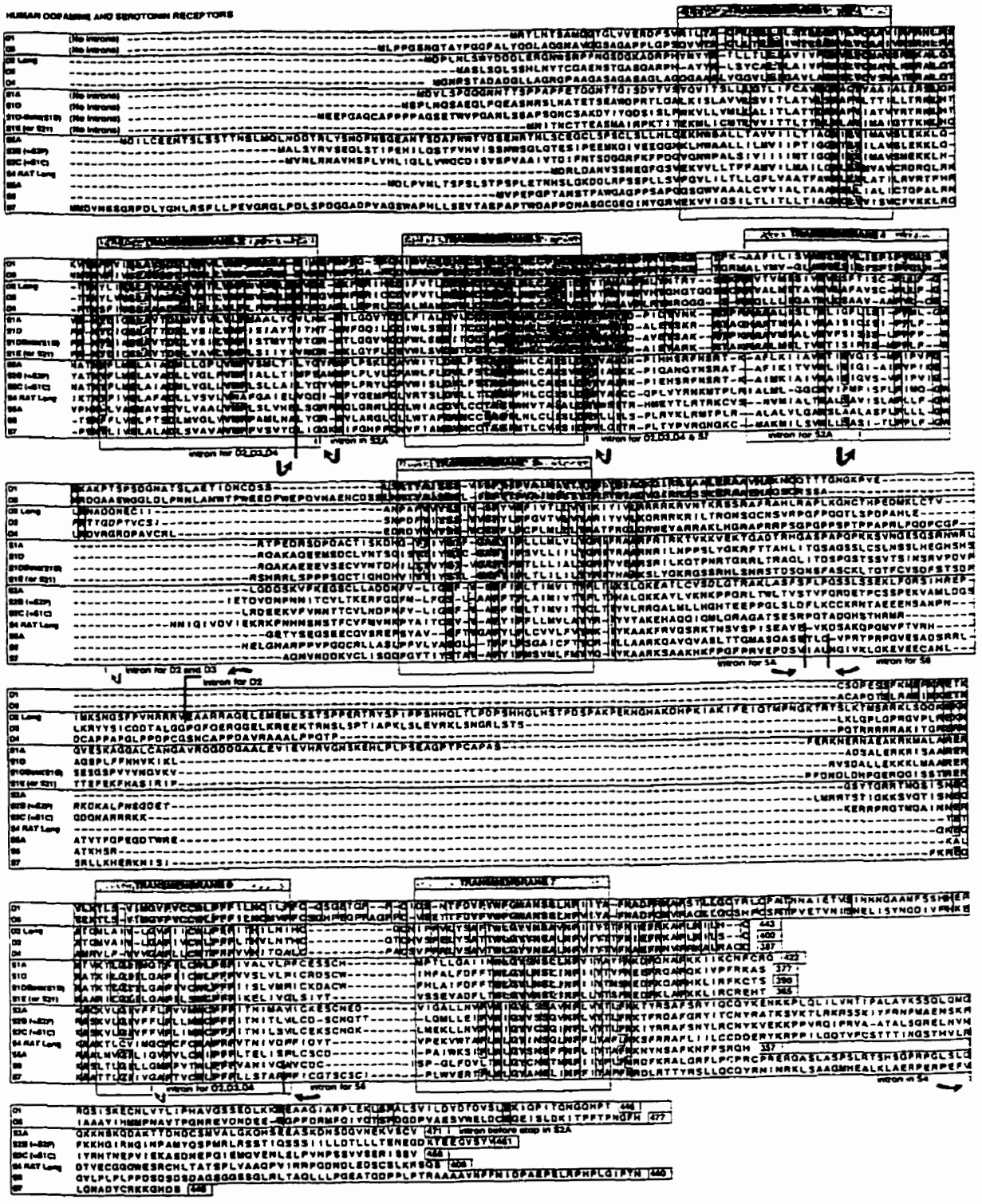


Figure 3 Alignment of human dopamine and serotonin receptor amino acids. Identical amino acids within each subgroup of receptors are shaded. Arrows indicate position of introns. Amino acid code: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; A, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y,tyrosine.

(a) Background: Amino acid sequences for human dopamine and 5-HT receptors:

In addition to binding to various types of dopamine receptors, neuroleptics also bind to 5-HT receptors (Table 2). The binding of antipsychotic drugs to both these two groups of receptors is related, to some degree, to certain identical amino acids found in the same critical position in these two groups of receptors, as illustrated in Figure 3.

For example, the key amino acid for the binding of amines (Mansour *et al*, 1992), such as dopamine and some neuroleptics, is aspartic acid (D) in the transmembrane (TM) region 3, as shown in Figure 3. This same aspartic acid is also important for the binding of the neurotransmitter amines, such as dopamine (Cox *et al*, 1992) and 5-HT. TM 5 contains a serine which is critical for the binding of agonists (Cox *et al*, 1992; Seeman and Van Tol, 1994), such as dopamine (Hibert *et al*, 1991), and presumably serotonin. Thus, the general three-dimensional structure of these receptors permits dopamine and/or serotonin to bind to TM regions 3 and 5 (Neve *et al*, 1991), as depicted in Figure 3.

The aspartic acid in TM 2 determines the sensitivity of the receptors to sodium ions, influences the existence of the high-affinity for the agonist, and thereby affects the G protein-coupling of the receptor to adenylyl cyclase (Metcalf *et al*, 1992; Ceresa and Limbird, 1994).

TM 7 of serotonin receptors S_{1D}, S_{1Dbeta} and S_{1E} contains threonine (adjacent to tryptophan, W, see Figure 3). The replacement of this threonine by asparagine enhances the affinity of these receptors for propranolol by 100- to 1000-fold, matching the affinity of S_{1A} for propranolol (Civelli *et al*, 1991; Oksenberg *et al*, 1992; Parker *et al*, 1993; Adham *et al*, 1994).

(b) Human variants of dopamine and 5-HT receptors:

The known amino acid variations of the human dopamine and 5-HT receptors are given in Tables 4 and 5. Of all the amino acid variations summarized in Tables 4 and 5, the only receptor variant which exhibits a significantly different sensitivity to drugs is the valine-to-glycine variation at position 194 in the human dopamine D_4 receptor (Seeman *et al*, 1994). Although the sensitivity of this receptor to spiperone is the same as that for the common dopamine D_4 receptor ($D_{4.4}$), the sensitivity of this variant to clozapine and olanzapine falls 100- to 1000-fold compared to the common D_4 receptor (Liu *et al*, 1996). Moreover, this receptor variant apparently has no functional high-affinity state for dopamine, and therefore, the receptor does not inhibit adenylyl cyclase, unlike the common D_4 receptor (Liu *et al*, 1996).

Thus, the strong homology between the two G protein-coupled class of dopamine and 5-HT receptors shows structural similarities which may translate into ligand binding similarities. These core similarities, which are susceptible to small amino acid variations through evolutionary pressures, may have diverged and evolved to account for the differences between typical and atypical neuroleptics in their relative occupancy of D_2 and $5\text{-}HT_{2A}$ receptors (Meltzer *et al*, 1989; Matsubara *et al*, 1993; Stockmeier *et al*, 1993). Attention has primarily focused on the $5\text{-}HT_{2A}$ receptor but other receptor subtypes may be involved.

Table 4: HUMAN DOPAMINE RECEPTORS AND VARIANTS

Receptors and variants	Amino acids	Characteristics	% Prevalence;
D1-like:			
D1	446		~100%
D5	477		~100%
D5 pseudo-1	154	Stops before TM 4	~100%
D5 pseudo-2	154	Stops before TM 4	~100%
D2-like:			
D2 long	443		~97-100%
D2 short	414	Loop 3 is missing 29 AA	~97-100%
D2 V96A	443	Valine replaced by alanine at 96 in TM2	~0.8%
D2 P310S	443	Proline replaced by serine at 310 (loop 3)	~0.4%
D2 S311C	443	Serine replaced by cysteine at 311 (loop 3)	~3%
D3	400		~72%
D3 S9G	400	Serine replaced by glycine at 9	~28%
D3nf	342	Stops before TM 6	in SZ & Aff.D.
D3(TM4 del)	138	Stops after TM 3; no binding	
D3(TM3 del)	109	Stops after TM 2; no binding	
D4			
D4.2	387	Loop 3 has 2 repeats of 16 amino acids each	
D4.3	403	Loop 3 has 3 repeats	
D4.4	419	Loop 3 has 4 repeats	
D4.5	435	Loop 3 has 5 repeats	
D4.6	451	Loop 3 has 6 repeats	
D4.7	467	Loop 3 has 7 repeats	
D4.8	483	Loop 3 has 8 repeats	
D4.9	499	Loop 3 has 9 repeats	
D4.10	515	Loop 3 has 10 repeats	
D4(del)		AlaSerAlaGly missing before TM 1	~8% (Italy)
D4 V194G		Valine replaced by glycine at 194	10-15% Africans

del = Has deletion; AA = amino acid

TM = hydrophobic transmembrane region.

nf = non-functional; SZ = schizophrenia; Aff.D. = affective disorder

There are over 20 different types of repeat units, each repeat unit being designated by a different Greek letter.

Table 5: HUMAN SEROTONIN RECEPTORS AND VARIANTS

Receptors and variants	Amino acids	Characteristics	% prevalence
5-HT1A	422		
5-HT1A I(28)V	422	Isoleucine replaced by valine at 28	2.7%
5-HT1A R219L	422	Arginine replaced by leucine at 219	~3%
5-HT1D	377		
5-HT1Dbeta (=5-HT1B)	390		
5-HT1Dbeta F124C	390	Phenylalanine replaced by cysteine at 124 in TM3	2%
5-HT1E (=5-HT31)	365		
5-HT1F	366		
5-HT2A	471		
5-HT2B	481		
5-HT2C (=5-HT1C)	458		
5-HT2C C23S	458	Cysteine replaced by serine at 23 in TM1	13%
5-HT4Rat long	406		
5-HT4Rat short	387		
5-HT5A	357		
5-HT5B mouse	370		
5-HT6	440		
5-HT7	445		

TM=hydrophobic transmembrane region

1.6 Research objective: Molecular cloning of a novel atypical antipsychotic receptor site(s)

The most widely accepted hypothesis for neuroleptic drug action is that antipsychotic effects are mediated by dopamine D_2 receptor blockade (Carlsson and Lindqvist, 1963; Van Rossum, 1996; Seeman *et al*, 1976; Creese *et al*, 1976; Peroutka and Snyder, 1980) . However, the risk of EPS is fairly high at dopamine D_2 receptor occupancies above 80% (Farde *et al*, 1992). Atypical neuroleptics show a low tendency to induce EPS in humans and reduces catalepsy in animals (Kostowski *et al*, 1972; Costall *et al*, 1975; Balsara, 1979;Korsgaard, 1985; Hicks, 1990). Extrapolation of the atypical effects of these drugs to selectively bind to 5-HT_{2A} receptors has implicated the serotonergic system in the ongoing work involving the receptor basis of atypical neuroleptic drug action. Recently, speculation of other such receptor sites contributing to the absence or low EPS of atypical neuroleptics has been the impetus for a novel receptor search. Thus, it is proposed that the receptor site of atypical neuroleptic action is a novel 5-HT receptor type or subtype yet to be identified. Cloning of such a receptor may not only have significant pharmacological and clinical effects but will also add to the knowledge of schizophrenia pathophysiology which has to date, remained unsolved.

1.7 Research strategy for the molecular cloning of novel atypical antipsychotic receptor(s)

Analysis of the putative TM domains of dopamine and serotonin receptor alignments of the primary amino acid structures can provide the basis for designing degenerate oligonucleotide primers for PCR amplification screening (Saiki *et al*, 1988). The regions of highest homology occurs in the TM regions of the coding sequence of G protein-coupled receptors. Thus, the conservation of amino acid sequences in these

regions is the key assumption for this approach. Using high and low stringency hybridization screening with radiolabelled DNA fragments, various commercial cDNA and genomic libraries were used to search for homologous fragments in the selected genome. Optimization of the conditions for these procedures greatly determines the cloning efficiency of homology screening and the detection of new genes.

