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FACULTY OF GRADUATE AND
POSTDOCTORAL STUDIES

Sylvie LEMONDE

AUTEUR DE LA THÈSE - AUTHOR OF THESIS

Ph.D. (Physiology)

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Department of Cellular and Molecular Medicine

FACULTÉ, ÉCOLE, DÉPARTEMENT - FACULTY, SCHOOL, DEPARTMENT

TITRE DE LA THÈSE - TITLE OF THE THESIS

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P. R. Albert

DIRECTEUR DE LA THÈSE - THESIS SUPERVISOR

CO-DIRECTEUR DE LA THÈSE - THESIS CO-SUPERVISOR

EXAMINATEURS DE LA THÈSE - THESIS EXAMINERS

S. Bennett

D. Bulman

D. Park

T. Young

J.-M. De Koninck, Ph.D.

LE DOYEN DE LA FACULTÉ DES ÉTUDES
SUPÉRIEURES ET POSTDOCTORALES

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AND POSTDOCTORAL STUDIES

**TRANSCRIPTIONAL REGULATION OF THE HUMAN
5-HT1A RECEPTOR GENE: IMPLICATIONS IN MAJOR
DEPRESSION AND SUICIDE**

By

SYLVIE LEMONDE

Thesis submitted to the School of Graduate Studies and Research
as a partial fulfillment of the requirements for the degree of
Doctor of Philosophy

Department of Physiology
Faculty of Medicine
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ABSTRACT

Major depressive disorder (MDD) constitutes the most commonly diagnosed mental illness affecting 16% of the population. Reduction in serotonergic tone is the most widely accepted etiological hypothesis for MDD and antidepressant treatments enhance serotonin (5-hydroxytryptamine, 5-HT) neurotransmission. Negative regulation of serotonergic raphe neurons is mediated by somatodendritic 5-HT_{1A} autoreceptors, which are increased in depressed suicides and become down-regulated before antidepressants take effect. I hypothesized that genetic variations in regulatory regions of this receptor that dictate its expression, could contribute to predisposition to depression and treatment responsiveness.

I initially addressed the basal mechanisms of human 5-HT_{1A} receptor gene regulation using transient transfections with luciferase reporter constructs of 5' flanking sequences. A region between -1624 and -1550 bp displayed strong repressor activity and contained at least three repressor elements: a consensus RE-1 and two copies of a novel dual repressor element (DRE). By yeast one-hybrid screening we identified a novel calcium-regulated repressor (Freud-1) that binds to DRE to reduce basal 5-HT_{1A} receptor expression in neurons. Using an inhibitor of histone deacetylase (HDAC), we have demonstrated that Freud-1 mediates HDAC-independent repression in neuronal 5-HT_{1A} positive cells, while REST or other DRE binding proteins recruit HDAC-dependant mechanisms to silence the receptor in non-neuronal 5-HT_{1A}-negative cells.

I also searched for sequence variations in 5-HT_{1A} regulatory regions that may associate with depression. Further downstream from this region, we have identified a functional C(-1019)G polymorphism in the human 5-HT_{1A} promoter that associates with

major depression and completed suicide. The occurrence of the G allele at -1019 bp prevents binding and repression by specific transcription factors NUDR and Hes5, identified by yeast one hybrid approach, and results in de-repression of the 5-HT1A receptor gene and hence, may contribute to the predisposition to depression.

In conclusion, I have identified important transcriptional regulatory elements and proteins of the 5-HT1A gene implicated in serotonin neurotransmission, and characterized the mechanism of a new functional 5-HT1A promoter polymorphism involved in both suicide and MDD. This study may provide an improved marker for diagnosis and treatment of depression and provide a model for correlation between polymorphisms, gene expression and mental illnesses.

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TABLE OF CONTENTS

ABSTRACT	i
DÉDICACE	iii
ACKNOWLEDGEMENTS.....	iv
TABLE OF CONTENTS	vi
LIST OF TABLES.....	ix
LIST OF FIGURES	x
LIST OF ABBREVIATIONS	xi
THESIS FORMAT	xvii
CHAPTER I – INTRODUCTION.....	1
1.1 Serotonin	2
1.1.1 Historical overview.....	2
1.1.2 Origin, biosynthesis and metabolism.....	5
1.1.3 Serotonergic system of the brain	8
1.1.4 Serotonin receptors	13
1.1.5 Serotonin autoreceptors as regulators of serotonergic activity	16
1.1.6 Serotonin transporter.....	27
1.1.7 Conclusion	28
1.2 Major depressive disorder (MDD): description	29
1.2.1 Epidemiology and diagnostic criteria	29
1.2.2 Genetic determinants and environmental causes	33
1.2.3 Pharmacotherapy	35
1.3 Major depressive Disorder: etiology and pathophysiology.....	38
1.3.1 Monoamine hypothesis of depression.....	38
1.3.2 Serotonin system in depression.....	40
1.3.3 5-HT1A receptor in mood disorders.....	42
1.3.4 HPA axis, corticosteroids and depression.....	48
1.4 Regulation of 5-HT1A receptor activity: mechanisms of desensitization.....	55
1.4.1 Rapid homologous versus heterologous desensitization.....	55
1.4.2 Long-term receptor downregulation	58
1.5 Gene transcription: general mechanisms.....	59
1.5.1 Transcription factor families.....	65
1.5.2 Neuron-specific gene transcription.....	71
1.6 Transcriptional regulation of the human 5-HT1A gene: implication in depression.....	75
1.6.1 Delayed onset of antidepressant action and regulated developmental expression	75
1.6.2 Insights from human and rodent 5-HT1A promoter studies	77
1.7 Rationale, goals and objectives	83

CHAPTER II – CELL TYPE-DEPENDENT RECRUITMENT OF TRICHOSTATIN A-SENSITIVE REPRESSION OF THE HUMAN 5-HT1A RECEPTOR GENE.....	85
2.1 Abstract	86
2.2 Introduction	87
2.3 Materials and Methods	89
2.3.1 Reporter Constructs	89
2.3.2 In vitro transcription/translation	93
2.3.3 Cell culture and transfection	93
2.3.4 Luciferase and β -galactosidase assays	94
2.3.5 Nuclear extract preparation	94
2.3.6 Electrophoretic mobility shift assay (EMSA)	95
2.4 Results	95
2.4.1 Core repressor of the human 5-HT1A receptor gene	95
2.4.2 REST-mediated repression via the 5-HT1A RE-1	103
2.4.3 Binding of specific proteins to identified repressor elements of the 5-HT1A promoter... ..	108
2.4.4 RE-1, but not FRE-mediated repression is HDAC-dependent	116
2.5 Discussion	122
2.5.1 Cell specific regulation of 5-HT1A genes	122
2.5.2 HDAC dependence of 5-HT1A repression	124
2.5.3 Functional implications of 5-HT1A repression	126
2.6 References	127
CHAPTER III – FREUD 1: A NEURONAL CALCIUM-REGULATED REPRESSOR OF THE 5-HT1A RECEPTOR GENE.....	134
3.1 Abstract	136
3.2 Introduction	137
3.3 Materials and Methods	138
3.3.1 Yeast one-hybrid screening	138
3.3.2 Plasmids	139
3.3.3 Freud-1 protein expression and purification	140
3.3.4 Cell culture	140
3.3.5 Electrophoretic mobility shift assay (EMSA)	141
3.3.6 Luciferase and β -galactosidase assays	141
3.3.7 Northern and Western blot analyses	142
3.3.8 Immunofluorescence	143
3.3.9 In situ hybridization	144
3.3.10 Statistical Analysis	145
3.4 Results	145
3.4.1 Molecular cloning of Freud-1	145
3.4.2 Freud-1 binding to 5-HT1A FRE	148
3.4.3 Freud-1 repression of raphe 5-HT1A receptor expression	151
3.4.4 Inactivation of Freud-1 by calcium/ATP	157
3.4.5 Freud-1 RNA and protein expression in brain	160
3.5 Discussion	168
3.5.1 Freud-1: a novel repressor of the 5-HT1A receptor gene expressed in brain.	168
3.5.2 Structural domains and calcium-dependent regulation of Freud-1.	170
3.6 References	174

CHAPTER IV – IMPAIRED TRANS-REPRESSION AT A 5-HT1A RECEPTOR GENE POLYMORPHISM ASSOCIATED WITH MAJOR DEPRESSION AND SUICIDE	180
4.1 Abstract	182
4.2 Introduction	183
4.3 Materials and Methods	184
4.3.1 Clinical samples	184
4.3.2 DNA analysis	186
4.3.3 Statistical analysis	186
4.3.4 Electrophoretic mobility shift assay	187
4.3.5 Yeast one-hybrid	187
4.3.6 <i>In vitro</i> transcription/translation and DNase I footprinting	188
4.3.7 Antibodies	189
4.3.8 Immunofluorescence	189
4.3.9 Western blot analysis	191
4.3.10 Plasmid constructs and transfections	191
4.3.11 Generation and analysis of stable clones	192
4.4 Results	193
4.4.1 Association of the C(-1019)G polymorphism with major depression and suicide ..	193
4.4.2 Specific protein-DNA interaction at an imperfect palindrome containing C(-1019)	194
4.4.3 Repressor activity of the C(-1019) palindrome sequence	202
4.4.4 Detection of NUDR in a protein-DNA complex from raphe nuclear extracts	207
4.4.5 Down-regulation of 5-HT1A receptor protein and binding by NUDR in raphe cells	211
4.4.6 Colocalization of NUDR and 5-HT1A receptors <i>in vivo</i>	214
4.5 Discussion	218
4.6 References	226
CHAPTER V – DISCUSSION	235
APPENDICES	256
A- Figure VI.1 – C(-1019)G sensitive repressor activity of the polymorphic region at the SV40 promoter.	258
B- Figure VI.2 – Reduced 5-HT1A receptor RNA in RN46A clones expressing NUDR.	260
C- Figure VI.3 – Freud-1 RNA in cortical progenitors and stem cells.	262
D- Figure VI.4 – Enhancer properties of NUDR and repressor activity of Hes5 in a post-synaptic model of 5-HT1A expressing cell line.	264
E- Figure VI.5 – Nuclear translocation of NUDR following 5-HT1A receptor agonist treatment	266
F- Figure VI.6 – C(-1019)G genotype-dependant responsiveness to antidepressant medication.	268
G- Figure VI.7 – Decreased response to antidepressant treatment in homozygous G(-1019) depressed patients	270
H- Figure VI.8 – Severity of depression according to genotype at the C(-1019)G polymorphic site ..	272
I- Figure VI.9 – NUDR and Hes5 -mediated repression at the rat 5-HT1A promoter.	274
J- Figure VI.10 – Tissue distribution of NUDR RNA expression.	276
K- Figure VI.11 – NUDR protein is expressed in the subcommissural organ (SCO)	278
L- Figure VI.12 – Schematic representation of the human 5-HT1A receptor promoter and key regulators that have been identified in this thesis project	280
REFERENCES	281
COPYRIGHT PERMISSION	332

LIST OF TABLES

Table I-1 – Classification of serotonin receptors.....	14
Table I-2 – The 17/21 version of the Hamilton Depression rating scale (HAM-D).....	32
Table II-1 – Oligonucleotide sequences for EMSA and mutagenesis.....	92
Table II-2 – Consensus sequences for DRE and RE-1 sites.....	102
Table IV-1 – Distribution of the 5-HT1A Receptor G(–1019) Gene Polymorphism in Depressed Patients and Control subjects from the Ontario cohort.	197
Table IV-2 – Distribution of the 5-HT1A Receptor G(–1019) Gene Polymorphism in Suicide Completers and Normal Controls from the Quebec cohort.	198

LIST OF FIGURES

Figure I.1 – Metabolic pathways for the synthesis and metabolism of serotonin.	7
Figure I.2 – Serotonergic System.	11
Figure I.3 – Schematic representation of the rat 5-HT1A G protein-coupled receptor.	18
Figure I.4 – Actions of long-term antidepressant drug treatment on pre- and post-synaptic 5-HT1A receptors.	46
Figure I.5 – The HPA axis/5-HT1A receptor relationship.	52
Figure I.6 – General structure of the most common transcription factor DNA-binding motifs. .	66
Figure I.7 – Sequence alignment of the 5' flanking region of the human, mouse and rat 5-HT1A receptor genes.	81
Figure II.1 – Deletion analysis of the human 5-HT1A receptor promoter.	97
Figure II.2 – Sequence alignment of the repressor regions of the 5-HT1A receptor gene. .	100
Figure II.3 – Deletion/mutation analysis of 5-HT1A repressor elements.	105
Figure II.4 – REST-mediated repression via the human 5-HT1A RE-1 site.	107
Figure II.5 – REST interacts with the 5-HT1A RE-1 site.	110
Figure II.6 – 5-HT1A-DRE-binding proteins in nuclear extracts from brain.	113
Figure II.7 – Freud-1 interacts with both 5-HT1A 5'- and 3'-DRE sites.	115
Figure II.8 – TSA blocks RE-1 but not DRE-mediated repression in RN46A cells.	118
Figure II.9 – Freud-1-mediated repression is TSA insensitive.	121
Figure III.1 – Protein structure of Freud-1.	147
Figure III.2 – Specific binding of Freud-1 to 5-HT1A-FRE requires an intact CalB motif. .	150
Figure III.3 – Freud-1 represses basal 5-HT1A receptor expression in raphe RN46A cells via FRE.	154
Figure III.4 – CAM kinase attenuates Freud-1-mediated repression.	159
Figure III.5 – Tissue distribution of Freud-1 RNA expression.	162
Figure III.6 – Co-localization of Freud-1 with 5-HT1A receptor.	165
Figure IV.1 – A C(-1019)G polymorphism of the human 5-HT1A receptor promoter.	196
Figure IV.2 – Allele-specific association of a nuclear protein complex with the C(-1019) palindrome of the 5-HT1A receptor gene.	201
Figure IV.3 – Trans-repression at the palindrome of the 5-HT1A receptor gene by NUDR and Hes5: differential sensitivity to the C(-1019)G polymorphism.	204
Figure IV.4 – Presence of NUDR bound to the C(-1019) allele in RN46A nuclear extracts. ...	210
Figure IV.5 – NUDR protein is expressed in 5-HT1A receptor-positive cells and brain regions and regulates 5-HT1A protein expression.	213
Figure IV.6 – Co-staining of NUDR with neuronal markers and specificity of immunohistochemical staining.	217
Figure IV.7 - Functional model for derepression at the 5-HT1A promoter by the C(-1019)G polymorphism.	221

LIST OF ABBREVIATIONS

-/-	knockout
Δ	deletion
[^3H]	tritiated
$[\text{Ca}^{2+}]_i$	intracellular calcium
5-HIAA	5-hydroxyindoleacetic acid
5-HT	5-Hydroxytryptamine, serotonin
5-HT1A	serotonin 1A receptor
5-HTP	5-hydroxytryptophan
8-OH-DPAT	8-hydroxy-2-(di- <i>n</i> -propylamino)tetralin
A	adenine
aa	amino acids
AC	adenylyl cyclase
Acc.	accession
ACTH	adrenocorticotropin hormone
Ala	alanine
Amp	ampicillin
ANOVA	analysis of variance
AP-1	activator protein-1
Asp	aspartate
Asn	asparagine
ATP	adenosine-5'-triphosphate
β ARK	β -adrenergic receptor kinase
BSA	bovine serum albumine
bp	base pairs
C	cytosine
C-	carboxy
C2	PKC conserved region 2 domain
Ca^{2+}	calcium ion
CalB	Calcium/phospholipids binding domain
CalC	Calphostin C
CAM	Ca^{2+} /calmodulin
CAMK	Ca^{2+} /calmodulin-dependant protein kinase
cAMP	cyclic adenosine-monophosphate
cDNA	complementary deoxyribonucleic acid
C/EBP	CCAAT/enhancer binding protein
Ci_3	carboxy-terminal portion of i_3
CIHR	Canadian Institutes of Health Research
Cli	caudal linear nucleus
CMZ	calmidazolium
CNS	central nervous system
CO_2	carbon dioxide

Con	consensus
cpm	counts per minute
CRE	cAMP-responsive element
CREB	cAMP-responsive element binding protein
CRF	corticotropin-releasing factor
CsCl	cesium chloride
CSF	cerebrospinal fluid
CtBP	C-terminal Binding Protein-1
CTF	CCAAT transcription factor
Ctrl	control
Ctx	cortex
d	day
D	aspartate
DAG	diacylglycerol
DBD	DNA binding domain
dCTP	deoxycytosine triphosphate
DEAF-1	Deformed epidermal autoregulatory factor 1
df	degree of freedom
DG	dentate gyrus
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
DNase I	deoxyribonuclease I
dNTP	deoxynucleotide triphosphate
DRE	Dual Repressor Element
DREAM	DRE-agonist modulator
DSM-IV	Diagnostic and Statistical Manual of Mental disorders, 4 th edition
DTT	dithiothreitol
DRN	dorsal raphe nucleus
E	embryonic day
ECT	electroconvulsive therapy
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis (aminoethyl ether) N, N, N', N'-tetraacetic acid
EMSA	electrophoretic mobility shift assay
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
FRE	Five-prime repressor element
Freud-1	Five-prime Repressor Element Under Dual Repression binding protein-1
g	gramme
G	guanine
G α	alpha subunit of G protein
G $\beta\gamma$	beta gamma subunit of G protein
GAL4-AD	GAL4 activation domain
GAL4-DBD	GAL4 DNA binding domain
GABA	γ -aminobutyric acid
GAP-43	growth-associated protein 43
GAPDH	glyceraldehydes-3-phosphate dehydrogenase

GDP	guanosine di-phosphate
GFAP	glial fibrillary acidic protein
GI	gastrointestinal
Gi	inhibitory G protein
GIRK	G protein-coupled inwardly rectifying potassium channel
GPCRs	G protein-coupled receptors
GR	glucocorticoid receptor
GRE	glucocorticoid responsive element
GRK(s)	G protein-coupled receptor kinases(s)
Gs	stimulatory G protein
GTP	guanosine tri-phosphate
H	histone
h	human
hr	hour
HAM-D	Hamilton Depression rating scale
HAT	histone acetyl transferase
HCl	chloridric acid
HDAC	histone deacetylase
HEK 293	Human Embryonic Kidney cells 293
Hes5	Hairy/Enhancer-of-split-5
Hip	hippocampus
His	histidine
HLH	helix-loop-helix
HPA	Hypothalamic-pituitary-adrenal
HRE	hormones responsive element
HRP	horseradish peroxidase
HSE	heat-shock element
HSF	heat-shock factor
HSP	heat shock proteins
HSV-TK	herpes simplex virus thymidine kinase
i ₁	first intracellular loop
i ₂	second intracellular loop
i ₃	third intracellular loop
IBMX	isobutyl 1-methylxanthine
Ile	isoleucine
Iono	ionomycin
IP ₃	inositol trisphosphate
Inr	initiator
K	lysine
kb	kilobases
KCl	potassium chloride
kDa	kilodalton
Leu	leucine
LSD	d-lysergic acid diethylamide
luc	luciferase
m	mouse

MAO-A	monoamine oxidase A
MAO-B	monoamine oxidase B
MAOI(s)	monoamine oxidase inhibitor(s)
MAP2	major microtubule associated protein
MAPK	mitogen-activated protein kinase
MAZ	Myc-associated zinc finger protein
MB	midbrain
MDD	major depressive disorder
MgCl ₂	magnesium chloride
min	minutes
MLF	medial longitudinal fasciculus
MR	mineralocorticoid receptor
MRN	median raphe nucleus
mRNA	messenger RNA
mut	mutated
MW	molecular weight
N (or n)	sample size
N	any nucleotide
N-	amino
NaChII	sodium channel type II
NaCl	sodium chloride
NARI(s)	noradrenaline reuptake inhibitor(s)
NBT/BCIP	nitro blue tetrazolium chloride/ 5-bromo-4-chloro-3-indolyl phosphate
NDRI(s)	noradrenaline and dopamine reuptake inhibitor(s)
NF1	nuclear factor 1
NF-κB	nuclear factor κB
NH ₂	amino
NOS	nitric oxide synthetase
NP-40	nonidet P-40
NRSE	Neuron Restrictive Silencing Element
NRSF	Neuron Restrictive Silencing Factor
NUDR	nuclear DEAF-1-related protein
OHRI	Ottawa Health Research Institute
ONPG	o-nitrophenyl β-D galactopyranosidase
P	probability
PBS	phosphate buffered saline
PCA	p-chloroamphetamine
pCMVBgal	plasmid cytomegalovirus promoter/β-galactosidase reporter gene
PCR	polymerase chain reaction
PET	positron emission tomography
PET-1	PC12 ets factor
Pfu	derived from <i>Pyrococcus furiosus</i>
PGK-puro	phosphoglycerate kinase-1/puromycine resistance gene
PIP ₂	posphatidylinositol-4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C

PLC	phospholipase C
PMDD	premenstrual dysphoric disorder
PMSF	phenylmethylsulfonyl fluoride
POU	after the first letters of Pit-1, Oct-1 and Oct-2 and Unc-86
PVN	paraventricular nucleus
PTEN	named for its homology to phosphatases and tensin
PTX	pertussis toxin
Py	pyrimidine
r	rat
R	arginine
RACE	5' rapid amplification of cDNA ends
RE-1	Repressor Element-1
REST	Repressor Element-1 Silencing Transcription factor
rFreud-1	recombinant Freud-1
RMg	raphe magnus
RNA	ribonucleic acid
RNase	ribonuclease
RNA pol II	RNA polymerase II
Rob	raphe obscurus nucleus
Rpa	raphe pallidus nucleus
rREST	recombinant REST
R.T.	room temperature
rtn	reaction
RT-PCR	reverse transcriptase-polymerase chain reaction
S	serine
SAND	Sp100, AIRE-1, NucP41/75, DEAF-1
SCID	Structured Clinical Interview for DSM-III
SCO	subcommissural organ
SD	standard deviation
SDS	sodium dodecyl sulphate
SDS-page	SDS polyacrylamide gel electrophoresis
sec	seconds
S.E.M.	standard error of the mean
Ser	serine
SERT	serotonin transporter
SNAP-25	synaptosomal-associated protein of 25
SNC	substantia nigra pars compacta
SNOG	for SNAP-25, NOS, GAP-43
SNR	substantia nigra pars reticulata
SNRI(s)	serotonin and noradrenaline reuptake inhibitors
SSC	3M NaCl and 0.3 M sodium citrate
SSRI(s)	selective serotonin reuptake inhibitors(s)
SV40	simian virus 40
TCA(s)	tricyclic antidepressant(s)
T	thymine
T	threonine

Taq	derived from <i>Thermococcus aquaticus</i>
TAFs	TBP-associated factors
TBP	TATA-binding protein
TBS	tris buffered saline
TF	transcription factor
TH	tyrosine hydroxylase
Thr	threonine
TK	thymidine kinase
TM	transmembrane domain
TME	75 mM tris pH 7.4, 12.5 mM MgCl ₂ and 1mM EDTA
TPH	tryptophane hydroxylase
TRE	Three-prime repressor element
TRH	thyrotropin-releasing hormone
TSA	trichostatin A
t-test	student t-test
TuJ1	β3 tubulin
U	unit
U.S.E.	unique site elimination mutagenesis
Ura	uracile
Val	valine
Veh	vehicle
VMAT	vesicular monoamine transporter
v/v	volume/volume
W	tryptophane
Way 100635	N-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-N-2-pyridinyl-cyclohexanecarboxamide
WHO	World Health Organization
X	any amino acid
X-Gal	5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside
χ^2	Chi-square

THESIS FORMAT

This thesis is presented as a collection of manuscripts. Chapter I is an overall introduction of historical and theoretical background. Chapter II is a paper entitled “Cell-type dependant recruitment of trichostatin A-sensitive repression of the human 5-HT1A receptor gene”. This manuscript had been submitted to the *Journal of Biological Chemistry* at the time of submission of the thesis and is currently published in the *Journal of Neurochemistry* 2004; 88(4):857-68. Chapter III consists of a second paper entitled “Freud-1: a neuronal calcium-regulated repressor of the 5-HT1A receptor gene”. This paper has been accepted for publication in the *Journal of Neuroscience* 2003; 23(19):7415-25. Chapter IV is a paper entitled “Impaired trans-repression at a human 5-HT1A receptor gene polymorphism associated with major depression and suicide”. The paper is published in the *Journal of Neuroscience* 2003; 23(25):8788-99. Chapter V is a general discussion of the work presented in relation to the actual knowledge in the field of serotonergic research.

CHAPTER I – INTRODUCTION

Overview of Thesis

Depression is a serious problem in today's society. The World Health Organization Global Burden of Disease Survey estimates that major depression will be the second leading cause of disability world-wide (after hypertension) by 2020. Serotonin reuptake inhibitors have proven to be very effective in the treatment of this disease and have strengthened the hypothesis that impaired serotonin (5-hydroxytryptamine, 5-HT) neurotransmission may contribute to the underlying cause of depressive disorders. Specifically, the 5-HT_{1A} receptor has been implicated as a key modulator of antidepressant activity (Blier and de Montigny, 1994; Artigas et al., 1996b), and gene knockout of the 5-HT_{1A} receptor in mice results in increased anxiety behaviours (Heisler et al., 1998; Parks et al., 1998; Ramboz et al., 1998). Importantly, in depressed suicide victims, the level of 5-HT_{1A} autoreceptors expressed on serotonin neurons is increased compared to control subjects (Stockmeier et al., 1998). Hence, this thesis focuses on understanding the transcriptional regulation of the human 5-HT_{1A} receptor gene, and identifying mechanisms that control the basal expression of this gene. The introduction presents background on the serotonin system, 5-HT receptors and their regulation and an overview of transcriptional regulatory mechanisms.

1.1 Serotonin

1.1.1 Historical overview

The field of serotonin research has its roots in the 1930's with the work of Erspamer and colleagues (Rome, Italy) and the detection of an amine substance contained in extracts of gastric mucosa responsible for gut motility. Believed to originate from enterochromaffin cells of the gastrointestinal tract, this substance was named 'enteramine' (Vialli and Erspamer, 1933). Meanwhile, during the 1930's and 40's researchers at the Cleveland Clinic (USA) were interested in characterizing the humoral agent responsible for the elevated blood pressure in hypertensive nephritic patients. Although the existence of an endogenous vasoconstrictor substance in blood was suspected over a century ago (Ludwig and Schmidt, 1868), its isolation only became possible 80 years later with the work of Rapport and his associates Green and Page (Rapport et al., 1948a, b). Due to its origin in serum and its constricting action on arteries, the isolated substance was named 'serotonin' (derived from a combination of the Latin words for blood, *serum*, and stretching, *tonus*). Following its discovery, serotonin was crystallized, a year later its chemical structure deduced (Rapport, 1949) and 5-HT (5-hydroxytryptamine) synthesized by the Upjohn Company and the Abbott Laboratories (Hamlin and Fisher, 1951; Speeter et al., 1951), which confirmed the proposed structure. The availability of 5-HT to all investigators allowed pharmacological and biological studies to advance rapidly. In 1952, enteramine was reported to be chemically identical to natural and synthetic serotonin (Erspamer and Asero, 1952), and the extensive pharmacology conducted by Erspamer soon confirmed their identity (e.g., contraction of rat and cat uterus, sheep carotid artery, and guinea-pig, mouse and rabbit jejunum) (Erspamer, 1954; Page, 1954).

Independent work indicated that serotonin was widely distributed in nature and occurred in several other tissues than blood and gut. In 1953, during a routine survey of various tissues, relatively high concentrations of 5-HT were found in the mammalian brain (Twarog and Page, 1953). Shortly thereafter, its heterogeneous distribution in the central nervous system (CNS) was demonstrated (Amin et al., 1954) and 5-HT was considered for the first time a putative neurotransmitter in the CNS (Bogdansky et al., 1956). The powerful hallucinogen LSD (d-lysergic acid diethylamide) was recognized to be structurally similar to 5-HT and found to antagonize the vasoconstrictor action of 5-HT on smooth muscle (Gaddum, 1953; Wooley and Shaw, 1953). The tranquilizing drug reserpine was observed to lower concentrations of 5-HT in the brain leading to profound behavioural depression, and based on these findings it was further suggested that 5-HT served as a neurotransmitter and attention focused on the possible role it might play in mental disorders such as schizophrenia and depression (Wooley and Shaw, 1954). An entire decade passed before histofluorescence and immunohistochemical techniques became available to provide evidence for the existence of serotonergic neurons in the CNS (Dahlstrom and Fuxe, 1964).

An initial classification of 5-HT receptors was achieved by Gaddum and Picarelli (1957) who suggested that the 5-HT-induced guinea-pig ileum contraction was mediated by two different receptors: one located on smooth muscle and one on nervous tissues (parasympathetic ganglia). These receptors were respectively called “D” and “M” receptors since dibenzyline (D) selectively antagonized the effect of 5-HT on smooth muscle and morphine (M) was selective for nervous tissues. This classification became widely accepted and remained for three decades. However, it was noticed that the “D”

receptor antagonists mianserine and cyproheptadine failed to block the vasoconstrictor effect of 5-HT in the canine carotid arterial bed and it was proposed that other 'special' receptors could mediate this effect (Saxena et al., 1971; Saxena, 1972). With the discovery of additional sites that seemingly failed to classify as "M" or "D" types and the emergence of specific ligand binding techniques, it became clear that more subtle classification could be obtained. The first successful radioligand binding study of 5-HT receptors was achieved in 1974 using [^3H]LSD (Bennett and Aghajanian, 1974). Further studies with [^3H]5-HT and [^3H]spiperone on rat cortical tissues enabled Peroutka and Snyder (1979) to establish the existence of two distinct receptors: a 5-HT₁ site with high (nM) binding affinity for 5-HT and a 5-HT₂ site with high (nM) affinity for spiperone but low (μM) affinity for 5-HT; LSD had high affinities for both. Later, the 5-HT₁ recognition site was subdivided into 5-HT_{1A} and 5-HT_{1B} subtypes on the basis of their high and low affinity for spiperone, respectively (Pedigo et al., 1981). In an attempt to organize the rapidly proliferating nomenclature of 5-HT receptors that included names such as "D", "M", 5-HT₁, 5-HT₂, S₁, S₂, S₃ and others, to a more uniform terminology, an international committee formulated specific definitions and criteria. This effort culminated in the Bradley et al., (1986) publication, classifying 5-HT receptors as follows: "5-HT₁-like" (corresponding to some "D" or 5-HT₁), 5-HT₂ (analogous to most "D" or 5-HT₂) and 5-HT₃ (equivalent to "M") receptors. With the development of molecular biological techniques, the serotonergic research community witnessed the cloning of the first 5-HT receptor in 1987 (Kobilka et al., 1987; Lubbert et al., 1987). Since that time, and especially in the past decade, there has been tremendous progress in 5-HT receptor identification.

1.1.2 Origin, biosynthesis and metabolism

It has been estimated that an adult human body contains about 10 mg of serotonin, which can be distributed in three main areas (Gershon, 1985; Lambert et al., 1995). Highest concentrations (approximately 90%) of total body 5-HT are found in enterochromaffin cells of the gastrointestinal tract. Between 8-9% is stored in circulating platelets where it is involved in wound healing responses/coagulation via platelet aggregation. The remaining 1-2% is localized in the brain. Traces of 5-HT have also been detected in other tissues, notably in heart, kidney, spleen, thyroid and retina (Essman, 1978). The synthesis of 5-HT is heavily dependent upon the uptake of the essential amino acid L-tryptophan, from which it is derived. Plasma tryptophan arises primarily from the diet, and elimination of dietary tryptophan can profoundly lower the levels of brain 5-HT. Serotonin and tryptophan belong to a group of aromatic compounds called indoles with a five-membered ring containing nitrogen joined to a benzene ring. Although peripheral cells may use circulating serotonin, since it cannot readily cross the blood brain barrier, the serotonergic neurons in the brain must synthesize their own neurotransmitter. The first step in the synthetic pathway of 5-HT is hydroxylation of tryptophan at the 5 position by the rate-limiting enzyme tryptophan hydroxylase (TPH) to form 5-hydroxytryptophan (5-HTP). Two isoforms of the enzyme are found in the brain that are encoded by two different genes: *Tph1* and *Tph2*. While TPH1 is also expressed in peripheral tissues, TPH2 is exclusive to the brain where it constitutes the most abundant isoform (Walther et al., 2003). Tryptophan hydroxylase is not saturated at normal CSF tryptophan concentrations, and as a result peripheral (and thus dietary) sources of tryptophan can exert a major influence on the level of 5-HT biosynthesis in brain. Once synthesized, 5-HTP is almost immediately

decarboxylated through the action of non-selective L-aromatic amino acid decarboxylase. Serotonin is the end point for synthesis in most of the brain except for the pineal gland, which contains considerable amounts of 5-HT, some of which is further metabolized to form the hormone melatonin (Figure I.1).

Upon synthesis in serotonergic neurons, 5-HT is cytoplasmic and requires transport by the vesicular monoamine transporter protein (VMAT) into secretory vesicles where it is accumulated for regulated exocytotic release (Liu and Edwards, 1997). Calcium-dependent exocytosis is the main mechanism used by neuronal cells for releasing neurotransmitter molecules. By this process, synaptic vesicles fuse with the plasma membrane and neurotransmitter(s) contained within them reach(es) the synaptic cleft. Exocytosis is induced by cell depolarization, which triggers the opening of voltage sensitive calcium channels. Ultimately, intracellular Ca^{2+} increase leads to docking and fusion of vesicles upon uncoupling from Ca^{2+} -sensitive vesicle membrane proteins such as synapsin I. Synapsin I fixes secretory granules to the cytoskeletal network, but once it undergoes Ca^{2+} -calmodulin/cAMP-dependent phosphorylation, it releases the vesicles from the cytoskeleton which allows them to move to the presynaptic membrane where they can be docked for release of their contents (Pineyro and Blier, 1999). Notwithstanding the vesicular, Ca^{2+} - and impulse-dependent release of 5-HT, it should be kept in mind that neuronal 5-HT can also be released from a non-vesicular para-chloroamphetamine (PCA)-dependent compartment reliant on tryptophan hydroxylase activity (Kuhn et al., 1985; Liu and Edwards, 1997), by means of a carrier-mediated mechanism (Levi and Raiteri, 1993). In addition to functioning as a storage depot for 5-HT, vesicles also sequester serotonin and protect it from metabolic inactivation by the mitochondrial enzyme monoamine oxidase A

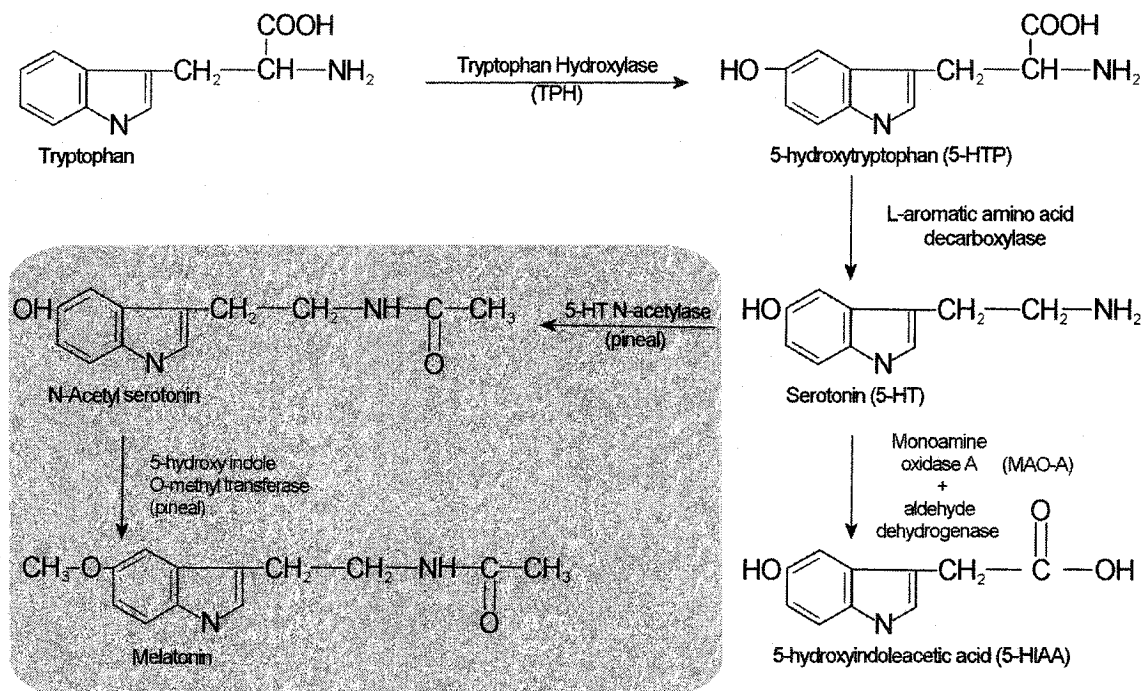


Figure I.1 – Metabolic pathways for the synthesis and metabolism of serotonin.

(MAO-A). MAO-A converts serotonin to the major metabolite 5-hydroxyindoleacetic acid (5-HIAA), which is excreted into the cerebrospinal fluid and detected in the urine (Youdim and Finberg, 1991).

1.1.3 Serotonergic system of the brain

Serotonin is perhaps the most actively researched neurotransmitter at present. The most widely studied effects of 5-HT have been those on the CNS, and this is not surprising given the importance of the 5-HT neuronal system and its involvement in more behaviours, physiological mechanisms and disease processes than any other known neurotransmitter system. Serotonin is part of an impressive neural circuitry that allows virtually all levels of the CNS to receive serotonergic innervation through extensive branching of 5-HT axonal projections. Serotonin can also diffuse to remote receptor sites influencing neurons in a non-synaptic but paracrine way, expanding the number of brain regions receiving serotonin input (Beaudet and Descarries, 1978; Tork, 1990; Bunin and Wightman, 1998). Therefore, it is not unforeseen that serotonergic signalling be involved in a broad array of functions that include perception, thermoregulation, control of appetite, sleep, learning and memory, mood and affect, behaviour (including sexual, aggressive and hallucinogenic), modulation of pain, response to stress, cardiovascular functions, muscle contraction and endocrine regulation (Fuller, 1990; Murphy, 1990; Jacobs and Azmitia, 1992; Buhot, 1997; Weiger, 1997).

The serotonergic system is comprised of a relatively small number of morphologically diverse multipolar neurons whose cell bodies are present largely within the midline raphe nuclei of the brainstem and particular regions of the reticular formation

(Tork, 1990). The raphe nuclei were first divided into nine groups (B1-B9) (Dahlstrom and Fuxe, 1964) and today consist of the raphe pallidus nucleus (Rpa, B1), the raphe obscurus nucleus (ROb, B2, B4), raphe magnus (RMg, B3), the median raphe nucleus (MRN, B5, B8), the dorsal raphe nucleus (DRN, B6, B7), and the caudal linear nucleus (Cli, B8). Outside the raphe nuclei there also are collections of 5-HT containing cell bodies in a region adjacent to the medial lemniscus called the supramedullary region (B9), in the ventrolateral medulla called the B3 cluster, in the central gray of the medulla oblongata (B4), and in the nucleus pontis oralis (B8, B9) (Tork, 1990). The raphe nuclei can further be divided into a rostral group (Cli, DRN, MRN, B9) located in the midbrain and pons and a caudal part (RMg, Rpa, Rob) in the medulla oblongata of the brainstem (Figure 1.2). From the “rostral raphe nuclei”, serotonin pathways project extensively through parts of the limbic system (amygdala, thalamus, hypothalamus, olfactory bulb, basal ganglia, hippocampal formation, cingulate gyrus, basal and limbic forebrains) into the forebrain (Tork, 1990; Jacobs and Azmitia, 1992). The limbic system is thought to be associated with the regulation of mood and emotion. The more “caudal raphe nuclei” provide important descending projections to the spinal cord and the cerebellum.