MATERIALS AND METHODS

2.0 Materials

Agarose	Bio-Rad Laboratories (Hercules, CA)
Alkaline Phosphatase	Boehringer Mannheim (Dorval, PQ)
Ammonium Persulphate	ICN (Dorval, PQ)
Ampicillin	Sigma (St. Louis, MO)
Bacto Agar	DIFCO Laboratories (Detroit , MI)
Calcium Chloride	Sigma (St. Louis, MO)
Casein Hydrolysate (NZ Amine)	Sigma (St. Louis, MO)
7-deaza-dGTP Sequenase Version 2.0 DNA Sequencing Kit	United States Biochemical (Cleveland, OH)
DNase I	Pharmacia Biotech (Baie d'Urfé, PQ)
Degenerate Oligonucleotides	General Synthesis & Diagnostics Co. (Toronto, ON);
Deionized Formamide	Fluka (Ronkonkoma, NY)
Dextran Sulphate	Caledon Lab (Georgetown, ON)
DIOB Competent Cells	Dr. B.F. O'Dowd (Univ. of Toronto, ON)
Geneclean II Kit	Bio 101 (Vista, CA)
Glucose	Sigma (St. Louis, MO)
Glycine	BDH Chemicals (Toronto, ON)
Human Fetal Brain cDNA Library	Clontech (Pasadena, CA) Gift from Dr. H.H.M. Van Tol (U of T)
Human Genomic Library	Clontech (Pasadena, CA)
Invitrogen Opimization Buffers	Invitrogen Corp. (San Diego, CA)
Liquid Scintillation Cocktail	ICN (Dorval, PQ)

Lysozyme	Boehringer Mannheim (Dorval, PQ)
Magnesium Sulphate	BDH Chemicals (Toronto, ON)
Maltose	Fisher Scientific (Nepean, ON)
Mouse Genomic Library	Clontech (Pasadena, CA)
NACS Prepac Columns	GIBCO BRL (Gaithersburg, MD)
Northern Blots of Human Tissues	Clontech (Pasadena, CA)
Nylon Membrane	Dupont NEN (Boston, MA)
Oakridge Tubes	Nalge Co. (Rochester, NY)
Original TA Cloning Kit	Invitrogen (San Diego, CA)
pBluescript	Strategene (La Jolla, CA) Gift from Dr. B.F. O'Dowd (U of T)
PCR Geneamp Kit	Perkin-Elmer Cetus (Norwalk, CT)
Polaroid Type 667 Film	Eastman Kodak Co. and Polaroid
Polyacrylamide Gel (6%)	Helixx (Toronto, ON)
Proteinase K	Sigma (St. Louis, MO)
Qiagen Plasmid Kit	Qiagen Inc. (Chatsworth, CA)
Rapid Ligation Kit	Boehringer Mannheim (Dorval, PQ)
Random Primer DNA Labelling System	GIBCO BRL (Gaithersburg, MD)
Restriction Endonucleases	Pharmacia/GIBCO BRL/Boehringer Mannheim
RNase A	Boehringer Mannheim (Dorval, PQ)
Salmon Testes DNA	Sigma (St. Louis, MO)
Schizophrenia Genomic DNA	Mount Sinai Hospital (Toronto, ON)
SDS	ICN (Dorval, PQ)
Sequencing Primers	BRL Life Technologies (Burlington, ON); ACGT Corp. (Toronto, ON)
Sodium Phosphate	BDH Chemicals (Toronto, ON)

Spin-X Centrifuge Tube Filter	Costar (Cambridge, MA)
T4 DNA Ligase	GIBCO BRL (Gaithersburg, MD); Pharmacia Biotech(Baie d'Urfé, PQ)
Taq Polymerase Enzyme	Perkin-Elmer Cetus (Norwalk, CT)
Tetrasodium Pyrophosphate	Sigma (St. Louis, MO)
Triton X-100	Sangon Ltd. (Scarborough, ON)
X-ray Diagnostic Film (BioMax)	Eastman Kodak Co. (Rochester, NY)
Yeast Extract	Oxoid (Basingstoke, Hampshire)
[α-^{32}P]dCTP	DuPont NEN (Boston, MA)
[α-^{35}S]dATP	DuPont NEN (Boston, MA)

All other chemicals not mentioned above were of analytical grade.

METHODS

2.1 Hybridization screening of human fetal brain cDNA library

A novel DNA sequence (named PCR 15) from a PCR amplification, obtained previously by a colleague in our lab, was used to make a radioactive probe to screen a human fetal brain cDNA library pooled from seven Caucasian fetuses, ages 20-26 weeks, using a λgt10 vector.

(a) Preparation of phage plating host cells:

A colony of the suppressing *E. coli* host strain, C600, was picked from LB agar plates to inoculate 5 ml of LB medium with 0.2% maltose and 10mM $MgSO_4$ to be grown overnight at 37°C rotating vigorously. The next morning, 50 µl of the culture were used to inoculate 5 ml of LB medium containing 0.2% maltose and 10 mM $MgSO_4$. The culture was incubated at 37°C rotating vigorously until an OD_{600}~0.5 was reached. The cells were centrifuged at 2500 rpm, 4°C for 10 min in a Beckman table-top centrifuge and resuspended in 10 mM $MgSO_4$. The C600 competent cells were stored at 4°C until needed for up to 4 days.

(b) Library titering and library plating:

Dilutions of the stock library were needed to determine the number of plaque-forming units per ml (pfu/ml). One µl from each of three dilutions in SM buffer, 10^{-3}, 10^{-4}, 10^{-5}, was used to infect 200 µl of host C600 cells (OD_{600}~0.5). SM buffer without phage was included as a negative control. The phage was allowed to adsorb to the bacteria during a 15 min incubation at 37°C. Immediately following the incubation, top agarose (3.5 ml at 55°C) was added, mixed and poured onto small (100 mm) prewarmed (37°C) NZY agar

plates and incubated overnight at 37°C. The number of plaques were counted to determine the titer of the cDNA library which was calculated to be 2 x 10^9 pfu/ml.

The library was plated such that 5 x 10^4 plaques/plate would be obtained for primary screening. Host cells were prepared as described above. The phage was allowed to adsorb to 600 µl of competent host cells, as described above, and plated onto 10 large (150 mm) prewarmed NZY plates in 7 ml of top agarose. The plates were allowed to set for 10 min and placed in a 37°C incubator for 8 h until phage plaques appeared. The plates were removed and chilled at 4 °C for at least 1 h to harden the top agarose for subsequent lifting of plaques onto nylon membranes for hybridization screening.

(c) Lifting of library onto nylon membranes:

The library was lifted onto nylon membranes by placing each one evenly onto the top agarose. The membrane was allowed to remain on the plate for 2 min. Three holes were poked through the membrane and into the agar plate with an ink-dipped needle for future alignment. The membranes were gently removed and placed plaque side up onto a Whatman 3MM blotting paper soaked in a pool of 0.5 M NaOH denaturing solution for 2 min. They were quickly blotted dry on blotting paper and placed onto Whatman 3MM blotting paper soaked in fresh denaturing solution for another 2 min. The membranes were again blotted dry and transferred onto Whatman 3MM blotting paper soaked in 1.0 M Tris-HCl (pH 7.5) neutralizing solution for 2 min. This was repeated in fresh 1.0 M Tris-HCl (pH 7.5) for another 2 min and finally set aside onto Whatman 3MM blotting paper to dry completely. This whole process was repeated for duplicate membranes, however, the membranes were allowed to remain longer on the plates for 3 min. The membranes were air-dried for at least 2 h in order for the phage DNA to bind onto the membranes permanently.

(d) Prehybridization:

A prehybridization solution for high stringency screening (Nguyen, 1993) was prepared consisting of 50% deionized formamide, 1X Denhardt's solution (2% w/v ficoll 400; 2% w/v polyvinylpyrrolidone; 20 mg/ml BSA), 100 mM sodium phosphate, 0.1% glycine, 3X SSC and 200 µg/ml denatured (boiled for 10 min) salmon sperm DNA. The air-dried membranes were presoaked in 2X SSC and soaked evenly in ~80 ml of prewarmed (42°C) prehybridization solution for incubation at 42°C, 50 rpm for 4 h.

(e) Purification of PCR DNA fragments for radiolabelling:

Radiolabelling with [α-^{32}P]dCTP requires purified DNA fragments. The novel PCR clone (PCR 15) which had been previously subcloned and miniprepped by a colleague in our lab, was digested with *Eco*RI (10 U/µl) in a 20 µl reaction volume of 5 µl DNA, 2 µl of GIBCO BRL 10X React 3 buffer (50mM Tris-HCl, pH 8.0; 10 mM $MgCl_2$; 100 mM NaCl) and 2 µl Rnase at 37°C for 1 h. It was electrophoresed on a 1.4% regular agarose gel (Ausubel, 1995); the DNA band of ~100 bp was excised. The DNA was extracted using Spin-X® purification microcentrifuge tube filters. Ample ddH_2O was added to cover the gel slice (usually 150 µl) placed in the upper compartment of the microcentrifuge tube. The tube was spun at 13,000 rpm for 3 min. The filtered DNA product collected in the lower compartment and dried. The upper compartment containing the filter, gel and other trapped PCR reagents was discarded. The DNA fragment (25 ng) was resuspended in 23 µl of ddH_2O and denatured into single-stranded DNA by boiling (100°C) for 5 min. It was pulse spun to bring down the condensate and iced immediately. According to a random-priming method described by Feinberg and Vogelstein (1983) a random-primed [α-^{32}P]-labelling kit from GIBCO BRL was used whereby 2 µl each of

dATP, dGTP, dTTP solutions and 15 µl of Random Primer Buffer solution were added and mixed thoroughly. Next, 5 µl of [α-^{32}P] (50 µCi) were carefully added and mixed with the pipette tip. Lastly, 1 µl of the Klenow Fragment was added and gently mixed. The labelling reaction was left to incubate at room temperature for at least 1 h.

An ion exchange resin column, NACS Prepac Column, was used to purify the radiolabelled DNA fragment. The purification column was hydrated with 3 ml of 2.0 M NaCl in TE buffer and equilibrated with 3 ml of low salt binding buffer (0.2 M NaCl in TE). To the labelling reaction, 950 µl of the binding buffer was added and the mixture was loaded into the column. The mixture was left to flow through the column by gravity for maximum binding of the radiolabelled DNA to the resin. The column was washed with 4 ml of binding buffer to remove unbound material (i.e. unincorporated nucleotides) and eluted with 1 ml of high salt solution (1.0 M NaCl in TE) by gravity flow. Two µl of the eluant were added to 4 ml of scintillation cocktail and counted using a scintillation counter. An approximate count of 10^6 cpm/ml was required for hybridization screening.

(f) Hybridization:

Hybridization solution was prepared by adding 10% dextran sulfate to the prehybridization solution described previously. The probe (~10^6 cpm/ml) was denatured by boiling (100°C) for 10 min and added immediately to the prewarmed (42°C) hybridization solution. Membranes were removed from the prehybridization solution and placed in hybridization tubes. Hybridization was carried out at 42°C in a carousel incubator at the lowest speed overnight.

(g) Washing and autoradiography:

The membranes were washed initially with two 15 min washings at room temperature in a solution of 2X SSC and 0.1% SDS to remove the excess probe. This was followed by two 30 min washings under high stringency conditions at 65°C in a prewarmed (65°C) solution of 0.1X SSC and 0.1% SDS.

After washing, the membranes were sandwiched in plastic wrap. In order to visualize the three holes on the membrane, as reference for alignment, old radioactive sequencing reactions were used to indicate the hole positions. These were subjected to autoradiography. After two overnight exposures at -70°C, positive signals were revealed on the X-ray film and this was consistent with the positive signals seen on duplicate membranes.

(h) Secondary and tertiary screenings:

It was necessary to isolate and purify the phage plaques through two additional rounds of screening. By reducing the density of the phage plaques through consecutive dilutions, pure phage plaques containing the hybridized sequence to the radiolabelled probe were obtained. The X-ray film and agar plates were aligned using the three holes previously marked. Primary phage plugs from the plates of interest were placed into a microcentrifuge tube containing 500 µl of SM and a drop of chloroform. The phage was allowed to diffuse out into the SM at room temperature for at least 2 h. One µl from each of the two dilutions (10^{-3}, 10^{-4}) of the phage stock was incubated with 200 µl of C600 competent cells, plated on small (100 mm) plates as previously described and incubated at 37°C overnight. The plates with ample well isolated phage plaques (10^{-4}) were chilled at 4°C before lifting. The membranes were subjected to the same screening procedure as the

primary high-stringency screening involving prehybridization, hybridization, washing and autoradiography.

The secondary autoradiographs were used, as described above, to locate well isolated phage plaques. Using the small end of a sterilized Pasteur pipette, plaques were picked for tertiary screening. The dilutions (10^{-3}, 10^{-4}) of secondary phage stocks were plated for a subsequent round of screening (same conditions and procedures as primary and secondary screening) until pure positive phage plaques were obtained.

2.2 Extraction of DNA from bacteriophage lambda

In order to identify and analyze the purified phage clones, a mid-scale preparation of the isolated phages was performed using a modified DNA extraction protocol as outlined by CLONTECH Laboratories Inc. An overnight culture (in LB medium, with 0.2% maltose) of the host bacteria strain C600 cells was diluted to an OD_{600}~0.6. In a 50 ml Falcon tube, 4 ml of this culture with 10 mM $MgSO_4$, 10 mM $CaCl_2$ and 200 µl of the appropriate phage stock were incubated at 37°C rotating at 250 rpm for ~9 h or until lysed. This was used to infect a larger culture (10 mM $MgSO_4$, 10 mM $CaCl_2$) of 25 ml of LB medium (0.2% maltose) inoculated with 25 µl of the overnight culture grown to an OD_{600}~0.6 (about 3.5 h). After approximately 5 h or until complete lysis, the culture was centrifuged at 5000 rpm for 10 min. The supernatant was treated with 5 µl each of 1 mg/ml DNase I and 1 mg/ml RNase A to incubate at 37°C for 15 min followed by the addition of 500 µl of chloroform (37°C, 15 min). The pellet from ultracentrifugation at 28,000 rpm for 45 min in an SW28 Beckman rotor was resusupended in 500µl of SM and transferred to microcentrifuge tubes. A solution of 20 mg/ml proteinase K in SM was prepared immediately before use and 5 µl were added to each phage suspension. This was incubated at 37°C for 20 min and followed by the addition of 20 mM EDTA and 0.5% SDS, also

incubated at 65°C for 20 min. The digests were extracted with 500 µl of phenol, twice with 500 µl of chloroform and precipitated with 1/10 volume of 3 M sodium acetate and 2.5 volumes of 100% EtOH. Samples were centrifuged at 14,000 rpm, washed in 70% EtOH, dried and resuspended in 100 µl TE buffer.