The major ascending projections to the forebrain areas come from the dorsal raphe nucleus which contains approximately 50-60% of the 5-HT producing neurons in the human brain, and from the median raphe nucleus which forms the second largest cluster of 5-HT neurons (Baker et al., 1990). From the DRN and MRN originate M and D fibers, respectively. While axons from the DRN (M-type) cell bodies appear thin with minute granular varicosities and are vulnerable to certain neurotoxic amphetamine derivatives such as PCA (para-chloroamphetamine), the MRN (D-type) axons are coarse, have large

Serotonergic System

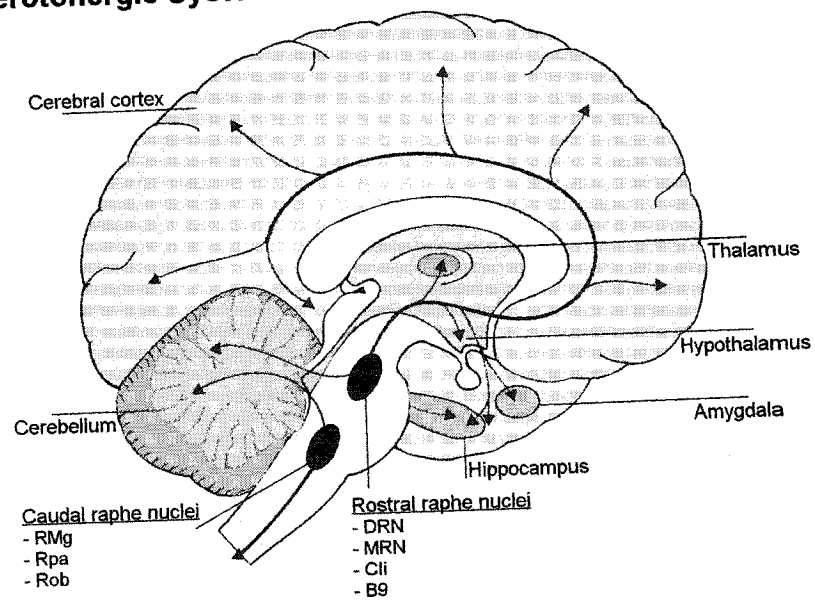


Figure I.2 – Serotonergic System.

Lateral view of the brain. The raphe nuclei which form a continuous collection of cell groups close to the midline throughout the brainstem can be divided into a caudal and a rostral portion. Neurons of the rostral raphe nuclei in the midbrain and pons project to a large number of forebrain structures. The caudal raphe nuclei located in the medulla oblongata of the brainstem send projections to the spinal cord and the cerebellum.

varicosities and are resistant to neurotoxic agents (Kosofsky and Molliver, 1987; Brown and Molliver, 2000). Both M and D fibers can be found together in most brain areas, but the cerebral cortex is the area where both systems co-exist extensively, whereas the striatum almost exclusively receives input from D-fibers and the dentate gyrus primarily M-fibers.

The largest serotonergic nucleus is the DRN, which is located in the ventral part of the periaqueductal gray matter of the midbrain, but its caudal sections extend well into the periventricular gray matter of the rostral pons. In cross sections of the midbrain, it displays a highly characteristic fountain shape and bilateral symmetry. In most species, the DRN is composed of several subregions distinguished by their different cell density, morphology and projections: 1) a medial portion subdivided in turn into dorso- and ventromedial components, just below the cerebral aqueduct and surrounding the medial longitudinal fasciculus (MLF), respectively; 2) dorso- and ventrolateral portions also called wings and 3) a caudal component. The largest neurons of the DRN are located within the dorsolateral subgroup, which extends into the lateral regions of the periaqueductal gray matter. Ventral to the DRN, the serotonergic cells of the MRN are arranged in two adjacent regions. On the midline, there are numerous small cells, with relatively short dendrites, many of which are aligned parallel to the mid-sagittal plane. Outside the midline region there are many additional cells loosely arranged and showing no particular orientation (Tork, 1990; Pineyro and Blier, 1999).

Within the DRN and MRN, it has been estimated that the serotonergic cells constitute 25-50% and 20-30% of the neuronal population respectively, the remainder being primarily GABAergic, peptidergic and dopaminergic. Adding to its intricate

network, the complexity of the serotonergic system is increased by the co-existence together with serotonin of other transmitter molecules including neuropeptides such as galanin, substance P, enkephalin and TRH as well as neurotransmitters such as dopamine and GABA (Pineyro and Blier, 1999). Thus, the nature of the cocktail that serotonergic neurons contain and/or use certainly contributes to the wide variety of functions in which these neurons are involved.

1.1.4 Serotonin receptors

Over the past decade, great excitement about understanding the diverse functions of serotonin has been precipitated by the appreciation that the neurotransmitter acts at multiple membrane-bound receptors. The advent of molecular techniques has provided an operational (function, antagonism, location), transductional (G protein, ion channel), and structural (gene sequence, chromosomal location) basis for sub-classifying and expanding this rather large family of serotonin receptors. Originally, receptors were grouped according to their pharmacological characteristics, although the currently accepted classification system relies primarily on amino acid sequence homology and signalling similarities. Thus far, at least fifteen different serotonergic receptors have been classified in seven distinct families (5-HT1 to 5-HT7) (Table I.1). Serotonin receptors all belong to the large multigenic family of seven-transmembrane G protein-coupled receptors (GPCRs) with the exception of the four-transmembrane 5-HT3 family which belongs to that of the ligand-gated ion channel receptors (Hoyer et al., 1994).

Signalling via GPCRs can lead to many cellular responses, ranging from regulation of intracellular levels of cAMP to stimulation of gene transcription. Heterotrimeric

Table I-1 – Classification of serotonin receptors.

Receptor	Previous name	Selective agonist	Selective Antagonist	Effector	Some responses	Comment
5-HT _{1A}	-	8-OH-DPAT	Way 100635	G _i /G _o ↓ AC	Behavioural changes, central hypotension	Well characterized Autoreceptor Intronless 7 TMD
5-HT _{1B}	5-HT ₁ -like 5-HT _{1X} 5-HT _{1Dβ}	Sumatriptan	GR 127935 SB224289	G _i /G _o ↓ AC	Vasoconstriction Trigeminal inhibition	Intronless Auto/hetero receptor 7 TMD
r5-HT _{1B}	5-HT _{1B}	CP93129	GR 127935 cyanopindolol	↓ AC	Vasoconstriction	Intronless Auto/hetero receptor 7 TMD
5-HT _{1D}	5-HT ₁ -like 5-HT _{1X} 5-HT _{1Dα}	Sumatriptan PNU 109291	GR 127935 BRL 15572	↓ AC	Inhibition of noradrenaline release	Intronless Autoreceptor 7 TMD
5-HT _{1E}	-	RU 24969	Methiothepin	G _i /G _o ↓ AC	-	Intronless 7 TMD
5-HT _{1F}	5-HT _{1Eβ} 5-HT ₆	LY344864 LY344370 Sumatriptan	Methiothepin	G _i /G _o ↓ AC	Trigeminal inhibition	Intronless 7 TMD
5-HT _{2A}	D 5-HT ₂	αMe5-HT MDL100907 DOI	Ketanserin MDL100907	G _q /G ₁₁ ↑ PLC	Platelet aggregation Vasoconstriction Increase capillary permeability Contraction of rat stomach	7 TMD Found both centrally and peripherally 7 TMD
5-HT _{2B}	5-HT ₁ -like 5-HT _{1F}	αMe5-HT	LY53857 S13200646 SB204741	G _q /G ₁₁ ↑ PLC	Endothelium- dependant relaxation	Low levels in brain High in periphery
5-HT _{2C}	5-HT _{1C}	αMe5-HT DOI	mesulergine	G _q /G ₁₁ ↑ PLC	Penile erection Hypophagia CSF production Neuronal depolarisation	7 TMD High in choroids plexus
5-HT ₃	M	SR57227 2Me5-HT	MDL72222 Ondansetron tropisetron	Ligand- gated cation channel	Mediates many neuronal reflex effects of 5-HT Pain response Affect cardiac function	4TMD Found both centrally and peripherally
5-HT ₄	-	Cisapride BIMU8 Renzapride	GR113808 SB204070	G _s ↑ AC	Cortisol secretion Bladder contraction Gastrokinetic action	7 TMD Found in areas associated with dopamine functions
5-HT _{5A}	-	-	-	G _s ↑ AC ?	-	7 TMD
5-HT _{5B}	-	-	-	-	-	7 TMD
5-HT ₆	-	-	RO046790 RO630563	G _s ↑ AC	-	7 TMD
5-HT ₇	5-HT ₁ -like 5-HT _{1Y} orphan	-	SB258719	G _s ↑ AC	Vasodilatation	7 TMD

guanine nucleotide-exchange proteins (G proteins) are composed of $G\alpha$ GTPase and $G\beta\gamma$ subunits. The $G\alpha$ family of G proteins comprises the G_s , G_i/o , G_z , G_q/G_{11} and G_{12}/G_{13} subfamilies, each mediating distinct and overlapping signal transduction pathways (Dohlman et al., 1991). The serotonergic GPCRs have been grouped into different categories, based on amino acid identities and depending on the particular G protein subtype that they predominantly interact with. Thus, receptors of the 5-HT₁ and possibly recombinant 5-HT₅ families couple to G_i/G_o proteins and inhibit adenylyl cyclase activity, decreasing the production of cAMP, while G_s -coupled receptors (5-HT₄, 5-HT₆, 5-HT₇) stimulate adenylyl cyclase to increase intracellular cAMP levels. All 5-HT₂ receptors, including, the previously named 5-HT_{1C} receptor (hence renamed 5-HT_{2C}, based on signalling similarities) are $G_{q/11}$ -coupled receptors that mobilize intracellular Ca^{2+} via activation of phospholipase C and hydrolysis of membrane phosphoinositides with the subsequent generation of the second messengers inositol trisphosphate (IP₃) and diacylglycerol (DAG). It is now proposed, however, that although one pathway often predominates over another, these receptors can couple to various G proteins depending on the tissue or cells in which they are expressed (Dickenson and Hill, 1998). Also, evidence is accumulating to suggest that activation of one particular signalling pathway by a GPCR can amplify intracellular signalling within a parallel but separate pathway (Selbie and Hill, 1998).

1.1.5 Serotonin autoreceptors as regulators of serotonergic activity

5-HT_{1A} receptor

One of the best-characterized binding sites for serotonin is the 5-HT_{1A} receptor. This is mainly due to the relatively early discovery of a selective ligand, 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT) that is specific for this receptor subtype (Arvidsson et al., 1981), which allowed for extensive biochemical, physiological and pharmacological characterization of the 5-HT_{1A} receptor. The human 5-HT_{1A} receptor was also the first 5-HT receptor to be cloned and fully sequenced (Kobilka et al., 1987; Fargin et al., 1988). The gene was found by screening a genomic library with a hybridizing probe to the full-length human β_2 -adrenergic receptor with which it shares striking sequence resemblance. In parallel, the rat 5-HT_{1A} was cloned using a probe to the coding region of the hamster β_2 -adrenergic receptor (Albert et al., 1990), and the cDNA coding for the murine 5-HT_{1A} receptor was isolated from a mouse brain library (Charest et al., 1993). The human 5-HT_{1A} gene is located on chromosome 5, locus 5q11.2-q13 and is intronless, a characteristic of members of the 5-HT₁ family (Kobilka et al., 1987).

The 5-HT_{1A} receptor, like the β_2 -adrenergic receptor, possesses a tertiary structure typical of GPCRs and consists of a single 422 amino acids polypeptide chain containing an extracellular amino terminus, a cytoplasmic C-terminal domain and seven stretches of 20-24 hydrophobic amino acids predicted to form transmembrane α -helices (TM₁-TM₇) linked by three intracellular (i₁, i₂, i₃) and three extracellular loops (Chanda et al., 1993; Martin et al., 1998) (Figure I.3). Of the three intracellular loops, the i₃ is typically the longest and least conserved. Potential asparagine-linked glycosylation sites are located in the amino-terminal domain of the receptor, and may account for the observed 63 kDa, as opposed to

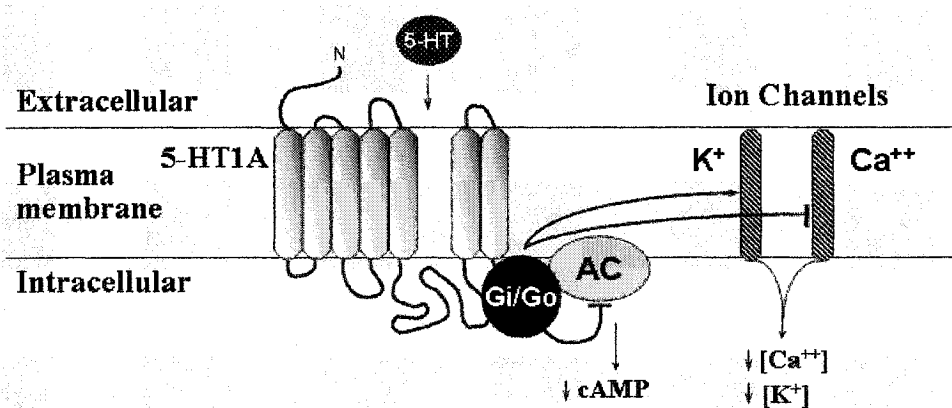
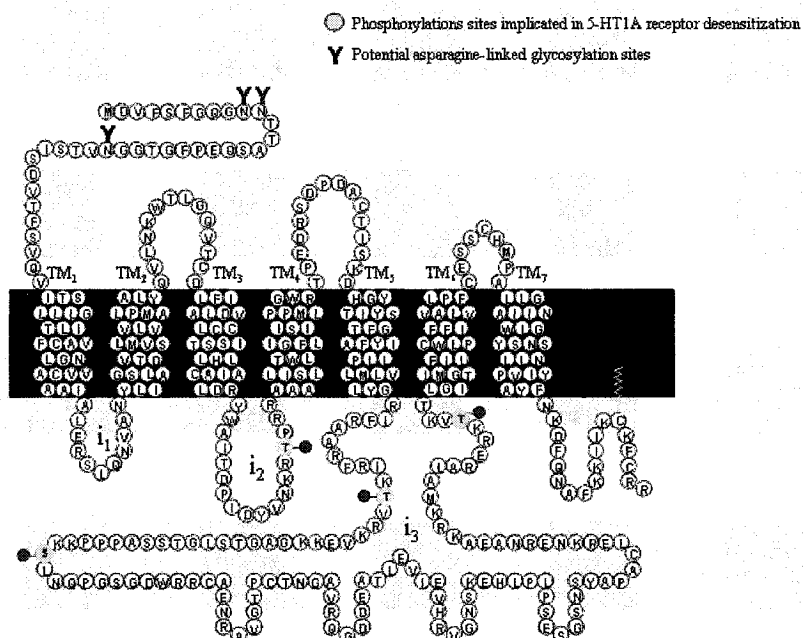


Figure I.3 – Schematic representation of the rat 5-HT1A G protein-coupled receptor.

The extracellular N- and cytoplasmic C-termini are separated by seven transmembrane domains (TM₁-TM₇) linked by three intracellular (i₁, i₂, i₃) and three extracellular loops. Putative Asn-linked glycosylation sites are located in the N-terminal domain of the receptor. Four consensus sites for phosphorylation by PKC (Thr²²⁹, Ser²⁵³, Thr³⁴³ in the i₃ and Thr¹⁴⁹ in the i₂ loop) are implicated in receptor desensitization. Agonist stimulation of the 5-HT1A receptor inhibits adenylyl cyclase activity (via G_i/G_o) to reduce intracellular cAMP, decrease the opening of voltage-dependant calcium channels and increase potassium conductance.

the predicted 43 kDa, molecular weight of the protein. Four consensus sites for phosphorylation by protein kinase C (PKC) (Thr²²⁹, Ser²⁵³, Thr³⁴³ in the i₃ loop and Thr¹⁴⁹ in the i₂ loop) have been implicated in receptor desensitization (Lembo and Albert, 1995; Lembo et al., 1997; Wu et al., 2002). Although the overall amino acid sequence identity between serotonergic receptors varies considerably, a relatively high degree of homology exists in the sequences of all 5-HT GPCRs at the level of their transmembrane domains (Hartig, 1989). Amino acid identity among receptor subtypes across species is >95% and up to 60% between subtypes such as rat 5-HT1A vs. 5-HT1B receptors (Albert et al., 1990; Voigt et al., 1991). Certain of these highly conserved residues of the TM regions participate in ligand binding and determine receptor-ligand specificity (Hartig, 1989; Ostrowski et al., 1992). By contrast, the amino acid sequence of the N-terminal portion and the third intracellular loop are rather unique for each given receptor type.

Insights from the crystal structure of the GPCR rhodopsin (Palczewski et al., 2000; Sakmar et al., 2002) as well as mutagenesis, chimeric receptors and computer-assisted modeling studies, have suggested that the ligand binding site lies within the cavity “pocket” formed by the bundling of the seven-transmembrane regions (Ostrowski et al., 1992; Strader et al., 1994). By contrast the interaction of the receptor with the G protein occurs at the level of the intracellular loops and the internal C-terminal tail. Sites for ligand binding appear to vary depending on receptor and ligand. For monoamine receptors, the conserved negatively-charged Asp residue in the TM₃ appears critical, and forms a salt bridge with the protonated NH₂ group of monoamines. In addition, ligand specificity is determined by the hydrophobic and hydrogen bonding interactions with residues in TM_{6/7} (Ostrowski et al., 1992; Albert et al., 1998). For the 5-HT1A receptor specifically, Asp82Asn (TM₂),

Asp116Asn (TM₃), Ser198Ala (TM₅) and Thr199Ala (TM₅) mutants have shown a 100-fold reduction in affinity for 5-HT without affecting the affinity for the antagonist pindolol (Ho et al., 1992). By contrast, the Asn385Val (TM₇) mutant has displayed low affinity for pindolol but no change in binding of other chemical classes of ligands (Guan et al., 1992).

Similar experimental design involving analysis of receptor mutants has been used to characterize regions of the 5-HT_{1A} receptor involved in coupling to G proteins. The 5-HT_{1A} receptor, like other members of the 5-HT₁ family, is an inhibitory receptor coupling mainly via the pertussis toxin (PTX)-sensitive G_i/G_o. Binding of the 5-HT_{1A} receptor by agonist induces a conformational change in the receptor that allows activation of the G protein complex, and mutational analyses have suggested that certain residues in the binding pocket of the receptor may be required to induce such change. In the inactive state, the G protein consists of a G α (GDP-bound), G β and G γ subunit. Upon activation, GDP is exchanged for GTP, which results in dissociation of the GTP-bound α subunit from the $\beta\gamma$ dimer (Gilman, 1987; Bourne et al., 1991; Neer, 1995). Both in turn, activate various effector molecules. Classically, the G α_i subunit inactivates adenylyl cyclase activity to reduced cAMP accumulation, while the G $\beta\gamma$ subunit activates its own signalling pathways (Clapham and Neer, 1997) to mediate effects such as activation of GIRK family of potassium channels (Luscher et al., 1997), inactivation of N-type calcium channels (Chen and Penington, 1997), activation of phospholipase C (PLC) β 2 and β 3 isoforms (Exton, 1996), adenylyl cyclase type II and IV (Sunahara et al., 1996) and phosphatidylinositol-3-kinase-dependent and tyrosine kinase-dependent activation of the MAPK cascade (van Biesen et al., 1996). G α subunits have intrinsic GTPase activity, which in

time results in hydrolysis of the GTP to GDP and reassociation of the inactive heterotrimeric complex.

The role of the receptor i_2 loop domain in receptor coupling was first suggested by experiments involving chimeric muscarinic/ β -adrenergic receptors to demonstrate that this region is critical in functional coupling to G_s proteins to mediate increase in adenylyl cyclase activity (Ostrowski et al., 1992; Strader et al., 1994). Point mutagenesis studies have also implicated specific residues in this loop that are critical for coupling. For instance, a conserved Thr¹⁴⁹ residue in the 5-HT1A i_2 loop corresponding to a consensus PKC phosphorylation site, is directly involved in $\beta\gamma$ -mediated phospholipase C pathway and coupling to Ca²⁺ entry and to Ca²⁺ mobilization, but plays a minor role in $G_{\alpha i}$ -mediated inhibition of cAMP accumulation (Lembo et al., 1997). Likewise, multiple phosphorylation sites (Thr²²⁹, Ser²⁵³, Thr³⁴³) in the i_3 loop are required for pathway-selective uncoupling of the 5-HT1A receptor by PKC (Lembo and Albert, 1995). The N- and C-terminal regions of the i_3 loop have also consistently been shown to play a critical role in receptor-G protein interactions (Albert et al., 1998). For example, deletion mutations of the N-terminal portion of the i_3 loop of the β -adrenergic receptor prevent coupling to G_s (Ostrowski et al., 1992; Strader et al., 1994). Mutation of the same region of the α_2 -adrenergic receptor to the corresponding 5-HT1A receptor sequence, was shown to prevent coupling to G_s but to preserve G_i coupling, suggesting a crucial role of this region in G_s and G_i coupling (Eason and Liggett, 1995). Consistent with a role for the C-terminal domain of the i_3 loop as an important determinant of receptor-G protein recognition, the four amino acid epitope (Val³⁸⁵, Thr³⁸⁶, Ile³⁸⁹, and Leu³⁹⁰, or VT-IL) on the $G_{i/o}$ -coupled m_2 muscarinic acetylcholine receptor can specifically recognize five

C-terminal amino acids of G α i (Liu et al., 1995). Coexpression studies with hybrid m₂/m₃ muscarinic receptors and mutant G protein α q subunits further suggest that this receptor/G protein contact site is essential for coupling specificity and G protein activation (Liu et al., 1995). Mutation of the corresponding threonine residue, conserved among several G_i-coupled receptors including the 5-HT_{1A} receptor (Albert et al., 1998), within the α ₂-adrenergic receptor, increases agonist-independent receptor coupling to G α i (Ren et al., 1993). This is consistent again with a role for the C-terminal part of the i₃ loop in controlling the extent of receptor coupling to G proteins. Labelling of both G α and G β subunits by cross linking analysis using a peptide against the i₃ C-terminal of the α ₂-adrenergic receptor also suggests a direct interaction of this domain with the G protein subunits (Taylor et al., 1996). Together, these studies stress the importance of the second and third intracellular loops in directing the specificity of coupling to different G proteins.

Another reason for the particular interest in the 5-HT_{1A} receptor over the past several years is attributed to its well-established role in the control of serotonergic activity. In the brain, 5-HT_{1A} receptors are located both presynaptically (i.e., on serotonergic cell bodies in midbrain dorsal and median raphe nuclei) and postsynaptically in limbic structures (e.g., on non-serotonergic neurons of the hippocampus, amygdala, entorhinal cortex and septum) (Sotelo et al., 1990; Thor et al., 1990; Chalmers and Watson, 1991; Pompeiano et al., 1992; Burnet et al., 1995). Presynaptic receptors located on the soma and dendrites of serotonergic raphe neurons act as autoreceptors to inhibit neuronal cell firing and 5-HT release onto post-synaptic sites (Verge et al., 1985; Albert et al., 1996; Evrard et al., 1999). As mentioned, apart from coupling to inhibition of adenylyl cyclase activity, 5-HT_{1A} receptors are coupled to two different ion channel effectors (i.e., potassium

channel activation and calcium channel inhibition). Specifically, 5-HT_{1A} receptor activation decreases the opening of voltage-dependent calcium channels to reduce 5-HT release and promotes potassium influx causing hyperpolarization at the cell membrane and reduction in action potential frequency (Penington and Kelly, 1990; Penington et al., 1991; Newberry, 1992; Penington et al., 1993; Chen and Penington, 1996; Langer, 1997). Accordingly, an increase in neuronal cell firing leads to the release of serotonin at the synapse, but also at the cell body, via recurrent collateral terminals (axonal varicosities of axonal 5-HT fibers) or dendro-dendritic release (Hery et al., 1982; Adell et al., 1993; Artigas, 1993; Artigas et al., 1996a). Serotonin in turn activates 5-HT_{1A} autoreceptors to suppress the discharge of serotonergic neurons and reduce the release of serotonin as part of a negative feedback regulatory mechanism.

5-HT_{1B/1D} receptors

Similarly to the 5-HT_{1A} autoreceptor, mRNA for both 5-HT_{1B} and 5-HT_{1D} subtypes is present presynaptically in the DRN, although proteins for these receptors are mainly localized at nerve terminals rather than cell body or dendrites. Originally, the 5-HT_{1B} receptor was thought to exist only in rodent brain (Pazos and Palacios, 1985; Hoyer et al., 1994). Subsequently, two structurally distinct genes encoding human 5-HT₁ receptors with pharmacological profiles most closely resembling the 5-HT_{1D} receptor were cloned (Weinshank et al., 1992). Since the operational profiles of these two new receptors were almost indistinguishable, they were called 5-HT_{1D α} and 5-HT_{1D β} . More recently, the amino acid sequence of the rodent 5-HT_{1B} receptor has been characterized, and found to be 93% overall and 96% identical within the TM domains with that of the 5-HT_{1D β} receptor (Hoyer et al., 1994). Hence, despite their important pharmacological differences,

it has recently been agreed to reclassify the receptors as species homologues of the same receptor termed h5-HT1B (formerly 5-HT1D β) and r5-HT1B with the h and r prefix referring to the human and rat species respectively (Hartig et al., 1996). Functionally, the difference in pindolol binding between the rodent and non-rodent 5-HT1B pharmacological profiles is due to the presence of a threonine residue at position 355 in TM₇ of the latter and the presence of an asparagine residue at the corresponding position in the rodent 5-HT1B receptors (Hoyer et al., 1994). The 5-HT1D α nomenclature was abandoned and simply replaced by the 5-HT1D receptor terminology (Hartig et al., 1996).

The 5-HT1B receptor is found primarily at sites of serotonin release (i.e., synaptic terminals or axonal varicosities) and activation of this receptor inhibits serotonin release in response to depolarization and reduces 5-HT synthesis independently of the degree of neuronal firing (Gothert, 1990; Hjorth et al., 1995). In addition to its actions as an autoreceptor the 5-HT1B receptor in non-serotonergic neurons may act as a terminal heteroreceptor to control the release of other neurotransmitters such as acetylcholine, glutamate, dopamine and GABA (Gothert, 1990; Hen, 1992; Galloway et al., 1993; Hoyer et al., 1994). Both 5-HT1B and 5-HT1D receptors have been shown to modulate negatively the release of 5-HT within the DRN and MRN (Davidson and Stamford, 1995; Pineyro et al., 1995; Adell et al., 2001; Hertel et al., 2001; Hopwood and Stamford, 2001). Endogenous 5-HT does not exert a tonic action upon 5-HT1B/1D autoreceptors in the raphe nuclei. Thus, it seems that these autoreceptors are only activated when an excess of serotonin is present in the extracellular space, similarly to 5-HT1A autoreceptors (Adell et al., 2002), and therefore “fine tune” the release of 5-HT. While *in vivo* and *in vitro* studies suggest that 5-HT1A receptors are involved in both controlling the firing of serotonergic

neurons and hence the release of 5-HT, 5-HT_{1B}/1D subtypes do not appear to influence 5-HT cell firing, but nevertheless clearly exert a role in the control of 5-HT release (Stamford et al., 2000). However, because 5-HT_{1A} receptor density in the raphe nuclei is much greater than that of 5-HT_{1B}/1D receptors, it is assumed that the 5-HT_{1A} receptor may play a more critical role in the regulation of serotonergic activity (Adell et al., 2002).

5-HT_{1A} and 5-HT_{1B} knockouts

Mice lacking the 5-HT_{1B} receptors were the first genetically engineered 5-HT-receptor-deficient mice to be described (Saudou et al., 1994). Four years later, three independent research groups reported the genetic disruption of the 1A subtype in three different mouse strains (Heisler et al., 1998; Parks et al., 1998; Ramboz et al., 1998). Although both 5-HT_{1A} and 5-HT_{1B} receptors control the tone of serotonergic activity and are involved in post-synaptic effects of 5-HT, knockouts of these receptors exhibit very different behavioural phenotypes in various tests of locomotion/exploratory behaviour (open field), anxiety (ultra-sonic vocalization, open field, novelty suppressed feeding, and elevated plus maze), and aggressiveness (resident-intruder test and maternal aggression test) (Zhuang et al., 1999; Gingrich and Hen, 2001). While the 5-HT_{1B} knockout mice are more aggressive, more reactive, less anxious, and have increased susceptibility to cocaine addiction, the 5-HT_{1A} knockouts are less reactive, more anxious, and possibly less aggressive than wild-type mice. These knockout mice have provided us with important animal models of genetic vulnerability to neuropsychiatric disorders including anxiety, aggression and drug abuse. However, the absence of these genes during development may lead to compensatory changes in other systems that can confound the interpretation of the resulting phenotype. Moreover, the observed phenotypes may be a consequence of effects

on serotonergic neurotransmission due to the lack of autoreceptors and/or heteroreceptors in either pre- or post-synaptic brain regions. It is difficult to distinguish between these possibilities using traditional knockout approaches but more recent genetic targeting strategies, called conditional knockouts, allow for the disruption of a gene in a time- (using an inducible tetracycline system) and tissue-specific (provided by the specificity of the promoter that drives the expression of the tetracycline transactivator) manner (Stark et al., 1998). Taking advantage of this approach, a group of researchers have created mice with conditional expression of the 5-HT_{1A} receptor in forebrain structures on a knockout background (Gross et al., 2002). Their results suggest that forebrain expression of the 5-HT_{1A} receptor is sufficient to rescue the normal anxiety phenotype in the 5-HT_{1A} knockout mice. They further suggest that the adult receptor is not required to maintain this phenotype but that its expression at critical time during development is essential in modulating a normal anxiety-like behaviour later in life (Gross et al., 2002).

In addition to the problems listed above, constitutive 5-HT_{1A} and 5-HT_{1B} knockouts do not entirely mimic the effects of pharmacological interventions. For example, while disruption of the 5-HT_{1A} gene in mice leads to anxiety-like behaviours (Sibille and Hen, 2001), WAY 100635, a specific 5-HT_{1A} receptor antagonist is not anxiogenic. Similarly, mice lacking the 5-HT_{1B} gene demonstrate an increased locomotor activity in response to cocaine and a greater tendency to self-administer the drug (Rocha et al., 1998), whereas injection of GR127935, a 5-HT_{1B} receptor antagonist, reduces locomotion attributed to cocaine and has no consequences on self-administration (Castanon et al., 2000). For this reason, the conventional knockout strategies may better serve as models of genetic disorders or chronic drug treatment, rather than as tools to study the

consequence of the acute blockade of the receptor. With time, new conditional 5-HT_{1A} and 5-HT_{1B} knockouts will be generated that will hopefully improve our understanding and knowledge of what role 5-HT_{1A} and 5-HT_{1B} receptors play as either autoreceptors or heteroreceptors in neuropsychiatric disorders. These modern strategies may also provide us with a better view of the various compensatory/adaptive regulatory mechanisms that may take place in these disorders (Stark et al., 1998).

1.1.6 Serotonin transporter

When 5-HT is released into the synaptic cleft, it is quickly removed, to terminate neurotransmission and prevent desensitization of post-synaptic receptors. The reuptake of 5-HT by serotonergic neurons is the principal mechanism of terminating the action of the transmitter on post-synaptic sites. This process is carried out by the serotonin transporter (SERT) located on presynaptic axon terminals as well as cell body and dendrites of serotonergic neurons (Blakely et al., 1994; Stahl, 1998b). The SERT belongs to a family of proteins with 12 putative transmembrane domains that include the dopamine, norepinephrine, GABA, glutamate and other transporters and has the same requirements for action, which is that the transport process is saturable, requires energy, is temperature and sodium dependent and is inhibited by specific uptake inhibitors (Amara and Kuhar, 1993; Blakely et al., 1994). The SERT is in fact the primary target of several antidepressants that work to block the transporter. In addition to its role in the reuptake of extracellular 5-HT, the SERT can promote the release of the neurotransmitter through a reversal of uptake mechanism. Stimulants such as para-chloroamphetamine and “ecstasy” (3,4-methylenedioxymethamphetamine) use this latter process to release 5-HT from

cytoplasmic (non-vesicular) pools in a Ca^{2+} -independent manner (Rudnick and Wall, 1992a, b).

Gene disruption of 5-HT uptake in SERT knockout mice leads to a six-fold increase in extracellular concentrations of 5-HT and reduces intracellular concentrations by 60-80% (Bengel et al., 1998; Fabre et al., 2000). The constant elevation of 5-HT in the SERT knockout has been shown to greatly alter the responsiveness of certain 5-HT receptors. For instance, electrophysiological studies have demonstrated that both presynaptic and postsynaptic 5-HT_{1A} are desensitized in mice lacking the SERT (Gobbi et al., 2001), and there is a significant decrease in 5-HT_{1A} receptor binding sites, mRNA and protein in the DRN (Li et al., 1999; Fabre et al., 2000). In these mice, 5-HT_{1B} receptors are also desensitized and have reduced levels in the substantia nigra compared to wild-types (Fabre et al., 2000). Such adaptive changes, in addition to the lack of 5-HT reuptake, certainly contribute to the marked increase in extracellular 5-HT levels, and decrease in brain 5-HT content. Although of larger amplitude, the changes observed in the SERT-deficient animals closely resembles those reported after chronic blockade of 5-HT reuptake by antidepressants such as selective serotonin reuptake inhibitors. Again, developmental alterations due to the lack of 5-HT reuptake during early life period may account for the differences observed between SERT gene disruption, and SERT inhibition by reuptake blockers.

1.1.7 Conclusion

Over the years, our view of serotonin has become tremendously complex, as we have learned that multiple specific proteins mediate each step of receptor binding, release,

reuptake and degradation. The past few years have witnessed an extraordinary number of publications ($\approx 3,000$ per year) in the serotonergic field, certainly strengthening the sentiment of Irvine Page, following his discovery of serotonin, and who stated that “No physiological substance has been discovered, of which I am aware, that is believed to have such diverse actions in the body”. The advent of molecular biological techniques for the cloning of GPCRs was an important step in revealing the multiplicity of 5-HT receptors that exist today. Despite its relatively simple structure, evidence continues to grow in support of the important roles for 5-HT and its receptors in various physiological processes and neuropsychiatric disorders.

1.2 Major depressive disorder (MDD): description

1.2.1 Epidemiology and diagnostic criteria

According to the World Health Organization (WHO), major depression (also called unipolar depression) affects at least 16% of the actual population, and is predicted to rise from fourth to second leading cause of disability, after heart disease, by the year 2020 (Doris et al., 1999; Holden, 2000). This mental illness is characterized by a large number of associated symptoms including depressed mood most of the day, diminished interest or pleasure (anhedonia), sleep disturbance, changes in appetite and weight, fatigue or loss of energy, decreased sex drive, psychomotor agitation or retardation, feelings of worthlessness or excessive or inappropriate guilt, difficulty in concentrating, indecisiveness, pessimistic thoughts and recurrent thoughts of death or suicide (American Psychiatric Association, 1994; Williams, 2001; Nestler et al., 2002a). Major depression is a major risk factor for suicide, the most devastating outcome of the disease, which accounts

for 24% of the deaths among 15-24 years old, and 16% among those aged 25-44 (<http://www.ontario.cmha.ca>). Risks for accidental death and cardiovascular disease are also elevated in MDD (Carney et al., 1988). Mortality however, certainly undervalues the social and economical impacts of the disease. Major depression is often a chronic or recurrent condition characterized by global individual impairment and usually by an early onset in life (Hyman, 2000). In a third of all patients, an episode of MDD will last for more than two years, the criteria for chronic depression. Patients who have had two episodes of MDD have a 60-90% chance of recurrence, and this percentage increases to 95% in patients who have had more than two episodes (Keller, 2002). Despite these factors, depression remains under-recognized and under-treated with less than 10% of patients receiving the correct treatment (Keller and Boland, 1998). Furthermore, as will be discussed later, a common characteristic of available antidepressants is their delayed onset of clinical efficacy and the high suicide risk associated with this period (Stockmeier, 1997; Gross-Isseroff et al., 1998; Verkes et al., 1998). Although pharmacological treatment results in clinically significant improvement in 65-75% of patients, only 40-50% will reach complete recovery (Frazer, 1997). MDD hence represents a life-threatening and debilitating disease costing billions through affecting workers productivity, increasing school dropout rates, and leading to familial dysfunction.

Women are about twice as likely as men to develop the disease (Kessler et al., 1993; Weissman et al., 1993; Lehtinen and Joukamaa, 1994; Kornstein et al., 2000). The reasons for this gender difference are not clear, but associations with women's greater tendency to report emotional distress, greater propensity to seek treatment for major depression as well as factors related to social situation and hormonal effects have been

proposed (Paykel, 1991). In support of the latter hypothesis, sex differences in the prevalence rate of depression begin to appear after puberty, a time of major change in the neuroendocrine reproductive axis, and are maintained throughout the reproductive years and dissipate only after menopause (Weissman et al., 1984; Angold et al., 1998). Many hormonal factors may therefore contribute to the increased rate of depression in women, particularly such factors as menstrual cycle changes, pregnancy, miscarriage, postpartum period, pre-menopause, and menopause.

The criteria for a major depressive episode is defined by the Diagnostic and Statistical Manual of Mental disorders (DSM-IV) which describes an individual as having MDD when at least two consecutive weeks of depressed mood or loss of interest are accompanied by no less than four additional symptoms of depression (listed above), and when the symptoms disrupt normal, social and occupational functioning (American Psychiatric Association, 1994). To be considered a major depressive disorder, psychotropic drugs or a general medical condition are not the cause of these symptoms and they do not occur within two months of the loss of a loved one. The Hamilton Depression rating scale (HAM-D) has been the most widely used scale for assessing depression severity. It was originally published by Max Hamilton in 1960, and was designated to measure severity of depression in previously diagnosed depressed inpatients (Hamilton, 1960), based on 21 different items (listed in Table I.2). As Hamilton himself reported, scores on the last four items (diurnal variation, depersonalization/derealization, paranoid symptoms, and obsessional and compulsive symptoms) should not contribute to the total score because they are either not considered part of the disease, are infrequent or are not

Table I-2 – The 17/21 version of the Hamilton Depression rating scale (HAM-D).

Item	
1.	Depressed mood
2.	Feelings of guilt
3.	Suicide
4.	Insomnia early
5.	Insomnia middle
6.	Insomnia late
7.	Work and activities
8.	Psychomotor retardation
9.	Psychomotor agitation
10.	Anxiety, psychic
11.	Anxiety, somatic
12.	Somatic symptoms, GI
13.	Somatic symptoms, General
14.	Genital symptoms
15.	Hypochondriasis
16.	Loss of weight
17.	Insight
18.	Diurnal variation
19.	Depersonalization/Derealization
20.	Paranoid symptoms
21.	Obsessive compulsive symptoms

thought to reflect reality. As a result, the 17-item version of the HAM-D is now the most commonly used, although many studies have reported total scores based on all 21 items and multiple other version of the scale, adapted to particular studies, have been created (Williams, 2001).

1.2.2 Genetic determinants and environmental causes

Major depression has long been believed to be a heterogeneous illness. Epidemiologic twin, adoption and family studies demonstrate that both genetic and environmental components, that likely manifest themselves through biochemical alterations in patients, contribute substantially to the liability for developing MDD. Approximately 40%-50% of the risk factor for depression is genetic (Fava and Kendler, 2000; Nestler et al., 2002a) characterizing it as a highly heritable disorder. Yet, the search for specific genes that confer this risk has been disappointing, with no robust genetic abnormality being identified to date with certainty (Nestler et al., 2002a). The complication in finding depression vulnerability genes parallels the difficulty in finding genes for other psychiatric disorders that are complex multigenic illnesses in which no single genetic variation will have a major impact on risk and would therefore be difficult to detect experimentally. Individual gene alterations may synergistically interact with each other to produce a strong predisposition to the illness. Moreover, various combinations of genes may contribute to depression in different families, which further complicates the search for candidate genes (Burmeister, 1999). In addition, vulnerability to depression is only partly genetic since major depressive disorders with 100% concordance between monozygotic twins are inexistent (Hyman, 2000). Thus, the multiple risk genes must interact in conjunction with non-

genetic/environmental factors to elicit the disease. Non-genetic factors that contribute to depression are diverse and, like the genetic components, have not been identified unequivocally. It has been reported that several viruses such as the Borna virus (Bode et al., 1995) can induce a depressive episode, but this possibility has remained controversial. Random processes during brain development have also been implicated in the etiology of depression (Nestler et al., 2002a). Still, the most commonly proposed factors involved in increased predisposition to MDD are psychological stressors related to loss (job, income, loved one), defeat, threat or humiliation (Kessler, 1997). However, causal links between stress and major depression have been difficult to establish as not all subjects who encounter a stressful life experience succumb to depression, and stress per se is not sufficient to induce depression in the vast majority of individuals. Other stress factors for depression involve physical and sexual abuse during childhood, poor parent-child relationships, as well as parental discord and divorce (Fava and Kendler, 2000). Certain personality traits such as neuroticism, which reflect the predisposition to develop emotional upset under stress, also contribute to the risk for MDD (Fava and Kendler, 2000). While studies on different rodent inbred strain or mutants suggest that genetic risk factors can dramatically alter an animal's response to stress, antidepressant treatment, and related perturbations (Nestler et al., 2002b), a recent epidemiological study has emerged to provide convincing evidence that an individual's response to stressful environmental insults is also influenced by genetic background (Caspi et al., 2003). In this study, a functional SERT polymorphism was found to moderate the influence of stressful life events on depression, with individuals carrying at least one copy of the short allele, exhibiting more depressive symptoms, diagnosable depression and suicidality than those homozygous for the long

polymorphic allele. Although evidence for an association between the short promoter variant and depression is inconclusive (Lesch, 2003), this study emphasizes the importance of genes in response to environment to modify behavioural phenotypes. Conversely, environmental factors can compensate for genetic-based abnormalities (Nestler et al., 2002b). The neuronal and molecular mechanisms by which the interplay between environmental adversities and genes modulate the susceptibility to depression are currently under intense investigation.