2.3 Subcloning of cDNA inserts into plasmid vector

(a) Excision of λDNA:

DNA phage preparations were digested with 1 µl *Eco*RI (10 U/µl) in a 20 µl reaction volume of 5 µl DNA, 2 µl of GIBCO BRL 10X React 3 buffer (50mM Tris-HCl, pH 8.0; 10 mM $MgCl_2$; 100 mM NaCl) and 2 µl RNase. The digestion was carried out at 37°C for 5 h. Accordingly, the vector pBluescript SK +/- was digested with 1 µl *Eco*RI (10 U/µl) at 37°C for 1 h, dephosphorylated with 7 U of alkaline phosphatase at 37°C for 30 min and heat shocked at 70°C for 10 min to terminate the dephosphorylation process. Both were electrophoresed on a 0.8% agarose gel. Appropriately sized DNA inserts (~1.6-2.0 kb) were excised from the gel and subjected to purification using the Geneclean II Kit. The excised DNA gel slice was dissolved at 55°C for 3-5 min in 3 volumes of NaI solution. Five µl of the GLASSMILK suspension were added, vortexed and iced for 5 min in order for the DNA to bind to the GLASSMILK beads. The suspension was pulse spun for 5 sec at 14,000 rpm and the pellet was washed 3 times in NEW WASH. The DNA was eluted out of the beads in 20 µl of ddH_2O for 5 min at 55°C, spun for 30 sec at 14,000 rpm and transferred into a fresh microcentrifuge tube.

(b) Preparation of competent cells:

A streak of frozen stock of *E. coli* strain DIOB was used to inoculate 5 ml of LB medium to grow overnight at 37°C, 225 rpm. The overnight culture was needed to

inoculate 75 ml of LB medium which was grown for 2 h at 37°C, 225 rpm or until an OD_{600}~0.6 was reached. The cells were pelleted at 2500 rpm, 4°C for 10 min in a Beckman table-top centrifuge and resuspended in 40 ml of ice-cold freshly prepared transformation buffer consisting of 60 mM $CaCl_2$, 20 mM MES (pH 5.8), 5 mM $MgCl_2$ and 5 mM $MnCl_2$. This was left to sit on ice for 1 h and centrifuged at 2500 rpm, 4°C for 10 min. The pellet was resuspended in 2 ml of ice-cold transformation buffer.

(c) Ligation and transformation:

A rapid ligation was carried out at room temperature for 30 min using an insert to vector ratio of 5:1 in a reaction containing 5X T4 ligase buffer (250 mM Tris-HCl, pH 7.6; 50 mM $MgCl_2$; 5 mM ATP; 5 mM DTT; 25% w/v polyethylene glycol-8000) and 1 µl of T4 DNA ligase in a total reaction volume of 14 µl. The ligation mixture was added gently to 100 µl of competent cells and allowed to sit on ice for 45 min. Following a 2 min heat shock at 42°C, the transformation mixture was put on ice for 30 min, allowed to recover in 200 µl of LB medium at 37°C for 20 min and plated onto LB-amp plates (100 µg/ml amp) with 40 µl of 20 mg/ml stock X-gal and 4 µl of 200 mg/ml stock IPTG. The plates were incubated at 37°C overnight.

2.4 Small-scale plasmid DNA preparation

Single white bacterial colonies of each cDNA clone were picked with sterile tips to inoculate an overnight culture of 3 ml of LB medium containing 100 µg/ml amp at 37°C, 225 rpm. The cultures were spun at 13,000 rpm for 1 min to pellet the cells and decanted. Using a rapid boiling method (Holmes and Quigley, 1981) for preparing small amounts of plasmid DNA, the cells were resuspended by vortexing in 200 µl of STET solution (0.1 M NaCl; 10 mM Tris, pH 8.0; 1mM EDTA; 5% Triton X-100) and treated with 20 µl of 10

mg/ml lysozyme (in 10 mM Tris, pH 8.0). The cells were placed in a boiling water bath (100°C) for 30 sec exactly. The tubes containing the lysed cells were microcentrifuged at 14,000 rpm for 10 min to pellet chromosomal DNA, protein complexes, high molecular weight RNA and cell debris. The supernatants were treated with 500 µl of cold absolute EtOH, incubated at -70°C for 30 min to precipitate the plasmid DNA and spun at 14,000 rpm, 4°C for 15 min. The DNA pellets were dried and resuspended in 30 µl of TE buffer .

Subsequently, the DNA samples were subjected to restriction endonuclease digestion to check for appropriately sized inserts and to discard false positives. *Eco*RI was used to excise the cDNA inserts out the λgt 10 vector. The digestion was carried out at 37°C for 1 h. Each sample was electrophoresed on a 1% agarose gel. Miniprep samples containing the proper insert size (~1.6-2.0 kb) were prepared for nucleotide sequencing.

2.5 Determination of nucleotide and amino acid sequences

The DNA samples were sequenced using the Sanger dideoxy chain termination method (Sanger *et al*, 1977). The method utilizes the ability of the DNA polymerase to use 2',3'-dideoxynucleoside triphosphates (ddNTPs) as substrates. Its incorporation at the 3' end of a growing chain prematurely terminates elongation.

(a) Denaturation of double-stranded plasmid DNA:

Double-stranded plasmid DNA (approximately 3-5 µg) resuspended in a volume of 20 µl was alkaline-denatured by adding 2 µl of 2 M NaOH and 2mM EDTA (pH 8.0). It was incubated for 5 min at room temperature and then neutralized with 3 µl of 3 M sodium acetate (pH 4.9). The DNA was precipitated with the addition of 80 µl of cold absolute EtOH and incubated in -70°C for 15 min. After centrifugation at 14,000 rpm, 4°C for 15

min, the DNA pellet was washed with 200 µl of cold 70% EtOH, dried and resuspended in 7 µl of sterile ddH_2O.

(b) Chain-termination DNA sequencing:

The denatured DNA was sequenced using the Sequenase Version 2.0 DNA Sequencing Kit and 7-deaza dGTP. This dGTP analogue was used to reduce compressions that commonly occur in GC-rich regions when sequencing reactions are electrophoresed.

Annealing mixtures were prepared with 7 µl of denatured DNA, 2 µl of 5X Sequenase buffer (200 mM Tris HCl, pH 7.5; 100 mM $MgCl_2$; 250 mM NaCl), and 1 µl (10 ng) of primer. An internal sequencing primer based on the TM 7 sequence of the previously identified PCR clone was used: 5'-CTCTCCACCCACAGTG-3'. The annealing reactions proceeded at 37°C for 15 min. To the template-primer annealed reactions, a labelling mix of 1 µl 0.1 M DTT, 2 µl of 1X 7-deaza dGTP mix (1.5 µM 7-deaza dGTP, 1.5 µM dCTP, 1.5 µM dTTP) and 0.5 µl of [α-^{35}S]dATP (1500 Ci/mmol), and 2 µl (3 U) of Sequenase enzyme (diluted 8X in sequenase dilution buffer) was added and incubated at room temperature for 2 min. The DNA synthesis and labelling reactions were terminated by transferring 3.5 µl of the labelling reaction to four tubes G, A, T, C (each containing their respective dideoxynucleotide: ddGTP, ddATP, ddTTP, ddCTP) incubated at 37°C for 5 min. The termination reaction was stopped by the addition of 4 µl of stop solution containing 95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol FF. The samples were stored at -20°C before loading onto a 6% polyacrylamide sequencing gel.

(c) Electrophoresis and autoradiography:

Denaturing polyacrylamide gels are capable of resolving labelled single-stranded DNA up to 500 bases in length which differ in size by a single deoxynucleotide. Thus, the four sequenced reactions (G, A, T, C) of each DNA sample can be resolved on adjacent lanes of a sequencing gel and visualized by autoradiography.

A 6% polyacrylamide gel mixture of 6% acrylamide, 7 M urea, 3 mM TEMED, 1X TBE and 0.1% ammonium persulfate was poured and set for at least 2 h to polymerize. The gel was prerun at 70W for 30-45 min on a GIBCO BRL sequencing gel apparatus. The sequencing reactions were denatured at 80°C for 3 min and placed on ice prior to loading on the gel in the order G, A, T, C. After a standard run of 2.5 h at 55W which would normally reveal ~200 bp for reading, the gel was removed and transferred onto a piece of Whatman 3MM blotting paper. The gel was dried in a BIO-RAD model 583 gel dryer for 1-1.5 h and subjected to autoradiography at room temperature overnight.

The sequences were read from the autoradiographs in the order G, A, T, C using a DNA and protein sequence analysis digitizer (HELIXX Technologies Inc.). A clear reading of approximately 180 bp upstream of the primer sequence was recorded. The preliminary sequences of the cDNA clones that could possibly code for G protein-coupled receptors were analyzed. Synthesizing more internal primers based on the revealed sequences, the full gene sequence of a novel cDNA clone was obtained.

2.6 Subcloning of human genomic λDNA clones

An earlier attempt was made by a colleague in our lab to screen a human genomic library with the novel PCR 15 fragment under high stringency conditions. Four positive signals, named G5, G7.2, G7.5 and G10, were eventually isolated and purified, however, all attempts by my colleague to subclone the 4 isolated phage plaques (average insert size

was 15-20 kb) into pBluescript SK +/- plasmid vector were unsuccessful. During the last subcloning attempt of these genomic clones, the screening and sequencing of clones from the human fetal brain cDNA library were completed (section 2.1-2.5). Using the same rapid ligation protocol previously outlined to subclone the cDNA clones, the 4 genomic clones were subcloned.

Genomic DNA phage preparations (5 µl) of G5, G7.2, G7.5 and G10 were digested with 1 µl *Sac* I (20 U/µl) at 37°C for 5 h. Accordingly, the vector pBluescript SK +/- was digested with 1 µl *Sac* I (20 U/µl) at 37°C for 1 h, dephosphorylated with 7 U of alkaline phosphatase at 37°C for 30 min and heat shocked at 70°C for 10 min to terminate the dephosphorylation process. Both were electrophoresed on a 0.8% agarose gel overnight at 20V for clear separation of bands. The following day, DNA inserts were excised from the gel and subjected to purification using the Geneclean II Kit (see section 2.7). Purified DNA inserts were rapid ligated in an insert to vector ratio of 4:1 and transformed into fresh DIOB competent cells for plating. Colonies were picked for small-scale plasmid DNA preparation. These were *Sac I* digested to check for the appropriate insert size and subsequently sequenced.

2.7 Northern blot analysis

Northern blots of poly A^+ RNA from various human tissues were purchased from CLONTECH Laboratories Inc. Following the protocol provided by CLONTECH Laboratories Inc., the Northern blots were placed in prehybridization solution at 68°C for 30 min, using 10 ml of prewarmed (68°C) Expresshyb (CLONTECH Laboratories Inc.) hybridization solution. A 1.8 kb cDNA containing the full gene of clone 8 was random-primed [α-^{32}P]-labelled, as previously described. However, the NACS Prepac Column was equilibrated with 0.5 M NaCl (in TE) binding buffer and eluted with high salt 2.0 M

NaCl in TE to accommodate for probe lengths greater than 1000 bp. The blots were then hybridized with the probe (~10^6 cpm/ml) in Expresshyb at 68°C for 1 h. The blots were washed at room temperature for 40 min in 2X SSC and 0.05% SDS. This was followed by a 40 min wash at room temperature in 0.1X SSC and 0.1% SDS. The blots were subsequently exposed to X-ray film at -70°C.

2.8 Hybridization screening of human genomic library

The 2 kb cDNA of clone 8, obtained from the human fetal brain library, was used to screen a human genomic library to detect other novel G protein-coupled receptors of homologous sequence. The details of the method used were outlined previously in section 2.1.

(a) Library titering, plating and membrane lifting:

The stock library was diluted in SM buffer (10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}). One µl of each was used to infect 200 µl of host K802 competent cells at OD_{600}~0.5. SM buffer without phage was also included as a control. This was mixed with 3.5 ml of top agarose (55°C) and plated onto small (100 mm) prewarmed (37°C) NZY agar plates. The plates were incubated at 37°C overnight. The number of plaques were counted and the titer of the library was calculated to be 4 x 10^9 pfu/ml.

The library was plated such that 5 x 10^4 plaques/plate would be obtained for primary screening. Phage adsorption to 600 µl of host K802 competent cells at 37°C for 15 min was plated in 7 ml of top agarose (55°C) onto 12 large (150 mm) prewarmed (37°C) NZY agar plates. After 8 h in a 37°C incubator, the plates were removed and chilled for 1 h before the library was lifted onto nylon membranes.

(b) Prehybridization, radiolabelling, hybridization, washing and autoradiography:

Under high stringency conditions, air-dried membranes were prehybridized at 42°C, 50 rpm for 4 h in a prehybridization solution of 50% deionized formamide, 1X Denhardt's solution, 100 mM sodium phosphate, 0.1% glycine, 3X SSC and 200 µg/ml denatured (boiled for 10 min) salmon sperm DNA. For hybridization, the 2 kb cDNA containing the full gene sequence of clone 8 was random-primed [α-^{32}P]-labelled and NACS Prepac Column purified, as previously described. The probe (~10^6 cpm/ml) was added to fresh prewarmed (42°C) prehybridization solution containing 10% dextran sulfate. Hybridization was carried out at 42°C in a carousel incubator at the lowest speed overnight.