Suicidal behaviour may also be regarded as an interplay between individual-specific environmental and neurobiological/genetic factors (Mann and Arango, 1992). A strong genetic predisposition appears to be involved in attempted and completed suicide with a higher concordance rate for suicide in monozygotic (13.2%) than dizygotic (0.7%) twins and in the families of suicide attempters (Roy et al., 1999).

1.2.3 Pharmacotherapy

Patients who meet the criteria for major depressive disorder may receive treatment options that include psychotherapeutic interventions, electroconvulsive therapy (ECT) and antidepressant medication. Although very effective, the use of ECT is usually used after failure of pharmacotherapy, because of its poor public image and its side effects on cognition with memory impairment and confused state that may last over a few weeks (Doris et al., 1999). Most patients respond effectively to a combination of psychotherapy and antidepressant drugs (Nestler et al., 2002a). The treatment of depression was revolutionized with the serendipitous discoveries in the 1950s, of two classes of agents: the tricyclic antidepressants (TCAs) and the monoamine oxidase inhibitors (MAOIs). The

original tricyclic agent, imipramine, was initially developed as an antihistamine, and then tested in schizophrenic patients as a potential treatment for psychosis (Kuhn, 1958). Although imipramine was of no evident use in schizophrenia, its mood-elevating effects in these patients led to its testing in clinical trials for the treatment of depression. Similarly, the early MAOI iproniazid arose from a failed attempt to develop effective antitubercular drug, and also proved to elevate mood in tubercular patients (Crane, 1956). The mechanism of action of these drugs was left misunderstood until it was demonstrated in the mid to late 1960s that imipramine and related TCA drugs blocked the neuronal uptake of noradrenaline and serotonin (Iversen, 1965; Cardenas et al., 1997) to provide one of the first indications into the type of chemical changes in the brain that may regulate depressive symptoms. Indeed, the discovery of their mode of action has been instrumental in developing the monoamine theory of depression.

Over the years, antidepressants have been classified according to their mode of action and into three categories: first, second and third generations. Because of their early discovery, TCAs and MAOIs constitute the first generation of antidepressants. TCAs (e.g., imipramine and clomipramine) at therapeutic doses are likely to inhibit noradrenergic and, to a lesser extent, serotonergic reuptake and to antagonize several neurotransmitter receptors (Richelson, 1991). TCAs are actually five or more drugs in one: (1) a serotonin reuptake inhibitor; (2) a noradrenaline reuptake blocker; (3) an anticholinergic-antimuscarinic drug; (4) an α_1 -adrenergic agonist; and (5) an antihistamine (Stahl, 1998a). The MAOIs comprise both the classical, irreversible and non-selective inhibitors of monoamine oxidase (e.g., tranylcypromine and phenelzine) and the more recent selective (for MAO-A or MAO-B) and reversible inhibitors of MAO-A (e.g., moclobemide).

Compared to TCAs, MAOIs are less widely used. In many people their antidepressant activity is weak, but some individuals respond to them much better than the TCAs, so they keep a small but real place in the treatment of depression. Although both types of drugs can be effective in the treatment of depression, their clinical use is limited by a number of adverse effects that include: unpleasant anticholinergic actions (constipation, blurred vision, dry mouth and drowsiness), sedation; weight gain; hypotension (for TCAs); serious cardiovascular side effects; life-threatening drug-drug interactions; requirement of tyramine-free diet to prevent hypertensive crisis (for MAOIs); and the fact that they both are lethal in overdose (Fava and Kendler, 2000). In an effort to identify compounds with more selective properties and fewer side effects, a second generation of medication that includes noradrenaline reuptake inhibitors (NARIs; e.g., reboxetine) and selective serotonin reuptake inhibitors (SSRIs) was produced. Developed in the 1980s, and today representing a market of over \$10 billion a year in sales, SSRIs (e.g., fluvoxamine-Luvox®, paroxetine-Paxil®, fluoxetine-Prozac®, sertraline-Zoloft® and citalopram-Celexa®) are among the most widely prescribed drugs for treating depression not only because of their effectiveness with relatively few side effects, but also because of their ability to alleviate several other disorders associated with serotonergic dysfunction. These disorders include anxiety, social phobia, premenstrual dysphoric disorder, obsessive-compulsive disorders, bulimia, aggression, premature ejaculation and post-traumatic stress disorder (Raap and Van de Kar, 1999). The SSRI-induced inhibition of 5-HT reuptake increases 5-HT levels in the synaptic cleft thereby prolonging the activation of post-synaptic receptors (Kreiss and Lucki, 1995; Hjorth et al., 1996; Moret and Briley, 1996; Sharp et al., 1997). The latest generation of antidepressants includes those with novel mechanisms of action and different

side effect profiles than the SSRIs. These newer medications comprise dual action compounds such as serotonin and noradrenaline reuptake inhibitors (SNRIs; e.g., venlafaxine, mirtazapine), serotonin uptake and 5-HT₂ receptor antagonists (e.g., nefazodone), noradrenaline and dopamine reuptake inhibitors (NDRIs; e.g., bupropion) and 5-HT_{1A} receptor partial agonists (e.g., buspirone, ipsapirone, gepirone). Although these medications are mostly still under clinical investigation, they are hoped to have a quicker onset of action. Overall, a universal mechanism of action of antidepressants can be proposed, which is that they all facilitate noradrenergic or serotonergic neurotransmission or both.

1.3 Major depressive Disorder: etiology and pathophysiology

1.3.1 Monoamine hypothesis of depression

Although many different etiological theories have been proposed, the most widely accepted for explaining the biological basis of depression has been the monoamine hypothesis. Simplistically, this theory proposes that depression is due to a deficiency in one or another of three biogenic monoamines, namely serotonin, noradrenaline and/or dopamine and came initially from three main lines of evidence (Bunney and Davis, 1965; Schildkraut, 1965; Coppen, 1967). First, early clinical studies demonstrated that treatment with reserpine, a medication for hypertension that interferes with storage of monoamines and thereby depletes their levels, inadvertently caused symptoms of depression in a small percentage of individuals (Quetsch et al., 1959; Peterfy et al., 1976). The second observation in support of the hypothesis was the discovery that TCAs and MAOIs, effective drugs in the treatment of depression, acutely increase the concentrations of monoamines and finally, that some

depressed patients have reduced levels of monoaminergic metabolites in some body fluids such as urine, plasma and CSF. The latter observation however, remains highly controversial with almost every report on 5-HT or other monoamines and related substances in brain and CSF of depressives being contested (Blier and de Montigny, 1994). Even if the monoamine hypothesis has received considerable support since it attempts to provide an etiological explanation for MDD, it remains a rather oversimplified view as it does not provide a complete explanation for the actions of antidepressants, and the pathophysiology of depression itself is left largely unknown. The major problems associated with a simple relationship between monoamines and mood relate to the fact that antidepressant drugs do not elevate mood in normal healthy subjects and that in depressed patients, even though they induce reuptake blockade and elevate monoamines levels relatively quickly, they require a long-term (typically 2-6 weeks) period to produce appreciable clinical effects (Jones and Blackburn, 2002). The late onset of the therapeutic activity is ubiquitous amongst the different classes of antidepressants, but is not a characteristic of the disease, since other treatment such as sleep deprivation and ECT appear to work more readily (Gillin, 1983; Daly et al., 2001). Furthermore, depletion of 5-HT or noradrenaline in healthy individuals does not induce clinically significant depressive symptomatology (Blier and de Montigny, 1994). The hypothesis has therefore evolved over the years to include, for example, adaptive changes in receptors to explain the delayed onset of action of antidepressant drugs (as will be discussed later) (Blier, 2003). Still, the monoamine hypothesis does not address key issues such as why therapeutic profile of antidepressants extends far beyond treatment of major depression, or why all drugs that enhance serotonergic or noradrenergic transmission are not necessarily effective

in depression. Despite these limitations, it is clear that the development of the monoamine hypothesis has been of great importance in understanding depression and in the development of safe and effective pharmacologic agents with a superior tolerability profile for its treatment.

1.3.2 Serotonin system in depression

While evidence exists for the contribution of both 5-HT and noradrenaline to the etiology of depression and the mechanism of action of antidepressants, more recently, the focus has shifted to the 5-HT system and the so called “indoleamine hypothesis” (Blier, 2003). The theory suggests that depressive illness may be associated with a deficit in central serotonergic transmission and supports the notion that an enhancement of 5-HT neurotransmission might underlie the therapeutic response to different types of antidepressants. The primary and most convincing evidence to support this hypothesis is provided by the fact that SSRIs, although devoid of any significant inhibition of noradrenaline uptake, are effective in the treatment of depression. Since SSRIs, NSRIs, TCAs, and MAOIs antidepressants belong to different chemical families that share the only common property of blocking 5-HT reuptake, it is undeniable that they exert their therapeutic effect essentially via the 5-HT system. Other clinical observations demonstrating that inhibition of 5-HT synthesis in drug-remitted depressed patients (using the tryptophan hydroxylase inhibitor para-chlorophenylalanine (Shopsin et al., 1975; Shopsin et al., 1976) and more recently the tryptophan depletion paradigm under rigorous controlled conditions (Delgado et al., 1990)) produces a rapid relapse of depression are also in agreement with the indoleamine hypothesis for depression. Depletion of plasma

L-tryptophan by dietary means leads to lowering of mood in healthy subjects and has been employed as an experimental model of clinical depression (Doris et al., 1999). Furthermore, reduced concentrations of 5-HT in whole blood or platelets of unipolar depressed patients (Coppen, 1967; Owens and Nemeroff, 1994) and low levels of 5-HIAA in the CSF and in brain tissue from depressed and suicidal patients have been demonstrated (Asberg and Traskman, 1981; Ohmori et al., 1992; Risch and Nemeroff, 1992; Mann and Malone, 1997). Measures of 5-HIAA metabolites in CSF are used to assess 5-HT turnover in the brain and thus, reduced concentrations of 5-HIAA are thought to reflect central serotonergic hypoactivity and a deficient 5-HT system. However, results obtained with this measure remain often controversial (Roggenbach et al., 2002). *In vivo* imaging (Malison et al., 1998; Willeit et al., 2000) and postmortem brain studies (Perry et al., 1983; Arango et al., 1995) also report less SERTs in brains of depressed and suicide victims. One potential cause for fewer SERT sites in depression could be a reduction in SERT gene expression or a degradation of SERT protein as a result of reduced overall levels of 5-HT and therefore less recycling processes. Alternatively, the diminution in SERT expression in the DRN of depressed suicides may be attributed to homeostatic changes (natural antidepressant produced by the body) to enhance the activity of serotonergic neurons (Arango et al., 2001). Another indicator of 5-HT neural function in humans is obtained by measurement of prolactin and growth hormone secretion in the blood. *In vivo* studies employing indirect (e.g., L-tryptophan or the serotonin releasing agent fenfluramine), or direct (e.g., buspirone) pharmacological challenges that activate brain 5-HT receptors invoking release of prolactin and growth hormone, indicate blunted neuroendocrine response to 5-HT in

depressed patients and suicide victims, again supporting the notion of abnormal serotonin functions in depression (Power and Cowen, 1992; Mann et al., 1995; Malone et al., 1996).

Although some 40-50% of all suicides are committed by depressed patients, and more than 70% of depressed people experience suicidal thoughts, it should be kept in mind that the criteria for major depression is not necessarily obligatory to suicidal profiles. Some authors in fact argue that suicidal ideation may be an independent psychiatric illness with its own phenomenological and epidemiological profile (Gross-Isseroff et al., 1998; Ahrens et al., 2000). For instance, suicide may be associated with personality traits such as impulsivity and aggression, rather than depressed mood (Roggenbach et al., 2002). Nevertheless, it is clear that in a large proportion of individuals, direct links between a deficient serotonergic system, depression and suicide can be established, and the indoleamine hypothesis in suicide remains highly attractive.

1.3.3 5-HT_{1A} receptor in mood disorders

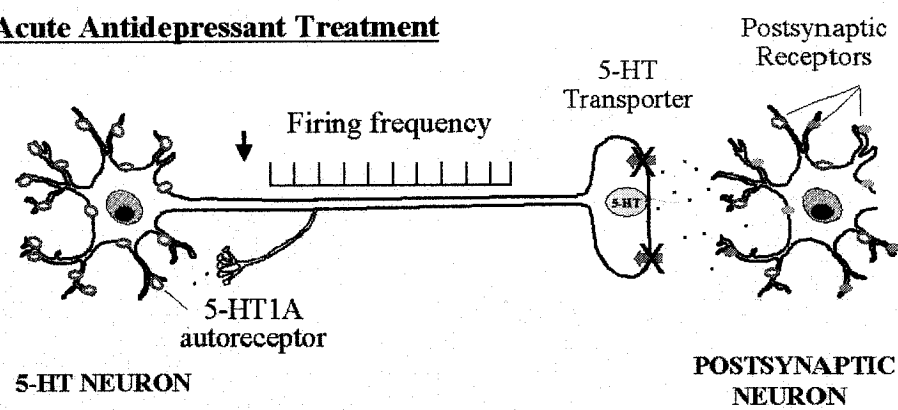
Over the past decade, the 5-HT_{1A} receptor in particular has been under intense investigation, mostly due to its putative role in both the etiology and treatment strategies for depression and anxiety-related behaviours. The distribution of this receptor subtype in cortical and limbic areas reflects well its implication in cognitive functions and emotional states (Verge et al., 1986; Hensler et al., 1994; Kia et al., 1996). Alteration in the level and function of 5-HT_{1A} receptors in the raphe and limbic system is associated with these mental disorders. Mice lacking the 5-HT_{1A} receptor display anxious phenotypes associated with increased serotonergic neurotransmission (Heisler et al., 1998; Parks et al., 1998; Ramboz et al., 1998; Sibille et al., 2000; Parsons et al., 2001; Richer et al., 2002).

Interestingly, 5-HT_{1A} receptor agonists exert anxiolytic properties in both humans and animal models (Feighner and Boyer, 1989; Menard and Treit, 1999), and partial agonists (e.g., buspirone and gepirone) are currently used clinically in the treatment of anxiety (Blier and de Montigny, 1994; Apter and Allen, 1999). It is believed that these drugs work to activate presynaptic 5-HT_{1A} receptors, inhibiting 5-HT neurotransmission and consequently reducing 5-HT release and signalling onto target sites to alleviate the anxious phenotype.

By contrast, elevated levels of 5-HT_{1A} autoreceptor have been observed in the raphe nuclei of postmortem brains from suicide victims diagnosed with major depression (Stockmeier et al., 1998). As described above, evidence supports the observation that inhibition of the serotonergic system is implicated in the pathogenesis of depressive illnesses and suicidal behaviours (Doris et al., 1999; Mann, 1999; Mann et al., 2001), and as such, it follows that reduction in serotonergic activity in depressed patients due to increased levels of 5-HT_{1A} autoreceptors likely contributes to their symptoms. Pharmacological agents used to treat major depression such as MAOIs, TCAs and particularly SSRIs enhance serotonin release by inhibiting its synaptic elimination (Blier and de Montigny, 1994; Stahl, 1998a). While these compounds are valuable in the treatment of major and related depressions, typically 2-6 weeks of drug regimen is required before clinical improvement may become apparent (Blier and de Montigny, 1994; Montgomery, 1997). This clearly indicated that antagonism of reuptake *per se* is not responsible for the antidepressant response, but rather that adaptive changes underlie their therapeutic effect (Artigas et al., 1996b). Electrophysiological approaches have shown that short-term administration of an SSRI or MAOI reduces the firing activity of 5-HT neurons

of the DRN in the rodent brain (Aghajanian et al., 1970; Quinaux et al., 1982; Blier and de Montigny, 1985). Therefore, the failure of acute antidepressant therapy resides in the fact that the prompt increase in synaptic, and in particular raphe extracellular serotonin levels such as seen following SSRI blockade of the SERT, is compensated by the reduction in firing frequency due to recurrent activation of 5-HT_{1A} autoreceptors (Pineyro and Blier, 1999). However, chronic SSRI treatment and sustained increases of 5-HT in the somatodendritic area results in the selective and progressive desensitization (or down-regulation) of the 5-HT_{1A} autoreceptors, which probably accounts for the late therapeutic effect of the treatment, i.e. by means of facilitation of 5-HT neurotransmission (Figure I.4) (Blier and de Montigny, 1994; Kreiss and Lucki, 1995; Le Poul et al., 2000). This notion has led to the idea that antagonism at the 5-HT_{1A} receptor, to prevent its acute feedback inhibition, may augment the ability of SSRIs to increase extracellular 5-HT levels by bypassing the period of time required to downregulate 5-HT_{1A} autoreceptors, therefore allowing for a faster onset of action (Artigas et al., 1996a; Trillat et al., 1998; Kinney et al., 2000). Preclinical studies showing that co-administration of SSRIs with the mixed β -adrenoreceptor/5-HT_{1A} receptor antagonist pindolol or the selective 5-HT_{1A} antagonist WAY 100635 prevents the suppressant effects of SSRI treatment on DRN cell firing in the rat (Romero et al., 1996), and produces a greater elevation of extracellular brain 5-HT levels compared with SSRI treatment alone (Hjorth et al., 1996; Hjorth et al., 1997), are supportive of this hypothesis (Artigas et al., 2001). In humans, clinical trials using both SSRIs and pindolol have shown accelerated (one week treatment instead of three) antidepressant effects (Artigas et al., 1994; Blier and Bergeron, 1995).

Acute Antidepressant Treatment



Chronic 3-Week Antidepressant Treatment

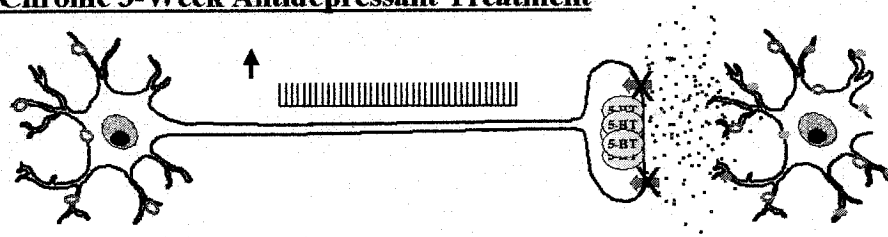


Figure I.4 – Actions of long-term antidepressant drug treatment on pre- and post-synaptic 5-HT_{1A} receptors.

Schematic diagram depicting the receptors and spike activity of neurons in the central serotonergic system after acute and chronic antidepressants (e.g., SSRIs) administration. Following 3-week of pharmacotherapy, selective desensitization of 5-HT_{1A} autoreceptors with no effect on responsiveness of post-synaptic receptors results in increase in serotonergic spike frequency and 5-HT release at the nerve terminal. Modified with permission from Albert et al., 1996.

Interestingly, pindolol does not modify the 5-HT_{1A}-mediated responses to 5-HT and 8-OH DPAT applied on to post-synaptic neurons in the rat hippocampus, suggesting different pharmacological profiles for pre- and post-synaptic receptors. Thus, the therapeutic benefit of enhancing the presynaptic component of 5-HT neurotransmission with pindolol is not likely to be cancelled out by the blockade of post-synaptic 5-HT_{1A} receptors. In contrast to the autoreceptors, post-synaptic 5-HT_{1A} receptors in target region such as the hippocampus also appear resistant to desensitization, and in fact may be slightly upregulated by antidepressant treatment (Blier and de Montigny, 1990; Beck et al., 1997; Haddjeri et al., 1998; Hensler, 2003 Le Poul et al., 2000). This is predicted to result in a net increase in 5-HT neurotransmission in forebrain areas (specifically the hippocampus) via the 5-HT_{1A} receptor subtype. Of considerable interest is the observation that SERT knockout mice, which constitute a model for whole-life treatment with SSRIs, also display reduced levels of 5-HT_{1A} receptor mRNA and protein in the DRN, increased levels in the hippocampus, and similar expression in other forebrain areas compared to wild-type mice (Fabre et al., 2000), supporting again a desensitization of pre- rather than post-synaptic receptors following SERT elimination. TCA antidepressants differ from SSRIs in that they increase post-synaptic 5-HT_{1A} receptor sensitivity, without altering autoreceptor sensitivity (de Montigny and Aghajanian, 1978; Chaput et al., 1991), at least potentially yielding the same (5-HT_{1A} receptor-mediated) net effect.

The reason for this disparity in desensitization susceptibility upon agonist activation between the two populations of 5-HT_{1A} receptors is not yet understood, but several hypotheses have been proposed. Differences in reserve between pre- and post-synaptic receptor pools have been suggested to be responsible for this phenomenon (Meller et al.,

1990; Castro et al., 2000). Other studies suggest that autoreceptors and post-synaptic 5-HT_{1A} receptors couple to different G protein subtypes (G_{αo} in the raphe vs. G_{αi3} in the hippocampus), which could have an impact on their respective desensitization (Hamon and Mannoury, 2001; Blier et al., 1993). The presence of more abundant 5-HT_{1B} autoreceptors on serotonergic terminals than in raphe nuclei may also better compensate for 5-HT release in target regions. Finally, one interesting hypothesis looks at the different NUDR activities in pre- vs. post-synaptic areas for longterm adaptation of 5-HT_{1A} gene expression. In fact, some of my preliminary results that are presented later in the thesis suggest that NUDR acts as a transcriptional repressor of 5-HT_{1A} gene expression in the raphe while it has enhancer activities at the 5-HT_{1A} receptor promoter post-synaptically.

1.3.4 HPA axis, corticosteroids and depression

In addition to the alterations observed in the 5-HT system, major depression has also repeatedly been associated with hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis. The HPA axis is an auto-regulating, endocrine system. By controlling the secretion of stress hormones (glucocorticoids), it functions to restore deviations from homeostasis and contributes to the regulation of energy metabolism on a daily basis. When activated, neurons in the paraventricular nucleus (PVN) of the hypothalamus secrete corticotropin-releasing factor (CRF), which stimulates the synthesis and release of adrenocorticotropin (ACTH) from the anterior pituitary. ACTH then stimulates the synthesis and release of glucocorticoids (corticosterone in rodents and cortisol in humans) from the adrenal cortex (Dallman et al., 1994). Glucocorticoids in turn bind with high affinity to the mineralocorticoid receptor (MR) located predominantly in the hippocampus and with

affinity six to ten-fold lower to the glucocorticoids receptor (GR) that is widely distributed in the brain. Occupation of hippocampal MRs maintains excitability and limbic inhibitory control over the HPA axis, while GR activation induced by higher levels of glucocorticoids negates the action of MR (de Kloet et al., 1993). Abnormalities in the HPA axis have been the most consistently demonstrated biological markers in depression, with patients suffering from endogenous depression frequently having high concentrations of plasma, CSF, and urinary cortisol, because of reduced sensitivity of the HPA axis to glucocorticoids-mediated feedback inhibition (Murphy, 1991). The Dexamethasone Suppression Test is therefore often used to diagnose depression, as dexamethasone (a potent synthetic glucocorticoid) fails to suppress plasma cortisol and ACTH level in at least 40% of depressed patients (Carroll, 1985; Murphy, 1991). Increased levels of CRF have also been detected in CSF of suicide victims and in patients with depression (Nemeroff, 1996; Arborelius et al., 1999; Holsboer, 2001). Furthermore, clinical and preclinical data suggest that unrestrained secretion of CRF in the CNS produces several signs and symptoms of depression through continuous activation of CRF-1 receptors, which has produced a clear rationale for the use of CRF receptor antagonists as novel antidepressant treatment (Zobel et al., 2000). In parallel with hypercortisolaemia, an increase in size of the adrenal gland can be observed, which is reversible upon clinical recovery from depression (Rubin et al., 1995). Other evidence implicating the HPA axis in MDD is that successful antidepressant treatment leads to normalization of cortisol and CRF levels and HPA axis feedback inhibition (Holsboer and Barden, 1996; Nestler et al., 2002a). TCA compounds have been shown to enhance GR gene expression (Pepin et al., 1992b; Rossby et al., 1995). Since GR are essential for the negative feedback action of glucocorticoids

hormones on the HPA axis, and because HPA axis hyperactivity returns to normal after successful TCA treatment, it is proposed that these agents, by inducing GR expression, could restore HPA axis sensitivity to circulating glucocorticoids hormones in major depression (Pepin et al., 1992b; Pepin et al., 1992a). However, this is not a common characteristic of all antidepressants, as the most widely used SSRI, fluoxetine, does not cause such an effect (Rossby et al., 1995).

A reciprocal relationship exists between the serotonergic and the HPA axis systems and again, the 5-HT_{1A} receptor appears to be critical for this interaction (Chaouloff, 1993; Chaouloff et al., 1999; Lanfumey et al., 2000; Lowry, 2002). The hippocampus has been demonstrated to be the site of serotonergic innervation associated with CNS control of the HPA axis, and there exists a good correlation between the concentrations of cellular receptors for 5-HT and glucocorticoids in other brain regions receiving 5-HT input. The strong suppressive effects of glucocorticoids on the serotonergic system and the marked sensitivity of the limbic system, to glucocorticoids are believed to involve 5-HT_{1A} receptors (Laaris et al., 1995; Lopez et al., 1998; McAllister-Williams et al., 1998). It has been demonstrated that activation of hypothalamic 5-HT_{1A} receptors by 8-OH-DPAT induces glucocorticoids levels through regulation of the HPA axis (Owens et al., 1990; Kelder and Ross, 1992; Pan and Gilbert, 1992) and that glucocorticoids in turn, can suppress 5-HT_{1A} receptor expression (Figure I.5) Adrenalectomy in rats leads to rapid increases in hippocampal 5-HT_{1A} receptor mRNA and radioligand binding, and these changes are completely suppressed by low concentrations of corticosterone that preferentially activate MR (Mendelson and McEwen, 1992; Chalmers et al., 1993; Liao et al., 1993; Meijer and de Kloet, 1994). Furthermore, this effect of glucocorticoids on

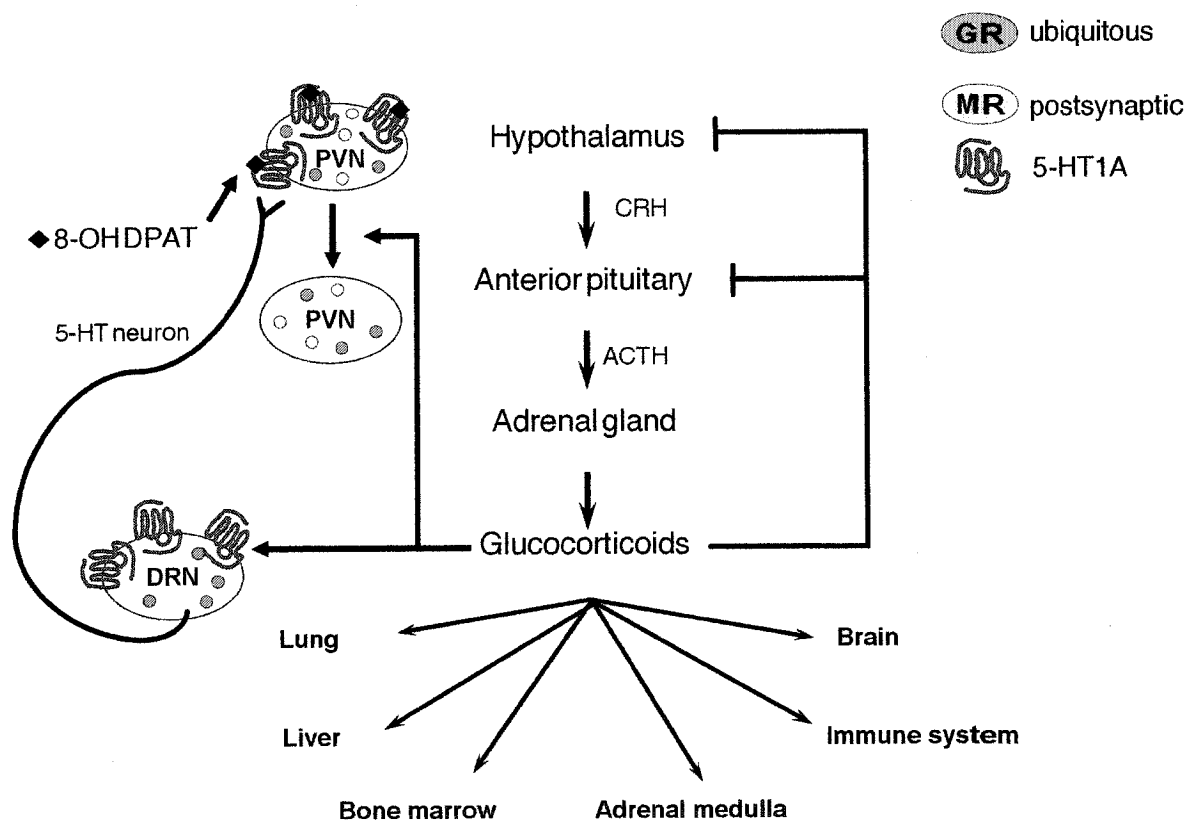


Figure I.5 – The HPA axis/5-HT1A receptor relationship.

The 5-HT1A receptor is implicated in the hypothalamic regulation of the HPA axis, and glucocorticoids in turn suppress 5-HT1A expression, predominantly in post-synaptic regions (e.g., hippocampus, septum, PVN) where both MR and GR are present, but to a lesser extent in presynaptic raphe neurons expressing only GR. A reduction in limbic 5-HT1A receptor number due to chronic stress would have a net effect of reducing serotonergic activity at these sites which may in part explain the link between HPA axis hyperactivity, increased glucocorticoid levels and depression.

inhibition of 5-HT_{1A} receptor gene transcription is pronounced to a greater extent at post-synaptic sites such as the hippocampus and the septum (Mendelson and McEwen, 1992; Chalmers et al., 1993; Chalmers et al., 1994; Meijer and de Kloet, 1994; Zhong and Ciaranello, 1995), where both glucocorticoid receptors GR and MR are expressed, but to a lesser degree at levels of the presynaptic raphe neurons expressing only GR (Laaris et al., 1997; Neumaier et al., 2000). This suggests a key role for MR in the negative regulation of limbic 5-HT_{1A} receptor levels and is consistent with the ability of MR-selective doses of dexamethasone to suppress 5-HT_{1A} mRNA following adrenalectomy. This is also supported by the maintenance of inhibitory action of glucocorticoids on 5-HT_{1A} expression in GR knockout mice (Meijer et al., 1997). Results from the Albert laboratory have shown that glucocorticoid-induced transcriptional repression of 5-HT_{1A} receptors involves dimerization of both MR and GR, which may account for the greater sensitivity of hippocampal 5-HT_{1A} receptors (Ou et al., 2001). The link between glucocorticoids and 5-HT_{1A} receptor transcription is suggestive that alterations in glucocorticoid-mediated repression of the 5-HT_{1A} receptor gene may account for the etiology/pathophysiology and symptoms of major depression (Lopez et al., 1998).

While the HPA axis/5-HT_{1A} receptor relationship constitutes an attractive hypothesis, other explanations have been given for the role of a hyperactive HPA axis in depression. For example, many studies have shown that stress hormones are toxic to neurons (McEwen, 1999; Young et al., 2002). Stress and glucocorticoids are reported to cause a rapid and long-lasting down-regulation of the neurotrophic factor BDNF in the dentate gyrus, CA1 and CA3 layers of the hippocampus. Studies have suggested that depressed animals have shunted neurogenesis in the hippocampus probably due to

reduction in BDNF. Also, because virtually all classes of antidepressants upregulate BDNF at the same time as they induce neurogenesis in the hippocampus, it is proposed that stress-induced diminution of hippocampal neurogenesis, probably through inhibition of BDNF, may be involved in the pathophysiology of depression. Antidepressant induction of BDNF is at least partly mediated via the transcription factor CREB (cyclic-AMP responsive element binding protein) (Thome et al., 2000), and glucocorticoids may interfere with CREB-induced control of BDNF expression. Preliminary studies demonstrate that activation of the cAMP-CREB cascade increases neurogenesis in adult hippocampus (Nestler et al., 2002b) and for this reason, phosphodiesterase inhibitors that inhibit the breakdown of cAMP are currently under investigation as candidate drugs to improve cell survival and plasticity (Holden, 2003). Moreover, chronic glucocorticoid treatment can cause atrophy of pyramidal CA3 neurons (Nestler et al., 2002b). Again down-regulation of BDNF could lead to this stress-induced atrophy. In addition, atrophy may result from glucocorticoid enhancement of excitotoxicity as well as reduced metabolic capacity of these neurons. Glucocorticoids could also make CA3 neurons more vulnerable to different types of neuronal insults. Neurotrophic factors such as BDNF also mediate their survival-promoting effects by inhibition of cell death cascades by increasing the expression of the anti-apoptotic protein Bcl-2 (Bonni et al., 1999). Therefore, atrophy and dysfunction of hippocampal neurons following chronic stress may be responsible for abnormalities observed in depression, including emotional and cognitive deficit that are thought to involve this brain region. Interestingly, recent data has implicated the 5-HT_{1A} receptor in antidepressant-induced changes in neurogenesis and behavioural improvement (Santarelli et al., 2003).

1.4 Regulation of 5-HT_{1A} receptor activity: mechanisms of desensitization

Upon ligand stimulation, heptahelical receptors undergo conformational changes that allow coupling to G proteins leading to the activation of different effectors and signalling pathways. Desensitization is a process whereby the waning of receptor responsiveness takes place to arrest G protein signalling as a result of an adaptive cellular response to prevent the potentially harmful effects that can result from persistent receptor stimulation. Short- and long- term desensitization of 5-HT_{1A} receptors and other GPCRs, occurs via multiple mechanisms that have been best characterized for the β ₂-adrenergic receptor and therefore serves as a prototype (Lefkowitz et al., 1990; Collins et al., 1991; Kobilka, 1992).

1.4.1 Rapid homologous versus heterologous desensitization

The initial step in the desensitization process involves the functional “uncoupling” of the G protein from the receptor, which occurs without any detectable change in the total number of receptors in a cell or tissue. This process is fairly rapid (seconds to minutes) and depends on receptor phosphorylation by the coordinate action of intracellular second-messenger regulated kinases (PKA and PKC) and by G protein-coupled receptor kinases (GRKs) ((Lefkowitz, 1998; Ferguson, 2001). Both PKA and PKC, activated by stimulation of G_s- and G_q-coupled receptors respectively, can phosphorylate and desensitize any given receptor containing appropriate PKA and/or PKC phosphorylation sites in a “heterologous”, or “non-agonist-specific” manner, since any stimulus that will elevate cAMP or DAG has the potential to activate these protein kinases. Therefore, heterologous desensitization typically results in the phosphorylation and uncoupling of multiple receptor families by the activation of only one. PKC phosphorylates serine/threonine residues on

minimal consensus sequence (S/T)-X-(K/R) or (K/R)-X-(S/T) (Kemp and Pearson, 1990; Kennelly and Krebs, 1991). Phosphorylation of these consensus sites found in the intracellular domains of the β -adrenergic receptor following phorbol-ester activation of PKC has been suggested to account for receptor uncoupling from G_s (Bouvier et al., 1991; Yuan et al., 1994). More recently, it has been demonstrated that PKA and PKC can also affect the desensitization of GPCRs by phosphorylating GRK2 and altering its activity (Winstel et al., 1996; Cong et al., 2001). The first evidence to suggest a potential role for PKC in 5-HT_{1A} receptor desensitization originates from the close concordance between the PKC-induced desensitization and phosphorylation of the 5-HT_{1A} receptor by phorbol-ester (Raymond, 1991). Since then, multiple putative PKC phosphorylation sites (three in the i_3 loop and one in the i_2 loop) have been identified and shown by mutagenesis studies to be involved in receptor uncoupling (Lembo and Albert, 1995; Lembo et al., 1997; Wu et al., 2002). Interestingly however, it appears that PKC-mediated desensitization uncouples 5-HT_{1A} receptor from some but not all signalling pathways. For instance, acute activation of PKC blocks 5-HT_{1A}-induced PLC activation and enhancement of calcium mobilization without affecting receptor-mediated inhibition of cAMP (Liu and Albert, 1991; Lembo and Albert, 1995), or uncouples the receptor from N-type Ca^{2+} channels via phosphorylation of T¹⁴⁹ in the i_2 loop (Lembo et al., 1997; Wu et al., 2002). Although the specific implication of PKA in 5-HT_{1A} receptor desensitization remains to be established, it has been determined that activation of PKA and PKC leads to enhanced uncoupling of the receptor from PLC activation, suggesting that both second messenger protein kinases may play a critical role (Liu and Albert, 1991; Albert and Tiberi, 2001).

In contrast to heterologous receptor desensitization that involves phosphorylation of GPCRs in both their ligand-activated and -inactive states, “homologous” or “agonist specific” desensitization is dependent on GRK-induced phosphorylation of activated (agonist-occupied) receptors only (Benovic et al., 1986; Hausdorff et al., 1990). GRKs belong to a family of serine/threonine kinases comprising seven members (GRK1-GRK-7), of which the most thoroughly investigated are rhodopsin kinase (GRK1) and β ARK1 (β -adrenergic receptor kinase 1, also known as GRK2) (Lefkowitz, 1998; Claing et al., 2002). In order to phosphorylate a receptor, GRKs must first be recruited to the plasma membrane where they can form a complex with the receptor. Of all GRKs, three are known to be constitutively associated with the plasma membrane through covalent attachment of either fatty acids or isoprenes to their carboxy termini: GRK4 and GRK6 are palmitoylated while GRK1 is farnesylated. The C-terminal domain of GRK7 has a CAAX sequence that predicts geranylgeranyl modification. GRK5 is also predominantly associated with the membrane via interactions between a positively charged domain in its C-terminus and a negatively charged head group of membrane lipids including phosphatidylinositol-4,5-bisphosphate (PIP₂). GRK2 and GRK3 are different in that they are primarily located in the cytosol. A pleckstrin homology domain in the carboxy terminus of these kinases exhibits G $\beta\gamma$ protein subunit specificity, and the coordinated interaction of free G $\beta\gamma$ and membrane PIP₂ with this domain promotes GRK2 and GRK3 membrane association and a synergistic enhancement of GPCR phosphorylation (Kohout and Lefkowitz, 2003). GRK phosphorylation of GPCRs on their C-terminal tail is not sufficient to induce desensitization, but rather serves to create high affinity sites to promote the binding of β -arrestin proteins which in turn guarantee desensitization by preventing further coupling

to G proteins (Lohse et al., 1990), and by scaffolding with proteins involved in receptor internalization such as clathrin-binding protein AP-2 (Ferguson, 2001; Claing et al., 2002). Several GPCRs undergo agonist-promoted endocytosis (internalization or sequestration). For certain GPCRs, this process utilizes the classical clathrin-coated pit and vesicles (e.g., β 2-adrenergic receptor) machinery (Ferguson et al., 1996), whereas other GPCRs make use of other mechanisms of endocytosis that are independent of arrestin-clathrin interaction (e.g., angiotensin II 1A receptor and endothelins) (Koenig and Edwardson, 1997). GPCR endocytosis has been the subject of intense study and controversy. However, it is well accepted that the rapid uncoupling and desensitization of GPCRs does not require endocytosis. Once internalized, receptors are targeted to recycling or degradative pathways. While it remains unproven whether endocytosis is the early step in the long-term downregulation of GPCRs following prolonged (hours or days) exposure to agonist leading ultimately to receptor degradation into lysosomes, experimental evidence supports a role for GPCR endocytosis in receptor resensitization and receptor signalling (Lefkowitz, 1998). Indeed, trafficking of an uncoupled receptor to low pH endosomal compartments allows dephosphorylation and recycling of the receptor to the cell surface. However, the processes involved in receptor recycling to the plasma membrane after dephosphorylation are yet not well understood (Lefkowitz, 1998).

1.4.2 Long-term receptor downregulation

Unlike the reversible mechanisms of phosphorylation and sequestration, which occur within seconds to minutes, downregulation operates over a much longer time scale of hours to days. Downregulation corresponds to a decrease in the total number of receptors present

in a cell or tissue and in that manner differs from the internalization process which can be viewed as a rapid physical redistribution of the receptor without any detectable change in receptor number. Multiple mechanisms involved at the transcriptional, translational and protein levels have been described for the downregulation of various GPCRs. For example, studies of the prototypic β 2-adrenergic receptor indicate that agonist-induced activation can modulate receptor gene transcription (Collins et al., 1989), RNA stability (Collins et al., 1989), and receptor proteolysis (Jockers et al., 1999; Galea et al., 2001).

1.5 Gene transcription: general mechanisms

The expression of the genetic material first requires the transcription of only one of the DNA strands into a complementary RNA strand. RNA polymerase I and III transcribe most structural RNA's, whereas RNA polymerase II transcribes genes that code for proteins. This primary RNA transcript is first modified at its 5' and 3' ends with a 5' cap and poly A tail respectively and subsequently spliced to remove introns, producing an mRNA molecule. Finally the mRNA is translated into a protein in a set of complex reactions that occur on the ribosome (Lewin, 1997). Factors controlling RNA synthesis, structure and stability all play a significant role in the regulation of eukaryotic gene expression. However, it is commonly accepted that the majority of regulatory events that control gene expression occur at initiation, i.e. the phase of transcription preceding elongation and termination which coordinates the assembly of the transcription apparatus, and more specifically, the positioning of RNA polymerase II (RNA pol II) at the promoter. The promoter region of a gene is defined as being the minimal DNA sequence capable of promoting and initiating transcription. Often enclosed within the first 100-200 bp immediately upstream of the transcription initiation site, this region contains "promoter

elements” of two types: a TATA box and/or initiator (Inr) sequence(s). These elements function as the core promoter at which the transcriptional initiation apparatus (including RNA pol II) is assembled. In addition, proximal promoter elements consist of short *cis*-acting elements recognized by various regulatory proteins that are required to enhance the efficiency of mRNA synthesis initiation (Maniatis et al., 1987; Smale and Baltimore, 1989; Lewin, 1997; Ogbourne and Antalis, 1998; Willy et al., 2000). Promoter elements in general bind proteins encoded by housekeeping genes and are involved in constitutive gene expression.

The most common promoter element in eukaryotic protein genes is the TATA box, an AT-rich sequence (consensus TATA(A/T)A(A/T)) centered at a point approximately 30 bp upstream of the initiation start site. The TATA-binding protein (TBP) is the only protein to make specific contact with the DNA at this site. The recruitment by TBP of TBP-associated factors (TAFs), together known as the transcription factor IID (TFIID), is the first step in the assembly of general transcription factors (TFIIA, B, F, E) and RNA pol II forming the preinitiation complex, a structure representing over 2000 kDa in size (Zawel and Reinberg, 1993). TFIIH, the last factor to adhere to the complex, has helicase and kinase activities that will allow separation of DNA strands to initiate transcription, and phosphorylation of RNA pol II in its C-terminal domain to relax the preinitiation complex and commence elongation (Ogbourne and Antalis, 1998).

Although binding to the TATA motif by TFIID has been shown to be the initial recognition step in transcription complex formation, many promoters that lack a traditional TATA motif have recently been described. In such TATA-less promoters, the Inr element is critical in positioning RNA polymerase II (Smale, 1997). Loosely conserved and

spanning the site of transcriptional initiation (+1), the Inr element is contained between positions -3 and +5 and may be commonly described as a central CA motif surrounded by several pyrimidines (Py₂CAPy₅), with transcription usually being initiated at the adenine residue (Smale et al., 1990). The Inr can act independently or in combination with the TATA box to synergistically direct the assembly of active transcription complexes. The TATA box is however usually the one responsible for the correct positioning of the polymerase to begin transcription. In its absence only, the Inr determines the precise location of the initiation start-point. Alternately, in a few genes that lack both a TATA box and an Inr, multiple initiation sites often surround 20-50 bp GC rich regions called CpG islands (Pedersen et al., 1999). Some genes also rely on Sp1 recognition elements, MAZ motifs (GGGG(C/A)GGGG), ETS motifs (GGCTTCCTGTCT) or the pyrimidine-rich initiator motif (CTCANTCT) to initiate transcription (Pugh and Tjian, 1990; Wasylyk et al., 1993; Parks and Shenk, 1996; Smale, 1997). It is generally thought that these sequences can bind one or more activating molecules (e.g., Sp1) that in turn recruit specific cofactors such as TAFs and ultimately TFIID, allowing interaction with other basic transcription factors and polymerase (Pugh and Tjian, 1990; Azizkhan et al., 1993; Bouwman and Philipsen, 2002). A promoter consisting uniquely of the Inr has the simplest possible form recognizable by RNA pol II. Like the TATA box, the Inr element binds TFIID, however it appears to be TAF-250 and TAF-150, and not TBP, which are responsible for this interaction. Specifically, TFIID containing a mutated TBP defective in DNA binding cannot function on TATA-only promoters, but supports transcription from Inr-containing promoters (Martinez et al., 1995). Alternatively, evidence suggests that other Inr-interacting proteins (such as YY1, (Usheva and Shenk, 1994)) may be capable of

directing the initiation of transcription even in the absence of TBP. However, this mechanism is as yet not clearly understood. TFII-I and RNA pol II also seem to have Inr-specific binding capacities (Smale, 1997).

The presence of a core promoter sequence (TATA box, or Inr) and assembly of the basal transcription apparatus is all that is required to initiate transcription of any given gene. However, the level of transcription achieved by such a promoter is minimal and upstream factors are necessary to augment transcriptional rates. Proximal promoter elements located within 200 bp of the initiation start-point bind to regulatory trans-acting proteins to either stabilize the preinitiation complex, or to augment its activity. These regulatory elements can operate in a position or orientation independent manner and they include elements such as the Sp1 box and the CCAAT box, both of which are important to promoter function. The GC motif at around -90 bp of the initiation site contains the sequence GGGCGG and recognizes the Sp1 protein. It is frequently found in multiple copies in many eukaryotic promoters. The CCAAT box named after its 9 bp consensus of 5'-GGCCAATCT-3', is usually located around -75 bp relative to the initiation start site and like the Sp1 site has been found in a wide variety of promoters. The CCAAT box interacts with CTF/NF1 (CCAAT transcription factor/ nuclear factor 1), stimulating transcription and DNA replication respectively. The CCAAT box also recognizes a family of C/EBP (CCAAT/enhancer binding protein) proteins that include C/EBP α , C/EBP β , and C/EBP δ with tissue-specific and distinct developmental patterns of expression. The existence of multiple CCAAT box binding proteins of both the CTF/NF1 and C/EBP type contribute to an important role for this sequence in gene regulation apart from acting simply as an activator of constitutive gene activation (Latchman, 1998).

As described, promoter elements have a relatively fixed location with regard to the transcription start-point and are usually located within 200 nucleotides of the initiation of transcription. When regulatory sequences are localized further away and lack themselves intrinsic promoter activity, they are instead usually termed "enhancer elements", or "silencer elements" if they inhibit gene expression (Goodbourn, 1990). These elements may in fact exert their effects at great distances of up to several thousand base pairs. Also, they can function in either orientation, operating either up- or down-stream from the promoter they are enhancing or silencing. This property may reflect the flexible nature of the DNA allowing regulatory elements to loop onto the promoter and interact with components of the transcription initiation complex to affect its assembly or disassembly. The importance of enhancer/silencer elements can vary considerably in response to physiological inputs in different cell types. The availability of DNA binding proteins which recognize the element and their activity in response to external stimuli such as hormone, growth factors or heat shock, can greatly contribute to diversity of gene regulation mediated by these proteins. Variation may also occur from promoter to promoter and may depend on the proximity of other elements, and the overall spatial arrangement of elements within a gene. Therefore, enhancers and silencers confer context and tissue-specificity for gene expression and their elements are important in regulating differential gene activity in higher eukaryotes.

Inducible gene expression is conferred by a large number of motifs called response elements, which respond to external signals and alter the rate of gene expression. The heat-shock element (HSE), cAMP-responsive element (CRE) and steroid-thyroid hormones responsive elements (HRE) which bind HSF, CREB and nuclear receptors, respectively,

are only a few examples of elements involved in inducible gene expression. In the classical model of nuclear receptor action, upon hormone binding, the occupied receptor undergoes a conformational change, displacing the inhibitory heat shock proteins (HSP90, HSP70 and p59), ultimately leading to the formation of a receptor homodimer or heterodimer. The activated receptor binds to a specific HRE consisting of an inverted repeat of a palindrome separated by 3-6 nucleotides in the target gene promoter. The HRE-bound nuclear receptor directly or indirectly, via recruitment of an array of transcriptional cofactors, interacts with the general transcription apparatus (Horwitz et al., 1996; Collingwood et al., 1999; McKenna et al., 1999). One mechanism by which cofactors influence transcription is by remodelling chromatin and thereby facilitating or preventing access of the general transcription factors to the transcription initiation site. In fact, the initiation of transcription in eukaryotes is greatly affected by the packaging of DNA by nucleosomes into chromatin. Nucleosomes contain two molecules of each of the core histones H2A, H2B, H3 and H4 (Lewin, 1997). Some coactivators and corepressors possess intrinsic histone acetyl transferase (HAT) or histone deacetylase (HDAC) activities respectively. HAT transfers the acetyl moiety from acetyl coenzyme A to the amino group of internal lysine residues, which neutralizes the positive charges, reducing the affinity of histones for the negatively charged DNA and facilitating thereby access of transcription factors to enhance gene expression (Utlei et al., 1998). Conversely, HDACs catalyze the removal of the acetyl group, re-establishing the positive charge of histones to prevent the accessibility of transcription factors to DNA and reduce gene expression (Grunstein, 1997; Kuo and Allis, 1998). The cell- and promoter-context of the bound receptor determine the resulting positive or negative effects on regulation of gene expression.

Nuclear receptors and other regulatory proteins may also modulate gene transcription indirectly via synergism or antagonism of other transcription factors activity at their cognate binding sites. Not all genes that are regulated by steroid hormones contain the appropriate HRE. Indeed, there is increasing evidence to suggest that glucocorticoids, for example, can also promote or repress transcription of genes lacking a consensus glucocorticoid responsive element (GRE). These include genes that utilize non-classical GREs (Ou et al., 2001) or genes that are regulated by GR through protein-protein interaction with other transcription factors, such as AP-1 (Diamond et al., 1990), NF- κ B (Almawi and Melemedjian, 2002) and CREB (Stauber et al., 1992).

1.5.1 Transcription factor families

Over the years, a large number of specialized transcription factors have been identified and characterized for three main functions: DNA-binding, transcription regulation, and responsiveness to regulatory signals. Transcription factors all share two common features, a DNA-binding domain, which recognizes specific DNA sequence and binds to it, and a transactivation domain which interacts with other proteins thereby modulating the rate of transcription. Despite the significant variation in the overall structure of these factors, recurrent structural motifs in their DNA-binding domains have provided the basis for their classification (Pabo and Sauer, 1992). Thus far, over twelve different DNA-binding motifs have been identified (He and Rosenfeld, 1991). However, more than 80% of transcription factors fall into one of the following categories: helix-turn-helix, zinc finger, leucine zipper or helix-loop-helix proteins (Figure I.6).

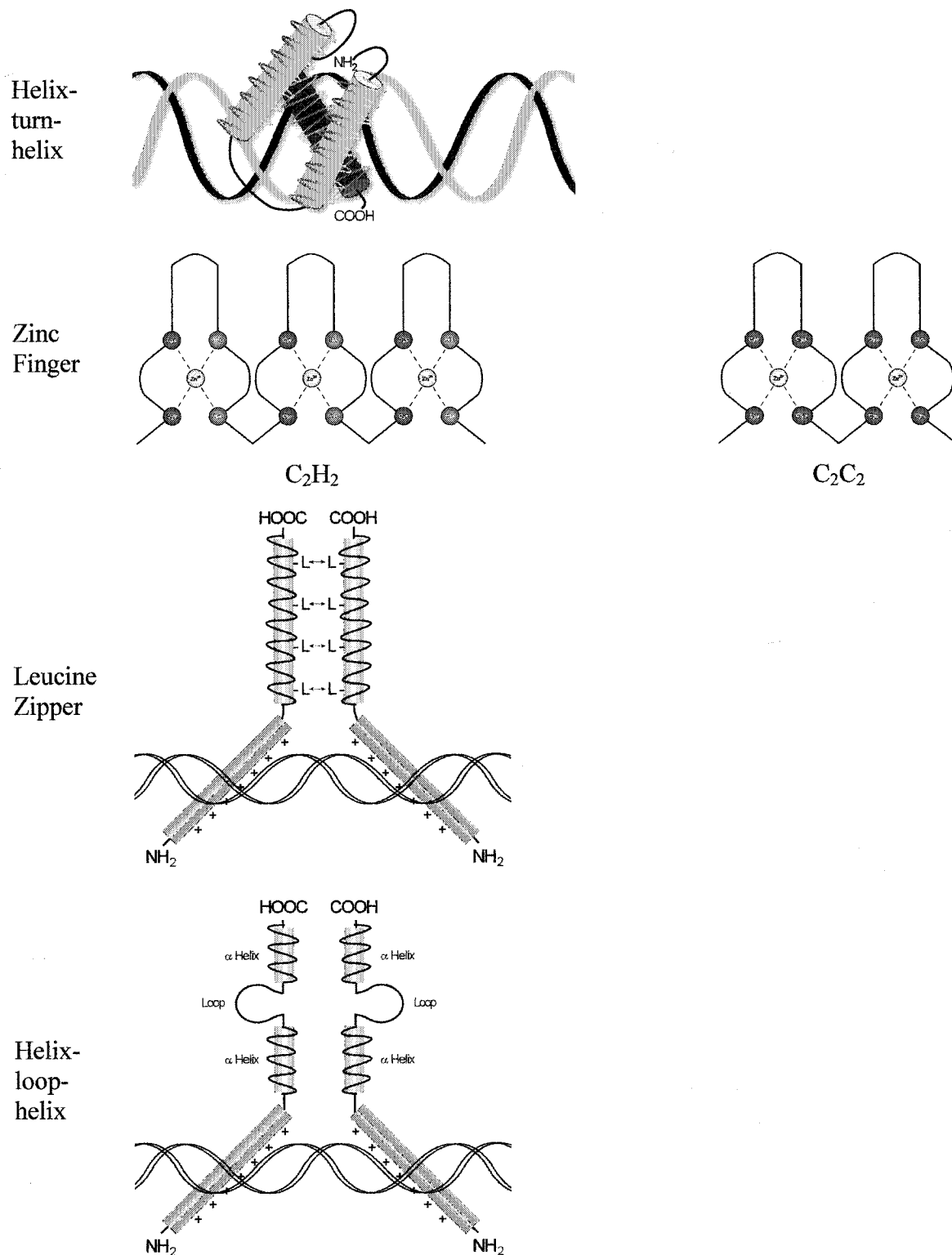


Figure I.6 – General structure of the most common transcription factor DNA-binding motifs.

The helix-turn-helix motif was the first being discovered in the phage 434 repressor and shown to consist of two alpha helices connected by a short amino acid sequence, the turn. One of these helices, the recognition helix, fits into the major groove of the DNA double helix and is responsible for sequence-specific binding. The other alpha helix lies at an angle across DNA. The most important type of eukaryotic helix-turn-helix motif is the highly conserved 60 amino acid homeodomain (with a fourth alpha helix). This motif was first characterized in several proteins coded by genes concerned with developmental regulation in *Drosophila*. Homeodomain-containing proteins have since been identified in a wide range of organisms including vertebrates and plants. The POU proteins are other helix-turn-helix DNA binding proteins, related to the homeodomain family, which contain an additional 75 amino acid sequence corresponding to the POU-specific domain located at the N-terminal side of a homeodomain (Ruvkun and Finney, 1991). The homeodomain of POU proteins can bind to DNA on its own but only weakly. The presence of the POU region is required for high affinity, sequence specific binding. POU proteins bind as monomers or dimers even though their binding site is not palindromic. It was called the POU domain after the first letters of Pit-1, Oct-1 and Oct-2 and Unc-86 where it was first observed (Rosenfeld, 1991; Ruvkun and Finney, 1991). The POU structure has since been found in a number of other proteins (He et al., 1989).

The zinc finger containing proteins constitute the largest group of transcription factors in the eukaryotic genome (Latchman, 1998). The zinc finger motif was originally recognized in factor TFIID, which is required for RNA polymerase II to transcribe 5S rRNA genes. It has since been identified in several other transcription factors, and a distinct form of the motif is also found in steroid receptors. The TFIID-like zinc finger

proteins have a ~30-residue peptide sequence that includes two cysteines and two histidines at specific locations in the polypeptide. These amino acids coordinate with ionic zinc, producing a loop, or “finger” structure, which binds to DNA. Similar sequences have been found in a range of other DNA binding proteins though the number of fingers varies (as many as 30 zinc fingers have been identified in one polypeptide). For example, the Sp1 transcription factor, which binds to the GC box found in the promoter proximal region of a number of genes, has 3 fingers (Kadonaga et al., 1987). This type of zinc finger that has 2 cysteines and 2 histidines is called a C₂H₂ zinc finger. The second class of zinc finger is mainly found in the DNA-binding domain of steroid receptors. It structurally differs from the C₂H₂ type by carrying two cysteine pairs instead of one cysteine and one histidine pair, and is therefore called C₂C₂ zinc fingers. Hence, while the C₂H₂ zinc finger contains a beta sheet and an alpha helix held together by one Zn²⁺ atom, the C₂C₂ motif consists of two alpha helices packed together by two zinc atoms thereby forming a pair of fingers. These two “sub motifs” are quite different, but both use zinc as structural feature and an alpha helix to recognize the major groove. Moreover, unlike the C₂H₂ finger which is present in multiple copies within the protein that contains it, the C₂C₂ motif present in steroid receptors is only found once in each receptor and is usually responsible for binding to short palindromic sequences. Steroid receptors also differ from other groups of transcription factors in that they have a third functional domain in addition to the two shared by all transcription factors: they have a steroid-binding domain, through which they bind their ligand, and have their activity regulated. The central region of steroid hormone receptors is required for DNA binding. Regions required for hormone binding, dimerization and transcriptional activation are located C-terminally to the DNA binding domain. The

dimerization domain allows for formation of receptor homodimers or heterodimers. In addition to the C-terminal domain, there is also an N-terminal region required for transcriptional activation.

The leucine zipper, one of the simplest motifs, consists of a stretch of 60-80 amino acids combining both a DNA binding and a dimerization region. A stretch of amino acids with a leucine residue in every seventh position forming an alpha helix is characteristic of this motif. Binding to DNA is accomplished via an adjacent region of 20-30 residues rich in basic (positively charged) lysine and arginine amino acids (Mitchell and Tjian, 1989; Pabo and Sauer, 1992). Leucine zippers were first described in factors such as C/EBP, c-Fos and c-Jun (Johnson and McKnight, 1989). Whereas C/EBP binds to DNA as a homodimer of identical subunits, Fos cannot form homodimers at all and Jun/Jun homodimers tend to be unstable. However Fos/Jun heterodimers are much more stable. These Fos/Jun heterodimers correspond to a general transcription factor called AP-1, which binds to a variety of promoters and enhancers and activates transcription. Dimerization is required for these polypeptides to bind DNA. The leucine zipper on one polypeptide interacts with the leucine zipper on another polypeptide, joining the two together in a mechanism reminiscent of the 'zipping up' of a zipper (hence the name). The two protein molecules have C-terminal alpha-helical regions which are coiled around each other, held together by interacting leucine side-chains. The N-terminal basic regions hook into the DNA major grooves. Dimerization leads to proper juxtaposition of this basic domain before sequence specific DNA binding can occur.

Helix-loop-helix (HLH) transcription factors, not to be confused with the helix-turn-helix proteins, contain a motif consisting of two amphipathic alpha helices (instead of

three), one short and one long, connected by a flexible loop, so that they can be packed together in one plane. The length of the connecting loop varies from 12-28 amino acids. Like the leucine zipper, the HLH motif mediates both DNA binding and dimerization, and helix-loop-helix containing proteins must dimerize in order to bind to DNA. A basic region near this motif is also required for DNA binding. Each amphipathic helix presents a face of hydrophobic residues on one side and charged residues on the other side. The motif enables proteins to dimerize, and occasionally heterodimers are formed, between a full-length helix-loop-helix protein and a truncated helix-loop-helix, which is unable to bind DNA tightly, and is therefore thought to enable inactivation of specific gene regulatory proteins (Lewin, 1997). The HLH motif has been identified in genes coding for eukaryotic DNA-binding proteins and some developmental regulators such as NeuroD (Lee, 1997).

More recently, a new family of DNA binding protein containing a SAND domain has been identified that comprise several nuclear proteins that have been linked to chromatin-dependant transcriptional regulation and human diseases such as promyelocytic leukemia and autoimmune poly-endocrinopathy-candidiasis-ectodermal dystrophy (Wojciak and Clubb, 2001). The conserved ~80-residue SAND domain was originally discovered in the DEAF-1 transcription factor from *Drosophila* and later in other proteins, hence the name SAND (for Sp100, Aire-1, NucP41/75 and DEAF-1). The presence of the SAND motifs in DNA-binding transcription factors has led to the suggestion that they were DNA binding domains (Gross and McGinnis, 1996; Huggenvik et al., 1998; Christensen et al., 1999). Work on the human homologue of DEAF-1, NUDR (Nuclear DEAF-1 Related Protein), has established that the SAND domain structure represents a novel compact α_4/β_5 fold in which a conserved KDWK sequence motif is found within an alpha-helical,

positively charged surface patch. The DNA-binding surface is localized to the alpha-helical region encompassing the KDWK motif, and contributes to sequence-specific recognition of TTTCG elements by NUDR (Bottomley et al., 2001).

The advance in bioinformatics has evolved to enable the prediction of structural motifs within protein. Structural models allow for the identification of DNA binding motifs from the primary structure of a protein. Based on their structure, it should be feasible to engineer protein domains with enhanced DNA binding ability compared to naturally occurring motifs. This in turn, may serve in developing drugs for silencing or enhancing certain genes that are over or under expressed in certain diseases.

1.5.2 Neuron-specific gene transcription

A complex transcriptional program involving both positive- and negative-acting factors governs the determination of neuronal fate. Positive helix-loop-helix regulators such as Mash1, NeuroD, Neurogenin and CD4 are thought to regulate neuronal development at the level of concomitant and post-mitotic differentiation (Guillemot and Joyner, 1993; Kageyama and Nakanishi, 1997; Lee, 1997; Kim and Siu, 1998). However, while positive transcriptional determinants of neuronal phenotype have been studied extensively, our understanding of the negative regulatory mechanisms involved in such determination has only started to emerge. The basic helix-loop-helix factors Hes1 and Hes5 are amongst the few transcription factors currently known to inhibit neuronal differentiation *in vivo*. They were originally isolated as mammalian homologues of hairy and Enhancer of Split, which negatively regulates neurogenesis in the *Drosophila*. Both constitute important effectors of the Notch signalling pathway, and act early in the transcriptional cascade to inhibit the

expression of the positive HLH genes by binding to E box (CANNTG) or N box (CACNAG) elements within those genes (Akazawa et al., 1992; Chen et al., 1997; Kim and Siu, 1998). In mammals, the Notch/Hes signalling cascade initiates when one of the four transmembrane Notch receptors (Notch 1-4) becomes activated by one of four ligands (Jagged-1, Jagged-3 and delta-like-1 and 3) (Mizutani et al., 2001). Upon ligand binding the Notch receptor undergoes conformational change, and the Notch intracellular domain of the receptor is cleaved, released from the receptor, and translocated to the nucleus. There, it is believed to interact with the DNA binding protein RBP-JK to activate transcription of target genes including those encoding Hes proteins. In neurons with low Notch activity, neurites extension proceeds, whereas high Notch activity is associated with inhibited extension or retraction of neurites, and repression of neuronal genes by Hes proteins (Sestan et al., 1999). Notch signalling appear to be critical for lateral inhibition, which ensures that cell differentiate to distinct fates from an initially homogeneous cell population (Beatus and Lendahl, 1998). Consistent with its role in the negative regulation of neuronal differentiation, Hes5 and Hes1 proteins are expressed exclusively in the developing nervous system, and are expressed at high levels throughout the ventricular zone where neuronal precursor cells are present (Ohtsuka et al., 2001).

Neuronal development and fate implies that brain-specific proteins are expressed not only in a temporal, but also spatially regulated fashion. The evolution of neuronal-specific patterns of expression of certain genes is generated in part by tissue-specific silencers that suppress expression outside of the nervous system. The Repressor Element-1 Silencing Transcription factor (REST, also known as Neuron-Restrictive Silencing Factor, or NRSE) is to date, the best-characterized transcriptional repressor of neuronal genes in

extra-neuronal tissues. Ubiquitously and abundantly expressed in non-neuronal cell types, REST/NRSF also acts as a silencer of neuron-specific gene expression in undifferentiated neuronal progenitor cells during embryogenesis (Chong et al., 1995; Schoenherr and Anderson, 1995; Chen et al., 1998). Independent research on the regulation of Sodium Type II Channel and SCG10 genes led to the simultaneous discovery of the 21-bp Repressor Element-1/Neuron Restrictive Silencing Element (RE-1/NRSE), that binds REST/NRSF, and both nomenclatures have prevailed (Maue et al., 1990; Mori et al., 1990). Subsequently, RE-1/NRSF has been identified in a number of neuronal genes encoding proteins of fundamental importance in brain function including neuronal receptors, synaptic vesicle proteins, adhesion molecules, signalling proteins, transcription factors and channel proteins (Maue et al., 1990; Li et al., 1993; Kallunki et al., 1995; Lonnerberg et al., 1996; Bai et al., 1998).

The consensus sequence of 5'-TTCAGCAC**CACGG**ACAGCGCC-3' was established from a comparison of RE-1 elements found in 19 different genes (Schoenherr et al., 1996). With the exception of the GG motif (in bold) which is critical for REST binding (Mori et al., 1992), RE-1 function tolerates variations from the consensus sequence with up to six base pairs of deviation being required to prevent REST/RE-1 interaction (Schoenherr et al., 1996). Typical of a silencer, this element can operate in an orientation and position independent manner. However, it has become increasingly evident that the RE-1/REST function is more complex than initially described. REST can act as either a repressor or an activator, depending on the cellular context (Bessis et al., 1997; Kallunki et al., 1998; Seth and Majzoub, 2001). For instance, when placed no further than 50 bp away from the transcriptional start site of the nicotinic acetylcholine receptor beta2-subunit gene, this

element behaves as an enhancer in neuronal cells, but always functions as a silencer in non-neuronal cells regardless of position (Bessis et al., 1997). Given these conflicting properties, the following model for REST function has been proposed: at low REST concentrations (e.g., in neurons), REST does not repress distant RE-1-containing promoters, but may enhance proximal RE-1 promoters while at high concentrations (e.g., in non-neuronal cells), REST elicits repression independently of the promoter context. However, REST silencing activity has now also been reported in the adult brain (Timmusk et al., 1999; Calderone et al., 2003) and multiple splice variants of REST have been identified in neurons (Palm et al., 1998), some with activating properties (Shimojo et al., 1999). Hence, REST appears to be capable to both repress and enhance neuronal gene expression in brain depending on the gene and its cellular context.

REST is a 116-kDa zinc finger protein of the C₂H₂ type that displays a modular structure. DNA-binding and transcriptional activities are attributed to different domains within the REST protein. In addition to the cluster of eight zinc fingers located at the N-terminus that functions as the DNA-binding domain for neuronal genes, two repressor domains were identified at the N- and C-termini (Tapia-Ramirez et al., 1997; Thiel et al., 1998). The repressor domain in the N-terminal part of the protein recruits the corepressor Sin3A/B that in turn associates with HDAC (Huang et al., 1999; Naruse et al., 1999; Grimes et al., 2000). The C-terminal repressor domain of REST recruits the CoREST/HDAC2 complex to mediate repression (Ballas et al., 2001)

Following the discovery of REST, and its implication as a neuronal-specific silencer, the search for other related regulators of neuronal phenotype has gained more and more attention. Recently, a new element called SNOG (for SNAP-25, NOS, GAP-43) was

shown to prevent the expression of the growth-associated protein (GAP-43) gene in non-neuronal cells, and was identified in the synaptosomal-associated protein of 25 kDa (SNAP-25) and nitric oxide synthetase (NOS) genes. This element is distinct in sequence (consensus (G/A)ATG(G/A)GGG(C/T)) and position dependence from the RE-1 element. Unlike the RE-1, the SNOG element must be located downstream of the TATA box and upstream of the initiation start site. Likewise however, the SNOG element exerts repression in cortical neurons, and the associated binding protein is expressed in neurons, although at a lower apparent level than in non-neuronal cells (Weber and Skene, 1997; Weber and Skene, 1998).

1.6 Transcriptional regulation of the human 5-HT1A gene: implication in depression

1.6.1 Delayed onset of antidepressant action and regulated developmental expression

Upon chronic treatment with antidepressants, desensitization of presynaptic 5-HT1A autoreceptors results in restoration of raphe firing rate and enhanced serotonergic neurotransmission that is associated with behavioural improvement (Blier et al., 1990; Blier and de Montigny, 1994; Jolas et al., 1994; Le Poul et al., 1995; Mongeau et al., 1997; Le Poul et al., 2000). Although the exact nature of the change in 5-HT1A receptor function following chronic antidepressant treatment probably involves multiple mechanisms, evidence tends to suggest that adaptive changes at the genomic level may occur. Firstly, the three-week delay in efficacy observed with antidepressants correlates well with the expected time course of transcriptional down-regulation of the inhibitory receptor. Secondly, the persistence of clinical effect suggests that antidepressant treatment may silence 5-HT1A gene transcription. The decrease in 5-HT1A mRNA levels in the rat

anterior raphe following chronic fluoxetine treatment is more direct evidence implying gene transcription in antidepressant-induced desensitization of 5-HT_{1A} autoreceptors (Le Poul et al., 2000). Moreover, in the SERT knockout mice, a model of lifelong treatment with SSRIs, reduced levels of 5-HT_{1A} protein in the anterior raphe area and concomitant increase in the hippocampus parallel the modification in 5-HT_{1A} mRNA in those regions. This suggests that changes at the transcriptional level probably account for the downregulation of DRN 5-HT_{1A} autoreceptors and upregulation of hippocampal 5-HT_{1A} receptors in the SERT ^{-/-} mutant mice (Fabre et al., 2000). Accordingly, desensitization of 5-HT_{1A} autoreceptors in the DRN in rats chronically treated with fluoxetine could be at least partly due to decreased 5-HT_{1A} gene transcription.

The mechanisms involved in regulating the 5-HT_{1A} receptor appear to be tightly synchronized. In adulthood, the expression of the receptor is restricted to the midbrain raphe nuclei and limbic regions involved in the control of mood and emotion such as the hippocampus and cerebral cortex (Chalmers and Watson, 1991; Miquel et al., 1992; Pompeiano et al., 1992). Moreover, the 5-HT_{1A} receptor appears to be developmentally regulated. It is only transiently expressed in the rat cerebellum (Daval et al., 1987), and forebrain receptor expression during the early postnatal period, but not in the adult, is necessary to establish normal adult anxiety-like behaviour (Gross et al., 2001). Reduction in forebrain 5-HT_{1A} receptor expression early in development is correlated with anxiety in the adult. By contrast, elevated 5-HT_{1A} receptor expression in the midbrain of depressed suicide victims has been reported (Stockmeier et al., 1998). Protein levels are likely to reflect changes in gene transcription. The highly regulated expression of 5-HT_{1A} receptor during development and in adulthood, as well as its brain regional distribution suggest that

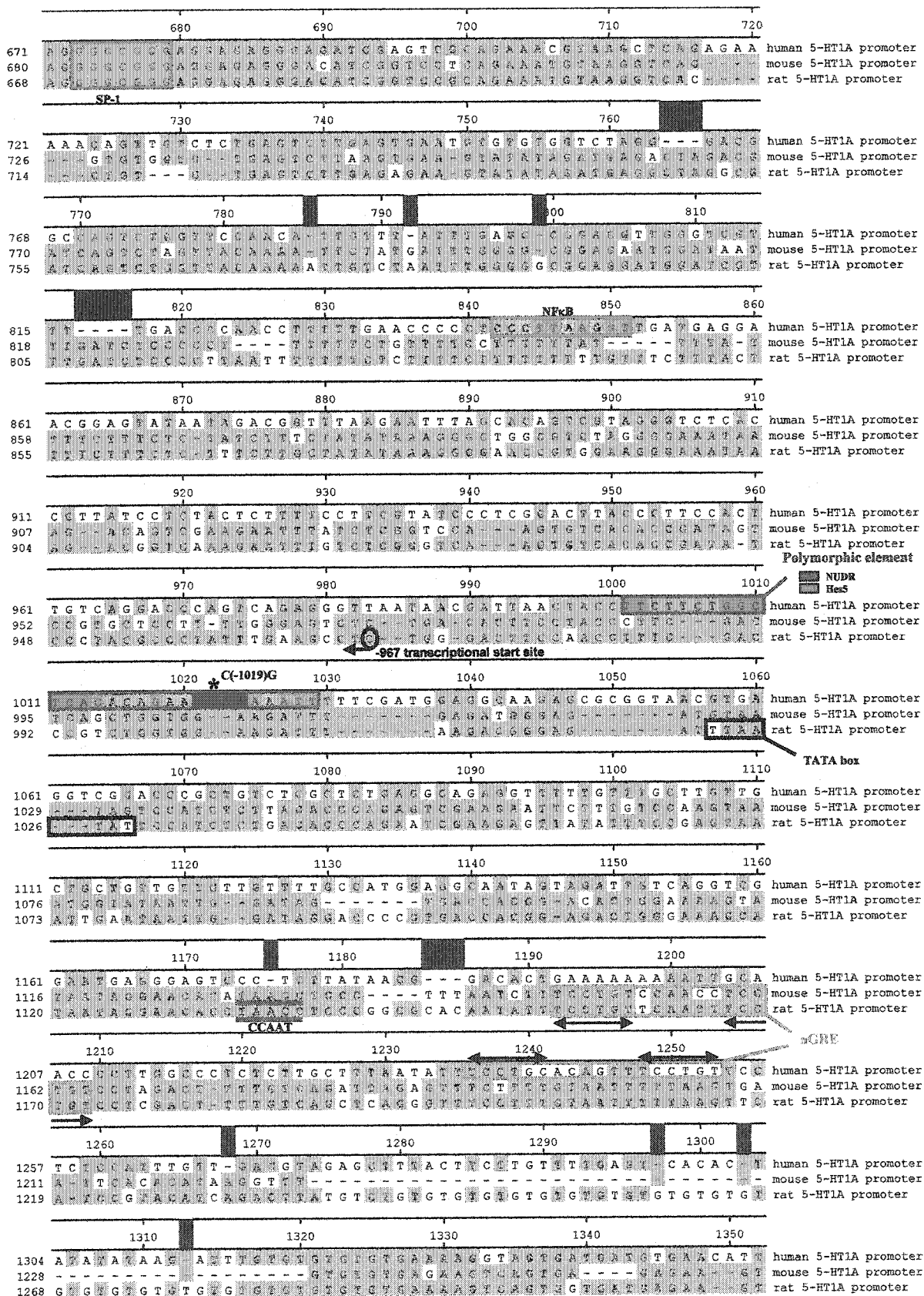
complex transcriptional mechanisms may be involved in control of 5-HT1A receptor gene expression, some of which may be compromised in mental illnesses.

1.6.2 Insights from human and rodent 5-HT1A promoter studies

The mechanisms underlying cell-specific and basal regulation of the rat 5-HT1A receptor have recently been investigated (Figure I.7) (Storring et al., 1999; Ou et al., 2000; Ou et al., 2001). The rat gene is regulated by a proximal TATA-driven promoter located 58 bp downstream of a single brain-specific transcriptional initiation site at -967 bp, and is repressed by an upstream region (from -1555 to -1524 bp) containing a novel 31-bp dual repressor element (DRE). The DRE is composed of a 14-bp 5'-portion (FRE) that mediates repression in neuronal cells expressing the 5-HT1A receptor, but is dispensable in receptor-negative cells, where an additional 12-bp 3'-element maintains repression (Ou et al., 2000). A non-selective enhancer region localized between -1519 and -426 bp contains consensus TATA, CCAAT, Sp1 and AP-1 elements. A promoter region between -426 and -117 from the initiator ATG codon contains AP-1 binding sites and several C-G rich sequences that possess the ability to drive transcription independently from the upstream TATA box (Storring et al., 1999). A novel nGRE half site 5-TGTCCT separated by six nucleotides (at -1169 bp) confers negative regulation of the rat 5-HT1A receptor promoter via interaction with GR/MR heterodimers. This element is also conserved in the murine (at -1161 bp) and the human (at -1250 bp) but their functionality in these gene homologues has yet to be investigated (Ou et al., 2001).