The membranes were washed with two 15 min washings at room temperature in a solution of 2X SSC and 0.1% SDS followed by two 30 min washings under high stringency conditions at 65°C in a prewarmed (65°C) solution of 0.1X SSC and 0.1% SDS. These were subjected to autoradiography overnight at -70°C.

(c) Secondary and tertiary screenings:

Positive signals from the primary screening were isolated and purified through two additional rounds of screening. Phage plugs from the plates of interest were placed into a microcentrifuge tube containing 500 µl of SM and a drop of chloroform. One µl from each of the three dilutions (10^{-3}, 10^{-4}, 10^{-5}) of the phage stock was subjected to the same screening procedure involving plating, lifting, prehybridization, hybridization, washing and autoradiography, as the primary high-stringency screening. This was repeated for the tertiary screening until pure positive phage plaques were obtained.

2.9 Extraction of λDNA

Extraction of λDNA from isolated phages was prepared using a modified DNA extraction protocol as outlined by CLONTECH Laboratories Inc. in section 2.6. Once the 25 ml culture of *E.Coli* host strain K802 cells had lysed completely, it was centrifuged at 5000 rpm for 10 min. The supernatant was treated (see section 2.6) and ultracentrifuged at 28,000 rpm for 45 min in an SW41 Beckman rotor. Pellets were resusupended in 500µl of SM and transferred to microcentrifuge tubes. The suspension was treated (see section 2.6) and extracted with 500 µl of phenol, twice with 500 µl of chloroform and precipitated with 1/10 volume of 3 M sodium acetate and 2.5 volumes of 100% EtOH. Samples were centrifuged at 14,000 rpm, washed in 70% EtOH, dried and resuspended in 60 µl TE buffer.

2.10 Analysis by PCR amplification

A pair of specific oligonucleotide primers were designed to diagnose the clones by the PCR method. The primers were taken from a region where they could be specific to both clone 8 and to the 5-HT_{7B} receptor pseudogene since clone 8 and the 5-HT_{7B} receptor pseudogene are 99% homologous to each other. They were as follows:

TPGS: 5'-GTGATGGATGTTAACAGC-3'

HSPG4: 5'-GACGAAGCACACGGAGAT-3'

The conditions for the PCR (Saiki *et al*, 1988) were as follows. Each PCR tube received 0.5 µl of λDNA (300 ng), 1 µg of each primer, 1 U of *Taq* DNA polymerase enzyme, 10 µl of 5X Invitrogen PCR buffer B (2.0 mM Mg^{2+}; 300 mM Tris, pH 8.5; 75 mM ammonium sulfate) and 5 µl of DMSO in a total reaction volume of 48 µl. The mixture was preincubated at 95°C for 4 min ("hot start"), and the reaction started with the final addition of 2 µl of 10mM dNTPs (dATP, dCTP, dGTP, dTTP). A total of 30

thermocycles were done (Perkin-Elmer-9600) each consisting of 30 sec at 94°C for denaturation, 30 sec at 55°C for annealing, 1 min at 72°C for extension and a final 7 min at 72°C for extension. This was repeated with serotonin-7 receptor pseudogene specific primer pairs.

2.11 Subcloning of PCR fragments into plasmid vector

The reactions were electrophoresed on 1% agarose. Amplified reactions showing a band size of approximately 300 bp were excised and directly ligated into the *Eco*RI cloning site of a plasmid vector (pCR™II), using the Original TA Cloning® Kit. Based on a PCR reaction artifact, this quick strategy makes use of the single deoxyadenosine (A) that is added to the 3' ends of PCR products by the nontemplate-dependent activity of the *Taq* polymerase. The linearized vector (pCR™II) included in the TA cloning kit has a single 3' deoxythymidine (T) residue which allows PCR inserts to ligate efficiently with the vector. Fresh PCR products were used due to degradation of the deoxyadenosine (A) 3' end over time. The vector contains restriction sites, the amp resistance gene and the *Lac*Za fragment gene for blue-white selection.

Briefly, 4 µl sterile water, 1 µl 10X ligation buffer (60 mM Tris-HCl, pH7.5; 60 mM $MgCl_2$; 50 mM NaCl; 1 mg/ml bovine serum albumin; 70 mM β-mercaptoethanol; 1 mM ATP; 20 mM DTT; 10 mM spermidine), 2 µl (50 ng) vector, 2 µl (~10 ng) of the insert and 1 µl of T4 DNA ligase were incubated overnight at 14°C. Vials of One Shot™ competent cells (*E. coli* strain: INVαF') were thawed on ice and treated with 2 µl of 0.5 µl β-mercaptoethanol with gentle mixing. Ten µl of the ligation mixture were added to the cells and incubated on ice for 30 min. The cells were heat-shocked for exactly 30 sec at 42°C and immediately chilled on ice for 2 min. The cells were allowed to recover in 450 µl of SOC medium (2% tryptone; 0.5% yeast extract; 10 mM NaCl; 2.5 mM KCl; 10 mM

$MgCl_2$; 10 mM $MgSO_4$; 20 mM glucose) for 1 h at 37°C while rotating at 225 rpm. The vials of cells were then placed on ice. The transformed cells were plated onto LB-amp plates (100 µg/ml) with 40 µl of 20 mg/ml stock X-gal and incubated at 37°C for at least 18 h before selection for blue colour.

2.12 Chromosomal and restriction mapping

(a) Maxi-preparation of plasmid DNA:

A large-scale rapid preparation of purified plasmid DNA was completed using the QIAGEN Plasmid Kit. The appropriate transformant (G5 and G7.2 from section 2.6) was used to inoculate 5 ml of LB medium with 100 µg/ml amp. Similarly, this was used to grow a 250 ml bacterial culture. The cells were centrifuged at 6000 rpm, 4°C for 10 min in Sorvall GSA rotors and resuspended in 10 ml of buffer 1 (100 µg/ml RNase A; 50 mM Tris-HCl, pH 8.0; 10 mM EDTA). Subsequently, these were transferred to Oakridge tubes and 10 ml of buffer 2 (200 mM NaOH; 1% SDS) was added for 3 min and inverted 4 times for alkaline lysis of cells. Chilled buffer 3 (3.0 M KAc, pH 5.5) was added to neutralize the lysis procedure and gently inverted for thorough mixing. The tubes were centrifuged at 16,000 rpm, 4°C for 30 min in Sorvall SS-34. The clear supernatant was added to equilibrated QIAGEN-tips/columns (Equilibration buffer: 750 mM NaCl; 50 mM MOPS; 15% EtOH, pH 7.0; 0.15% Triton X-100) and allowed to flow by gravity for binding to the resin. The QIAGEN-tip was washed twice with 30 ml of wash buffer (1.0 M NaCl; 50 mM MOPS; 15% EtOH, pH 7.0). The plasmid DNA was eluted by gravity flow with 15 ml of elution buffer (1.25 M NaCl; 50 mM Tris-HCl, pH 8.5; 15% EtOH) and precipitated with 0.7 volumes of isopropanol. This was immediately centrifuged at 12,000 rpm, 4°C for 30 min, washed in 15 ml of cold 70% EtOH and air-dried for 5 min before resuspending in 250 µl of sterile ddH_2O.

(b) Chromosome localization:

The chromosome localization of the clone 8 gene and the serotonin-7 receptor pseudogene was prepared by See DNA Biotech Inc. (Downsview, ON). The method was as follows:

Lymphocytes isolated from human blood were cultured in α-minimal essential medium (α-MEM) supplemented with 10% fetal calf serum and phytohemagglutinin at 37°C for 68-72 h. In order to synchronize the cell population, the lymphocyte cultures were treated with 0.18 mg/ml 5-bromodeoxyuridine. The cells were washed with serum-free medium and recultured at 37°C for 6 h in α-MEM with 2.5 μg/ml thymidine. The cells were harvested and prepared for hybridization on slides. The slides were treated with RNase before being denatured in 70% formamide in 2X SSC at 70°C for 2 min.

The genomic preparations were biotinylated with dATP using the BioNick Labelling Kit at 15°C for 1 h (Heng *et al*, 1992). Prehybridization was carried out at 37°C for 15 min in a solution of 50% formamide, 10% dextran sulfate and human cot I DNA (Heng and Tsui, 1994). The probe was denatured (at 75°C for 5 min) and added to hybridize overnight. By superimposing fluorescence *in situ* hybridization (FISH) signals with 4-6-diamidino-2-phenylindole (DAPI) banded chromosomes, localization of the gene (s) was determined (Figure 10).

(c) Restriction mapping:

The recombinant DNA of clone 8 and the serotonin-7 receptor pseudogene was each cleaved with a variety of restriction endonucleases, either individually or in combination. The resulting products were electrophoresed on a 1% agarose gel. The restriction map was deduced progressively by determining the sizes of the DNA fragments

produced by enzymatic cleavage. There were 19 single enzyme reactions and 2 reactions with 2 enzyme combinations. From the single enzyme digestions producing few but distinct banding patterns, 2 sets of enzyme combinations were devised. The first combination used restriction endonucleases *Acc* I and *Hinc* II. The second set used *Not* I in conjunction with *Acc* I. From the data compiled, a restriction map of clone 8 and the 5-HT_{7B} receptor pseudogene was obtained (Figure 12). Specifically, *Not* I, *Acc* I, *Dra* I, *Sph* I, *Hinc* II and *Kpn* I were used. *Not* I *Acc* I, and *Dra* I were GIBCO BRL enzymes and *Sph* I, *Hinc* II and *Kpn* I were Pharmacia enzymes.

2.13 Hybridization screening of mouse genomic library

The ~100 bp PCR fragment (section 2.1) obtained from amplifying schizophrenia genomic DNA and the 2 kb cDNA of clone 8 obtained from the human fetal brain library, were used to screen a mouse genomic library. The details of the method used were outlined previously in section 2.5.

(a) Library titering, plating and membrane lifting:

The library stock was diluted in SM buffer (2.5 x 10^{-5}). Two, five, ten and zero µl of each were used to infect 200 µl of host K802 competent cells at OD_{600}~0.5. This was mixed with 3.5 ml of top agarose and plated onto small prewarmed NZY agar plates. The plates were incubated at 37°C overnight. The titer of the library was calculated to be 5 x 10^8 pfu/ml.

The library was plated such that 3 x 10^4 plaques/plate were obtained for primary screening. Phage and 600 µl of host K802 competent cells were allowed to adsorb at 37°C for 15 min. This was then plated in 7 ml of top agarose (55°C) onto 20 large prewarmed

NZY agar plates. After 8 h in a 37°C incubator, the plates were removed and chilled for 1 h before the library was lifted onto nylon membranes.

(b) Prehybridization, radiolabelling, hybridization, washing and autoradiography:

Under low stringency conditions, air-dried membranes were prehybridized at 42°C, 50 rpm for 4 h in a prehybridization solution of 30% deionized formamide, 1X Denhardt's solution, 100 mM sodium phosphate, 0.1% glycine, 3X SSC and 200 µg/ml denatured (boiled for 10 min) salmon sperm DNA. For hybridization, the ~100 bp fragment and the 2 kb cDNA (containing the full gene sequence of clone 8) were random-primed [α-^{32}P]-labelled and NACS Prepac Column purified, as previously described. Both probes (~10^6 cpm/ml each) were added to fresh prewarmed (42°C) prehybridization solution containing 10% dextran sulfate. Hybridization was carried out at 42°C in a carousel incubator at the lowest speed overnight.

The membranes were washed in a solution of 2X SSC and 0.1% SDS at room temperature for 15 min twice. This was followed by two 30 min washings in the same solution under low stringency conditions at 55°C. These were subjected to autoradiography overnight at -70°C.

(c) Secondary and tertiary screenings:

Positive signals from the primary screening were isolated and purified through two additional rounds of screening. Phage plugs from the plates of interest were placed into a microcentrifuge tube containing 500 µl of SM and a drop of chloroform. Five and ten µl of a 10^{-2} dilution of the phage stock were subjected to the same screening procedure involving plating, lifting, prehybridization, hybridization, washing and autoradiography, as the

primary high-stringency screening. Similarly, this was repeated for the tertiary screening using 1 and 5 µl of a 10^{-2} dilution until pure positive phage plaques were obtained.

2.14 Extraction of λDNA

Extraction of λDNA from isolated phages was prepared using a modified small-scale DNA extraction protocol as outlined in *Current Protocols in Molecular Biology* (Ausubel, 1995). A 5 ml culture of K802 cells with 0.2% maltose was grown overnight. The following morning, 150 µl of the saturated culture, 150 µl of 10 mM $MgSO_4$/10mM $CaCl_2$ binding solution and 200 µl of the purified phage stocks were allowed to incubate at 37°C for 15 min in microcentrifuge tubes. This was transferred to 50 ml Falcon tubes with 5 ml of NZY medium and allowed to grow and lyse at 37°C, 300 rpm for 3-6 h. Once lysed, 5 drops of chloroform was added and incubated again at 37°C, 300 rpm for 15 min. The cultures were centrifuged at 5000 rpm for 10 min. Five ml of the supernatant were transferred to 14 ml Falcon round-bottom tubes and treated with 10 µl chloroform, 1 µg/ml DNase I and RNase A. This was incubated at 37°C for 30 min. An equal volume of 20% polyethyleneglycol-8000 was added and incubated at 4°C for 1 h. Samples were centrifuged at 8000 rpm, 4°C for 30 min in an SM24 Sorvall rotor. The pellets were resusupended in 500µl of SM and transferred to microcentrifuge tubes. The suspension was treated with 5 µl of 10% SDS and 0.5 µl of 0.5 M EDTA and incubated at 65°C for 15 min. The phage DNA was extracted with 500 µl of phenol, 500 µl of phenol/chloroform, 500 µl of chloroform and precipitated with 1/10 volume of 3 M sodium acetate and 0.7 volumes of isopropanol (-20°C, 30 min). Samples were centrifuged at 14,000 rpm, dried and resuspended in 40 µl TE buffer. Subsequently, each sample was digested with *Sac* I at 37°C for 4 h to excise the insert.