Recent studies have implicated Pet-1, an ETS domain transcription factor of the helix-loop-helix DNA binding protein family involved in regulation of cell proliferation,

[illegible]



1353	AAAGGCACTGTGACGGTGAAGGCTATCGAAGACCGGACCTTTTAACTCCG	human 5-HT1A promoter
1254	ATAGTTACATAAATTAAGGCTATCGAAGACCTTTTAACTCCG	mouse 5-HT1A promoter
1316	AGAGTTAGG-CAATTTGCTGAGGCTATCGAAGACCTTTTAACTCCG	rat 5-HT1A promoter
1403	IACCTTTATATAAAGCTTATACACAGATTCACCTCGGACCCGTAAAGTGT-A	human 5-HT1A promoter
1304	TTTCTTTAAACAAAGCTTATACACAGATTCACCTCGGACCCGTAAAGTGTGA	mouse 5-HT1A promoter
1365	TTCTTTAA-CAAGTAGATACATAAAGCTTATCGGACCCGTAAAGTGT-A	rat 5-HT1A promoter
1452	CGGCAAA-----ACCTGAAGAAATGGCTCGGACCTTTGAGACCCCT	human 5-HT1A promoter
1354	CCAGCAAGCTTGAATACAGAGAGAGTGGCTCGGACCCGTAAAGTGTGA	mouse 5-HT1A promoter
1413	CCTGAAGCTTGAAGACCGGAGAGAGTGGCTCGGACCCGTAAAGTGTGA	rat 5-HT1A promoter
1495	TTGAACCACTTTTGGGSCCAAGCCATCGGCCATCGGAGAGCAATTAGTGC	human 5-HT1A promoter
1402	TTGAATCACTTTAGGGAGAGAGAGTGGCTCGGACCCGTAAAGTGTGA	mouse 5-HT1A promoter
1461	TTAAACCACTTTAGGGAGAGAGAGTGGCTCGGACCCGTAAAGTGTGA	rat 5-HT1A promoter
1545	TTTGACCTTAACTGAGGACCTTGGCAATTCCTTACTCC--AA--AATA	human 5-HT1A promoter
1445	TTTAACCTTAACTGAGGACCTTGGCAATTCCTTACTCC--AA--AATA	mouse 5-HT1A promoter
1504	TTTAAACCTTAACTGAGGACCTTGGCAATTCCTTACTCC--AA--AATA	rat 5-HT1A promoter
1591	ACCTTTGGGGAATAC	human 5-HT1A promoter
1495	GGCTTTTATAAAGCAG	mouse 5-HT1A promoter
1554	CTTTTTCAAACAG	rat 5-HT1A promoter
1641	G--TTTTCCTTCTGTTTATGTTGAAATCTTTCTC	human 5-HT1A promoter
1545	TAAATTTTAAATTCATTAATTCCTTTGGGTTCTGAGA--	mouse 5-HT1A promoter
1604	ARTTTCCTTTAATTCATTAATTCCTTTGGGTTCTGAGA--	rat 5-HT1A promoter
1688	CTTCTGTCAATAATTTTCTGTTCCATCATAACTCAAAACAGAGGAAGGAT	human 5-HT1A promoter
1586	CTTCTGTCAATAATTTTCTGTTCCATCATAACTCAAAACAGAGGAAGGAT	mouse 5-HT1A promoter
1645	CTTCTGTCAATAATTTTCTGTTCCATCATAACTCAAAACAGAGGAAGGAT	rat 5-HT1A promoter
1738	AAAAGAAATTTTAAATTAATCAACCGGGAGAAACGAGACCTTGGGAATATA	human 5-HT1A promoter
1603	GG--TTTAAATTAATTAATCAACCGGGAGAAACGAGACCTTGGGAATATA	mouse 5-HT1A promoter
1668	GG--TTTAAATTAATTAATCAACCGGGAGAAACGAGACCTTGGGAATATA	rat 5-HT1A promoter
1788	TAGTCCGGATATATACAAATTCGAAAGGAACCTTTCCGGACTTTCATAGACA	human 5-HT1A promoter
1638	AGGTTTATATATACAAATTCGAAAGGAACCTTTCCGGACTTTCATAGACA	mouse 5-HT1A promoter
1703	AGGTTTATATATACAAATTCGAAAGGAACCTTTCCGGACTTTCATAGACA	rat 5-HT1A promoter
1838	GTAAGTGTTCAGAGATTGAACCTTCCGAAATTAATTTGATCCGATGCTGG	human 5-HT1A promoter
1670	ATAC--TTTATTCGTTCTTATTAATTAATTAATTTGATCCGATGCTGG	mouse 5-HT1A promoter
1750	ATAC--TTTATTCGTTCTTATTAATTAATTAATTTGATCCGATGCTGG	rat 5-HT1A promoter
1888	GAAAGTGGACACAAACAAAGACAGTATTGGACTTGAACCTTGGACT	human 5-HT1A promoter
1713	G-TGTTGATAC--TCTTGGACAGGATTTCTTCTAGTGTCTGAATCTGCTCC	mouse 5-HT1A promoter
1793	GATGTTGATAC--TCTTGGACAGGATTTCTTCTAGTGTCTGAATCTGCTCC	rat 5-HT1A promoter
1938	GTATTAAATGGGAAACAACTCTTTTGCCTATGATTAACCTAAAGGTTTA	human 5-HT1A promoter
1759	G-ATCTTA--TTTAAATTAATTAATTAATTTGATCCGATGCTGG	mouse 5-HT1A promoter
1839	G-ATCTTA--TTTAAATTAATTAATTAATTTGATCCGATGCTGG	rat 5-HT1A promoter
1988	CTAAATTTGTAACCTTTTACAACTTATGAGTGAATTTTCTTGTGATGTTTC	human 5-HT1A promoter
1795	GTGAAATTTTAAACCTTTTACAACTTATGAGTGAATTTTCTTGTGATGTTTC	mouse 5-HT1A promoter
1876	GTGAAATTTTAAACCTTTTACAACTTATGAGTGAATTTTCTTGTGATGTTTC	rat 5-HT1A promoter

Figure I.7 – Sequence alignment of the 5' flanking region of the human, mouse and rat 5-HT1A receptor genes.

The nucleotide sequence of the proximal 2.037 Kb of the 5' flanking region of the human 5-HT1A receptor gene was determined using Sanger dideoxy sequencing techniques. The murine (NCBI, AC# U33820) and rat (NCBI, AC# AF087675) 5-HT1A receptor sequences are aligned below. Sequence identity is shaded in grey and ruler corresponds to human 5-HT1A receptor promoter numbering. DNA sequences begin with the initiator ATG codon and important regulatory regions are as indicated. The DREs and RE-1 elements are discussed in Chapter II. The NUDR and Hes5 binding sites around the C(-1019)G polymorphisms are described in Chapter IV.

cell-type specific differentiation, programmed cell death and oncogenic transformation, as an important regulator of serotonergic phenotype. Pet-1 expression is highly restricted to the entire rostro-caudal extent of rat serotonergic hindbrain raphe nuclei. This transcription factor is expressed shortly before 5-HT appears in the hindbrain and constitutes a marker of developing and adult 5-HT neurons. Conserved Pet-1 binding sites are present in or near the promoter region of several markers of serotonin neurons including the serotonin transporter, tryptophan hydroxylase, L-amino acid decarboxylase and the 5-HT_{1A} receptor gene. In the 5-HT_{1A} receptor gene, Pet-1 ETS domain interacts at the AGCAGGAAGTT enhancer sequence from -137 to -127 bp to activate gene transcription in serotonergic neurons (Hendricks et al., 1999). Interestingly, Pet-1 knockout mice display aggressiveness and an anxious behaviour comparable to that seen in 5-HT_{1A} deficient animals. The forebrain 5-HT_{1A} receptors are thought to mediate the anxiety phenotype in 5-HT_{1A} gene knockouts, since tissue-specific rescue of the 5-HT_{1A} receptor in these areas results in mice that display normal anxiety behaviour. Therefore, the anxiety of Pet-1 nulls is likely to result in part from a deficiency of serotonergic innervation in the forebrain (in PET-1 ^{-/-} mice (Hendricks et al., 2003)) or a lack of 5-HT_{1A} receptors in the forebrain (as in 5-HT_{1A} ^{-/-} mice).

Although the rat 5-HT_{1A} promoter shares high sequence homology (especially within the first kb of 5' flanking sequences) with the human promoter, differences in terms of gene regulation have been identified (Figure I.7). For instance, the TATA initiation site present in the rat 5-HT_{1A} promoter is not conserved in the human and mouse homologues (Storring et al., 1999). Nevertheless, DNA elements similar to the four MAZ and SP-1 sites (from -6 to -225 bp) that are involved in regulation of the human and mouse 5-HT_{1A}

receptor gene expression (Parks and Shenk, 1996), may also contribute to the promoter or enhancer activity of the rat gene. Mitogen-induced rat 5-HT_{1A} receptor expression in B and T lymphocytes involves transcriptional regulation by NF κ B (Abdouh et al., 2001), which also regulates the human gene in Chinese Hamster Ovary cells (Cowen et al., 1997). A few consensus NF κ B sites are present in the rat and human promoters, but the contribution of each to 5-HT_{1A} gene regulation still needs to be determined.

While the identification of specific regulatory elements in the rat 5-HT_{1A} gene provides important insights into the basic structure of the 5-HT_{1A} promoter, small differences in their sequences between species homologues gives rise to the possibility of species-specific regulation of the gene. It is therefore important to functionally identify analogous or novel gene regulatory elements in the human 5-HT_{1A} promoter for understanding the transcriptional regulation of this receptor in man and its possible aberrations in mental illness.

1.7 Rationale, goals and objectives

The delayed therapeutic effect of antidepressants suggests that long-term adaptive changes in the neuronal system, possibly at a genomic and 5-HT_{1A} levels, may be important to explain the mechanism of action of antidepressants. Furthermore, evidence suggests that genetic factors partially influence overall risk of illness and the sensitivity of individuals to the depression promoting effect of environmental adversity (Champoux et al., 2002; Caspi et al., 2003). The large variability in inter-individual pharmacological response pattern could be ascribed in part to heritable factors (Zanardi et al., 2001; Serretti et al., 2002; Mancama and Kerwin, 2003; Serretti et al., 2003). Since the 5-HT_{1A} receptor is a key

element in regulating serotonergic tone, a crucial determinant of mood and emotion, genetic variation in this receptor or its regulation could contribute to the genetics of psychiatric diseases and to individual differences in response to medication. Given the discernible drawbacks associated with SSRI use, knowledge of the biological factors that may predispose to MDD and predict outcome would be beneficial to improving existing levels of patient care.

My major hypothesis is that transcriptional regulation of the human 5-HT_{1A} receptor gene is implicated in predisposition to mental illnesses that involve abnormal regulation of the serotonin system, such as major depression. This thesis sought to examine the transcriptional regulation of the human 5-HT_{1A} receptor gene, and its implication in mood disorders, more specifically major depression and suicide. In Chapter 2, I have characterized the regulatory elements involved in basal 5-HT_{1A} gene expression in neuronal and non-neuronal cells. In Chapter 3, I present the cloning of one repressor (Freud-1) that is a critical regulator of basal 5-HT_{1A} receptor expression in neurons. In Chapter 4, I have identified a novel polymorphism in the 5-HT_{1A} gene that is associated with depression and completed suicide, and identified the regulatory proteins that regulate the gene and whose functions are affected by the polymorphism. Thus, the transcriptional activity of the 5-HT_{1A} receptor gene is altered by a promoter polymorphism, consistent with a crucial role for altered levels of expression of the 5-HT_{1A} autoreceptor in the etiology and possibly treatment response of anxiety and MDD.

CHAPTER II – CELL TYPE-DEPENDENT RECRUITMENT OF TRICHOSTATIN A-SENSITIVE REPRESSION OF THE HUMAN 5-HT1A RECEPTOR GENE

Sylvie Lemonde

Anastasia Rogaeva, Paul R. Albert

Authors contribution: Sylvie Lemonde conducted the research presented in this paper as well as preparation of the manuscript. Gal-4 constructions and related transfections were performed by Anastasia Rogaeva to generate Fig. II.9 of this manuscript. All work and manuscript editing was supervised by Paul R. Albert.

Abbreviated Title: Silencing of the human 5-HT1A receptor gene

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2.1 Abstract

Regulation of 5-HT_{1A} receptor expression in brain is implicated in mood disorders such as depression and anxiety. Transcriptional activity of the human 5-HT_{1A} receptor was strongly repressed by a negative regulatory region containing a consensus repressor element-1 (RE-1) and two copies of the dual repressor element (DRE) identified in the rat 5-HT_{1A} receptor gene (Ou et al., 2000). REST, a silencer of neuronal genes, bound the 5-HT_{1A} RE-1 and repressed the 5-HT_{1A} promoter. Inactivation of RE-1 completely abolished REST-mediated repression, but resulted in only partial (15-50%) de-repression of basal 5-HT_{1A} promoter activity. The human 5-HT_{1A} DRE sequences bound specifically to the novel repressor Freud-1 (Ou et al., 2003) and conferred repressor activity at 5-HT_{1A} or SV40 promoters. In 5-HT_{1A}-negative cells (L6, HEK 293), histone deacetylase (HDAC) inhibitor trichostatin A (TSA) abolished repression mediated by both RE-1/REST and DRE/Freud-1, and induced almost complete de-repression of the 5-HT_{1A} gene. By contrast, in 5-HT_{1A}-expressing neuronal cells (RN46A, SN-48) TSA blocked RE-1/REST repression, but did not affect DRE/Freud-1-mediated repression. Thus Freud-1 mediates HDAC-independent repression of the 5-HT_{1A} receptor promoter in neuronal 5-HT_{1A}-positive cells, while REST or DRE-binding proteins recruit HDAC-dependent mechanisms to silence the 5-HT_{1A} gene in non-neuronal 5-HT_{1A}-negative cells. These results suggest a new level of regulation for neuron-specific gene expression involving dependence on HDAC activation.

2.2 Introduction

Serotonin (5-HT) and 5-HT_{1A} receptors are implicated in the regulation of mood and in the etiology of various psychiatric disorders such as anxiety and depression (Blier et al., 1990; Murphy, 1990; Jacobs and Azmitia, 1992; Blier and de Montigny, 1999). Serotonin-containing neurons of the brain originate in the raphe nuclei and project throughout the brain to innervate the cortex, hippocampus, septum, and hypothalamus (Tork, 1990), areas that are enriched in 5-HT_{1A} receptors and implicated in the regulation of mood and emotion. The activity of 5-HT neurons is controlled in part by somatodendritic 5-HT_{1A} autoreceptors that inhibit their firing activity by opening potassium channels (Zgombick et al., 1989; Penington and Kelly, 1990; Penington et al., 1993). Recent studies suggest that increase in 5-HT_{1A} autoreceptor expression is associated with major depression (Mongeau et al., 1992; Blier and de Montigny, 1994; Albert et al., 1996; Stockmeier et al., 1998). Conversely, desensitization of the 5-HT_{1A} autoreceptor upon chronic (2-3 week) antidepressant treatment correlates with enhanced 5-HT neurotransmission and clinical improvement, suggesting that these drugs may down-regulate 5-HT_{1A} gene transcription. However, the regulatory mechanisms that determine 5-HT_{1A} receptor expression in raphe neurons remain incompletely understood (Blier et al., 1990; Albert et al., 1996).

We have previously investigated the mechanisms underlying basal regulation of the rat 5-HT_{1A} receptor (Storring et al., 1999; Ou et al., 2000) in 5-HT_{1A}-negative non-neuronal cells compared to 5-HT_{1A}-expressing RN46A and SN-48 neuronal cells. RN46A cells are derived from rat E13 raphe cells and express presynaptic serotonergic markers such as tryptophan hydroxylase, 5-HT reuptake transporters, and 5-HT_{1A} autoreceptors (White et al., 1994). SN-48 cells are derived from rat post-natal day 21 septum and express

neurofilament proteins, glutamic acid decarboxylase (GABAergic phenotype) and 5-HT1A receptors (Lee et al., 1991; Charest et al., 1993). In these cells, the rat 5-HT1A receptor gene is regulated by a proximal TATA-driven promoter and is strongly repressed by an upstream region containing a novel 31-bp dual repressor element (DRE). The DRE is composed of a 14-bp 5'-portion (FRE) that mediates repression in neuronal cells that express the 5-HT1A receptor, but is dispensable in 5-HT1A receptor-negative cells, where an additional 12-bp 3'-element maintains repression. We have recently cloned a novel helix-loop-helix transcriptional repressor, Freud-1 (Five-prime Repressor Element Under Dual repression binding protein-1) described in chapter III, that binds to the 14-bp FRE to repress the rat 5-HT1A promoter (Ou et al., 2003). Thus, repression induced by Freud-1-FRE assists in silencing the 5-HT1A gene in non-neuronal cells, but is a key determinant of the basal level of transcription in 5-HT1A-expressing cells.

Since 5-HT1A-expressing cell lines of human origin have yet to be reported, we have compared the transcriptional regulation of the human 5-HT1A gene in rodent and human cell lines to that known for the rat gene. In the human 5-HT1A receptor we localized an important cluster of *cis*-acting regulatory elements between -1624 and -1550 bp that silenced transcriptional activity. This region shares 81% nucleotide identity with the corresponding region of the rat 5-HT1A gene, and contains a consensus RE-1/NRSE element (Maue et al., 1990; Mori et al., 1990; Schoenherr et al., 1996) and two tandem DRE-like elements (Ou et al., 2000). Unlike the rat gene that is repressed primarily by the 3'-DRE, the human gene was repressed by both 5'- and 3'-DRE, and RE-1 sites. Consistent with the importance of these elements, both Freud-1 and REST repressed the

human 5-HT1A gene, but mediated differential silencing in non-neuronal vs. neuronal cell types.

2.3 Materials and Methods

2.3.1 Reporter Constructs

The human 5-HT1A receptor was cloned from a human genomic λ EMBL3 library by screening with the *DbaI* fragment of the rat 5-HT1A receptor gene (Albert et al., 1990). The DNA sequence of the clone corresponded to the 5-HT1A sequence (Acc. #AC008965). The luciferase plasmid -6061-luc was obtained by insertion of a *SalI/BssHII* fragment of the human 5-HT1A promoter into a *XhoI/MluI* site of a modified pGL3-Basic vector (Promega, Madison, WI) containing a repeated *KpnI/XhoI* cassette in the reverse orientation. From -6061-luc, all subsequent deletion constructs were generated. The -3446-luc, -1598-luc and -224-luc were constructed by digestion with *EcoRV*, *Van911* and *PvuII* respectively and vector site *SmaI* or *MluI*, followed by internal ligation. The -1128-luc was obtained by insertion of a *KpnI/BssHII* fragment into pGL3-Basic vector digested with *KpnI* and *MluI*. A *Bst1107I/HindIII* fragment and a *StuI/HindIII* fragment were subcloned into a *SmaI/HindIII* cut pGL3-Basic to generate -1790-luc and -2561-luc. Digestion with *HincII* and *SmaI* generated a fragment that was inserted into the *SmaI* site of pGL3-Basic to produce -723-luc construct. Similarly, the DNA segments obtained by digestion with *XbaI* and *NheI* or *NheI* alone were inserted into the *NheI* site of pGL3-Basic and were called -391-luc and -1517-luc respectively. PCR amplification of a proximal 164-bp fragment gave a product that was then digested with *HindIII* and subcloned into a *SmaI* and *HindIII* digested pGL3-Basic vector to generate -139-luc. Plasmids pGL3P(5'-DRE),

pGL3P(3'-DRE), pGL3P(5'+3'-DRE) and pGL3P(RE-1) were generated by annealing complementary oligonucleotides (Table II-1) flanked by *SacI* and *NheI* sites, and subcloning into *SacI/NheI*-cut pGL3-Promoter (Promega). The 200bp-SV40 construct was obtained by insertion of a 273-bp *StuI/NheI* (-1790/-1517) fragment into *NheI* site of PGL3P. Constructs of -1790-luc: Δ RE-1, Δ 3'-DRE, Δ 3'-DRE Δ RE-1 and Δ 5'+3'-DREs were generated by deletion mutagenesis using the QuikChangeTM XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the two-stage procedure described previously (Wang and Malcolm, 1999). The -1624-luc construct was made by insertion of the 5'-DRE sequence (Table II-1) to the -1598-luc construct using the same mutagenesis strategies. The RE-1 mutated constructs were also generated using the same procedure, and the sense oligonucleotide sequences used for mutations are indicated in Table II-1. The -1790 Δ 5'+3'-DREs construct was used to generate -1790 Δ 5'+3'-DREs mut RE-1 with RE-1 mut2 oligos and the 200bp-SV40 as well as the -2561 RE-1-mutated constructs were generated using the RE-1 mut1 primers. For Gal4 fusion constructs, Freud-1 (Ou et al., 2003) was subcloned into the *EcoRI* site of pBXG-1 (Bremner et al., 1995) (gift from Dr. R. Bremner, Univ. of Toronto) in frame with the Gal4 DNA binding domain (1-147aa). LexA-expressing vector (SLN3) was used as a positive control for repressor activity. Reporter construct X2G2P, containing two copies each of Gal4 and LexA DNA elements upstream of the SV40 promoter, was generated by digesting the X2G2P vector with *MluI/Sall* and ligating the insert into *MluI/XhoI*-cut pGL3-Promoter vector. All plasmids were purified by CsCl equilibrium gradient centrifugation, quantified spectrophotometrically, and verified by dideoxynucleotide DNA sequencing.

Name	Sequence
5'-DRE	5'-AGATGGCACTCTAAAACATTGCCAGA-3'
3'-DRE	5'-AGGTGGCGACATAAAACCTCATTGCTTAGAACT-3'
5'-DRE +3'-DRE	5'-AGATGGCACTCTAAAACATTGCCAGAAGGTGGCGACATAAAACCTCATTGCTTAG AACT-3'
5-HT1A RE-1	5'- <u>AGA</u> ACTGTCCCAGGTGCTGAA-3'
Rat NaChII RE-1	5'-TTCAG <u>A</u> ACCACGGACAGTGCC-3'
5-HT1A RE-1 mut1	3'-AATCTTGACA <u>CTGTGA</u> ACGACTTGGG-5'
5-HT1A RE-1 mut2	3'-CGTATTCCCAAAGGTTGACAT <u>TGT</u> CCACGACTTGGGTCAAAG-5'
Rat E2F	5'-CGGCATAAGCAAGCCCTTATTGCACAGAGCT-3'

Table II-1 – Oligonucleotide sequences for EMSA and mutagenesis.

Sequences of oligonucleotides for the rat E2F, Sodium channel type II (NaChII) RE-1 or human 5-HT1A receptor DNA elements used for reporter construction, mutagenesis and EMSA. Underlined nucleotides correspond to bases that deviate from the RE-1 consensus and bold nucleotides in the human 5-HT1A RE-1 mut sequences are those that have been mutated.

2.3.2 In vitro transcription/translation

Recombinant REST and Freud-1 proteins were synthesized using the TNT® Coupled Reticulocyte System (Promega) and 1 µg of human REST cDNA in pcDNA1-Amp plasmid ((Chong et al., 1995), gift of Dr. Gail Mandel, SUNY Stony Brook, NY) or Freud-1 in pcDNA3 (Ou et al., 2000), respectively.

2.3.3 Cell culture and transfection

Rat myoblast L6, rat raphe RN46A and human embryonic kidney HEK 293 cells were cultured and transfected as described previously (Ou et al., 2000). Mouse septal SN-48 and neuroblastoma-glioma NG108-15 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL) supplemented with 10% v/v heat-inactivated fetal bovine serum (FBS) at 37°C in 5% CO₂. Cells (except RN46A) were transfected by calcium-phosphate coprecipitation as described (Charest et al., 1993) with 20 µg of luciferase construct and 5 µg of pCMVβgal per 10-cm plate. For repressor assays, HEK 293 cells were transfected with 10 µg of luciferase expressing vector, indicated amounts of protein-expressing constructs, keeping the total DNA transfected at 25 µg using an empty vector and 0.1 µg of pCMVβgal to normalize transfection efficiency. RN46A cells grown in 3.5-cm Primaria 6-well plates were transfected using 1.5 µg/plate of luciferase plasmid, 0.5 µg of pCMVβgal/plate and 12 µg/plate pFx-7 (Invitrogen, San Diego, CA), as described (Ou et al., 2000). NG108-15 cells were differentiated with 10 µM forskolin, 100 µM IBMX for 6 hours prior to lysis. In some experiments, 200 nM TSA or vehicle (0.75% ethanol) was applied to the culture medium for 24 hours prior to lysis.

2.3.4 Luciferase and β -galactosidase assays

48 hours after transfection, cells were washed once with phosphate buffered saline (PBS) and lysed in 200 μ l of Reporter Lysis Buffer (Promega). After one freeze-thaw cycle, cellular debris was collected and supernatants assayed for luciferase and β -galactosidase activities as described previously (Ou et al., 2000). Activities were obtained from at least six independent experiments in which triplicate transfections were performed and corrected for transfection efficiency by establishing the ratio of Luciferase/ β -galactosidase activity, and normalizing to vector-transfected (pGL3-Basic) extracts. Data are presented as mean $\pm$ SD. Statistical significance was evaluated using two-tailed unpaired t-test and 95% confidence intervals with GraphPad Prism software (San Diego, CA).

2.3.5 Nuclear extract preparation

Cells (10^7) were washed once in PBS and once in buffer A (10 mM Hepes (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl). Cell pellet was re-suspended in buffer A containing 0.5 mM DTT, 0.1% Nonidet P-40 and incubated on ice for 10 min. After centrifugation (4', 500g, 4°C), the nuclear pellet was resuspended in 20 μ l buffer C (20 mM Hepes pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT) and incubated on ice for 10 min. Cells were centrifuged (3', 18,000g, 4°C) and the supernatant containing nuclear extracts was diluted with 75 μ l buffer D (20 mM Hepes pH 7.9, 20% glycerol, 0.05M KCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT). Tissues from adult Sprague Dawley rats were homogenized in buffer A containing 0.5 mM DTT,

0.5 mM PMSF, 0.5 mM benzamidine and 20 μ M leupeptin, and nuclear extracts were prepared as described above.

2.3.6 Electrophoretic mobility shift assay (EMSA)

Probes and respective competitors were made by annealing synthesized complementary oligonucleotides corresponding to the human 5-HT1A sequences: 5'-DRE, 3'-DRE, RE-1, or 22-bp element, or other sequences: rat E2F or rat type II sodium channel RE-1 (see Table II-1). Probes with 5'-overhangs were filled-in with [32 P]- α -dCTP (150 μ Ci/reaction) using 2.5 U of Klenow fragment DNA polymerase and purified over Sephadex G-50 column. Labeled probe (100,000 cpm/sample) was incubated with 25 μ g of nuclear protein extracts or 4 μ l of *in vitro* transcribed and translated protein in a 25 μ l reaction containing DNA binding buffer pH 7.9 (20 mM Hepes, 0.2 mM EDTA, 0.2 mM EGTA, 100 mM KCl, 5% glycerol) and 2 μ g poly [d(I-C)] (Sigma St.Louis, MO) for 20 min at room temperature. Samples were electrophoresed on a 5% acrylamide/tris-glycine gel at 4°C.

2.4 Results

2.4.1 Core repressor of the human 5-HT1A receptor gene

In order to analyze the transcriptional regulation of the human 5-HT1A receptor gene rodent and human cells were transfected with luciferase reporter constructs and compared to assess the utility of rodent cell lines (Fig. II.1). In all cell types a similar pattern of activity was observed, as reported for the 2.7-kb rat 5-HT1A gene (Storring et al., 1999; Ou et al., 2000). A basal promoter (up to -391 bp) containing multiple Sp1 and MAZ sites

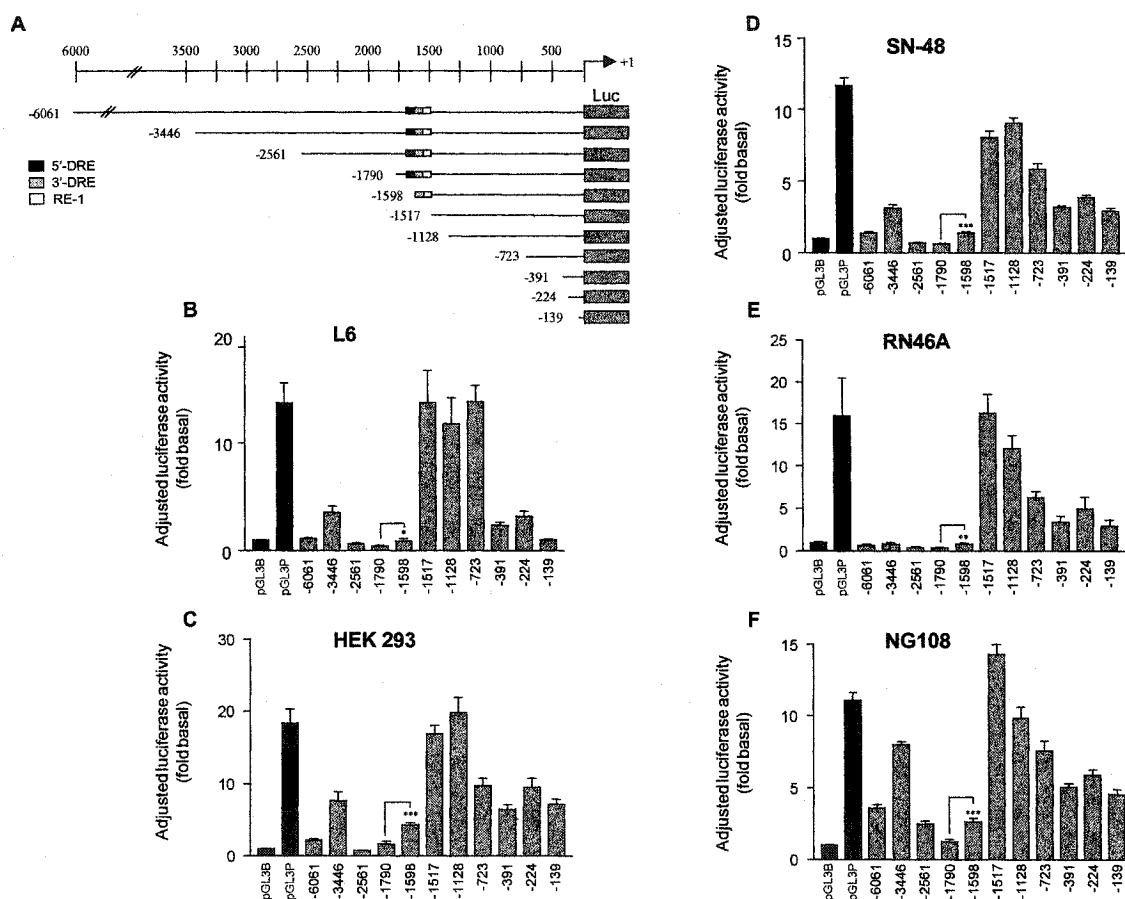


Figure II.1 – Deletion analysis of the human 5-HT1A receptor promoter.

A, A figurative representation of human 5-HT1A receptor gene reporter luciferase constructs is illustrated. Shown are the translational initiation site (solid arrow), 5'-DRE, 3'-DRE and RE-1 sites (boxes). Numbers indicate the distance (in bp) from the initial coding ATG. **B-F**, Transcriptional regulation of the human 5-HT1A receptor gene. Luciferase reporter constructs were transfected in 5-HT1A-expressing (SN-48, RN46A, NG108-15) or non-expressing (L6, HEK 293) cells. Luciferase activity of each reporter construct is normalized to that of basal activity of the promoter-less vector (pGL3B), with pGL3P as a positive control. Activities were obtained from at least eight separate experiments in which triplicate transfections were performed and corrected for transfection efficiency with a co-transfected pCMV β gal plasmid. Data are presented as mean $\pm$ SD. Significant ($*P < 0.05$, $**P < 0.005$, $***P < 0.0005$) derepression was observed in all cell lines when the fragment between -1790 and -1598 bp was deleted.

(Parks and Shenk, 1996) drove transcription in all cells. An enhancer region was located adjacent to the 5-HT1A promoter (-1517/-391 bp) and conferred two- to three-fold greater activity. The most salient feature was a strong repressor region in constructs greater than -1517 bp that completely silenced the 5-HT1A promoter, with most repressor activity residing between -1598/-1517 bp (Figs II.1B-F). Importantly, this repressor region between -1624/-1550 bp shares 81% identity with a corresponding region of the rat 5-HT1A promoter (Fig. II.2). Finally, significant but weak enhancer activity was detected upstream in the -3446 and -6061 human 5-HT1A reporter constructs. This pattern of activity for the human 5-HT1A gene strongly resembles that of the rat 5-HT1A gene suggesting common regulatory mechanisms that are shared in both human and rat cell lines.

The repressor region located between -1790/-1517 bp of the human 5-HT1A gene contains sequences homologous to the DRE (Dual Repressor Element) of the rat 5-HT1A gene (Ou et al., 2000) (Fig. II.2). Alignment of human, mouse and rat 5-HT1A genes revealed a pair of adjacent copies conforming to consensus 5'-DRE and 3'-DRE sequences (Table II-2). Although 5'- and 3'-DRE sequences were most highly homologous within each family, the two types of DRE shared 55-61% nucleotide identity to each other (Table II-2), suggesting that both DREs could be functionally active. Downstream from the DRE sites was a RE-1/NRSE (Repressor Element-1/Neuron Restrictive Silencer Element) sequence that differed from the consensus sequence by only five nucleotides (Fig. II.2, Table II-2), four of which are non-essential for REST/NRSF binding (Schoenherr et al., 1996), suggesting that the 5-HT1A RE-1 may be functional. Thus at least three distinct elements (5'-DRE, 3'-DRE and RE-1) were implicated in silencing the human 5-HT1A receptor gene.

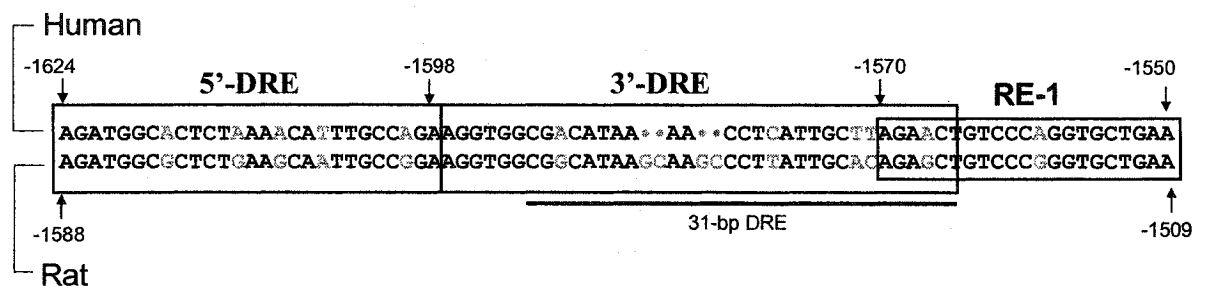


Figure II.2 – Sequence alignment of the repressor regions of the 5-HT1A receptor gene.

Shown are the alignments of the two consecutive repeats of DREs (5'-DRE and 3'-DRE) and RE-1 from human and rat 5-HT1A promoters. The repressor region between -1624 and -1550 of the human 5-HT1A gene shares 81% homology to the corresponding region (-1588 to -1509) of the rat sequence. Bold nucleotides represent sequences identity. The sequence corresponding to the previously characterized 31-bp DRE in the rat 5-HT1A gene is identified.

Name	Sequence
3' DREcon	 AGGTGGCGGCAT.AAGCAAGCCCTTATTGCACAGAGCT
3' rDRE	-----
3' mDRE	-----T-----C-A-----
3' hDRE	-----A--A--A-.....-C-----TT--A--
5' rDRE	--A-----CTC-G-----.....C-----.-G--...
5' mDRE	-AA-----CTC-G-----.....C-----.-G--...
5' hDRE	--A---ACTC-A--A-.....T-----.-A--...
5' DREcon	--A-----CTC-G-----.....C-----.-G--...
RE-1con	 TTCAGCACCACGGACAGCGCC
rNaChII	-----A-----
rSCG10	-----G-----
rSyn	-C-----GT-----A---
rm5-HT1A	-----CG-----T-T
h5-HT1A	-----TG-----TT-T

Table II-2 – Consensus sequences for DRE and RE-1 sites.

Shown are the alignments of 5-HT1A DRE and RE-1 sites to consensus sequences (con). The DRE sequences are from alignments of the rat (r), mouse (m), and human (h) 5-HT1A genes. Gaps (.), conserved (–) and non-conserved nucleotides are as indicated, and sites with 2 or more changes to the consensus sequence are flagged (*).

We examined the contribution of each putative repressor to transcriptional activity of the human 5-HT1A gene by deletion or mutation of RE-1 or deletion of 5'-DRE or 3'-DRE, or a combination of these in the -1790-luc construct containing the complete repressor region (Fig. II.3). The mutated form of the RE-1 element contains a GG to TT change at its center that has been reported to disrupt REST/NRSF binding (Mori et al., 1990). Deletion of the sequence 5' to the identified (RE-1, DRE) elements in the -1624-luc did not affect repression in any cell type, further localizing the repressor region to a 107-bp fragment between -1624 and -1517 bp. Conversely, deletion or inactivation of all three elements (bottom lane) completely de-repressed the 5-HT1A promoter to reach maximal activity of the -1517 construct in all cell lines. Deletion of individual elements induced a partial derepression (to 15-50% of maximal activity), with inactivation of RE-1 and 3'-DRE having the strongest effect in 5-HT1A-expressing SN-48 or RN46A cells. Deletion of two elements resulted in greater derepression (to >50% maximal activity) compared to constructs with single deletions. However, significant repression remained with only a single element (5'-DRE or RE-1) present, indicating that these elements confer repressor activity.

2.4.2 REST-mediated repression via the 5-HT1A RE-1

To address the role of REST/NRSF in RE-1-mediated repression of the human 5-HT1A receptor gene, we co-transfected REST or p73, an inactive form of REST (Tapia-Ramirez et al., 1997) with constructs containing an intact or mutated RE-1 (Fig. II.4). In RN46A cells, the 200-bp repressor region containing the RE-1 conferred strong repressor activity (to 35% of control) at the heterologous SV40 promoter, which was further repressed (to

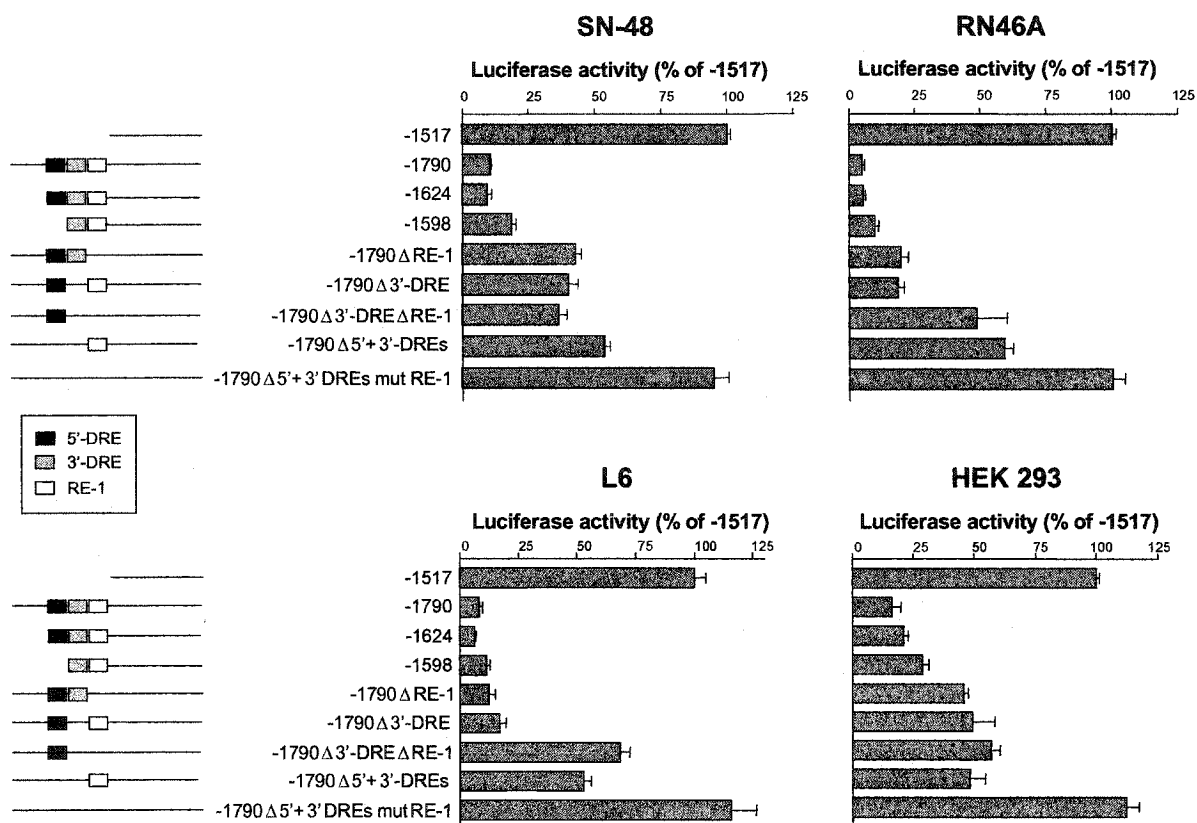


Figure II.3 – Deletion/mutation analysis of 5-HT1A repressor elements.

Deletion analysis localized the repressor region to a 107-bp fragment between -1624 and -1517 bp from the initiator ATG containing tandem DREs and a RE-1 (boxed). The transcriptional activity of the -1790 bp reporter construct with deletion (Δ) or mutation (mut) of either the 5'-DRE, 3'-DRE, RE-1, or combinations indicated were transfected in both neuronal 5-HT1A expressing (SN-48, RN46A) cells and non-neuronal (L6, HEK 293) cells. Deletion of all three repressors (-1790 Δ 5'+3'-DREs Δ RE-1) was required for full derepression. Luciferase activity is presented as % of -1517-luc construct (with highest promoter activity). Data were obtained from at least five separate experiments in which triplicate transfections were performed and normalized for transfection efficiency using pCMV β gal. Data are presented as mean $\pm$ SD.

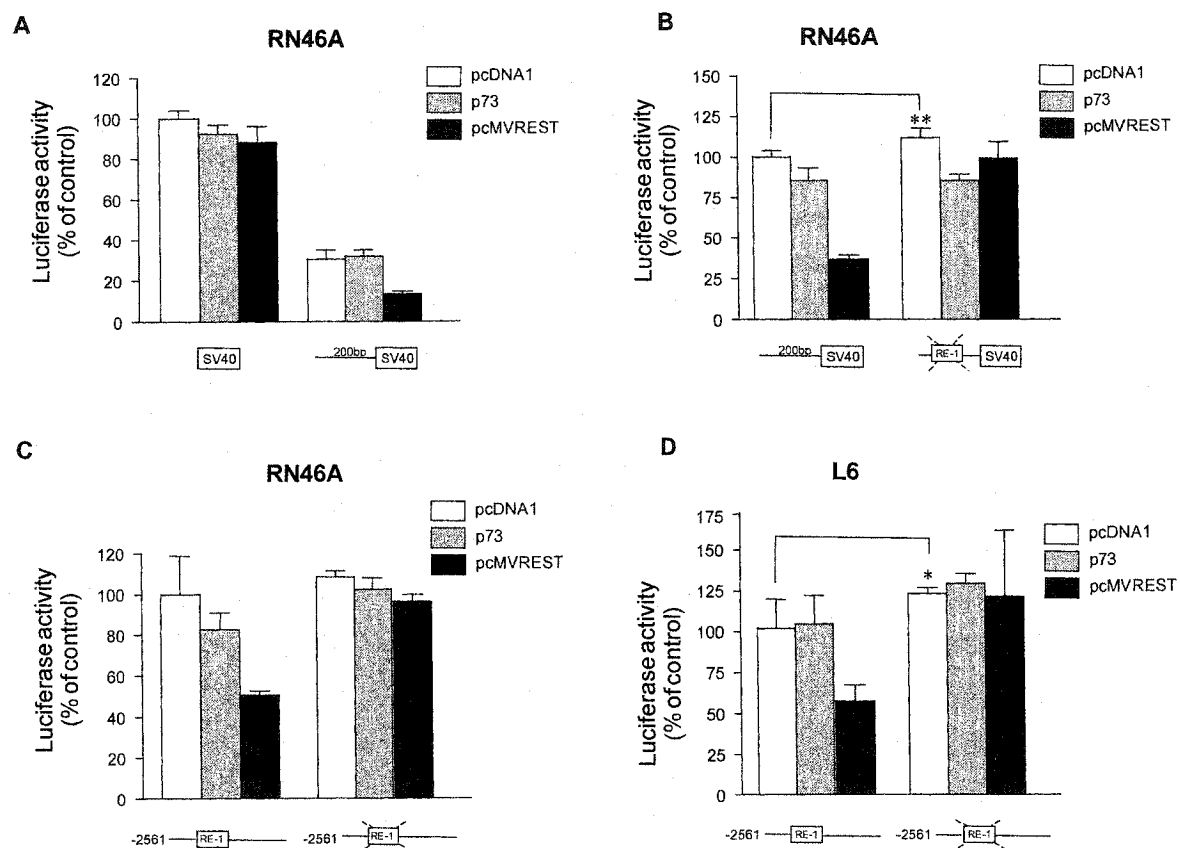


Figure II.4 – REST-mediated repression via the human 5-HT1A RE-1 site.

A 200-bp fragment containing the repressor region (between -1790 to -1517 bp) with an intact or mutated RE-1 element was subcloned upstream of the SV40 promoter (**A**, **B**) or examined in the human 5-HT1A promoter (**C**, **D**) and cotransfected with either pcDNA1, p73 (inactive REST) or pCMVREST in RN46A or L6 cells. **A**, REST-mediated repression. The repressor region mediated a 65% reduction in luciferase activity and REST further repressed reporter activity by 20%. **B**, Requirement of intact RE-1 site. Inactivation of the RE-1 significantly increased basal activity, and prevented REST-mediated repression. **C**, REST-mediated repression of 5-HT1A gene in RN46A cells. REST, but not p73, repressed the -2561 bp 5-HT1A reporter construct in neuronal RN46A and mutagenesis of the RE-1 site completely abolished this effect. **D**, REST-mediated repression in L6 myoblast cells. In 5-HT1A-negative non-neuronal L6 cells, REST also mediated repression and mutagenesis of the RE-1 site completely abolished REST-induced repression and induced significant derepression of basal activity, consistent with the presence of endogenous REST in those cell lines. Results are representative of five independent experiments in which triplicate transfections were performed and corrected for transfection efficiency using pCMV β gal. Data are presented as mean $\pm$ SD, * P < 0.05, ** P < 0.005.

15% of control) upon cotransfection with REST expression vector, while p73 had no effect (Fig. II.4A). No effect of REST or p73 was observed in the SV40 promoter vector control lacking RE-1 sites (pGL3P). Mutational inactivation of the RE-1 in the 200-bp 5-HT1A repressor completely abolished REST-mediated repression and significantly increased basal SV40 promoter activity, consistent with the presence of endogenous REST activity in these cells (Fig. II.4B). To directly test REST-mediated repression at the 5-HT1A promoter, the -2561-luc construct was examined (Figs II.4C, D). In both RN46A and L6 cells, cotransfection of REST mediated a 50% reduction in luciferase activity of this construct, which was blocked upon mutagenesis of RE-1 (Figs II.4C, D). The weak or non-significant tendency toward derepression of basal 5-HT1A promoter activity following RE-1 disruption again suggests a role for endogenous REST, but that most repression is maintained by other DNA elements (e.g., DRE). REST also repressed the -1598-luc 5-HT1A reporter construct, which was blocked by mutation of the RE-1, but had no activity at the -1517-luc construct lacking the RE-1 (data not shown). Thus, REST-mediated repression of the 5-HT1A gene requires interaction at the RE-1 located at -1570 to -1550 bp.

2.4.3 Binding of specific proteins to identified repressor elements of the 5-HT1A promoter

We initially identified protein-DNA interactions at the human 5-HT1A RE-1 by electrophoretic mobility shift assay (EMSA) using nuclear extracts from raphe RN46A and myoblast L6 cells (Fig. II.5A). A single major protein-DNA complex was detected, which was competed by a 20-fold molar excess of unlabelled RE-1 oligonucleotides, but not by unrelated E2F oligonucleotides, indicating a specific interaction. The 21-bp human

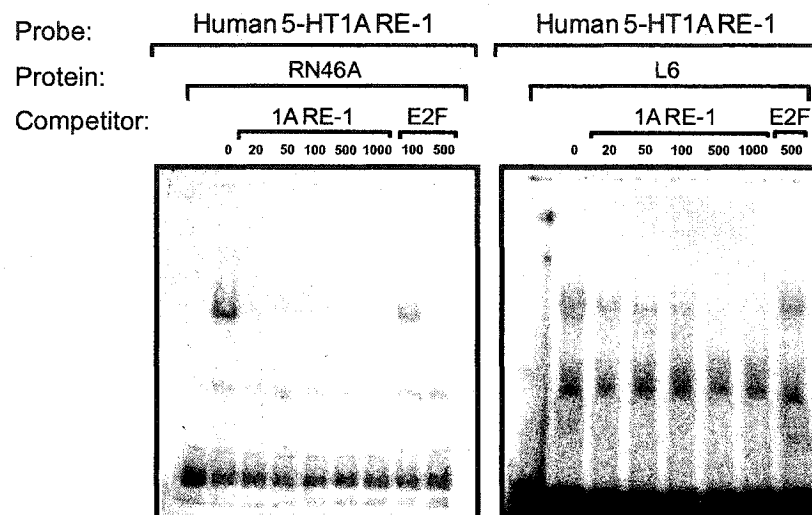
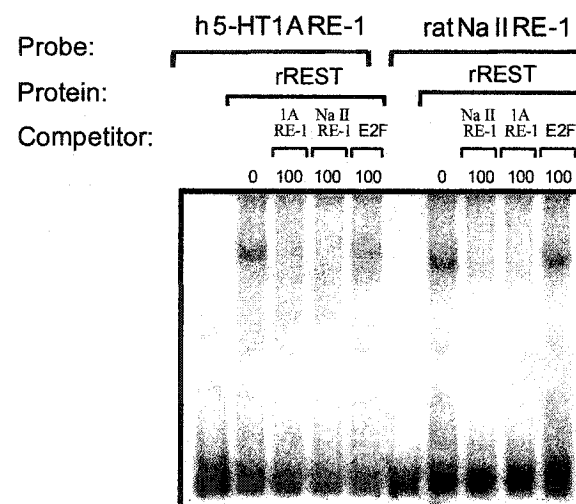
A**B**

Figure II.5 – REST interacts with the 5-HT1A RE-1 site.

A, Specific association of a nuclear protein complex with the human 5-HT1A RE-1 element. EMSAs were performed using nuclear extracts (25 µg) from L6 or RN46A cells. Unlabelled 5-HT1A, RE-1 or E2F primers were added at 20, 50, 100, 500 or 1000-fold molar excess to the incubation as indicated. **B**, REST specifically binds to the human 5-HT1A RE-1. EMSA was done using *in vitro* transcribed/translated REST. Labeled oligonucleotide probes were present as either the 21-bp 5-HT1A RE-1 or the 21-bp Na type II channel gene RE-1 site. Unlabelled 5-HT1A RE-1, Na II RE-1 or unrelated E2F were added at 100-fold molar excess to the incubation as indicated.

5-HT1A RE-1 was compared to the rat sodium channel type II gene RE-1 (Table II-1) (Kraner et al., 1992) for its ability to bind REST (Fig. II.5B). EMSA was performed using *in vitro*-synthesized REST (Chong et al., 1995). A specific REST/RE-1 complex of similar mobility was formed with both probes that was completely competed by cold 5-HT1A receptor or sodium type II channel genes RE-1 oligonucleotides, indicating that REST binds with equal efficiency to both elements. The presence in L6 and RN46A cells of a specific protein-DNA complex that recognizes 5-HT1A RE-1 is consistent with a role for REST in RE-1 mediated repression of the 5-HT1A receptor gene in these cells.

The presence of DRE-binding proteins in the brain and their specificity for human 5-HT1A 5'-DRE or 3'-DRE was assayed by EMSA. EMSA was performed using rat brain nuclear extracts of midbrain, hippocampus and cortex (Fig. II.6). One major protein complex associated specifically with the 5'-DRE, whereas three different protein/DNA complexes were formed with 3'-DRE, suggesting the presence of additional repressor complexes in brain tissue. We investigated whether Freud-1 (see chapter III), which was previously shown to interact with the rat DRE (Ou et al., 2000; Ou et al., 2003), also interacts with the human DRE elements in EMSA. *In vitro* transcribed and translated mouse Freud-1 bound specifically to both 5'-DRE and 3'-DRE (Fig. II.7). While the unrelated competitor E2F oligonucleotides failed to compete, a 100-fold molar excess of cold 5'-DRE and cold 3'-DRE were sufficient to compete for the 5'-DRE/Freud-1 and 3'-DRE/Freud-1 complexes, respectively. Thus, both 5'-DRE and 3'-DRE recognize Freud-1, while the 3'-DRE appears to interact with additional proteins from rat brain nuclei.

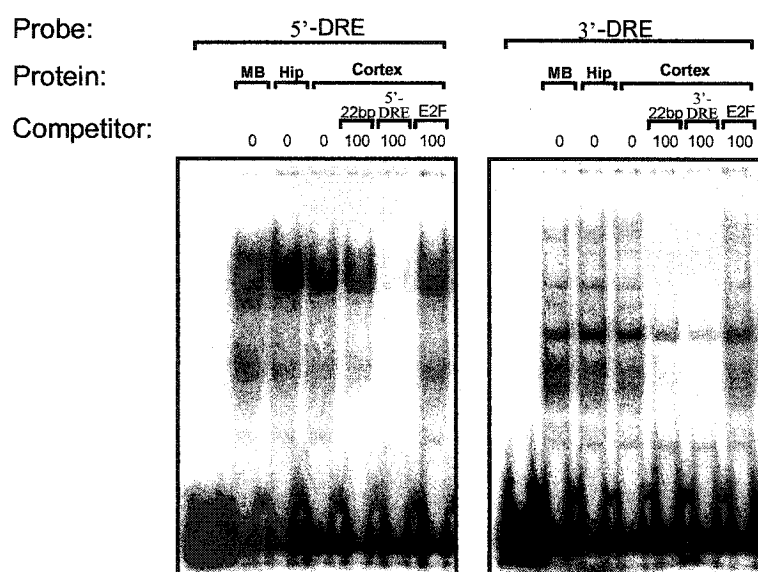


Figure II.6 – 5-HT_{1A}-DRE-binding proteins in nuclear extracts from brain.

EMSA was done using the 5'-DRE or the 3'-DRE as probes and nuclear extracts (25 µg) from rat brain tissues, including raphe/midbrain (MB), hippocampus (Hip) and frontal cortex. Competition with unlabelled oligonucleotides was performed as indicated using 100-fold molar excess of 22-bp element, 3'-DRE or unrelated E2F. One major protein complex was shown to specifically associate with 5'-DRE whereas at least three different protein/DNA complexes were detected with 3'-DRE.

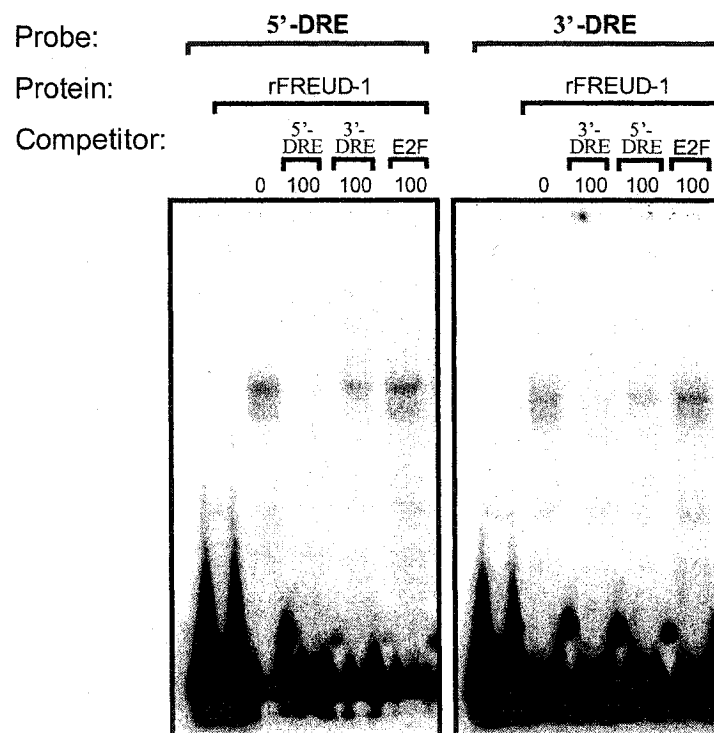


Figure II.7 – Freud-1 interacts with both 5-HT1A 5'- and 3'-DRE sites.

EMSA was performed using *in vitro* transcribed and translated Freud-1. Labeled oligonucleotide probes were present in all samples as either 5'-DRE or 3'-DRE. Unlabelled 5'-DRE, 3'-DRE or unrelated E2F were added at 100-fold molar excess to the incubation as indicated. Freud-1 bound specifically to both 5'-DRE and 3'-DRE.

2.4.4 RE-1, but not FRE-mediated repression is HDAC-dependent

Acetylation of core histones is closely linked to transcriptional activation (Grunstein, 1997; Kuo and Allis, 1998), and activation of HDAC is implicated in gene silencing by REST (Huang et al., 1999; Roopra et al., 2000; Abderrahmani et al., 2001; Ballas et al., 2001; You et al., 2001). The role of histone deacetylation in the activity of specific repressor elements was examined in cells transfected with SV40 promoter-luciferase constructs flanked by 5-HT1A DRE or RE-1 elements and treated for 24 hr with 200 nM TSA, a specific HDAC inhibitor (Fig. II.8). Without TSA treatment, the three elements (5'-DRE, 3'-DRE, RE-1) displayed similar activity to repress SV40 promoter activity, except in RN46A and L6 cells where RE-1 was the strongest and 5'-DRE the weakest as observed for 5-HT1A deletion constructs (Fig. II.3). However, in 5-HT1A-expressing cells (RN46A and SN-48), repressor activity diminished with both DREs when compared to the activity of the 3'-DRE alone, suggesting antagonism by the weaker 5'-DRE in these neuronal cells. Consistent with observations in other RE-1-containing genes, REST/RE-1-mediated repression at the SV40 promoter was nearly completely abolished upon TSA treatment indicating a requirement for HDAC activity.

Freud-1 binds to the 5' portion of DRE (FRE) and the Freud-1/FRE interaction is required for transcriptional repression in neuronal cells, but is dispensable in non-neuronal cells where an additional protein complex binds to the 3' portion of DRE (Ou et al., 2000). Unlike REST, FRE-mediated repression (mediated by Freud-1) was insensitive to TSA treatment in 5-HT1A-expressing RN46A cells, and weakly affected in SN-48 cells (Figs II.8 C, D). However, TSA induced almost complete derepression in 5-HT1A receptor-

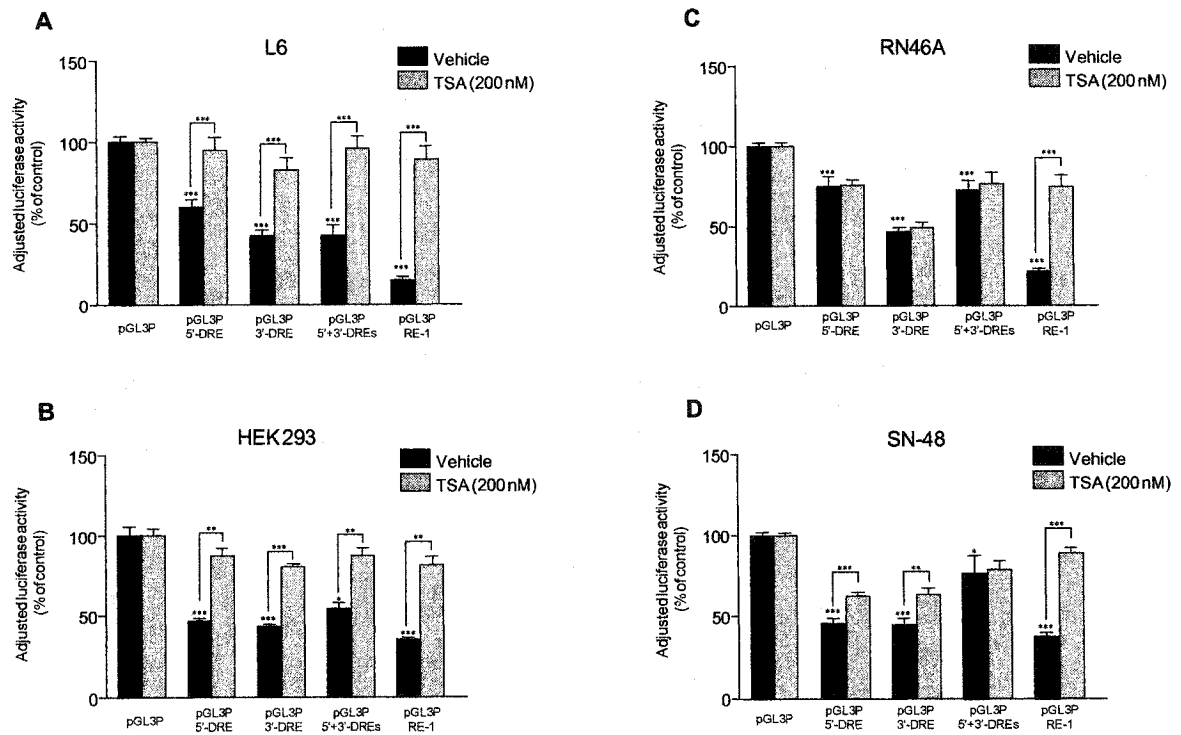


Figure II.8 – TSA blocks RE-1 but not DRE-mediated repression in RN46A cells.

Human 5-HT1A DRE (5'-DRE and 3'-DRE or both) or RE-1 sites were placed 5' to SV40 promoter-luciferase and tested for repressor activity in non-neuronal L6 or HEK 293 or 5-HT1A-expressing SN-48 and RN46A cells. **A-D**, Both 5' and 3'-DREs conferred strong and similar repressor activity at the SV40 promoter that was not further repressed by the combination of both DREs. In non-neuronal L6 (**A**) and HEK 293 (**B**) cells, treatment with HDAC inhibitor TSA almost completely relieved the inhibition mediated by RE-1, 5'-DRE, 3'-DRE or both DREs (when compared to vehicle treated cells). In contrast, neuronal RN46A (**C**) and SN-48 (**D**) cells showed repression of SV40 activity by RE-1 (REST-mediated) but not by DRE (Freud-mediated) in the presence of 200 nM TSA treatment suggesting that Freud-1 has a distinct repressor mechanism from REST in neurons. Data were obtained from at least ten separate experiments in which triplicate transfections were performed and corrected for transfection efficiency with a co-transfected pCMV β gal plasmid. Data are presented as mean $\pm$ SD (statistical significance * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$).

negative (L6, HEK 293) cells, where additional protein(s) mediate the dual repression mechanism (Fig. II.8 A, B). In the absence of repressor elements in the pGL3P vector, no significant effect of TSA on the SV40 promoter activity was observed indicating that TSA is specific to abrogate repressor activity. Together, these results suggest that Freud-1 mediates repression in neuronal cells through a HDAC-independent mechanism. By contrast, in non-neuronal cells another protein-DRE complex appears to mediate HDAC-dependent repression at the DRE.

To assess directly whether Freud-1-mediated repression is insensitive to TSA, the DNA binding domain (DBD) of Gal4 was fused to Freud-1 (Gal4-Freud-1) to recruit Freud-1 to Gal4 elements and the repressor activity of this construct was assayed (Fig. II.9). The Gal4-Freud-1 fusion construct or Gal4-DBD alone were cotransfected in HEK 293 cells with a luciferase reporter construct containing two copies of Gal4 and LexA DNA elements upstream of the SV40 promoter (X2G2P) and luciferase activity measured. In the presence of increasing amounts of Gal4-Freud-1, transcriptional activity was decreased up to 50% compared to Gal4-DBD vector, demonstrating the repressor activity of Freud-1. By contrast, Gal4-Freud-1 did not alter transcriptional activity of the pGL3P construct lacking the Gal4 sites (data not shown), consistent with the requirement of DNA binding for Freud-1-induced repression. For comparison, the prokaryotic repressor LexA (Brent and Ptashne, 1981) which also represses in mammalian Ltk- cells (Smith et al., 1988) mediated strong repressor activity at the LexA elements in this assay (Fig. II.9A). Treatment of the cells with HDAC inhibitor TSA did not alter the concentration-dependent repression induced by Gal4-Freud-1 compared to untreated cells. Together, these results demonstrate that Freud-1 has strong repressor activity that does not depend on HDAC

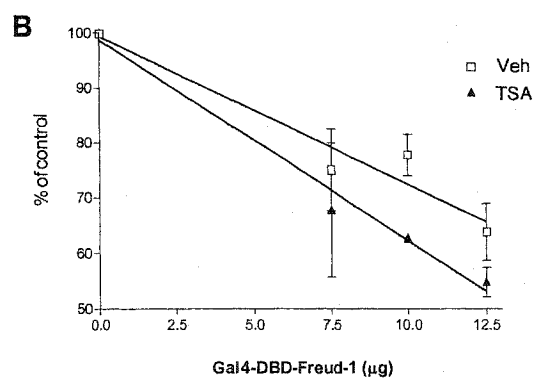
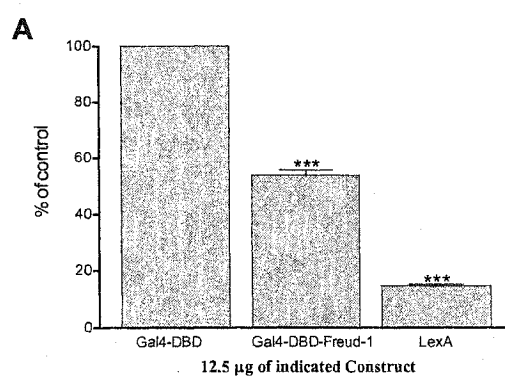
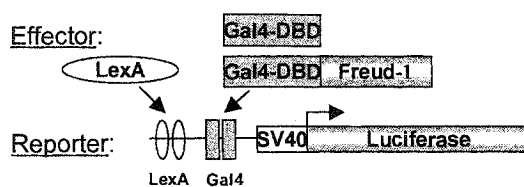


Figure II.9 – Freud-1-mediated repression is TSA insensitive.

The indicated amounts of Gal4-DBD (Gal vector), Gal4-Freud-1, or LexA (positive control) plasmids were cotransfected with the X2G2P reporter construct containing two LexA and Gal4 sites upstream of SV40 promoter-luciferase (shown above) in HEK 293 cells and luciferase activity measured in triplicate. Luciferase activity was corrected for transfection efficiency by cotransfection of pCMV β gal and normalized to the Gal vector (100%). **A**, Repressor activity of Gal4-Freud-1. Freud-1 reduced transcriptional activity to 50%, while LexA reduced activity to 20% of control. **B**, TSA-insensitive repression by Gal4-Freud-1. Gal4-Freud-1 transfected at the indicated amounts induced a concentration-dependent repression of X2G2P transcriptional activity which was the same in cells treated with vehicle (*Veh*) or 200 nM of TSA (*TSA*) for 24 hrs. Data was fit by linear regression analysis for vehicle (open squares) or TSA (closed triangles) and no significant difference was observed. Data represents average from 3 independent experiments and is presented as mean $\pm$ SEM (statistical significance *** $P < 0.001$).

recruitment or activation. Thus Freud-1-FRE dependent repression could mediate TSA-insensitive repression of 5-HT1A receptor gene in neuronal cells.

2.5 Discussion

2.5.1 Cell specific regulation of 5-HT1A genes

This study reveals a similar overall pattern of regulation of rat and human 5-HT1A promoters. Within the promoter region of the rat and human 5-HT1A genes, conserved MAZ and Sp-1 sites drive basal transcription (Parks and Shenk, 1996; Storrington et al., 1999), and a conserved PET-1 enhancer element (-137/-127 bp of human 5-HT1A) is thought to confer cell-specific regulation. PET-1, an ETS transcription factor that is enriched in serotonergic neurons (Hendricks et al., 1999) is required *in vivo* for development of most serotonergic cells in the brain, and activates a number of serotonergic genes including 5-HT transporters (Hendricks et al., 2003). Although cell lines may not completely recapitulate regulation *in vivo*, the limited specificity of the -224 bp and -139 bp-5-HT1A promoter constructs for 5-HT1A-positive cells suggests that additional regulatory elements must play a role to regulate its expression in serotonergic and non-serotonergic neurons. In rat and human 5-HT1A genes, positive regulatory elements were detected between -1517 and -391 bp and further upstream between -3500/-2500 bp of the 5-HT1A gene, suggesting that additional enhancers remain to be identified that may participate in directing neuron-specific expression *in vivo*.

Another similarity between human and rat 5-HT1A genes was the presence of a homologous strong repressor region located between -1600/-1500 bp that silences 5-HT1A receptor expression (Storrington et al., 1999; Ou et al., 2000). For both genes, this silencer is

especially strong in non-neuronal cells that do not express the receptor, but is also very active in neuronal cells, including raphe RN46A cells. However we identified some interesting differences between rat and human repressor regions. The present studies suggest that both 5'-DRE and RE-1 collaborate with 3'-DRE to silence the human 5-HT1A receptor gene, whereas the rat gene was primarily repressed by the 3'-DRE (Ou et al., 2000). Despite their sequence similarity, the human 3'-DRE bound additional complexes from brain extracts (Fig. II.6) and conferred greater repressor activity in neuronal cells (Fig. II.8) compared to the 5'-DRE. Thus in both rat and human genes, the 3'-DRE appears more important. The conserved duplication of DRE and its strong repressor activity in the cells examined suggests a predominant role in determining the basal level of 5-HT1A receptor expression *in vivo*.

The functionality of the 5-HT1A RE-1 indicates that 5-HT1A receptor is a neural-restricted gene that is silenced by REST. REST is a zinc finger transcription factor that recognizes RE-1 to silence the expression of a variety of neuronal genes in non-neuronal cells and tissues, contributing to their specific expression in neurons (Chong et al., 1995; Schoenherr and Anderson, 1995; Schoenherr et al., 1996; Chen et al., 1998). REST recruits co-repressors such as the Sin3A-HDAC1/2 complex (Huang et al., 1999; Naruse et al., 1999; Grimes et al., 2000; You et al., 2001) and CoREST-HDAC2 complex (Ballas et al., 2001) to mediate HDAC dependent repression of multiple neuronal genes. Similarly, REST-mediated repression of the 5-HT1A receptor gene in neuronal and non-neuronal cells was HDAC dependent. However, recently REST has been shown to recruit HDAC-independent mechanisms for genomic silencing by DNA methylation (Lunyak et al., 2002). Although classically thought to silence neural genes in non-neuronal cells, REST mRNA or

protein is detected in adult hippocampus, pons/medulla and midbrain and REST represses target genes in hippocampal CA1 cells (Palm et al., 1998; Calderone et al., 2003). Thus REST may in part determine the level of expression of 5-HT1A receptors in a subset of neurons, such as hippocampal CA1 pyramidal cells.

The repression of the human 5-HT1A receptor by tandem DRE elements serves to further regulate basal 5-HT1A expression in neuronal cells. The DRE is repressed by Freud-1 in neurons, and Freud-1 is co-expressed with 5-HT1A receptors in most 5-HT1A-containing brain regions including raphe nuclei, hippocampus and cortex (Ou et al., 2003). The present findings demonstrate that Freud-1 functions as a true repressor when tethered to a heterologous DNA element by GAL4-DBD. Unlike REST, Freud-1 recruits HDAC-independent mechanisms distinct from the HDAC dependent silencing of the 5-HT1A receptor observed in non-neuronal cells. The co-expression in raphe neurons of cell-specific enhancers (PET-1) and repressors (Freud-1) suggests that cell-specific regulation of 5-HT1A receptor expression will involve an interplay between both positive and negative transcriptional regulators.

2.5.2 HDAC dependence of 5-HT1A repression

The mechanism of Freud-1 action remains incompletely understood and could involve recruitment of corepressors. The C-terminal Binding Protein-1 (CtBP-1) functions as a co-repressor in both HDAC-dependent and -independent fashions (Chinnadurai, 2002). Since Freud-1 protein has at least one consensus CtBP-1 recognition sequence, CtBP may be recruited by Freud-1 to mediate HDAC-independent repression of 5-HT1A receptor transcription. HDAC-independent repression by CtBP may occur by interference with the

basal transcription machinery or recruitment of the polycomb complex of repressors (Chinnadurai, 2002). Corepressors such as CtBP or CoREST can recruit additional HDAC-independent mechanisms, such as DNA and histone methylation or chromatin remodelling complexes, for gene silencing (Battaglioli et al., 2002; Lunyak et al., 2002; Shi et al., 2003). Recently, CoREST was shown to mediate TSA-insensitive global silencing of several genes within a chromosomal segment by recruiting MeCP2 which binds to methylated CpG sequences and recruits histone methylase activity (Lunyak et al., 2002; Fuks et al., 2003). By analogy, Freud-1 may recruit co-repressors such as CtBP that can induce histone methylation to regulate chromatin remodelling.

The present studies indicate that one important aspect of cell-specific gene regulation is likely to include the cell-specific recruitment by repressors of different co-repressor complexes leading to TSA-sensitive or TSA-insensitive repression. This concept is exemplified by the TSA sensitivity of repression of the 5-HT1A receptor in non-neuronal vs. neuronal cell types. The importance of histone deacetylation in silencing activity of the 5-HT1A repressor complex in non-neuronal cells is consistent with the predominant role of REST and perhaps additional factors to silence neuronal genes in non-neuronal tissue. However, histone deacetylation does not play a major role in activity of the repressor in neuronal cells, consistent with the importance of Freud-1 to repress 5-HT1A expression in a TSA-insensitive manner. Thus, recruitment of distinct, cell-specific co-repressor complexes provides a tertiary level of regulation that may supercede primary (DNA sequence elements) and secondary (repressors) mechanisms to provide a highly refined determination of cell specificity.

2.5.3 Functional implications of 5-HT1A repression

Alterations in the expression of 5-HT1A receptors in the raphe and limbic system are associated with mental disorders such as major depression and anxiety. Mice lacking the 5-HT1A receptor display increased anxiety related behaviours (Heisler et al., 1998; Parks et al., 1998; Ramboz et al., 1998). Moreover, the basal levels of 5-HT1A receptors are critical during development to establish normal anxiety-like behaviour in the adult mouse (Gross et al., 2002). We have recently identified the repressor function of a C(-1019)G polymorphism in the human 5-HT1A promoter that is significantly associated with major depression and suicide (Lemondé et al., 2003). De-repression of the 5-HT1A receptor gene may contribute to elevated levels of the 5-HT1A autoreceptor observed in postmortem brains from depressed suicide victims (Stockmeier et al., 1998). These studies suggest that regulation of the basal level of 5-HT1A receptors by gene repression is an important determinant of 5-HT1A receptor function implicated in predisposition to mental disorders. Since Freud-1 is strongly co-expressed with 5-HT1A receptors in neuronal tissue, the regulation of Freud-1 by calcium-dependent (Ou et al., 2003) and transcriptional mechanisms could have a large impact to determine the level of 5-HT1A receptor expression and serotonergic activity in the brain.

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CHAPTER III – FREUD 1: A NEURONAL CALCIUM-REGULATED REPRESSOR OF THE 5-HT_{1A} RECEPTOR GENE

Xiao Ming Ou and Sylvie Lemonde

Hamed Jafar-Nejad, Christophe D. Bown, Aya Goto, Anastasia Rogaeva

Paul R. Albert

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Authors contribution: Xiao Ming Ou and Sylvie Lemonde are recognized as co-first authors on this paper and each contributed about 40% to the overall effort. All perfusions, preparation of frozen brain sections, *in situ* hybridization, immunohistochemical and immunocytochemical staining (including Ds-red experiments) and generation of the -1519 construct were performed by Sylvie Lemonde. The manuscript was written by Xiao Ming Ou and Sylvie Lemonde. Freud-1 protein expression and purification, plasmid constructions, luciferase/ β -galactosidase assays, EMSAs, Northern and Western blot analysis were carried out by Xiao Ming Ou. Yeast one-hybrid cloning of Freud-1 was performed by Hamed Jafar-Nejad. Colocalization of Freud-1 and tyrosine hydroxylase in the substantia nigra was demonstrated by Aya Goto. Rat hippocampal and cortical cultures were provided by Christopher D. Bown. All work and manuscript editing was supervised by Paul R. Albert.

Abbreviated Title: Freud-1: A calcium-regulated repressor of the 5-HT1A gene.

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3.1 Abstract

Altered regulation of 5-HT_{1A} receptors is implicated in mood disorders such as anxiety and major depression. To provide insight into its transcriptional regulation, we previously identified a novel DNA element (14-bp 5'-repressor element, FRE) of the 5-HT_{1A} receptor gene that mediates repression in neuronal and non-neuronal cells (Ou et al., 2000). We have now cloned a novel DNA binding protein (named Freud-1) that binds to FRE to mediate repression of the 5-HT_{1A} receptor or heterologous promoters. Freud-1 is evolutionarily conserved and contains two DM-14 basic repeats, a predicted helix-loop-helix DNA binding domain and a C2/CalB calcium/phospholipid binding domain. An intact CalB domain was required for Freud-1-mediated repression. In serotonergic raphe cells, over-expression of Freud-1 repressed the 5-HT_{1A} promoter and decreased 5-HT_{1A} receptor protein levels, while transfection of antisense to Freud-1 de-repressed the 5-HT_{1A} gene and increased 5-HT_{1A} receptor protein expression. Calcium-dependent signaling blocked Freud-1-FRE binding and de-repressed the 5-HT_{1A} promoter. Treatment with inhibitors of calmodulin (CAM) or CAM-dependent protein kinase (CAMK) reversed calcium-mediated inhibition of Freud-1. Freud-1 RNA and protein were present in raphe nuclei, hippocampus, cortex and hypothalamus, and Freud-1 protein was colocalized with 5-HT_{1A} receptors, suggesting its importance in regulating 5-HT_{1A} receptors *in vivo*. Thus, Freud-1 represents a novel calcium-regulated repressor that negatively regulates basal 5-HT_{1A} receptor expression in neurons and may play a role in the altered regulation of 5-HT_{1A} receptors associated with anxiety or major depression.

3.2 Introduction

The 5-HT_{1A} receptor is widely expressed at post-synaptic sites including cortex, hippocampus, limbic system and hypothalamus (Albert et al., 1990; Törk, 1990; Pompeiano et al., 1992) and is implicated in major depression, anxiety, and suicide (Mann, 1999; Pineyro and Blier, 1999; Veenstra-VanderWeele et al., 2000). Importantly the 5-HT_{1A} receptor functions as a somatodendritic autoreceptor (Riad et al., 2001) to inhibit the firing of serotonergic raphe neurons (Pineyro and Blier, 1999). 5-HT_{1A} receptor knockout mice display enhanced serotonergic neurotransmission due to the loss of presynaptic 5-HT_{1A} receptors (Ase et al., 2000) and have increased anxiety-related behaviours (Heisler et al., 1998; Parks et al., 1998; Ramboz et al., 1998). Down-regulation of 5-HT_{1A} autoreceptors is implicated in the clinical efficacy of chronic antidepressant treatments, which enhance serotonergic neurotransmission (Albert et al., 1996; Pineyro and Blier, 1999). Acute desensitization by receptor uncoupling (sec), internalization (sec-min) or degradation (hrs) occurs too rapidly to explain the 2-3 week time course for 5-HT_{1A} autoreceptor downregulation *in vivo* (Albert et al., 1996; Riad et al., 2001), suggesting a role for transcriptional silencing. Conversely 5-HT_{1A} autoreceptor levels are increased in the midbrain of suicide victims with major depression compared to control subjects (Stockmeier et al., 1998) suggesting that the level of basal expression of this receptor may predispose subjects to depression or suicide. Therefore, we have addressed the transcriptional mechanisms that regulate the basal expression of the 5-HT_{1A} receptor gene in raphe cells.

Transcriptional regulation is an important determinant of the basal neuron-specific expression of 5-HT_{1A} receptors (Parks and Shenk, 1996; Hendricks et al., 1999; Storrington

et al., 1999; Ou et al., 2000). We have used rat RN46A cells as a model of raphe neurons to examine the transcriptional regulation of the rat 5-HT1A receptor gene (Storring et al., 1999; Ou et al., 2000). RN46A cells are E13 rat raphe neurons that are transformed reversibly and retain essential characteristics of raphe neurons including expression of tryptophan hydroxylase, 5-HT transporters, and 5-HT1A receptors (Eaton et al., 1995; Storring et al., 1999). Analysis using 5-HT1A promoter-luciferase reporter constructs revealed that rat, mouse and human 5-HT1A receptor genes contain a conserved dual repressor element (31-bp DRE, -1555/-1524 bp) that conferred strong basal repression in non-neuronal and neuronal cells (Ou et al., 2000). Deletion or inactivation of the DRE resulted in 10-fold induction of basal 5-HT1A transcriptional activity. The 5-HT1A DRE is composed of two elements: a 14-bp 5'-repressor element (FRE) and an adjacent 12-bp 3'-repressor element (TRE). The FRE is active in neuronal cells that normally express the 5-HT1A receptor such as raphe RN46A or septal SN-48 cells, as well as in non-neuronal cells. The TRE is active only in 5-HT1A receptor-negative cells such as rat L6 myoblasts. Here, we identify a novel FRE-binding protein Freud-1 as the first transcriptional repressor for neuronal regulation of a serotonin system gene, the 5-HT1A receptor.

3.3 Materials and Methods

3.3.1 Yeast one-hybrid screening

The MATCHMAKER One-Hybrid system (Clontech, Palo Alto, CA) was used to screen for DRE-binding cDNA clones. A concatenated probe with three copies of 31-bp 5-HT1A DRE (-1555/-1524 bp) was subcloned into *EcoRI/XmaI*-digested pHISi and pLacZi vectors (Clontech). The constructs were linearized and stably integrated into the genome of

YM4271 and plated onto synthetic dropout -His-Ura plates to establish the dual-construct yeast strain YM4271-LZ3-HIS3. Subsequent transformation with a mouse brain MATCHMAKER cDNA library ($>1 \times 10^6$ clones) fused to GAL4-AD allowed for selection of resistant clones, which were tested by the β -galactosidase plate assay, retransformed for verification, and sequenced. 5' RACE was used to isolate full-length Freud-1 using the primer, 5'-GCACGAATGGCGTCTTGGTATTGCTTGACA-3'. 5' RACE was performed using the Mouse brain Marathon-ready cDNA and the Advantage 2 PCR Kit (Clontech). PCR products were gel-purified, subcloned into the pGEM-T Easy vector (Promega, Madison, WI) and sequenced using the ABI/PRISM automated system.