RESULTS

3.0 Hybridization screening of human fetal brain cDNA library

(a) Identification and isolation of positive signals:

A colleague in our lab had previously synthesized a pair of degenerate oligonucleotide primers based on the highly conserved amino acid sequence of transmembrane domains TM6 and TM7 of human serotonin S_{5A} (Rees *et al*, 1994) and S_7 (Bard *et al*, 1993) and dopamine D_2 (Grandy *et al*, 1989), D_3 (Giros *et al*, 1990), D_4 (Van Tol *et al*, 1991) receptors (Figure 4). Using these primers, PCR amplification of human schizophrenia genomic DNA revealed an electrophoretic band of approximately 100 bp. This band was isolated and the DNA subcloned.

From the 90 transformants that were analyzed by members of our lab, most were nonspecific or dopamine receptors. However, 20% were identified as the $5HT_{1A}$ receptor, 15% were identified as the $5\text{-}HT_{7B}$ receptor pseudogene (Qian *et al*, 1997), and 4% (named PCR 15) were identified as the functional gene of the $5\text{-}HT_{7B}$ receptor pseudogene, predicted as the $5\text{-}HT_{7B}$ receptor. The sequence of PCR 15 was shown to be 86% homologous (Figure 5) with the corresponding TM6 to TM7 regions of the cloned $5\text{-}HT_7$ receptor (Bard *et al*, 1993). Thus, under high stringency conditions, a human fetal brain cDNA library in the λgt 10 vector was screened with [α-^{32}P]-labelled PCR 15 in an effort to obtain the full gene sequence of PCR 15.

Approximately 500,000 pfu's of the cDNA library on primary screening plates were lifted onto nylon membranes to be screened. Confirmed by duplicate membranes, four positive phage plaques carrying the corresponding hybridizing signal were identified: 2, 7, 8 and 9. These plaques were purified through a second and third round of hybridization screening. A mid-scale phage preparation was prepared from the final

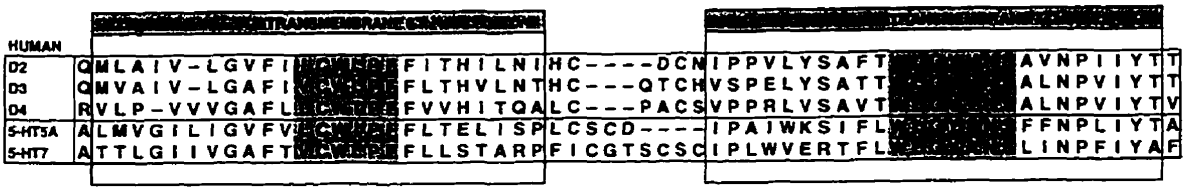


Figure 4 Amino acid sequence alignment of transmembrane domains TM6 and TM7 of human dopamine D_2, D_3, D_4 and serotonin 5-HT_{5A} and 5-HT_7 receptors. The highly conserved amino acid sequences (shaded) were used to design degenerate oligonucleotides for PCR amplification.

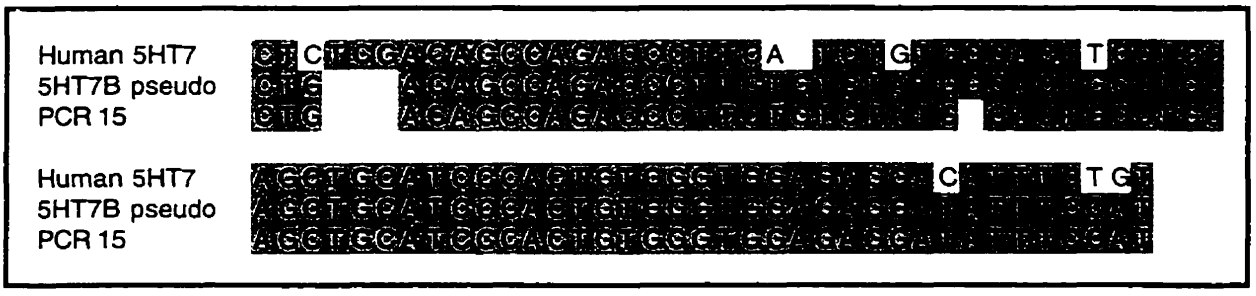


Figure 5 Nucleotide sequence alignment of the 5-HT_7 and 5-HT_{7B} pseudogene receptors as compared to the amplified ~100 bp sequence of PCR 15. PCR 15 shares 86% homology with the 5-HT_7 receptor and 99% homology to the 5-HT_{7B} pseudogene receptor in the corresponding TM6 to TM7 regions.

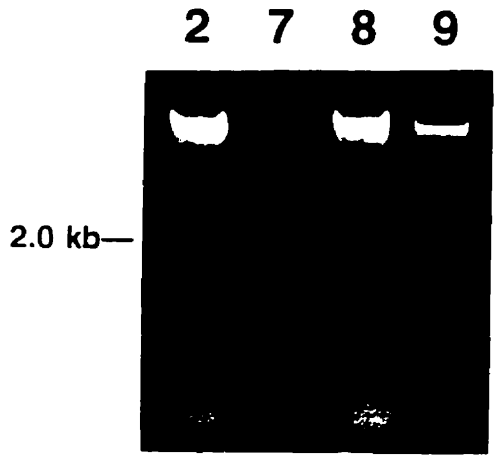


Figure 6 Hybridization screening of human fetal brain cDNA resulted in the isolation of four positive clones, 2, 7, 8 and 9. Only clone 8 revealed a novel G protein-coupled receptor sequence of 2 kb upon excision from the λgt 10 vector with *Eco* RI restriction endonuclease.

purified phage plaques. The purified phage DNA of each clone was cleaved from the λgt 10 vector (Figure 6), subcloned into pBluescript SK +/- plasmid vector and transformed into *E. coli* competent cells. Four transformants from each clone were picked for small-scale plasmid DNA preparation.

(b) DNA sequence analysis of the cDNA clones:

The miniprepped DNA was sequenced using the Sanger dideoxy chain termination method. The initial DNA sequence analysis indicated that clones 2 and 7 had the DNA sequence for the 5-HT_7 receptor, while clone 9 had that for the 5-HT_{7B} receptor pseudogene.

The 2 kb subcloned fragment of clone 8 (Figure 6) showed a novel sequence very similar to that of the 5-HT_{7B} receptor pseudogene. Oligonucleotide primers (18-22 bp) were sythesized from the available sequences. The full DNA sequence of this clone revealed a coding sequence of 1325 bases (Figure 7). Both strands were sequenced completely to ensure accurate reading of the sequence. In comparison to the 5-HT_7 receptor, clone 8 had 19 deleted bases and 12 inserted bases, but no intron after the TM3 region which was present in the 5-HT_7 receptor sequence. The deletions resulted in a frameshift which led to a premature stop codon at nucleotide 250. The clone 8 coding sequence was different than the 5-HT_{7B} pseudogene (found by Qian *et al*, 1997) by 1 nucleotide deletion and 4 nucleotide substitutions. Frameshifts and the premature stop codon of this gene, however, precluded it from producing a functional receptor. The coding sequence was, therefore, "pseudogene-like". Whether clone 8 was a new gene or a polymorphism of the 5-HT_{7B} receptor pseudogene was yet to be determined. Therefore, it continued to be referred as clone 8.

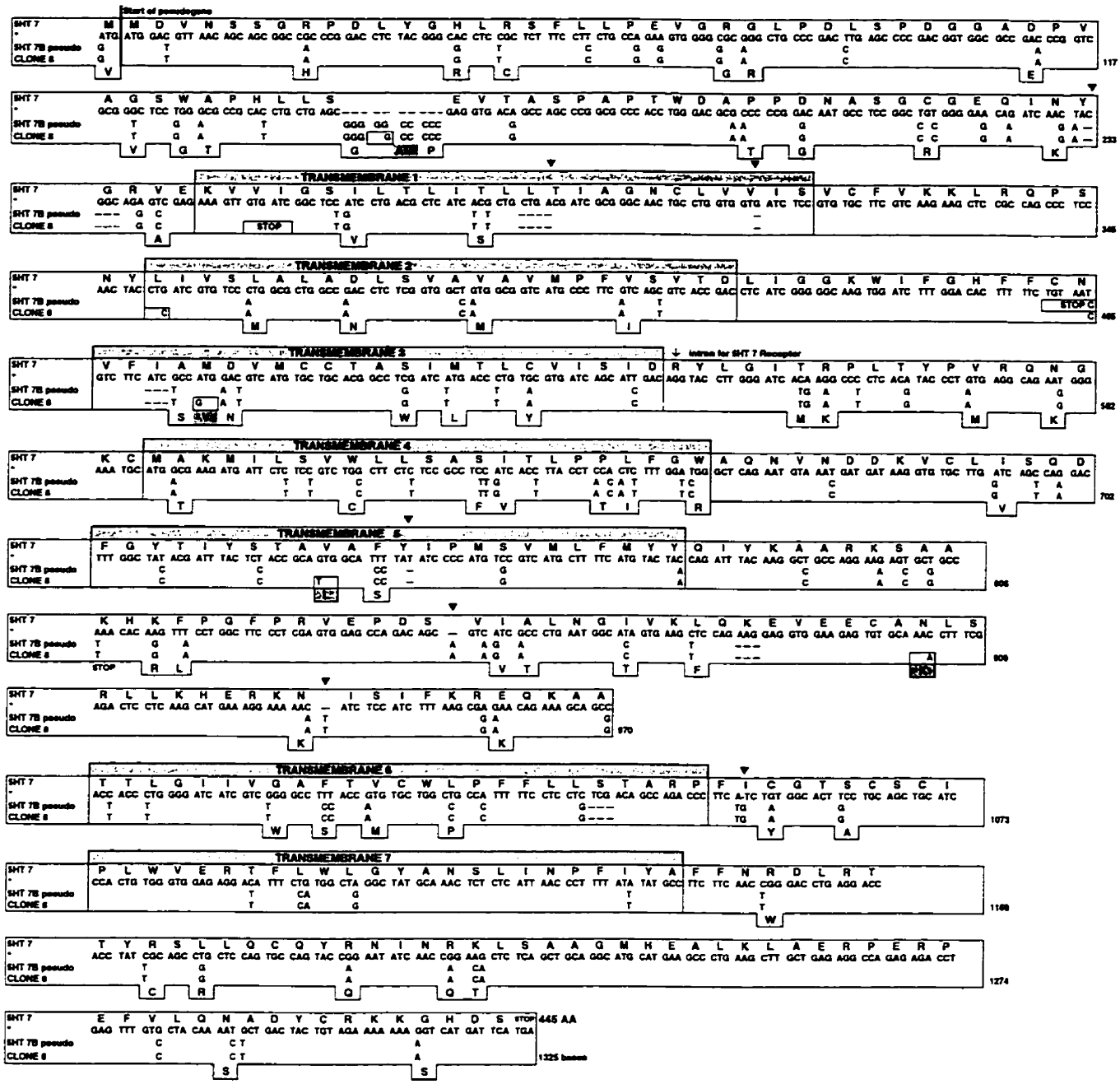


Figure 7 Nucleotide and deduced amino acid sequence of clone 8. The sequencing of clone 8 revealed a coding sequence of 1325 bp and 445 amino acids. The presumed transmembrane regions are identified by the shaded areas. Nucleotide changes specific to clone 8 are boxed. The resulting amino acid changes of clone 8 are indicated below the clone 8 nucleotide sequence shaded. Frameshift locations are indicated by the dark arrow points.

3.1 Subcloning of human genomic λDNA clones G5, G7.2, G7.5, and G10

Shortly after the sequence of PCR 15 was revealed by members of our lab, this novel fragment was used to screen a human genomic library. Seven positive signals were initially detected from the primary screening of which only 4 signals remained after tertiary screening. These 4 clones carrying the hybridizing signal to PCR 15 were named G5, G7.2, G7.5 and G10. Phage preparations were made of each clone and digested with restriction enzymes to cleave the λDNA insert (ranging in size between 15-20 kb) out of its EMBL3 SP6/T7 vector for subcloning into pBluescript SK +/- plasmid vector. Several attempts were made but subcloning was unsuccessful. The results of the human genomic library screening remained incomplete.

Concomitantly, the sequencing of the human fetal brain cDNA clones, using PCR 15, was being completed. Since the human fetal brain cDNA library screening did not detect the functional 5-HT_{7B} receptor, the 4 genomic clones (G5, G7.2, G7.5, G10) were given to me to subclone. The analysis of these genomic clones was necessary to determine if the functional 5-HT_{7B} receptor or other novel G protein-coupled receptor sequences were present.

Using a rapid ligation protocol and standard transformation techniques, as previously described, the 4 genomic clones were digested with *Sac* I to excise the λDNA out of the EMBL3 SP6/T7 vector. The inserts were purified and subcloned into pBluescript SK +/- plasmid vector. Minipreparations and sequence analysis of the putative regions showed that clones G5 and G7.2 were identical to clone 8 and clones G7.5 and G10 were identical to the 5-HT_7 receptor gene. The functional 5-HT_{7B} receptor gene remained elusive.

3.2 Northern blot analysis of clone 8

In order to localize the tissue region of highest expression, clone 8, which contained the whole coding sequence of ~1.3 kb and ~700 bp of noncoding 3' sequence, was [α-^{32}P]-labelled. Northern blot analysis of polyadenylated mRNA of human tissues revealed the presence of two mRNA transcripts of 4.3 and 4.7 kb (Figure 8). Levels of clone 8 mRNA were seen in brain tissue. The specific regions of relatively high expression were in the cerebral cortex, frontal, occipital and temporal lobes and putamen. In peripheral tissues, clone 8 was most expressed in the placenta and to a lesser extent in the kidney and heart. However, it is important to note that the overall mRNA levels were very low. After an overnight exposure at -70°C, the signals were visualized but only faintly. An exposure of 14 days was necessary to see the present autoradiograph (Figure 8). A common problem in detecting some classes of 5-HT receptors [e.g. 5-HT$_4$ (Ford and Clarke, 1993; Gerald *et al*, 1994; 1995) and 5-HT$_7$ (Bard *et al*, 1993; Ruat *et al*, 1993; Lovenberg *et al*, 1993; Meyerhof *et al*, 1993)] is very low expression of these transcripts. This may also be the problem for 5-HT$_{7B}$-like receptors.

3.3 Hybridization screening of human genomic library

Approximately 600,000 pfu's of the phage library was lifted onto nylon membranes from a 12 large primary screening plates and probed with [α-^{32}P]-labelled clone 8 under high stringency conditions. Using the larger (2 kb) DNA fragment, cloned from the human fetal brain cDNA library, to probe the human genomic library, it was hypothesized that homologous sequences would have a longer region of DNA for hybridization. A longer DNA probe would also bind more stably to the DNA. After an overnight exposure at -70°C, 19 positive signals were seen on the primary autoradiographs. The positive signals were isolated and purified through two additional rounds of screening. All 19 clones were

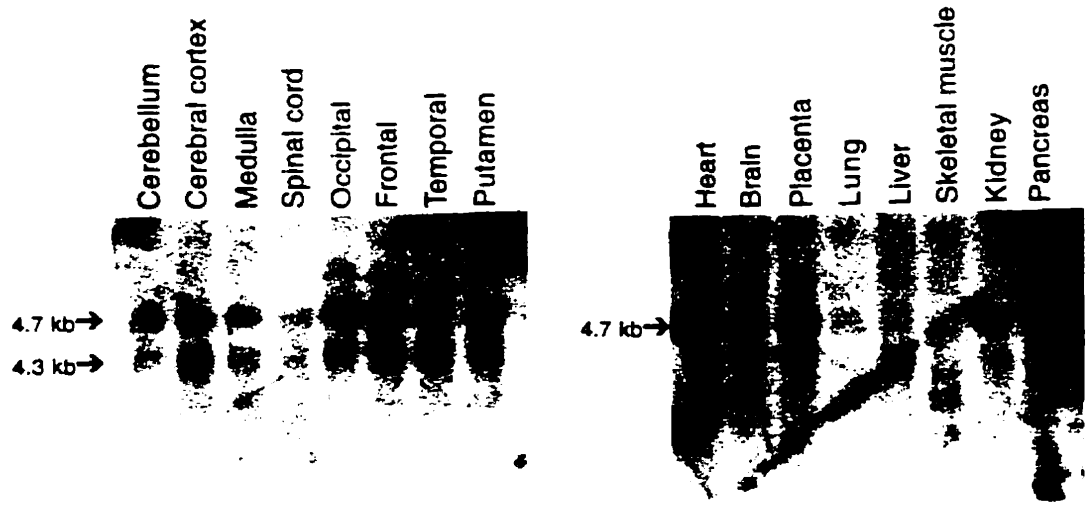


Figure 8 Northern blot analysis of polyadenylated mRNA of various human tissue regions. Hybridization with [α-^{32}P]-labelled clone 8 cDNA revealed the presence of two mRNA transcripts of 4.3 and 4.7 kb.

chosen for λDNA phage preparation from the isolated and purified phage plaque stocks.

3.4 Analysis by PCR amplification

The 19 phage preparations were subjected to PCR amplification with clone 8 and 5-HT_{7B} receptor pseudogene specific primers in order to determine the presence of clone 8 or the 5-HT_{7B} receptor pseudogene sequence. In search of the functional 5-HT_{7B} receptor, this would allow the detection and elimination of clone 8 and the 5-HT_{7B} receptor pseudogene. Thus, all 19 clones would not have to be fully subcloned and analyzed. Attention could be afforded to those clones which were not amplified by these specific primers. Such clones would be homologous to the probe ([α-^{32}P]-labelled clone 8) used to screen the genomic library but would not be the pseudogene or clone 8.

From 19 separate PCR reactions with these specific primers, all 19 clones were identically amplified when electrophoresed on a 1% agarose gel. To verify that all 19 clones were indeed the pseudogene or clone 8, six were randomly chosen for quick subcloning directly from PCR amplification using the Original TA Cloning Kit. Sequence analysis confirmed that the amplified sequences were of the 5-HT_{7B} receptor pseudogene. The remaining 13 clones were eventually sequenced with the same outcome. None of the 19 clones had the clone 8 sequence.

3.5 Chromosome localization

In order to map the location of clone 8 and the 5-HT_{7B} receptor pseudogene to specific chromosomes, a much larger genomic fragment, between 7-12 kb, was required to adequately bind for the mapping.

Previously in section 2.6, the clone 8 genomic fragment had already been subcloned into pBluescript SK +/- plasmid vector. From G5 and G7.2, only G7.2 was

digested with *Sac* I into a large enough fragment of 7.5 kb to be considered for the chromosome mapping. G5 was digested into fragments smaller than 5.5 kb, therefore, was not used (Figure 9). The 5-HT_{7B} receptor pseudogene genomic fragment was taken from one of the 19 clones (P2) from the human genomic library screening in section 2.8. P2 was also *Sac* I digested and the largest fragment was 7 kb. Similarly, the 7 kb P2 fragment was subcloned into pBluescript SK +/- plasmid vector and used for the chromosome mapping.

Large quantities of pure DNA plasmid preparations of clone 8 and the 5-HT_{7B} receptor pseudogene were needed. QIAGEN maxi-preparations were performed and yielded 1.4 μg/μl of the purified recombinant 5-HT_{7B} receptor pseudogene and 1.7 μg/μl of purified recombinant clone 8.

The chromosome mapping was prepared by See DNA Biotech Inc. (Downsview, ON) using the genomic fragment of clone 8 and the 5-HT_{7B} receptor pseudogene. Nonisotopic *in situ* hybridization was used to determine the chromosome location of each dATP-biotinylated probe by superimposing fluorescence *in situ* hybridization (FISH) signals with 4-6-diamidino-2-phenylindole (DAPI) banded chromosomes. Under the conditions used, the hybridization efficiency was approximately 91% for the 5-HT_{7B} receptor pseudogene (P2). Among the 100 mitotic figures that were analyzed, 91 showed signals. The DAPI banding was used to identify the specific human chromosome to chromosome 12 (Figure 10B). The exact locus was picked by FISH detection on the short arm of human chromosome 12 (Figure 10A). The 5-HT_{7B} receptor pseudogene (P2) was located at human chromosome 12, region p12.3-p13.2 (Figure 11). Under these same conditions, clone 8 (G7.2) was mapped to the same locus as the 5-HT_{7B} receptor pseudogene with an efficiency of 89% (Figures 10 A,B). No other hybridizing signal was observed under these experimental conditions.

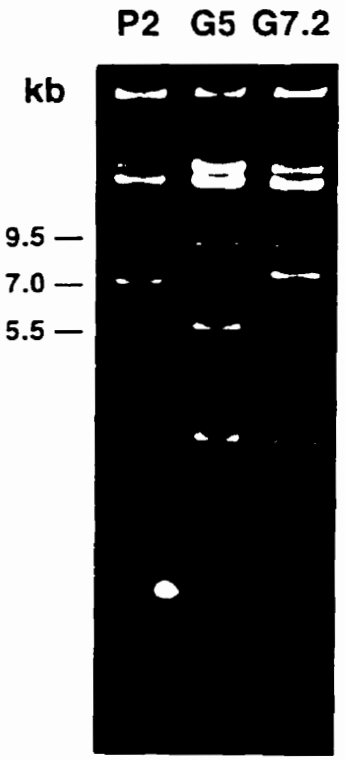
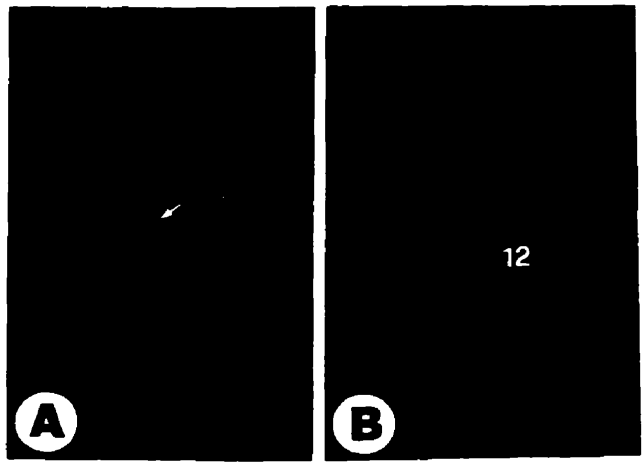


Figure 9 Genomic DNA fragment *Sac* I digestions of the 5-HT_{7B} pseudogene receptor (P2) and clone 8 (G5 and G7.2). Only the 7 kb fragment of P2 and the 7.5 kb fragment of G7.2 were excised and maxi-prepped for the chromosome mapping. The 5.5 kb fragment of G5 was not used.

P2



G7.2

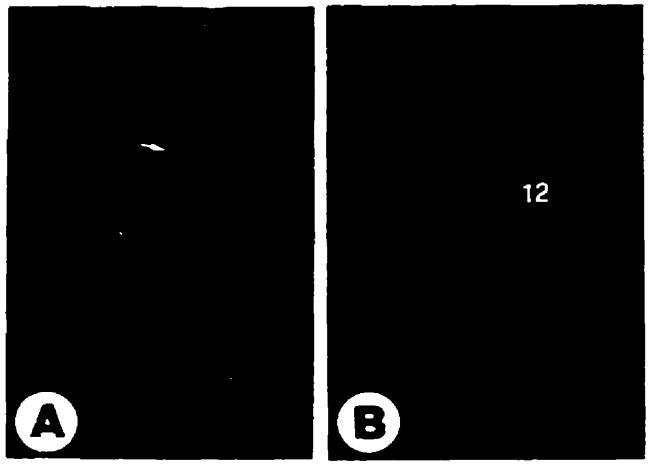


Figure 10 Fluorescence *in situ* hybridization (FISH) mapping of dATP-biotinylated probe P2 and G7.2. Panel A shows the FISH signals on the chromosome; panel B shows the same mitotic figure stained with DAPI to identify chromosome 12.

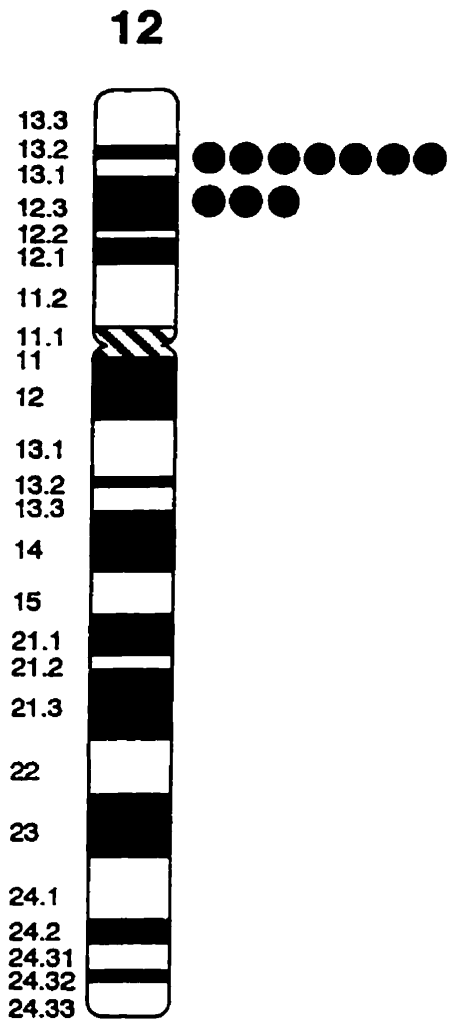


Figure 11 Schematic representation of FISH mapping results for probe P2 and G7.2. Each dot represents a pair of FISH signals detected on human chromosome 12p12.3-p13.2.