3.3.2 Plasmids

5-HT1A reporter constructs were derived from a 2.7-kb fragment of the rat 5-HT1A receptor promoter (-2719-luciferase), which has been described previously (Storring et al., 1999; Ou et al., 2000). Freud-1 expression plasmids were subcloned by digestion of pACT2 clones obtained from yeast one-hybrid screening with *Bgl*II and subsequent ligation with *Bam*HI-digested pcDNA3 (Invitrogen, Burlington, ON). The Freud-1 coding sequence was obtained by PCR and verified by DNA sequence analysis. Subsequently, the PCR product was subcloned into the pGEM-T Easy vector (Promega) prior to subcloning in the *Eco*R1 site of pET30A (Invitrogen) for protein expression and purification. A Freud-1 deletion mutant (Freud-1 del), lacking amino acids 341 to 348 within the CalB portion of the C2 domain, was made by site-specific mutagenesis of the Freud-1 sequence in pcDNA3, Flag-pcDNA3 or pET30A plasmids using QuikChangeTM XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The Flag epitope (DYKDDDK) was

incorporated at the N-terminus of Freud-1 or Freud-1 del and subcloned into the *EcoRI* site of pcDNA3.

3.3.3 Freud-1 protein expression and purification

Escherichia coli BL21 (DE3) cells were transformed with Freud-1 expression plasmid pET30A-Freud-1, grown overnight and induced with 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) at 37°C for 3-4 h. Cells were harvested and purified by TALON metal affinity resin (Clontech). Fractions containing Freud-1 were dialyzed against a buffer containing 20 mM HEPES; pH 7.9, 150 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 20% glycerol and stored frozen at -80°C.

3.3.4 Cell culture

Rat L6 myoblast and rat raphe RN46A cells were maintained and transfected as described previously (Ou et al., 2000). For Western blots of 5-HT_{1A} receptor, 75% confluent RN46A cells were differentiated at 39°C and the medium changed to 1:1 DMEM/Ham's F12 medium containing 1% (v/v) fetal bovine serum, 1 μ g/ml insulin, 1 μ g/ml transferrin, and 30 ng/ml nerve growth factor for 3 days (White et al., 1994). Differentiated RN46A cells were transiently transfected using *Superfect* (Qiagen, Mississauga, ON) for 72 h and cell extracts harvested. Transfection efficiency was determined in separate cultures by counting X-gal-stained cells upon transfection of pCMV- β Gal as described (Albert et al., 1999). Rat hippocampal and cortical cells were dissected from Sprague Dawley fetuses at 18 days of gestation (Banker and Cowan, 1977). Cells were dispersed, resuspended in NeurobasalTM media containing B27 supplement (Invitrogen), 0.5 mM L-glutamine, 1%

penicillin/streptomycin and 25 μ M glutamate (Brewer et al., 1993), plated on poly-D-lysine (MW 30,000-70,000) coated vessels at 37°C in 5% CO₂ for 13 days and then fixed in 4% paraformaldehyde. There was less than 0.2% GFAP-positive cells present suggesting that the cultures were 99.8% neuronal (Brewer et al., 1993).

3.3.5 Electrophoretic mobility shift assay (EMSA)

Nuclear proteins were extracted and EMSA performed as previously described (Ou et al., 2000). Nuclear extracts (20 μ g/sample) or purified Freud-1 (4 μ g/sample) were preincubated with or without competitor 5-HT1A DNA oligonucleotides, antibody, peptide, CaCl₂ (100 μ M) and/or ATP (150 μ M) or different protein kinase inhibitors (50 μ M) in a 30 μ l reaction containing DNA binding buffer (20 mM HEPES, 0.2 mM EDTA, 0.2 mM EGTA, 100 mM KCl, 5% glycerol and 2 mM DTT, pH 7.9), 2 mg of poly (dI-dC) at R.T. for 25 min. ³²P-labeled DRE or FRE probe (60,000 cpm/sample) was added and incubated for an additional 20 min. The locations, sizes and forward sequences of rat 5-HT1A receptor gene primers were: DRE (-1555/-1524 bp, 31-bp): 5'-CGGCATAAGCAAGCCCTTATTGCACAGAGCT-3'; FRE: (-1554/-1540 bp, 14-bp): 5'-GGCATAAGCAAGCC-3'; TRE (-1543/-1532 bp, 12-bp): 5'-GCCCTTATTGCA-3' (Ou et al., 2000). The mutation in the 2300m1 and DREmut/SV40 constructs changes the FRE sequence to: 5'-GGACATAGAGCACC-3'.

3.3.6 Luciferase and β -galactosidase assays

Cells were extracted with 150 μ l Reporter Lysis Buffer (Promega) 48 hr following transfection with promoter-luciferase, pCMV- β Gal and expression plasmids. The extracts

were centrifuged, collected and assayed for luciferase and β -galactosidase activities as described previously (Ou et al., 2000). The ratio of luciferase/ β -galactosidase activity was determined in triplicate samples, and normalized to vector-transfected (pGL3-basic) extracts. All data are presented as the mean $\pm$ SEM of at least three independent experiments.

3.3.7 Northern and Western blot analyses

For Northern blot analysis, tissues were dissected from Sprague Dawley rats (Gispen et al., 1972) and RNA isolated using "RNA Isolation Reagent" (Ambion, Austin, TX), electrophoresed, blotted, and probed using a full-length Freud-1 cDNA probe (Albert et al., 1990). For production of antibodies, the peptide KEALYRRNLVESELQR (Freud-1 amino acids 577-592) was synthesized as a multiple-antigenic peptide to immunize rabbits (Cedarlane, Hornby, ON). Freud-1 antiserum was purified by Affi-Gel blue 50-100 mesh (Bio-Rad Laboratories Inc., Hercules, CA) for albumin removal, followed by purification on peptide-conjugated Affi-Gel 15 (Bio-Rad). For Western blot, nuclear proteins (20 μ g) or purified Freud-1 (1 μ g) were separated by SDS-PAGE on a 10% polyacrylamide gel. Immunoblotting was performed as described previously (Ghahremani et al., 1999). Freud-1 antibody was used at a dilution of 1:1000 followed by a 1:2000 dilution of the secondary horseradish peroxidase (HRP)-linked rabbit antibody (Bio/Can Scientific, Mississauga, ON). Anti-Flag M2 mouse monoclonal antibody (Sigma, Oakville, ON) was used at 1:1000, and HRP-conjugated anti-mouse (Amresco, Santa Cruz) as the secondary antibody at a 1:8000 dilution. For detection of 5-HT_{1A} receptor, whole cell lysates (60- μ g) were electrophoresed on a 12% Tris Glycine polyacrylamide gel

(Invitrogen/Novex Electrophoresis, Carlsbad, CA), transferred onto a nitrocellulose membrane, incubated with Tris-buffered saline (TBS) and 5% milk for 1 hr, and overnight at 4 °C with primary rabbit polyclonal anti-5-HT1A antibody (Santa Cruz Biotechnology, Santa Cruz, CA; cat. no. sc 10801) at a 1:300 dilution. The blots were then washed three times and incubated with HRP-conjugated donkey anti-rabbit secondary antibody (Amersham, Arlington Heights, IL) at 1:3000. The reactive bands were visualized by chemiluminescence using the ECL-kit (Amersham) after exposure to Hyperfilm (Amersham). An antiserum to β -actin (Santa Cruz Biotechnology) was also tested on blots and served as a control for equal loading of samples.

3.3.8 Immunofluorescence

Adult male (250-300g) Sprague-Dawley rats were anaesthetized (Somnatol, 100 mg/kg, I.P.) perfused with saline, 4% paraformaldehyde and decapitated. Brains were removed and post-fixed overnight at 4°C, transferred to 10% sucrose/0.02% sodium azide and frozen. Tissues were sectioned (12 μ m), mounted on slides and kept at -80°C, or stored as floating sections at 4°C in PBS containing 0.02% sodium azide. Cultured cells were fixed in 4% paraformaldehyde for 1 hr at 37°C. Brain sections or cells were permeabilized and blocked in 1% BSA, 5% goat serum, and 0.05% Triton X-100 and incubated for 48 hr at 4°C with rabbit anti-Freud-1 primary antibody in combination with guinea pig anti-5-HT1A (Chemicon International Inc., Temecula, CA), guinea pig anti-5-HT (Dr. A.A. Verhogstad, Nijmegen, Sweden), or mouse anti-tyrosine hydroxylase (TH) (Chemicon) (1:100, 1:500 and 1:100 dilutions in blocking buffer, respectively). Immunostaining was detected using corresponding secondary antibodies, Texas Red-coupled anti-rabbit

(Calbiochem, San Diego, CA), anti-guinea pig FITC (Chemicon) and anti-mouse FITC (Sigma) (1:100, 1:500 and 1:100 dilutions respectively, in 1% BSA, 1% goat serum, 0.05% Triton X-100 for 1hr at R.T.). RN46A cells transfected with DsRed (pDsRed2-C1, Clontech) were fixed, stained for 5-HT1A receptor (as above) and the Red Fluorescent Protein was detected under the red channel by fluorescence microscopy. Immunofluorescence was visualized under a Zeiss Axioscop 2 microscope and images were captured using a Sony Hyper Had color camera and Northern Eclipse software (Emphix imaging).

3.3.9 In situ hybridization

Sense (5'-CAGCGGAAGGCACGCATGCATGAACGAATTGTCAAGCAAT-3') or antisense (5'-ATTGCTTGACAATTCGTTTCATGCATGCGTGCCTTCCGCTG-3') Freud-1 oligonucleotides (1 µg/rtn) were labeled using 30 U terminal transferase, 5 nmol digoxigenin-11-dUTP in tailing buffer/CoCl₂ (Roche, Laval, Qc) in 50 µl for 90 min at 37°C, and stored at -20°C. Slides were heated (45°C, 10 min), permeabilized in 5 µg/ml proteinase K/0.1M Tris-HCl, pH 7.5 (37°C, 4 min), rinsed and hybridized overnight at R.T. with 75 µl of hybridization buffer/slide (500 pg/µl of sense or antisense oligonucleotide in 50% deionised formamide, 6X SSC, 50mM Tris-HCl pH 7.0, 2X Denhardt's solution, 0.2% SDS). Sections were washed in 2X and 0.5X SSC (2 x 10 min, R.T.), and incubated with anti-digoxigenin-AP (Roche) (1:500, 2 hr, R.T.). The alkaline phosphatase reaction was developed in NBT/BCIP (10mg/5mg, Boehringer-Ingelheim, Laval, QC) and 8 mg levamisole (15 hr, R.T.) in 30 ml of 100 mM Tris-HCl (pH 9.5)/NaCl/MgCl₂. After

washing, sections were dehydrated in a series of ethanol washes, mounted, and images captured as described above.

3.3.10 Statistical Analysis

Significance of values compared to control was set at $p < 0.05$ as determined by t-test using Prism 3.0 (GraphPad Software, San Diego, CA).

3.4 Results

3.4.1 Molecular cloning of Freud-1

To identify regulatory proteins that target the 5-HT_{1A} DRE, three copies of the DRE sequence were placed upstream of LacZ or various selectable markers to screen a mouse brain cDNA library for DRE-binding proteins by the yeast one-hybrid approach. Three different positive clones were identified, two of which encoded a novel 595-aa protein (NP_666082; predicted molecular weight-66.5 kDa) that we named Freud-1 (Five-prime Repressor Element Under Dual repression binding protein-1). By Genbank search, human (BAA91194), *Drosophila melanogaster* (NP_609488), *Anopheles gambiae* (EEA09979) and *C. elegans* (NP_493412) homologues were identified with 85%, 33%, 31% or 28% amino acid identity to the murine Freud-1, respectively. As shown in Figure III.1A, Freud-1 contains a number of known protein domains, including two DM-14 domains (*boxed*) and a PKC conserved region 2 (C2 domain) (*dashed box*). The DM-14 domain is a conserved 60-a.a. repeat sequence of unknown function that is specific to Freud-1-species homologues (Fig. III.1B). The C2 domain (via its central 43-aa CalB domain) mediates Ca²⁺-dependent lipid binding of PKC, phospholipases and synaptotagmin and protein-

A

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1  MERYHVAAAQAKAKGDQRKARMHERIVKQY
31 QDAIRAHKAGRAVDVAELPVPPGFPPMQGL
61 ESAEPSQQSLVGVLETAMRLANHDEGSDDDE
91 EEEI PKK HNTPAASTTQLKSSPSKAPPSGP
121 APAGKAAPKGT SN RA QQQ LAFLEGRKKQLL
151 QAALRAKQKNDVEGAKMHLRQAKGLEPMLE
181 ASRNGLPVDIAKVPPAPV NKDDFVLVQRP G
211 PGLSQEAVRRYGELTKLLRQQHEMCLNHST
241 QFTHLGNIAE I K FEKLAEDCKRSM DI LK Q
271 AFARSLPTPAARFEQRTFSVIKVFPDLSNS
301 DMLLFIVKGINLPTPTGLSPSDLDAFVRFD
331 FPYPNVEEAQKDKTSVIKNTDSPEFKEQFK
361 LGINRGHRGFRRAIQTKGIKFEVVHKGGLF
391 K I Q RV LG T AQLKLG TLETACEVHEILEVLD
421 G R R P T G GRLEV MVRI REPLTAQQLETT I E R
451 WLVIDHIPAAMP T VTGPKAKAPLI PAS S RE
481 AGNRS A RP LHSLSVLA F DQERLERKILALR
511 QAARR S VPPEVAQQYQDVVQRSQWQRAQLEQ
541 GGAALRREYASHLERQLHFYTEAARRLGYD
571 GSREAAKEALYRRNLVESELQRLRR

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B

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          AA  AK  D  AR
LAX LXX RX EQYX QAA LQA KX KGD X EKA RMH
          10      20      30
1  LALLQQRCEQYKQAALQA KRA GDEEKA RRI
139 LAFLEGRKKQLLQAALRAKQKNDVEGAKMI
1  MERYHVAAAQAKAKGDQRKARMH
          R  K  A  G  VD  P  P
LRI AKQXX DAI X AX RAG RPV DX AEL PPP P
          40      50
31 LRI AKQFD DAI KAARAGRPV DLS ELP PPP
160 LRI QAKGLE PMLEASRNGLPVDIAKVPPAP
24 ERI VKQYQDAIRAHKAGRAVDVAELPVPP

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Consensus s
Majority

DM14-CONSUS:
Freud-DM14-2
Freud-DM14-1

Consensus s
Majority

DM14-CONSUS:
Freud-DM14-2
Freud-DM14-1

Figure III.1 – Protein structure of Freud-1.

A, Predicted structural motifs in Freud-1 are shown. Indicated are consensus sequences for DM-14 (boxed) and C2 domains (dashed box), helix-loop-helix DNA binding domain (underlined), cAMP-phosphorylation site (bold and outlined), PKC phosphorylation sites (italic and underlined). *B*, Alignment of Freud-1 DM-14 sequence with consensus sequence.

protein interactions (Clark et al., 1991). The C2 domain is present in all Freud-1 species homologues, suggesting a conserved calcium-dependent regulation of Freud-1. Freud-1 also contains a putative helix-loop-helix (HLH) DNA binding domain (HELIXTURNHELIX, <http://bioinfosoftware.phls.org.uk/pise/helixturnhelix.html>) that could mediate its DNA binding function (Fig. III.1A). In addition, a consensus PKA/CAMK-phosphorylation site and nine putative PKC phosphorylation sites were identified (ProSearch; Fig. III.1A). Although a consensus nuclear localization signal was not identified, Freud-1 localization is predicted to be nuclear (ProtComp program) consistent with a transcriptional regulatory function.

3.4.2 Freud-1 binding to 5-HT1A FRE

We examined the DNA binding specificity of purified recombinant His-tagged Freud-1 protein to double-stranded primers of 5-HT1A repressor elements by EMSA (Fig. III.2). A single specific species (*band 1*) was observed that was competed by unlabeled 5-HT1A-FRE or -DRE oligonucleotides (Fig. III.2A, *lanes 3 and 5*), indicating that Freud-1 binds specifically to FRE. Incubation of purified Freud-1 with an antibody against its N-terminal supershifted the protein-DRE complex, *band 2* (Fig. III.2A, *lane 4*) and this supershifted complex was partially disrupted by Freud-1 peptide antigen leading to reappearance of the original complex, *band 1* (Fig. III.2A, *lane 6*). Purified Freud-1 also bound as a single complex to labeled FRE that was competed by unlabeled FRE oligonucleotides, but not by TRE primers (Fig. III.2B). To address the role of the C2 domain in Freud-1, a deletion mutant (Freud-1 del) lacking amino acids 341-348 within the CalB domain was examined. Mutation of the C2 domain attenuated but did not abolish Freud-1 DNA binding activity,

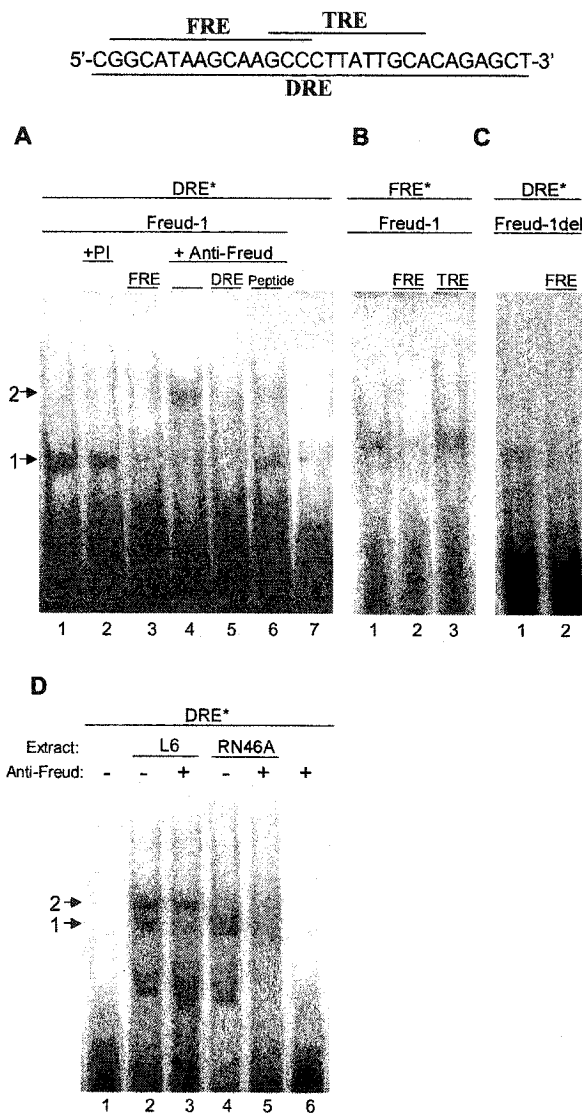


Figure III.2 – Specific binding of Freud-1 to 5-HT1A-FRE requires an intact CalB motif.

The sequences of oligonucleotides for DRE (31-bp), FRE (14-bp) and TRE (12-bp) are indicated.

A, Recombinant Freud-1-DRE binding was assessed by EMSA using 5-HT1A-DRE (31-bp) as a probe incubated with 4 µg of purified Freud-1 (*lanes 1-6*) or without (*lane 7*) as indicated. A single specific species (*band 1*) was retarded in the presence of 10 µg of anti-Freud-1 antibody (*band 2, lane 4*). The retarded complex was blocked by co-incubation with 4 µg of peptide antigen (*lane 6*). Double-stranded unlabelled competitor oligonucleotides (at 100-fold molar excess) were included as indicated. FRE was sufficient to compete for Freud-1-DRE complex.

B, Recombinant Freud-1-FRE binding: 4 µg of purified Freud-1 interacted specifically with FRE (*band 1, lane 1*). Freud-1-FRE interaction was abolished by pre-incubation with 100-fold unlabelled FRE (*lane 2*) but not by TRE (*lane 3*).

C, Recombinant mutated Freud-1-DRE binding: EMSA using DRE as a probe incubated with 8 µg of purified Freud-1 with a disrupted CalB domain (Freud-1 del) alone (*lane 1*) or with unlabelled 14-bp FRE (*lane 2*) as indicated. A disrupted CalB motif reduced affinity of Freud-1 for the DRE.

D, Presence of Freud-1 in nuclear extracts of L6 and RN46A cells. EMSA using the 31-bp DRE as a probe incubated without (*lane 1*) or with nuclear extracts from L6 (*lanes 2, 3*) or RN46A cells (*lanes 4, 5*) as indicated. The higher mobility complex (*band 1*) was displaced, rather than retarded in the presence of anti-Freud-1 antibody in extracts from both cell lines (*lanes 3 and 5, respectively*). The second protein/DRE complex present in L6 cells (*band 2*) was not affected by Freud-1 antibody (*lane 3*) and represents an additional unknown repressor.

suggesting a role for this domain in Freud-1-DNA interaction (Fig. III.2C). Thus recombinant Freud-1 binds specifically to the FRE of the 5-HT1A receptor gene.

To determine whether Freud-1 is present in cells and can bind to the 5-HT1A DRE, EMSA was done using nuclear extracts (Fig. III.2D). As we previously reported (Ou et al., 2000), in 5-HT1A-expressing raphe RN46A nuclear extracts only one protein-DRE complex was observed (band 1, lane 4), while in 5-HT1A receptor-negative L6 myoblasts two complexes were present (lane 2). Nuclear extracts were pre-incubated with anti-Freud-1 antibody prior to EMSA to assess the presence of Freud-1 in protein-DRE complexes. Addition of anti-Freud-1 specifically displaced the lower FRE-specific complex (*band 1*) in both RN46A and L6 cells but did not affect the upper protein-TRE complex (*band 2*) present in L6 cells (Fig. III.2D, lane 3). Previous studies showed that in 5-HT1A-negative cells such as L6 myoblasts, this second protein-DRE complex maintains repression of the 5-HT1A receptor gene upon mutation of the FRE (Ou et al., 2000). Antibody alone did not bind the DRE probe (Fig. III.2D, lane 6). In contrast to the supershift of recombinant Freud-1 by anti-Freud-1 (Fig. III.2A), incubation with anti-Freud-1 inhibited DRE binding by the protein complex from nuclear extracts (Fig. III.2D, lanes 3 and 5), suggesting that anti-Freud-1 destabilizes the Freud-1 complex present in cell nuclei. These results indicate that Freud-1 is present in nuclear extracts and exhibits sequence-specific DNA binding to the 31-bp DRE.

3.4.3 Freud-1 repression of raphe 5-HT1A receptor expression

To test whether Freud-1 represses transcriptional activity of the rat 5-HT1A receptor gene, the Freud-1 expression plasmid was cotransfected with the DRE-containing -2300 or -1590

5-HT1A promoter-luciferase reporter constructs in RN46A and L6 cells (Fig. III.3). In both cell types, Freud-1 decreased the transcriptional activity of the -2300 5-HT1A construct, but the effect was greater in the 5-HT1A-expressing raphe RN46A cells (Fig. III.3A). Transrepression of the 5-HT1A promoter by Freud-1 was abolished by disruption of the CalB domain in the Freud-1 del mutant, although this mutant could still bind weakly to the DRE (Fig. III.2C). Importantly, both Freud-1 and Freud-1 del were expressed at similar levels in L6 and RN46A cells as detected by Western blot (Fig. III.3A, blot). Thus Freud-1 represses the 5-HT1A receptor gene, especially in raphe RN46A cells, and the CalB domain is required for this repression.

The importance of an intact FRE for Freud-1-induced repression was examined using the -2300m1 and DREmut/SV40 constructs containing mutations that specifically inactivate the FRE, while the downstream TRE remains intact (Ou et al., 2000). In contrast to the -2300 construct, transfection of Freud-1 did not affect the luciferase activity of the -2300m1 construct in either L6 or RN46A cells (Fig. III.3B). The DRE/SV40 construct contains the 5-HT1A DRE upstream of a heterologous promoter (SV40) and mediates repression in RN46A cells (Ou et al., 2000). Cotransfection of Freud-1 induced a further 50% repression of this construct (Fig. III.3C, DRE/SV40), but Freud-1 had no effect when the FRE was inactivated (DREmut/SV40). These results demonstrate that Freud-1 requires an intact FRE to mediate repression of the 5-HT1A or heterologous promoters.

To further study its importance in transcriptional regulation of the 5-HT1A receptor gene, Freud-1 protein expression was modulated using sense and antisense Freud-1 cDNA constructs (Fig. III.3D-G). Upon cotransfection with sense Freud-1, transcriptional activity of the DRE-containing -1590 5-HT1A promoter-luciferase construct was significantly

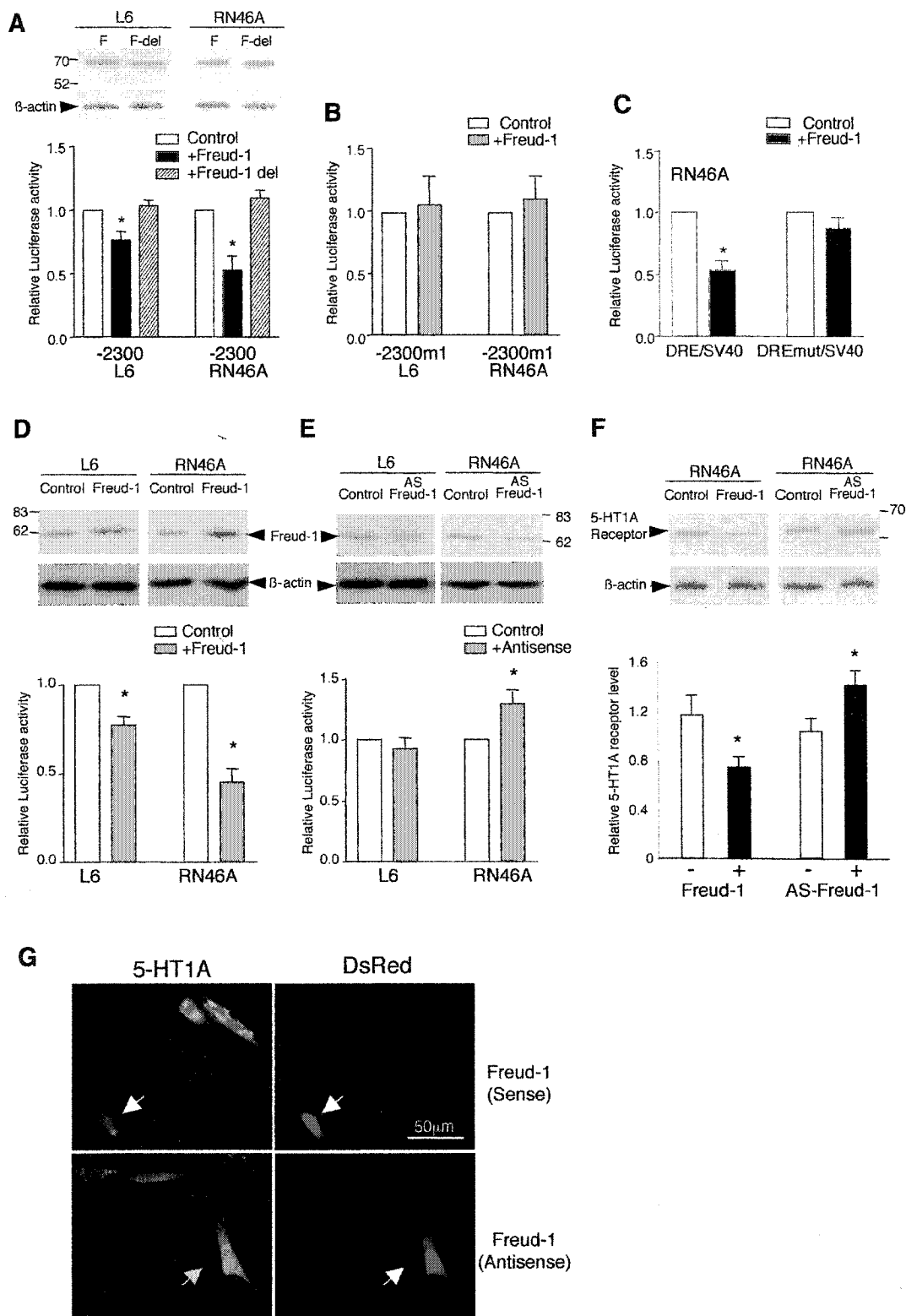


Figure III.3 – Freud-1 represses basal 5-HT1A receptor expression in raphe RN46A cells via FRE.

Luciferase activity was normalized to that of β -galactosidase and is expressed as relative light units and normalized to Control. * $p < 0.05$ compared to control by t-test.

A, CalB-dependent Freud-1 repression. The FRE-containing -2300 5-HT1A-luciferase was transiently co-transfected in L6 myoblast or raphe RN46A cells with vector (pcDNA3, Control), Freud-1 or Freud-1 del mutant of the C2 (CalB) domain. Above: A Western blot of 30 μ g of nuclear extracts was probed with anti-Flag to show equal expression of Flag-Freud-1 or Flag-Freud-1 del in these experiments.

B, FRE-dependence of Freud-1 repression. Transfections as above were done with FRE-inactivated mutant 2300m1, which was insensitive to Freud-1.

C, FRE-dependent repression of SV40 promoter by Freud-1. The 31-bp DRE (DRE/SV40) or the FRE-mutant DRE (DREmut/SV40) were placed upstream of the SV40 promoter and were co-transfected with vector (pcDNA3) or Freud-1 expression construct.

D, E, Freud-1 inhibits transcriptional activity of the 5-HT1A receptor gene. L6 myoblast or raphe RN46A cells were transiently co-transfected with the DRE-containing (-1590luc) 5-HT1A-luciferase reporter or vector (pcDNA3, Control), and sense (*D*) or antisense (*E*) Freud-1 expression vectors. Freud-1 protein expression in each cell line after transfection was detected by Western blot using specific anti-Freud antibody (shown above). β -actin immunoreactivity was tested to confirm equal loading.

F, G, Freud-1 protein inhibits 5-HT1A receptor expression in raphe RN46A cells.

F, Proteins prepared from RN46A cells transfected with Freud-1 sense or antisense expression vectors and differentiated for 72 hr were subjected to anti-5-HT1A receptor immunoblotting. β -actin immunoreactivity was tested to confirm equal loading. Shown is a representative blot of three independent experiments. The relative intensity was quantified by an Automated Digitizing System (UN-SCAN-IT, Silk Scientific Inc.). Data represent the mean $\pm$ S. E. of three independent experiments.

G, RN46A cells cotransfected with 1 μ g of Red Fluorescent Protein (DsRed) and 5 μ g of Freud-1 sense or antisense expression plasmids as indicated were stained for 5-HT1A immunoreactivity (see Methods). Arrows indicate representative DsRed-positive cells that express either high or low 5-HT1A immunoreactivity (for antisense or sense Freud-1, respectively) compared to cells not transfected with DsRed.

decreased compared to control in both L6 and RN46A cells (by 25% or 50% respectively), as observed for the larger -2300 5-HT1A construct (Fig. III.3D). The DRE-deficient -1519-luciferase construct lacked Freud-1-induced repression (data not shown). Transfection of Freud-1 plasmid increased Freud-1 protein content by 1.8- and 2.2-fold in L6 and RN46A cells, respectively (Fig. III.3D, blot). The greater repression by Freud-1 in RN46A cells may reflect the importance of the single Freud-1-DRE complex in these cells compared to two protein-DRE complexes in L6 (Fig. III.2D) and other non-neuronal cells (Ou et al., 2000). Cotransfection of an antisense Freud-1 cDNA construct depleted endogenous Freud-1 protein levels in L6 and RN46A by approximately the same extent (50% vs. 55%, Fig. III.3E, blot). Cotransfection of antisense Freud-1 derepressed the 5-HT1A promoter construct only in RN46A cells, indicating that endogenous Freud-1 plays a role in basal regulation of the 5-HT1A receptor gene in these neuronal cells.

To address the role of Freud-1 in regulating receptor expression, we measured 5-HT1A receptor levels in raphe RN46A cells transiently transfected with sense or antisense Freud-1 cDNA constructs (Fig. III.3F). The level of endogenous 5-HT1A receptor protein in differentiated RN46A cells was decreased significantly upon transient transfection with sense Freud-1, but increased with antisense to Freud-1, parallel to the changes observed in 5-HT1A gene transcription. Since the transfection efficiency in these experiments was 30% (see Methods), alterations in Freud-1 and 5-HT1A receptor expression would occur in a maximum of only 30% of cells. To examine changes specifically in transfected cells, co-transfection of sense or antisense Freud-1 constructs was done with a DsRed construct as a fluorescent marker for transfected cells (Fig. III.3G). DsRed fluorescence filled the transfected cells consistent with its cytosolic localization,

whereas 5-HT1A staining was strongest in perinuclear regions perhaps reflecting internalized receptors. Upon transfection of sense Freud-1, decreased 5-HT1A immunostaining was observed in DsRed-positive cells relative to DsRed-negative cells, whereas antisense to Freud-1 led to increased 5-HT1A receptor staining in DsRed-labeled cells. While dramatic effects were seen in a majority of DsRed-positive cells, immunofluorescence represents a qualitative method that complements the quantitative findings observed using Western blot (Fig. III.3F). These experiments indicate that Freud-1 represses 5-HT1A gene transcription to decrease 5-HT1A protein expression in raphe RN46A cells.

3.4.4 Inactivation of Freud-1 by calcium/ATP

Since the CalB domain is thought to confer Ca^{2+} -dependent lipid binding, we examined the action of calcium and ATP on protein-DRE binding activity in nuclear extracts from L6 and RN46A cells by EMSA. Upon addition of Ca^{2+} and ATP, the specific interaction of the Freud-1-containing nuclear protein-DRE complex (*band 1*) was decreased in RN46A or abolished in L6 extracts (Fig. III.4A). By contrast, the protein-TRE complex from L6 cells (*band 2*) was sensitive to ATP alone. Thus the combination of calcium and ATP specifically interferes with Freud-1 binding to DNA. The role of calcium-dependent phosphorylation in Ca^{2+} /ATP-mediated inhibition of the Freud-1-DRE interaction was assessed by addition of protein kinase inhibitors (Fig. III.4B-C). The inhibitors of CAMK (KN93) and CAM (calmidazolium, CMZ) blocked the inhibitory action of Ca^{2+} /ATP on Freud-1-DRE binding leading to a recovery of the Freud-1-DRE complex (Fig. III.4B, lanes 4 and 7). In L6 cells, these inhibitors specifically rescued the Freud-1-DRE complex (*band 1*, Fig. III.4C), whereas the second protein-TRE complex (*band 2*) was not rescued.

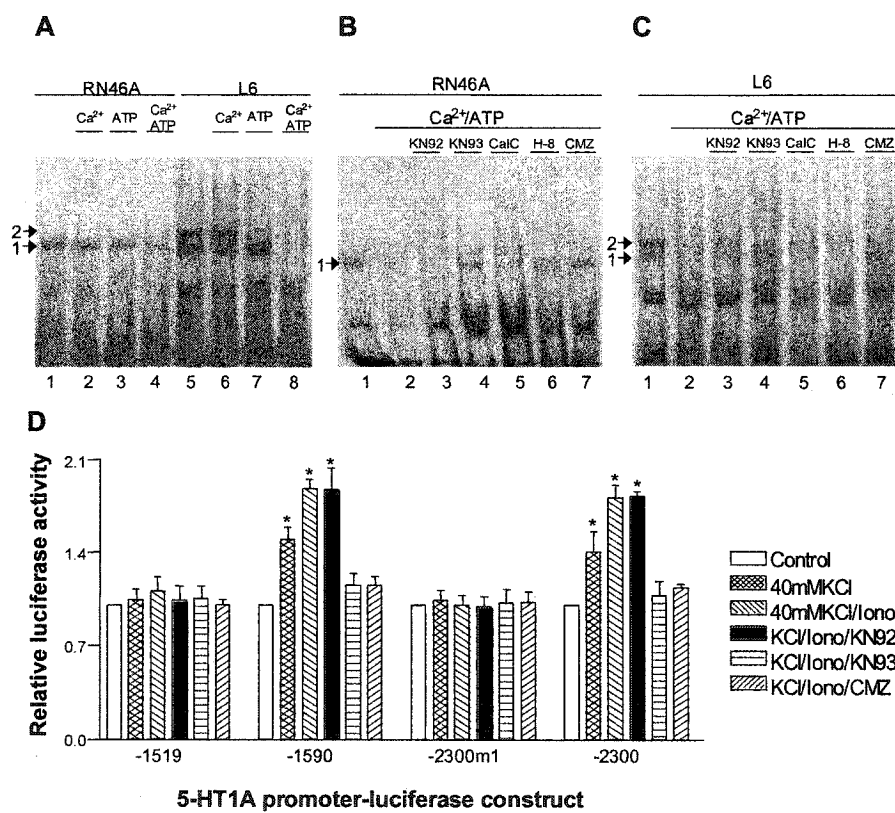


Figure III.4 – CAM kinase attenuates Freud-1-mediated repression.

A, Ca^{2+} and ATP in combination interfere with Freud-1/DRE interaction. EMSA using the 31-bp DRE as a probe incubated with nuclear extracts from RN46A (lanes 1-4) or L6 cells (lanes 5-8) as indicated. CaCl_2 (lanes 2, 6), ATP (lanes 3, 7) or both (lanes 4, 8) were added into incubation buffer for 20 min prior to incubation with DRE probe.

B, *C*, CAM kinase activation in nuclear extracts interferes with Freud-1-DRE binding. EMSA using DRE as a probe and nuclear extracts from RN46A cells (*B*) and L6 cells (*C*). Both CaCl_2 and ATP were added into the incubation before adding probe (*lanes 2-7*). Treatment was with 10 μM (except 50 nM CalC) of KN92 (negative control for KN93; lane 3), KN93 (CAM kinase inhibitor; *lane 4*), CalC (Calphostin C, PKC inhibitor; *lane 5*), H-8 (PKA inhibitor, lane 6) and CMZ (calmidazolium, CAM inhibitor; *lane 7*), as indicated. In L6 cells (*C*), CAM or CAMK inhibitors rescued only the Freud-1-containing species (*band 1*).

D, Calcium signaling enhances 5-HT1A promoter activity in a Freud-1-FRE-dependent manner. 5-HT1A promoter-luciferase reporter constructs containing FRE (-1590 or -2300) or lacking FRE (-1519, -2300m1) were transfected into RN46A cells. 24 hr after transfection, the cells were treated with 40 mM KCl or 40 mM KCl and 1 μM ionomycin without or with 10 μM KN92, KN93 or CMZ in the medium for 16 h. Luciferase activity is expressed as relative light units normalized to control (untreated) samples. * $p < 0.05$ in comparison with control. Note that calcium mobilizing agents had no effect in FRE-lacking reporter constructs (-1519 or -2300m1) and were blocked by CAM or CAMK inhibitors.

However, the inactive analogue of KN93 (KN92), PKC inhibitor Calphostin C (CalC) or PKA inhibitor H-8 failed to influence Freud-1-DNA binding. These results suggest that CAMKIV (the predominant nuclear CAMK), but not PKC or PKA, mediated the inhibitory action of calcium/ATP on Freud-1 interaction with FRE.

We tested whether calcium-mediated inhibition of Freud-1 binding to FRE can activate 5-HT1A receptor gene transcription. RN46A cells were treated with agents to increase $[Ca^{2+}]_i$ and the activity of FRE-containing (-1590- and -2300-luciferase), FRE-deleted (-1519-luciferase) or FRE-inactivated (-2300m1) 5-HT1A reporter constructs was examined (Fig. III.4D). Upon incubation with 40 mM KCl to induce calcium influx the luciferase activity of -1590 and -2300, but not the -1519 or -2300m1 constructs, was increased significantly. Co-addition of ionomycin (1 μ M), a calcium ionophore that induces calcium influx and releases calcium stores (Albert and Tashjian, 1986; Szonyi et al., 2001), further enhanced the luciferase activity of the -1590 and -2300 5-HT1A promoter constructs. Thus calcium signaling enhances 5-HT1A receptor transcription in raphe cells via a Freud-1-FRE dependent mechanism. Inhibitors KN93 and CMZ, but not the inactive analogue KN92 blocked calcium-mediated induction of the 5-HT1A promoter constructs, indicating a specific role for CAMK and CAM. Thus, calcium/CAM-dependent activation of CAMK inhibits Freud-1 function resulting in enhanced transcriptional activity of the 5-HT1A receptor gene.