3.6 Restriction mapping

Plasmid DNA obtained from the maxi-preparations in section 2.12 of clone 8 and the 5-HT_{7B} receptor pseudogene were digested with various restriction endonucleases to create a partial restriction map. This information, aside from facilitating the subcloning and sequencing of genomic clones, also allows different hybridizing clones to be distinguished from one another. Depending on the restriction map pattern of different genomic clones, unique clones will have unique restriction sites. Both clone 8 and the 5-HT_{7B} receptor pseudogene plasmid DNAs were digested at 37°C for 1 h each. In all, 21 digests were performed per gene of which 8 did not cleave the DNAs. Analyzing the banding patterns of each digestion in relation to the corresponding DNA size and its position to restriction sites in the coding sequence, a partial restriction map was gradually deduced (Figure 12). Three restriction endnucleases cleaved the DNAs outside of the coding sequence exclusively. These were *Not* I, *Dra* I and *Acc* I. Two enzymes, *Hinc* II and *Sph* I, cleaved only in the coding sequence and one, *Kpn* I, cut in both the coding and noncoding regions. The restriction endonuclease map of clone 8 and the 5-HT_{7B} receptor pseudogene were identical.

3.7 Hybridization screening of mouse genomic library

After screening two different human libraries, the search for the functional 5-HT_{7B} receptor was expanded to a more primitive species. It was thought that the pseudogene, being a more recent evolutionary event, may not be present in rodents. With the possible elimination of confounding pseudogenes, the detection of the functional 5-HT_{7B} receptor homologue in the mouse genome may shed some light on the search for the human counterpart.

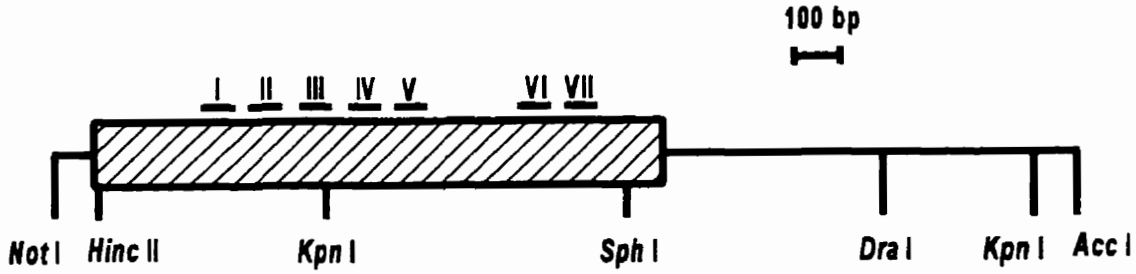


Figure 12 Restriction map of clone 8. Unique restriction endonuclease sites are indicated below. The hatched area denotes the coding region of the clone. Putative transmembrane domains are demarcated above the coding area by Roman numerals. The restriction map for the 5-HT_{7B} pseudogene receptor was identical to that of clone 8.

This screening involved the use of two probes. The ~100 bp PCR 15 fragment and the 2 kb cDNA of clone 8 were both [α-^{32}P]-labelled and prepared for low stringency hybridization screening using K802 *E. Coli* host cells. Approximately 700,000 pfu's were lifted onto nylon membranes from 20 plates. The primary screening produced 24 hybridizing signals on the autoradiographs after an overnight exposure at -70°C. After secondary and tertiary screening, 20 signals remained positive. All 20 clones were isolated and purified for λDNA phage preparation.

Small-scale phage preparations of each sample were digested with *Sac* I at 37°C for 4 h and electrophoresed for analysis. The inserts were excised out of the EMBL3 SP6/T7 vector and visualized under UV light. All but two clones, M6.2 and D1.1, carried the appropriate inserts. The remaining 18 clones were grouped into similar digest patterns to avoid redundancy. Clones M4, M5, M7.1 and D2.2 all had the same digest pattern (Figure 13). All unique restriction patterns will need to be further analyzed.

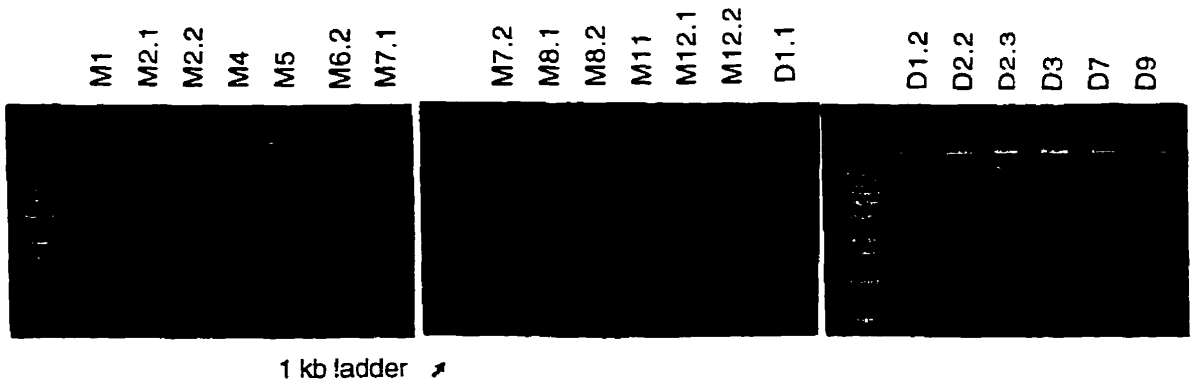


Figure 13 Twenty mouse genomic DNA clones were digested with *Sac* I and electrophoresed for analysis.

DISCUSSION

Antipsychotic drugs have been central to the treatment of schizophrenia and psychosis. Neuroleptics do not eliminate the fundamental disorder of thinking but they have provided most patients with some semblance of "normal" existence within society. At the same time, antipsychotic drugs and their proposed mechanism of action have increased our understanding of brain abnormalities in schizophrenia. This has led to important strategies for isolating the target sites of neuroleptic drug action.

The search for a selective mechanism shared by all antipsychotic drugs focused on the dopaminergic system of the brain and has since established the dopamine D_2 receptor as the primary mediator of neuroleptic drug action (Seeman *et al*, 1975; 1976; Farde *et al*, 1992; 1994; Nordström, 1994). However, as Parkinsonian side effects suggested that antipsychotic drugs might interfere with dopamine transmission (Janssen, 1967; Hornykiewicz, 1973), alleviation of these same side effects by atypical antipsychotic drugs have implicated certain modulatory neurotransmitter systems. Of these, the 5-HT system is thought to be involved (Malmberg *et al*, 1993; Leysen *et al*, 1994; Huttunen, 1995; Meltzer *et al*, 1995).

Similar in primary structure, connected closely anatomically (Törk *et al*, 1991) and functionally interactive (Kelland *et al*, 1990), it is difficult to view the dopamine and 5-HT systems in isolation. The possible role of 5-HT in schizophrenia and in atypical antipsychotic drug action is most likely intertwined with dopaminergic abnormalities, thus, emphasis on studying both systems was important. Hence, strategies to search for other receptor sites included analysis of homologous regions of amino acid sequences of both receptor types. In this way, the basic premise of the search accounted for novel binding sites with similar characteristics of both dopamine and 5-HT receptors. This type of

approach was successful in detecting novel DNA fragments through PCR amplification, a sensitive method to search for new genes using degenerate oligonucleotide primers.

It was not surprising that from this initial search undertaken by colleagues in the lab, a novel 5-HT-like G protein-coupled receptor structure was identified (PCR 15). Further analysis of the ~100 bp sequence showed 86% homology with the corresponding region of the 5-HT_7 receptor (Ruat *et al*, 1993; Bard *et al*, 1993; Lovenberg *et al*, 1993; Meyerhof *et al*, 1993; Shen *et al*, 1993; Plassat *et al*, 1993; Tsou *et al*, 1994) and 99% homology to the recently cloned pseudogene (Qian *et al*, 1997) of the 5-HT_{7B} receptor which has not been isolated. The sequence of PCR 15 revealed the functional 5-HT_{7B} receptor sequence. However, in an effort to isolate the functional 5-HT_{7B} receptor through colony/plaque hybridization screening, a sequence 99% homologous to the cloned 5-HT_{7B} receptor pseudogene (Qian *et al*, 1997) was isolated. Sequence analysis of this clone (clone 8) revealed frameshifts, insertions and deletions producing a premature stop codon at 250 bp. The predicted amino acid sequence of clone 8, if translated from the coding DNA sequence of 1325 bp and if any stop codon is ignored, is 88% identical to the 5-HT_7 receptor. The seven frameshifts and the premature stop codon of this sequence, however, preclude it from producing a functional receptor. Clone 8 was sufficiently different from the 5-HT_{7B} receptor pseudogene by 1 deletion and 4 nucleotide substitutions resulting in 4 amino acid changes (Figure 7). These changes produced a truncated protein of 84 amino acids (Figure 14) as compared to the truncated protein of 154 (Figure 14) amino acids processed by the 5-HT_{7B} receptor pseudogene. The gene was, therefore, initially thought to be another pseudogene since a similar situation was seen with the dopamine D_5 receptor which has two pseudogenes of its own that are 98% homologous to each other (Nguyen *et al*, 1991; Weinshank *et al*, 1991; Grandy *et al*, 1991).

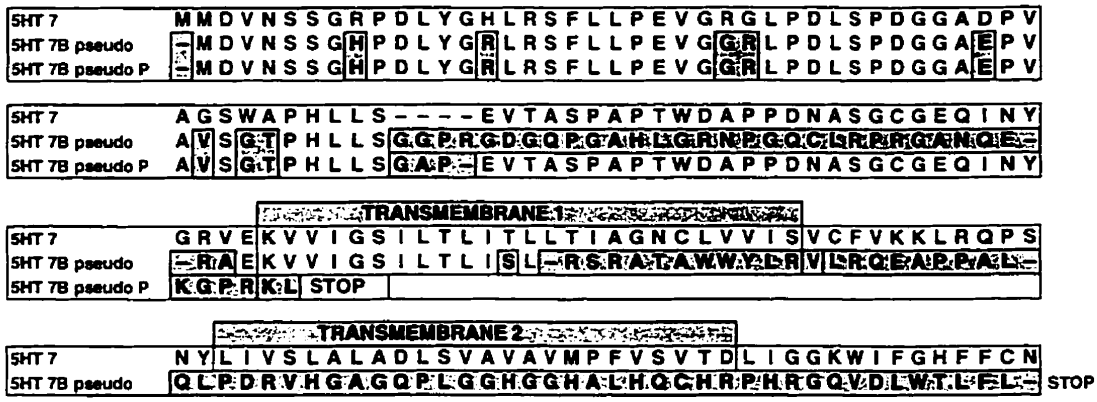


Figure 14 The predicted amino acid sequence of the truncated protein from the DNA sequence of clone 8 (5HT 7B pseudo P - 84 amino acids) and the 5-HT_{7B} receptor pseudogene (154 amino acids). The homologous amino acids are shaded.

Results of the chromosome mapping concluded that clone 8 and the 5-HT_{7B} receptor pseudogene were at the same locus on chromosome 12. However, often with chromosome mapping, it is difficult to resolve separate genes that are directly adjacent to one another (Heng *et al*, 1992; 1993). A technique called restriction endonuclease mapping confirmed the chromosome mapping results and eliminated the possibility of clone 8 to be a new gene. Hence, clone 8 was a polymorphism of the 5-HT_{7B} receptor pseudogene.

This result conferred with the Northern blot analysis of human tissues which localized two mRNA transcripts of 4.3 and 4.7 kb in the placenta, heart and kidney and in specific regions of the brain at very low levels of expression. Presuming that one of the two transcripts coded for the pseudogene, the other transcript was thought to be the functional 5-HT_{7B} receptor. If clone 8 had been a second 5-HT_{7B} receptor pseudogene, a third transcript would have been evident (assuming the tissue distribution to be the same) since the sequence homologies were so high. However, it is uncertain as to which band is the 5-HT_{7B} receptor pseudogene and which band is the functional 5-HT_{7B} counterpart. Since the pseudogene has been more commonly detected, it is presumed to be more highly expressed than the functional 5-HT_{7B}. Most likely, then, the 4.7 kb transcript is speculated to be the pseudogene due to the stronger band density seen at this size on the autoradiogragh.

The widespread brain regional localization of the pseudogene mRNA (cerebellum, cerebral cortex, medulla, occipital pole, frontal lobe, temporal lobe, and putamen) differed from the more selective brain regional mRNA localization found for the 5-HT_7 receptor in other species at similar transcript sizes (hypothalamus, thalamus and hippocampus) (Lovenberg *et al*, 1993; Meyerhof *et al*; 1993; Tsou *et al*, 1994). This may suggest a role for 5-HT_{7B}-like receptors in modulating cortical and higher thought functions found to be abnormal in some neuropsychiatric disorders (Shen *et al*, 1993). Whatever the implications

may be with regards to the pseudogene and its polymorphism, 5-HT's role in antipsychotic drug action still required the identification of the functional 5-HT_{7B} receptor.

Efforts to detect and isolate the functional 5-HT_{7B} receptor have so far unsuccessful. Possible factors contributing to the difficulty in identifying the full coding sequence of the 5-HT_{7B} receptor may be attributed to three reasons. First, it was predicted that the expression of this receptor in tissues would be very low if the scarcity of the 5-HT_4 (Ford and Clarke, 1993; Gerald *et al*, 1994; 1995) and 5-HT_7 receptor gene (Ruat *et al*, 1993; Bard *et al*, 1993; Lovenberg *et al*, 1993; Meyerhof *et al*, 1993; Shen *et al*, 1993; Plassat *et al*, 1993; Tsou *et al*, 1994) expression was any indication of the levels for the 5-HT_{7B} receptor gene. Cloning techniques, such as PCR amplification of RNA by reverse transcriptase polymerase chain reaction (RT-PCR) and strategies using cDNA libraries of specific tissue regions, have been employed in the past to increase the chances of detecting genes of low expression. The fetal brain cDNA library is commonly used presumably because it provides a richer source of transcripts. This latter approach was taken to search for the 5-HT_{7B} receptor gene using human fetal brain cDNA and yielded only the polymorphic 5-HT_{7B} receptor pseudogene. It could be that the expression of the 5-HT_{7B} receptor gene may not be substantially increased during fetal development and remains at low levels. RT-PCR is also another method to compensate for poorly expressed transcripts. As seen by Northern blot analysis of human tissues, only the placenta showed adequate levels of mRNA. However, since we were presently only interested in CNS (central nervous system) anomalies, this path was not pursued. [Peripheral tissues were included in the Northern blot analysis for future considerations and was not focused on at the time but may now be a useful alternative.]

To bypass the issue of low gene expression, the whole genome can be screened using genomic DNA. In a genomic DNA library, all genes have equal representation.

This, however, may also be difficult depending on the number of introns and the length of the introns in the gene. The number of introns and their lengths could play an important role in hybridization screening with homologous probes. The larger and more frequent introns will destabilize hybrids. Clone 8 may be a polymorphism of a processed pseudogene (Jacq *et al*, 1977; Vanin, 1985). The processed pseudogene is an intronless contiguous sequence of exons mutated directly from mature mRNA of the functional counterpart (Vanin, 1985). Although clone 8 is 2 kb in length, only the 1.3 kb coding sequence would be homologous to the functional receptor since all 5' and 3' intervening sequences are lacking. As a probe for hybridization screening, it may be unable to adequately form stable hybrids if the 5-HT_{7B} receptor gene has large and/or numerous introns. This may be the second reason as to why the 5-HT_{7B} receptor gene has been so difficult to isolate.

In addition to these problems, the search for the 5-HT_{7B} receptor is confounded by the presence of pseudogenes and polymorphic pseudogenes. This third obstacle could be solved by screening genomic DNA but the problem of potentially large introns would still exist. Therefore, by screening mouse genomic DNA at low stringency, it was hoped that a mouse homologue of the 5-HT_{7B} receptor would be detected.

This approach was based on the evolution of pseudogenes (Figure 15) and polymorphisms. Comparison of a number of pseudogene sequences with those of their functional counterparts indicated that processed pseudogenes were formed recently (Proudfoot *et al*, 1980; Li *et al*, 1981; Gwo-Shu *et al*, 1983; Freytag *et al*, 1984; Hanauer *et al*, 1984). Even though pseudogenes (Vanin, 1985) and polymorphisms (Conneally, 1994) are common within multigene families in higher eukaryotes, there are only four reports of pseudogenes in the superfamily of the G protein-coupled receptors (O'Dowd, 1991) and this is the first report of a polymorphic pseudogene in this family of receptors.

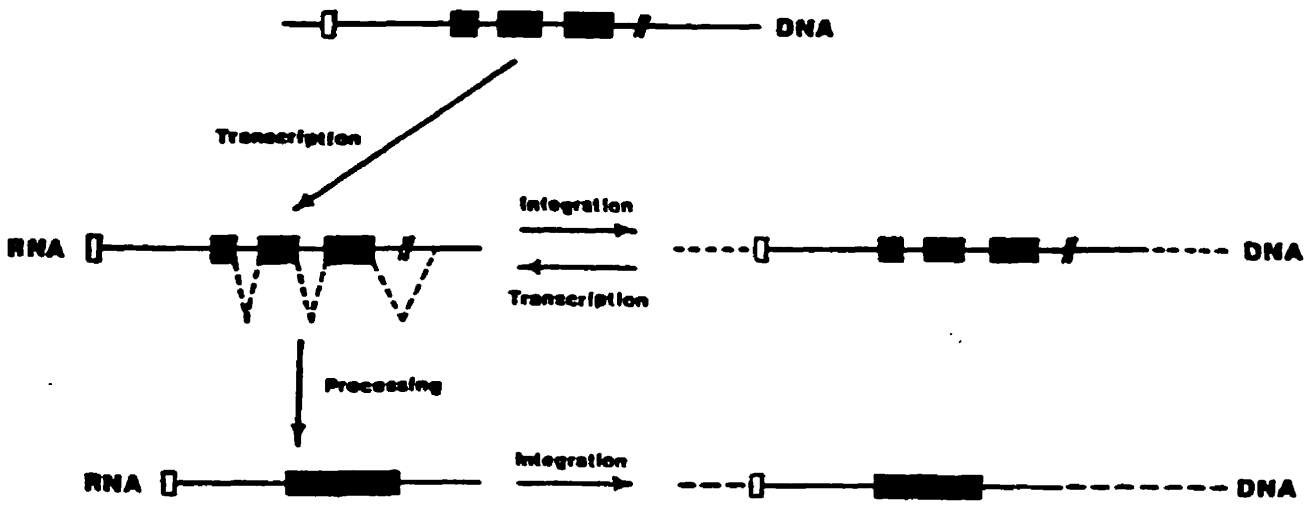


Figure 15 Possible mechanism for the formation of pseudogenes. The solid lines represent exons of the functional gene, while the open box represents the RNA polymerase III promoter. The solid and dashed lines indicate various flanking and intervening sequences. The diagonal dashed lines are intervening sequences which are removed. The DNA, although represented as a single line, is double stranded while the RNA is single stranded.
[Adapted from Vanin, 1985]

All five reports are from the dopamine and 5-HT systems. Two are pseudogenes of the dopamine D_5 receptor (Nguyen *et al*, 1991; Weinshank *et al*, 1991; Grandy *et al*, 1991) and the other is a 5-HT_{1D} receptor pseudogene (Bard *et al*, 1995; Nguyen *et al*, 1993) and the 5-HT_{7B} receptor pseudogene (Qian *et al*, 1997). These pseudogenes are present in primates and humans; none have been isolated from lower mammalian species. Without knowing the putative coding sequence of the functional 5-HT_{7B} receptor, it is difficult to assess evolutionary divergence from a common precursor. However, as indicated by the 7-10% divergence of the human dopamine D_5 receptor pseudogenes and the 5-HT_{1D} receptor pseudogene with their functional counterparts, the emergence of pseudogenes may have occurred approximately 7 million years ago (Vanin, 1985; Bailey *et al*, 1991; Nguyen *et al*, 1991; O'Dowd, 1991). In fact, at least one processed pseudogene, the human dihydrofolate reductase gene (DHFRΨ1) (Chen *et al*, 1982), has been formed so recently that it is polymorphic. This may also be the case with the 5-HT_{7B} receptor pseudogene. This would not be unprecedented with evidence of other genes, such as, the human metallothionein II gene which has a related processed pseudogene and a polymorphism associated with the processed gene (Karin and Richards, 1982).

In humans, it is probable that the majority of beneficial mutations have been available to the genome and selected during evolution. Thus new mutations occurring in humans are most likely deleterious and contribute to a large extent on our genetic load (Conneally, 1994). Such mutations in humans which occur at a rate as high as 10^{-5} per locus per generation (McKusick, 1992; Conneally, 1994) may lead to polymorphic changes through nucleotide point mutations, insertions and deletions. The loss of gene function as a result of gene silencing from such mutations are postulated to be a recent evolutionary event.

Taking all this into consideration, it is highly likely that a lower species, in this case the mouse, will not contain the 5-HT_{7B} receptor pseudogene or its polymorphism. The confounding effects of the pseudogene, therefore, would be eliminated. Hybridization screening at low stringency will help to stabilize homologous hybrids and hopefully detect a 5-HT_{7B}-like mouse receptor. The mouse 5-HT_7 receptor (Plassat *et al*, 1993) has been identified and comparative sequence analysis will aid in determining novel G protein-coupled receptor clones.

Presently, 18 purified clones are being investigated from this initial mouse screening, the results of which are as yet undetermined. It is an ongoing project that will require further work, analysis and modification. Once isolated and characterized, it is hoped that the 5-HT_{7B} receptor will shed some new light on 5-HT's role in atypical antipsychotic drug therapy. As to speculate on the role of the 5-HT_{7B} receptor pseudogene and its polymorphism, no functional purpose exists. However, it has been hypothesized that pseudogenes may have a role in developmental regulation (Nguyen *et al*, 1993; Qian *et al*, 1997) or in modulating the expression or function of its functional gene. This is all still very uncertain.

The potential for the 5-HT_{7B} receptor site to have clinical relevance in antipsychotic drug therapy needs to be pursued. Whether it will prove to have significance in explaining the unique actions of atypical neuroleptics is unknown but the preliminary data from Roth and coworkers seem promising. They illustrate that some atypical antipsychotic drugs (e.g. clozapine and loxapine) are selective for the 5-HT_7 receptor (relative to the dopamine D_2 receptor) and elicit less catalepsy in rats (Roth *et al*, 1994; Seeman *et al*, 1996). Hence, the cloning and analysis of 5-HT_{7B}-like receptors may provide important information on the mechanisms of atypical neuroleptic action. Ultimately, this new knowledge is hoped to define a more distinct role for 5-HT receptors in the pathophysiology of schizophrenia and

extend its role in the development of new atypical antipsychotic drugs which elicit low levels of Parkinsonism in psychotic patients.

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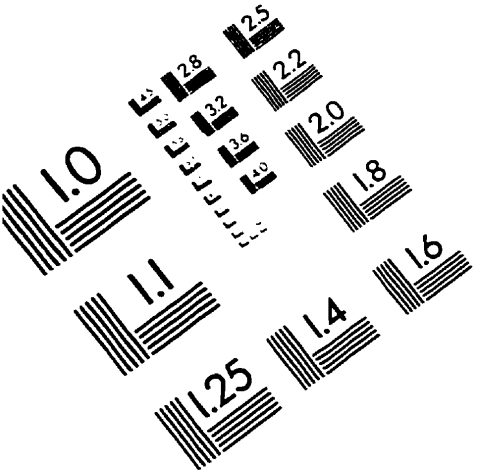
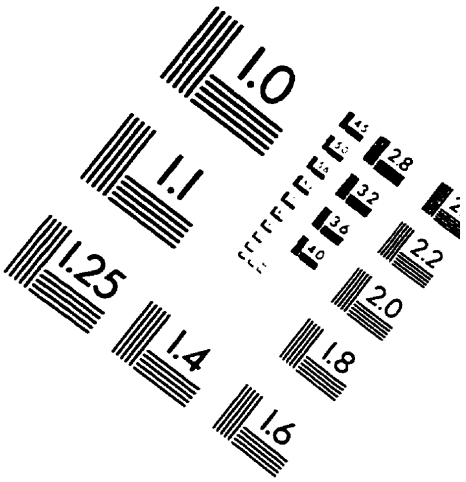
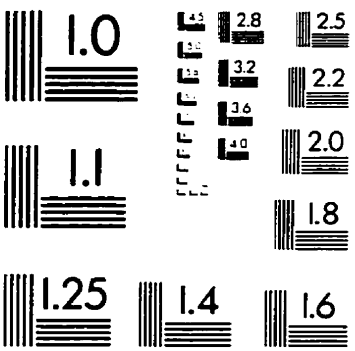
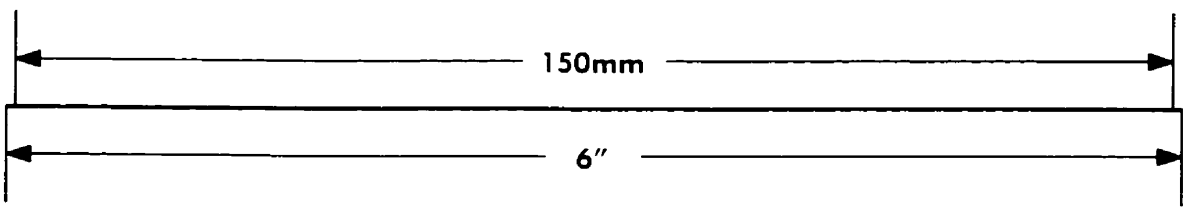
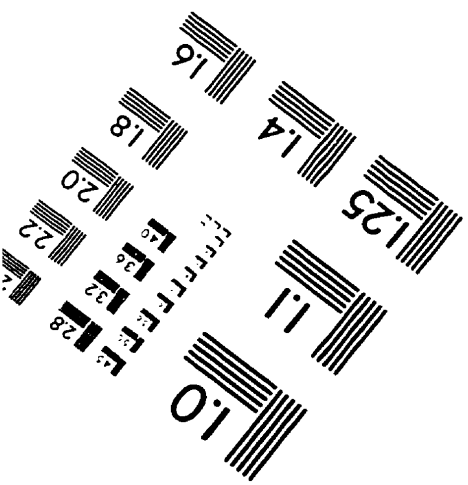
IMAGE EVALUATION TEST TARGET (QA-3)

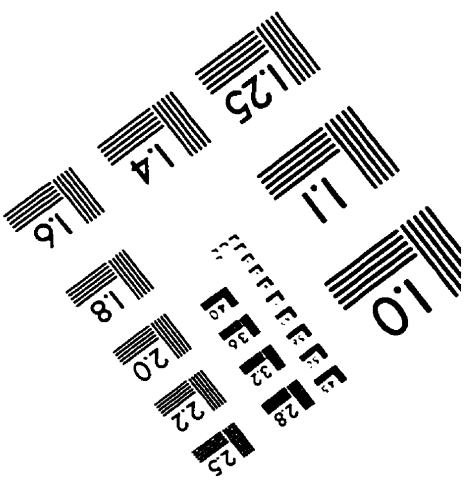












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