3.4.5 Freud-1 RNA and protein expression in brain

The role of Freud-1 *in vivo* was addressed initially by examining the expression profile of Freud-1 mRNA by Northern blot analysis of rat tissues and brain regions (Fig. III.5). A

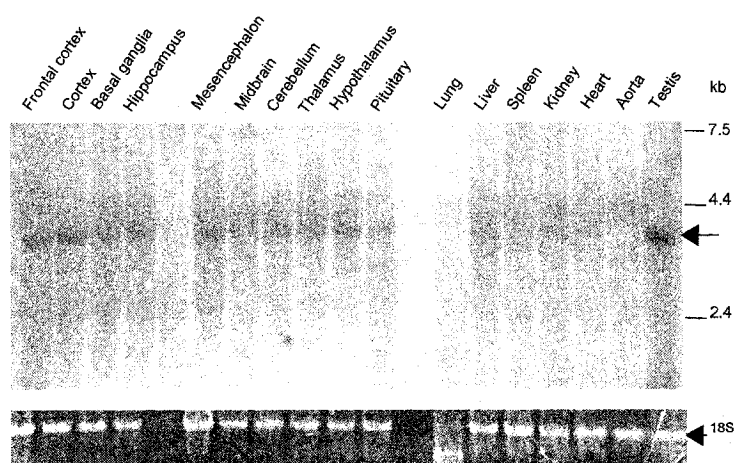


Figure III.5 – Tissue distribution of Freud-1 RNA expression.

Freud-1 mRNA expression in rat tissues. RNA prepared from 17 different rat tissues was used for Northern blot analysis. The position of molecular mass markers is shown on the right. An arrow indicates hybridization to Freud-1 probe. Ethidium bromide-stained 18S RNA was used as a loading control.

single 3.8-kb mRNA species was strongly expressed in several rat brain areas, including frontal cortex, cortex, mesencephalon, hypothalamus, hippocampus and midbrain. Freud-1 RNA was also detected in peripheral tissues such as testis and at low levels in pituitary, liver and kidney. Freud-1 RNA and protein expression in rat brain was analyzed further by *in situ* hybridization and immunohistochemistry (Fig. III.6A-C). Hybridization of antisense (*a'-c'*) versus sense (*a-c*) Freud-1 oligonucleotide probes demonstrated specific Freud-1 mRNA expression that corresponded with regions of strong Freud-1 immunoreactivity (*a''-c''*). The highest levels of Freud-1 RNA were detected in hippocampal pyramidal cells (Fig. III.6B) and raphe nuclei (including dorsal, medial (Fig. III.6A), and magnus (not shown). Discrete subsets of cells were labeled intensely in the hypothalamus, cortex, with weaker hybridization in the thalamus. In pyramidal cells of hippocampal CA1, CA2, CA3 and dentate gyrus regions, Freud-1 RNA was present in the cell body and proximal dendrites, whereas Freud-1-like immunoreactivity was predominant in the nucleus (Fig. III.6B). A few pyramidal cells of the prefrontal cortex also displayed intense Freud-1 RNA staining and immunoreactivity (Fig. III.6C). Freud-1 was also strongly expressed in cells of the dorsal and intermediate nuclei of the lateral septum, medial septum and hypothalamus (data not shown). Each of these brain areas that express Freud-1 RNA and protein also express 5-HT1A receptor RNA (Pompeiano et al., 1992), consistent with a role for Freud-1 to regulate the 5-HT1A gene *in vivo*.

In order to address directly whether Freud-1 protein is expressed in 5-HT1A receptor-positive cells dual immunofluorescent staining of cells in culture and brain sections was done. In RN46A cells as well as primary hippocampal and cortical cultures (Fig. III.6D), Freud-1 protein was present in the nucleus of cells expressing 5-HT1A

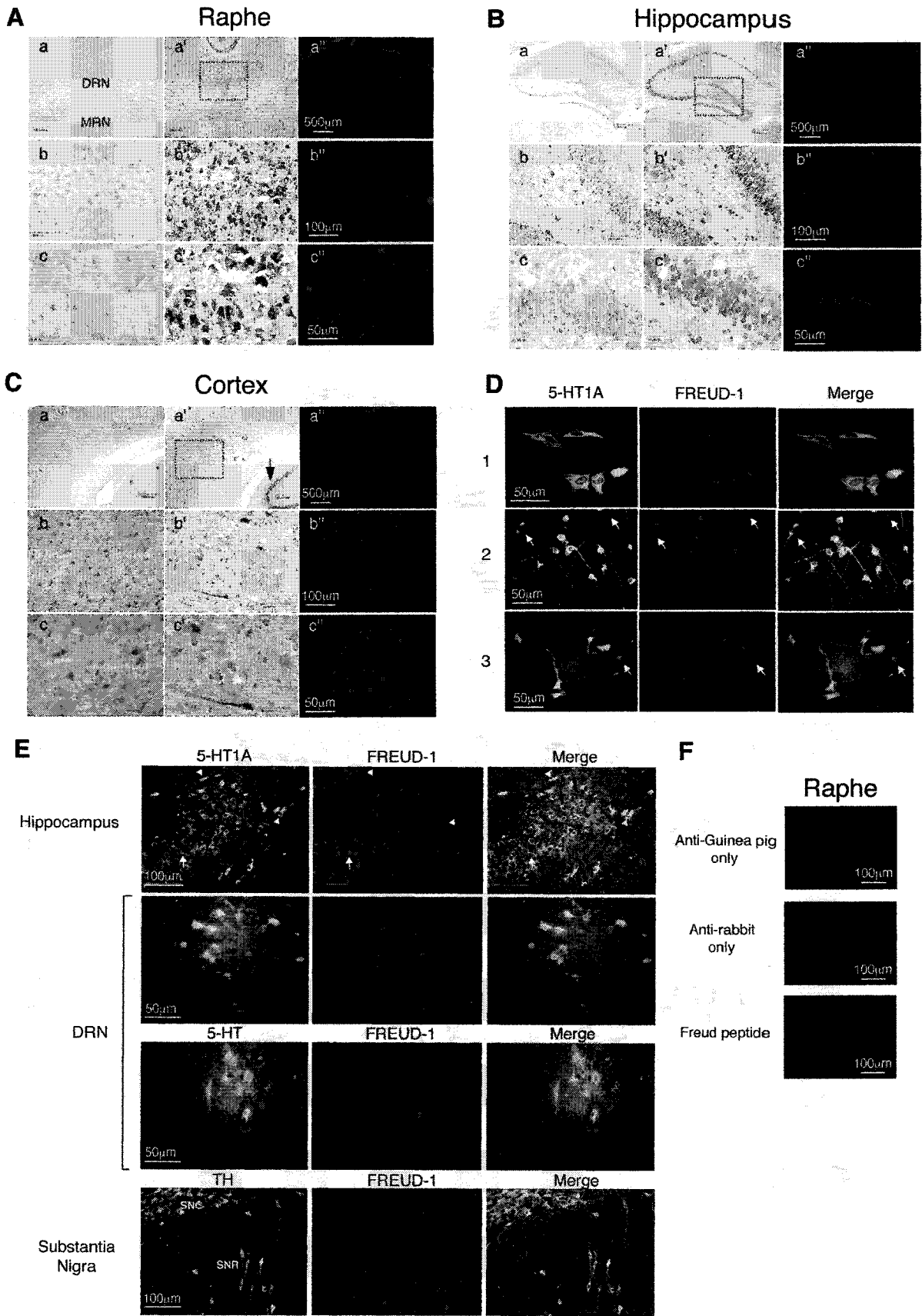


Figure III.6 – Co-localization of Freud-1 with 5-HT1A receptor.

A-C, Coronal brain sections were hybridized with sense (*a-c*) or antisense (*a'-c'*) digoxigenin-labeled Freud-1 oligonucleotides and stained as described in Methods, or assayed for Freud-1 immunoreactivity (*a''-c''*). Boxed regions are displayed at successively higher magnification in *a-c*, *a'-c'*, and *a''-c''*. Scale Bars = 500 μ m (*a*, *a'*, *a''*), 100 μ m (*b*, *b'*, *b''*), and 50 μ m (*c*, *c'*, *c''*). Freud-1 staining was observed in the following regions:

A, Raphe nuclei. Staining was most intense in cells of the dorsal raphe nucleus (DRN), but also present in the medial raphe nucleus (MRN) and raphe magnus (not shown). The dorsal raphe nucleus is magnified showing cytoplasmic Freud-1 RNA and nuclear Freud-1 protein.

B, Hippocampus. Staining is prominent in pyramidal cells of CA1, CA3 and the dentate gyrus. Dentate gyrus cells are magnified to show pyramidal cell morphology.

C, Primary sensory cortex. The arrow indicates hippocampus for comparison.

D, Nuclear localization of Freud-1. Colocalization of Freud-1(*red*) and 5-HT1A receptors (*green*) in raphe RN46A cells (*1*), and primary cultures of embryonic cortical (*2*) and hippocampal (*3*) cells. Arrows indicate cells expressing high levels of Freud-1 and low levels of 5-HT1A receptor.

E, Colocalization studies. Dual immunofluorescence was used to detect colocalization of Freud-1 with various markers. Freud-1 was colocalized with 5-HT1A receptor staining in sections from hippocampus and dorsal raphe nucleus (DRN), as indicated. In DRN, Freud-1 was also colocalized with staining for 5-HT, a marker of serotonergic neurons. In

the substantia nigra pars compacta (SNC) or especially pars reticulata (SNR), Freud-1 was colocalized with TH, a marker for dopaminergic neurons. Arrowheads indicate cells expressing low Freud-1 and high 5-HT_{1A} receptor levels.

F, Specificity of 5-HT_{1A} and Freud-1 staining. Raphe tissue sections that were incubated with anti-guinea pig or anti-rabbit secondary antibodies alone or in the presence of 100 µg of Freud-1 peptide antigen displayed only background immunoreactivity.

receptors in the cell body and processes. Especially in the cortical cultures, some cells expressed Freud-1 but not 5-HT1A receptors (*arrows*). However, the majority of 5-HT1A-expressing cells also expressed Freud-1. In tissue sections (Fig. III.6E), Freud-1 staining was predominantly nuclear and was colocalized by dual immunofluorescence with extranuclear staining for 5-HT1A receptors (in hippocampus and dorsal raphe, DRN), 5-HT (dorsal raphe, DRN) or TH (substantia nigra, SN). In the raphe, Freud-1 staining was weaker than in the hippocampus and was largely colocalized with 5-HT1A receptors and 5-HT staining, suggesting its presence in 5-HT1A-positive serotonergic neurons. Interestingly, some cells that stained weakly for Freud-1 displayed strong 5-HT1A staining (Figs III.6D-E, *arrowheads*), and vice-versa (*arrows*), suggesting that Freud-1 protein inhibits 5-HT1A receptor expression *in vivo*, as we observed upon transfection of sense or antisense Freud-1 in RN46A cells (Fig. III.3G). Staining for Freud-1 in substantia nigra was strongest in TH-positive cells of the pars reticulata, suggesting a role in a subset of dopaminergic neurons. In control studies, fixed sections and cells that were incubated with primary or secondary antibody alone or preincubated with Freud-1 peptide antigen displayed only background immunoreactivity, demonstrating the specificity of Freud-1 staining (Fig. III.6F and data not shown). Co-localization of Freud-1 and 5-HT1A receptors in pre- and post-synaptic neurons of the serotonin system is consistent with a role for Freud-1 as a repressor to negatively regulate 5-HT1A gene expression *in vivo*.

3.5 Discussion

3.5.1 Freud-1: a novel repressor of the 5-HT1A receptor gene expressed in brain.

Previous studies have suggested the importance of regulation of 5-HT1A receptor expression in mental illnesses such as anxiety, major depression and in suicide (Albert et al., 1996; Mann, 1999; Pineyro and Blier, 1999; Artigas et al., 2001). Antidepressant compounds target the serotonin system and chronic down-regulation of the somatodendritic 5-HT1A autoreceptor on raphe neurons has been proposed as an obligatory step to their clinical efficacy. Conversely, genetic ablation of the 5-HT1A receptor results in increased anxiety behaviours in three different strains of mice (Saudou et al., 1994; Heisler et al., 1998; Parks et al., 1998; Ramboz et al., 1998). Importantly, in postmortem brains from depressed suicides the basal level of 5-HT1A receptor expression was increased specifically in the raphe nuclei, suggesting that altered regulation of the 5-HT1A autoreceptor may predispose subjects to depression or suicide (Stockmeier et al., 1998). Thus, we have studied the transcriptional mechanisms of 5-HT1A receptor regulation in raphe cells and identified the neuronal repressor element FRE (Ou et al., 2000). We have now used the yeast one-hybrid approach to identify a novel protein, Freud-1, that binds to the FRE site of the 5-HT1A promoter to repress 5-HT1A receptor expression.

Several pieces of evidence indicate that Freud-1 acts as a neuronal repressor of the 5-HT1A gene by binding to the FRE site. First, the two independent Freud-1 clones were isolated based on trans-activation of the DRE in the yeast system. Second, recombinant purified His-tagged Freud-1 bound specifically to the 5' FRE 14-bp segment of the DRE and this complex was retarded by a specific antibody directed against Freud-1. Additionally in RN46A or L6 nuclear extracts, the formation of the FRE-protein complex

was inhibited by this antibody, indicating the presence of Freud-1 in the complex. Third, Freud-1 repressed a variety of 5-HT_{1A} or heterologous promoter-luciferase constructs in a FRE-dependent manner in raphe RN46A cells to a greater extent than in non-neuronal L6 cells, suggesting a greater role for Freud-1 in raphe cells. In combination with other repressors such as the TRE-specific protein complex, Freud-1 mediates silencing of the gene in non-neuronal cells (Ou et al., 2000). Depletion of Freud-1 protein using an antisense construct derepressed the 5-HT_{1A} receptor gene in RN46A cells but not L6 cells, suggesting that Freud-1 is a crucial regulator of this gene in raphe cells. Fourth, Freud-1 expression decreased 5-HT_{1A} receptor protein levels while antisense to Freud-1 increased receptor expression in RN46A cells, indicating that Freud-1 is a key repressor of the 5-HT_{1A} receptor in raphe cells. Thus Freud-1 mediates neuronal repression of the 5-HT_{1A} receptor gene by binding to the FRE.

The subcellular and brain regional distribution of Freud-1 further supports its role in neuronal regulation of the 5-HT_{1A} receptor gene. The co-localization of Freud-1 with both 5-HT and the 5-HT_{1A} receptor in raphe nuclei strongly suggest that Freud-1 is involved in regulation of the expression of somatodendritic 5-HT_{1A} autoreceptor on serotonergic neurons. Interestingly, Freud-1 was also expressed in TH-positive cells of the substantia nigra, not known to express 5-HT_{1A} receptors, suggesting other roles for Freud-1 in addition to regulation of the 5-HT_{1A} receptor. The substantia nigra is known to express the dopamine-D₂ receptor and an FRE-like sequence is present in the second intron of the human D₂ receptor gene, suggesting that D₂ receptors could be an additional target for regulation by Freud-like proteins.

The presence of a human Freud-1 homologue suggests a role in regulation of the human 5-HT1A receptor. Within a strong repressor region of the human 5-HT1A gene located between -1624/-1570 bp we have identified two adjacent functional DRE's with 80% identity to the rat DRE (Lemondé et al., in preparation). The human DRE binds to Freud-1 and mediates repression of the human 5-HT1A gene, suggesting a role for Freud-1 in humans. For example, altered regulation of Freud-1 in the raphe nuclei could mediate the increase in 5-HT1A autoreceptors observed in depressed suicide victims (Stockmeier et al., 1998) perhaps implicating Freud-1 in predisposition to suicide or depression. Expression of Freud-1 in serotonergic and 5-HT1A-positive neurons of the raphe nuclei suggests that Freud-1 could play a role in regulating human 5-HT1A autoreceptor expression *in vivo*.

3.5.2 Structural domains and calcium-dependent regulation of Freud-1.

Freud-1 represents a novel DNA binding protein with no significant homology to other families of known DNA binding proteins. All Freud-1 species homologues contain multiple repeats of the basic DM-14 domain, a predicted HLH domain and a conserved C2 domain. By analogy with known basic HLH transcription factors, the HLH domain of Freud-1 may mediate DNA binding in combination with the basic DM-14 repeats. The nuclear localization of Freud-1 could also be mediated in part by the basic DM-14 domains, given the lack of a consensus nuclear localization sequence. Deletion of the first nine amino acids of the C2 domain reduced DNA binding of Freud-1 and abolished its repressor activity, suggesting its role in DNA binding but especially in repressor function of Freud-1. Interestingly, the calcium-independent C2 domain of PTEN mediates protein-

protein interaction with the tumor suppressor p53 (Freeman et al., 2003). By analogy the C2 domain of Freud-1 may also interact with nuclear proteins to localize Freud-1 to the nucleus or to recruit co-repressors. The Freud-1 C2 domain may be calcium-insensitive since calcium alone did not alter Freud-1 function and the Freud-1 C2 domain lacks several acidic residues that mediate calcium binding of the PKC C2 domain. In addition, the Freud-1 C2 domain contains a poly-basic insert (5/8 basic residues between residues 365-372) that is not present in calcium-dependent C2 domains and may function as a nuclear localization signal as reported for the double-C2 gamma protein (Fukuda et al., 2001). Further experiments will be required to identify the precise function of the Freud-1 C2 domain.

We found that although not directly regulated by calcium, Ca^{2+} -ATP inactivated Freud-1 binding to DNA in nuclear extracts. Pharmacological analysis of calcium-mediated inhibition of Freud-1 implicated CAMKII or more likely CAMKIV, which is located predominantly in the nucleus and known to mediate transcriptional activation associated with long-term memory (Soderling, 2000; Kasahara et al., 2001; Impey et al., 2002). It remains unclear whether CAMK directly phosphorylates Freud-1 or phosphorylates an associated protein to inactivate Freud-1 function. Unlike repressors that are inhibited directly by calcium binding such as DREAM (Carrion et al., 1999; Mellstrom and Naranjo, 2001; Cheng et al., 2002), Freud-1 provides a new example of a repressor that is inactivated by CAMK-mediated phosphorylation. Consistent with this, calcium mobilization by high K^+ depolarization and calcium ionophore increased 5-HT1A transcriptional activity in RN46A cells. Calcium signaling in raphe neurons can be initiated via glutamatergic input, which activates NMDA receptors to enhance calcium-

dependent 5-HT release (Celada et al., 2001; Lee et al., 2003). Conversely, activation of 5-HT_{1A} autoreceptors decreases $[Ca^{2+}]_i$ (Chen and Penington, 1996; Bayliss et al., 1997a, b), which would activate Freud-1 to repress the 5-HT_{1A} receptor gene. Activation of 5-HT_{1A} autoreceptors by antidepressant treatment could utilize calcium- and Freud-dependent mechanisms to desensitize the 5-HT_{1A} autoreceptor. Thus, Freud-1 can regulate 5-HT_{1A} receptor transcription in two ways: the level of Freud-1 protein negatively regulates basal 5-HT_{1A} expression, while CAMK-mediated signaling enhances 5-HT_{1A} transcription by inactivation of Freud-1. Thus, either Freud-1 protein levels or calcium-mediated signaling determine Freud-1 activity to regulate 5-HT_{1A} receptor expression.

It should be emphasized that Freud-1 is one of several transcription factors that regulate 5-HT_{1A} receptor expression including enhancers, such as Sp1/MAZ protein (Parks and Shenk, 1996), the ETS factor PET-1 (Hendricks et al., 1999), and NFkB (Abdoun et al., 2001) that positively regulate the gene. Other negative regulators include the putative TRE-binding protein (Ou et al., 2000), the neural restriction silencer REST/NRSF (Lemonde et al., in preparation), and a novel protein complex that recognizes a palindrome DNA sequence in the repressor region of the 5-HT_{1A} gene (Lemonde et al., 2003). In addition, a negative mineralocorticoid response element confers inhibitory regulation of 5-HT_{1A} transcription by glucocorticoids (Ou et al., 2001). However, Freud-1 appears to play an important role in neuronal regulation of the 5-HT_{1A} receptor, whereas factors like REST or TRE-binding proteins appear more important to silence the gene in non-neuronal tissues. The recent finding that PET-1 regulates the differentiation of precursor raphe cells to a serotonergic phenotype (Hendricks et al., 2003) suggests that in raphe cells Freud-1 and PET-1 may coordinately regulate 5-HT_{1A} receptor expression,

whereas in non-serotonergic neurons, Freud-1 may coordinate with other as yet unknown factors. Thus, the transcription factors that underlie the tissue-specific and regulated expression of 5-HT_{1A} receptors may include, but are not limited to Freud-1.

In summary, Freud-1 is a novel transcription factor that negatively regulates the basal expression of the 5-HT_{1A} receptor gene in neuronal cells. The importance of Freud-1 in repressing basal 5-HT_{1A} transcription and receptor levels in raphe cells suggests a potential role in altered regulation of 5-HT_{1A} receptors in disorders such as anxiety or major depression.

3.6 References

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CHAPTER IV – IMPAIRED TRANS-REPRESSION AT A 5-HT_{1A} RECEPTOR GENE POLYMORPHISM ASSOCIATED WITH MAJOR DEPRESSION AND SUICIDE

Sylvie Lemonde

[‡]David Bakish, [‡]Lisheng Du, [‡]Pavel D. Hrdina,

[†]Gustavo Turecki, [†]Adolfo Sequeira

*Christopher D. Bown, *Neena Kushwaha, *Stephen J. Morris, *Xiao-Ming Ou, [§]Ajoy Basak,

*Paul R. Albert

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Authors contribution: Over 90% of all research presented in this paper as well as preparation of all manuscript revisions was conducted by Sylvie Lemonde. Blood samples from depressed patients and controls were obtained through collaboration with David Bakish, Lisheng Du and Pavel D. Hrdina who performed psychiatric evaluations and collected blood samples. Result relative to suicide victims were obtained by our collaborators Gustavo Turecki and his graduate student Adolpho Sequeira. Rat hippocampal and cortical cultures were prepared and maintained by Christopher D. Bown. Initial cloning and sequencing of the human 5-HT_{1A} receptor promoter was performed by Stephen J. Morris. Ajoy Basak generated the NUDR multi antigenic peptide for production of NUDR antibody. Neena Kushwaha and Xiao Ming Ou helped with some of the

sequencing of genomic DNA and NUDR antibody purification, respectively. All work and manuscript editing was supervised by Paul R. Albert.

*Ottawa Health Research Institute (Neuroscience), University of Ottawa, Canada

†Douglas Hospital Research Center, McGill University, Montreal, Canada

‡Institute for Mental Health Research, Royal Ottawa Hospital, Ottawa, Canada

§Loeb Research Building, Ottawa Health Research Institute, Ottawa, Canada

Abbreviated Title: Derepression at a 5-HT_{1A} polymorphism

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Keywords: Promoter, polymorphism, major depression, suicide, serotonin, transcription.

4.1 Abstract

Inhibition of serotonergic raphe neurons is mediated by somatodendritic 5-HT1A autoreceptors, which may be increased in depressed patients. We report an association of the C(-1019)G 5-HT1A promoter polymorphism with major depression and suicide in separate cohorts. In depressed patients, the homozygous G(-1019) allele was enriched two-fold versus controls ($P = 0.0017$ and 0.0006 for G/G genotype and G allele distribution, respectively), and in completed suicide cases the G(-1019) allele was 4-fold enriched ($P = 0.002$ and 0.00008 for G/G genotype and G allele distribution, respectively). The C(-1019) allele was part of a 26-bp imperfect palindrome that bound transcription factors NUDR/DEAF-1/suppressin and Hes5 to repress 5-HT1A or heterologous promoters, whereas the G(-1019) allele abolished repression by NUDR, but only partially impaired Hes5-mediated repression. Recombinant NUDR bound specifically to the 26-bp palindrome and endogenous NUDR was present in the major protein-DNA complex from raphe nuclear extracts. Stable expression of NUDR in raphe cells reduced levels of endogenous 5-HT1A protein and binding. NUDR protein was colocalized with 5-HT1A receptors in serotonergic raphe cells, hippocampal and cortical neurons, and adult brain regions including raphe nuclei, indicating a role to regulate 5-HT1A autoreceptor expression. Our data indicate that NUDR is a repressor of the 5-HT1A receptor in raphe cells whose function is abrogated by a promoter polymorphism. We suggest a novel transcriptional model in which the G(-1019) allele derepresses 5-HT1A autoreceptor expression to reduce serotonergic neurotransmission, predisposing to depression and suicide.

4.2 Introduction

The serotonin system of the brain originates from neurons of the raphe nuclei that project widely throughout the brain to innervate a variety of cortical, limbic, and hypothalamic areas that are involved in regulation of mood, emotion, stress, etc. (Törk, 1990; Jacobs and Azmitia, 1992). Reduced serotonergic neurotransmission is implicated in the pathogenesis of depressive illnesses and suicidal behaviors (Doris et al., 1999; Mann, 1999). Negative feedback inhibition of serotonergic raphe neurons is mediated by somatodendritic 5-HT_{1A} autoreceptors (Mongeau et al., 1997; Pineyro and Blier, 1999). Several antidepressant compounds desensitize raphe 5-HT_{1A} autoreceptors resulting in enhanced 5-HT neurotransmission (Albert et al., 1996; Artigas et al., 1996; Pineyro and Blier, 1999). Conversely, postmortem brains from depressed suicide victims versus non-depressed control individuals display elevated 5-HT_{1A} receptor density in the raphe nuclei, but not at post-synaptic sites (Stockmeier et al., 1998), which would decrease serotonergic activity in depressed patients.

Given the high lifetime prevalence of major depression of 16.1% (Doris et al., 1999; Hyman, 2000; Nestler et al., 2002), this illness is likely to involve multiple genetic loci, complicating linkage analysis. In an alternate approach, polymorphisms identified in candidate genes have been correlated with traits of mental illness. The short allele of the long polymorphic repeat of the serotonin transporter gene has been associated with reduced transcriptional activity and anxiety-related behavior in man (Lesch et al., 1996), whereas the long allele is associated with obsessive-compulsive disorder (Bengel et al., 1999) and completed suicide (Du et al., 1999). Polymorphisms identified in the 5-HT_{1A} receptor gene have yet to be associated with mental illness (Erdmann et al., 1995; Nakhai et al.,

1995; Kawanishi et al., 1998; Wu and Comings, 1999; Arias et al., 2002). The C(-1019)G polymorphism of the 5-HT_{1A} receptor gene is prevalent in the normal population (Wu and Comings, 1999) and is located in a region associated with significant repressor activity (Lemondé et al., in preparation). We hypothesized that variation in the sequence of the repressor region could lead to impaired repression of the 5-HT_{1A} receptor and might correlate with depression and/or suicidal behavior (Albert et al., 1996).

4.3 Materials and Methods

4.3.1 Clinical samples

Major Depression

Blood samples were obtained from a total of 129 (53 males; age: 43 ± 11 years; 95% Caucasian) unrelated patients and 134 (55 males; age: 36 ± 12 years; 96% Caucasian) normal healthy volunteers recruited by local advertising or from a random pool of individuals. Patients suffering from major depressive disorder (unipolar) were selected from those attending the Royal Ottawa Hospital Psychopharmacology Unit as described (Du et al., 2000), and bipolar patients were excluded. Briefly, the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) criteria for major depression was used. Only subjects with a minimal score of 18 on the Hamilton Rating Scale for Depression (HAM-D, 17 items) were included in this study consistent with moderately severe clinical depression. All control subjects were asked to complete a self-rating Beck's Depression Inventory, had no history of serious medical or mental illness, were drug free, and had no family history of psychiatric illness. According to origin, gender and age, subjects from the patient and control groups were comparable and all drawn from the Ontario area. Exclusion of non-

Caucasian samples did not alter the conclusion drawn. Genotype distribution in both samples was in Hardy-Weinberg equilibrium.

Suicide Completers

Consecutive cases of suicide were collected as part of an ongoing collaboration with the Coroner's Office of Quebec. Controls for the suicide study were living subjects without a history of suicidal behavior or a major psychiatric diagnosis. A total of 102 suicide completers (98 males) and 116 normal controls (116 males) were investigated. The mean ages were respectively 32.20 ± 9.38 and 34 ± 10.56 years old for suicide cases and controls. Both suicide cases and controls cases were of French-Canadian origin, which is an isolated population with a well-characterized founder effect (Heyer and Tremblay, 1995). All subjects included in this study (or their families for suicide cases) provided written informed consent, as approved by the local Institutional Review Board. Following consent, DNA was extracted from blood or a tissue sample. Psychological autopsies were carried out using a version of the SCID (Spitzer et al., 1992) adapted for interviews with informants. Best-estimate DSM-IV diagnoses were made by a blind panel of psychiatrists and mental health professionals. Forty-three percent ($N = 44$) of the suicide cases had available information on psychiatric diagnoses based on best-estimate psychological autopsies. Of these, 52% ($N = 23$) met criteria for major depression. Given the low N values, no differences were observed in either allelic ($P = 0.63$) or genotypic ($P = 0.82$) distributions when depressed suicide cases were compared to non-depressed suicides. Detailed diagnostic procedures are described elsewhere (Lesage et al., 1994).

4.3.2 DNA analysis

Amplification was between -1595/-879 bp of the human 5-HT1A gene and was performed in 25 μ l using 100 ng of genomic DNA, 200 μ M dNTP's, 1 mM MgCl₂, 0.5 U of Taq and Pfu, and 2 μ M of primers: 5'-GTGGCGACATAAAACCTCA-3' and 5'-TTCTTAAATCGTGTTCAGCATC-3'. The amplification cycles were: 92 °C for 5 min.; 92 °C, 45 sec., 69 °C, 45 sec.; -0.5 °C/cycle, 72 °C for 90 sec (10 cycles); 92 °C for 45 sec., 64 °C for 45 sec., 72 °C for 90 sec. (30 cycles); and terminated at 72 °C for 10 min. The 716-bp PCR product was gel-purified and sequenced (T7 sequencing kit, Amersham, Pharmacia Biotech, Milwaukee, WI). Genotyping of the two sample populations was carried out independently by two different laboratories.

4.3.3 Statistical analysis

Chi-square (χ^2) analysis with two-tailed *P* values was used to compare genotype frequencies between patients and controls, and between suicide completers and controls. Allele frequencies were also compared by Fisher exact test with two-tailed *P* values. In cell studies, unpaired t-tests with two-tailed *P* values were carried out to compare transcriptional activities of the C versus G alleles, while all other statistical significance were obtained by one-way ANOVA analysis with a post-hoc Dunnett's test. All above statistical analysis were performed using the GraphPad Prism software (San Diego, CA), and calculated using 95% confidence intervals.

4.3.4 Electrophoretic mobility shift assay

Nuclear extract preparation and EMSA were done as described (Ou et al., 2000). For EMSA, complementary oligonucleotides from the normal human 5-HT_{1A} sequence were:

Sense -1021/-998 bp 5'-AACGAAGACACACTCGGTCTTCTT-3'

Antisense -996/-1021 bp 5'-GGAAGAAGACCGAGTGTGTCTTCGTT-3'

The probe contains the polymorphic site (double underlined) and the imperfect palindrome sequence (bold). The unrelated rat E2F sequence: 5'-ATTTAAGTTTCGCGCCTTTTC-3' was used as non-specific competitor. The ³²P-labeled probe (60,000 cpm/sample) was incubated with or without competitor DNA, in 25 µl of DNA binding buffer containing 15 µg of nuclear protein extract, 2 µg poly (dI-dC) (Sigma, Oakville, ON), at room temperature for 20 min. Samples were electrophoresed on a 5% acrylamide/Tris-glycine gel at 4°C for 3 hours. For supershift experiments, anti-Hes5 was used at 1:10 dilution, anti-NUDR at 1:5.

4.3.5 Yeast one-hybrid

Six repeats of the C-allele of the 26-bp element were subcloned into *KpnI/XhoI*-cut pHISi or *SacI/SacII*-cut pLacZi vectors (Clontech, Palo Alto, CA), which were linearized by *ApaI* or *AflIII* digestions, and stably integrated into the YM4271 yeast strain. The transformants were selected on synthetic dropout His, Ura plates containing 20 mM 3-amino-1, 2, 4-triazole for integration of the target-reporter constructs into the HIS3 and URA3 loci. Subsequent transformation with a human brain MATCHMAKER cDNA library (Clontech) fused to GAL4 activation domain and containing a LEU2 selectable marker, allowed for

selection of transformants on synthetic dropout His, Ura, Leu plates containing 20 mM 3-amino-1, 2, 4-triazole. Resistant clones were tested by β -galactosidase plate assay, retransformed for verification, and sequenced for identification by database search (<http://www.ncbi.nlm.nih.gov/BLAST/>). To compare C and G alleles, 6 tandem repeats of each were cloned into the pLacZi vector and integrated into YM4271 as described above. Plasmid DNA from clones 76D, 18C and 33B was transformed into the two yeast strains, and transformants selected on synthetic dropout Ura, Leu plates. Assays of β -galactosidase activity were done in liquid culture using ONPG as a substrate (Clontech) or by plate assay using X-Gal (Duttweiler, 1996).

4.3.6 *In vitro* transcription/translation and DNase I footprinting

Recombinant proteins were synthesized using the TNT Coupled Reticulocyte Lysate System (Promega, Madison, WI) and 1 μ g of the plasmid hNUDR (from ATG to Stop codon) in pcDNA1. The Core Footprinting System (Promega) was utilized following manufacturer's recommendations. A gel-purified 434-bp *KpnI/BlnI* (-1128/-694) DNA fragment was dephosphorylated and labeled with polynucleotide kinase and [γ - 32 P]ATP. *HincII* digestion generated a single end-labeled probe (20,000 cpm) that was preincubated with 0.03, 0.15, 0.3 or 0.45 U of RQ1 RNase-free DNase and 0, 10, 20 μ g of raphe/midbrain nuclear extract or 3 μ l of a 25 μ l preparation of *in vitro*-transcribed/translated NUDR protein. The reaction products were purified by phenol-chloroform extraction and ethanol precipitation, and loaded on a 6% polyacrylamide sequencing gel with known DNA sequence as position marker.

4.3.7 Antibodies

Rabbit anti-Hes5 affinity purified polyclonal antibody was purchased from Chemicon International, Inc (Temecula, CA). The hNUDR peptide EAEEPVLSRDEDSEED (amino acids 36-51) was synthesized as a multiple-antigenic peptide and injected in rabbits for production of antibodies (Cedarlane, Hornby, ON). NUDR antibody was applied to the Affi-Gel blue gel 50-100 mesh, (Bio-Rad Laboratories Inc. Hercules, CA) for albumin removal. Subsequently the antibody was purified by Affi-Gel 15 Gel (Bio-Rad Laboratories Inc.) following manufacturer's instructions. Specificity to endogenous NUDR was assessed by Western blot analysis in which a single specific 59-kDa species was identified (Huggenvik et al., 1998) and was completely competed by preincubation with 100 µg of hNUDR (36-51) peptide. Guinea pig anti-5-HT1A receptor antibody (Chemicon International, Inc) was tested for its specificity by Western blotting of extracts (60 µg) from rat myoblast L6 cells transfected with vector or the rat 5-HT1A expression plasmid (Lembo and Albert, 1995).

4.3.8 Immunofluorescence

Immunohistochemistry was performed on rat brain primary cultures, RN46A cells and 12 µm frozen sections from adult male (250-300g) Sprague Dawley rat brains. Rats were anesthetized by intraperitoneal injection of 1 ml somnatol, perfused with saline, followed by fresh fixative (4% paraformaldehyde with 0.4% picric acid in PBS). Animals were decapitated, brains removed and post-fixed overnight at 4°C in the same fixative, transferred to 10% sucrose/0.02% sodium azide, sectioned and stored frozen at -80°C. Rat hippocampal and cortical cells were dissected from Sprague Dawley fetuses at 18 days of

gestation, as described (Banker and Cowan, 1977; Dichter, 1978). Cells (0.02% glia, 99.98% neurons (Bown et al., 2003)) were dispersed and cultured following the procedure detailed previously (Brewer et al., 1993). Briefly, dispersed cells were resuspended in Gibco Neurobasal™ media (Invitrogen, Burlington, Canada) containing B27 supplements (Invitrogen), 0.5 mM L-glutamine, 1% penicillin/streptomycin and 25 μ M glutamate. Cells were plated on poly-D-lysine (MW 30,000–70,000) coated vessels and maintained at 37°C, 5% CO₂ for 13 days. Cells were fixed in 4% paraformaldehyde in PBS for 1 hour at 37°C. Brain sections and cells were blocked and permeablized in 1% BSA, 5% goat serum, 0.3% Triton X-100 and incubated overnight with 1:50 rabbit anti-NUDR primary antibody in combination with 1:500 guinea pig anti-5-HT1A (Chemicon) or guinea pig anti-5-HT (A.A. Verhofstad, Nijmegen, Sweden) followed by secondary goat Texas-Red-coupled anti-rabbit (Calbiochem, San Diego, CA) antibody (1:400) and goat anti-guinea pig Alexa Fluor 488 (Molecular Probes Inc, The Netherlands) antibody (1:400) in 1% BSA, 5% goat serum, 0.3% Triton X-100 for 30 min at room temperature. Primary cortical and hippocampal cells were immunostained as above for the major microtubule associated protein (MAP2) and the β 3 tubulin early neuronal marker (TuJ1) using 1:250 mouse anti-MAP2 (Sigma) and 1:100 anti-TuJ1 (D. Brown, University of Ottawa) followed by 1:400 secondary goat anti-mouse Cy3 (Jackson Immuno Research laboratories, Inc., West Baltimore Pike, PA). Immunofluorescence was visualized under a Zeiss Axioscop 2 microscope and images were captured using a Sony Hyper Had color camera and Northern Eclipse software (Emphix imaging).

4.3.9 Western blot analysis

Rat brain tissues were homogenized in buffer A (10 mM Hepes pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT and 0.1% NP-40). Nuclear extract were obtained as described (Ou et al., 2000). 20 µg of nuclear proteins from RN46A, rat midbrain, cortex and hippocampus were separated by SDS-page electrophoresis on a 12% gel. After transfer to nitrocellulose membrane and blockage with TBS containing 5% dry milk and 0.01% Tween 20, immunoreactive proteins were detected with rabbit anti-Hes5 or anti-NUDR antibodies at a dilution of 1:1000 followed by a 1:2000 dilution of the secondary horseradish peroxidase-linked (HRP) anti-rabbit antibody. To assess for 5-HT1A and NUDR expression in RN46A or NUDR overexpressing clones, whole cell extracts were prepared by sonication in NP-40 basic solution (1% NP-40, 50mM Tris pH 7.4, 150 mM NaCl) containing a mixture of protease inhibitors (Roche Diagnostics, Mannheim, Germany). 80 µg of total protein were loaded on 12% SDS-page gels and immunoreactivity detected using anti-NUDR and anti-5-HT1A antibodies at a dilution of 1:1000 followed by a 1:2000 dilution of the secondary HRP-conjugated anti-rabbit and anti-guinea pig antibodies respectively. Anti-β-actin antibody (Sigma) was used at a 1:10,000 dilution followed by HRP-conjugated anti-mouse secondary antibody at a dilution of 1:2000.

4.3.10 Plasmid constructs and transfections

The luciferase plasmid 5-HT1A(C) was obtained by insertion of a *KpnI/BssHII* fragment of the human 5-HT1A promoter (cloned from human genomic library) into pGL3-Basic (Promega) digested with *KpnI* and *MluI*. The 5-HT1A(G) construct contained the

C(-1019)G mutation that was generated by U.S.E. mutagenesis (Amersham, Pharmacia Biotech) of 5-HT1A(C). Plasmids 26bp-C(TK) and 26bp-G(TK) were generated by annealing complementary oligonucleotides of the 26-bp polymorphic element flanked by *SacI* and *NheI* sites, and subcloning into *SacI/NheI*-cut pGL3-Basic containing the HSV-TK promoter between *SalI/XhoI* sites. Appropriate oligonucleotides of the 26-bp element flanked with CC and GG 3'-overhangs were annealed, concatenated in six copies using T4 DNA ligase, blunted with Klenow and subcloned into *SmaI*-cut pGL3-Promoter to generate 26bp-C(6) and 26bp-G(6). All constructs were verified by DNA sequence analysis. HEK 293 or RN46A cells were maintained and transfected as described previously (Storring et al., 1999; Ou et al., 2000). NUDR and Hes5 expression plasmids were subcloned by digestion of pACT2 clones 76D (lacking the first sixteen amino acids) or 18C (full-length) with *BglII* and subsequent ligation with *BamHI*-digested pcDNA3 (Invitrogen). Full-length NUDR was obtained by PCR amplification using the human brain Marathon-Ready cDNA kit (Clontech). The PCR product was subcloned in pGEM-T Easy vector (Promega) prior to subcloning in *EcoRI* site of pcDNA1 (Invitrogen).

4.3.11 Generation and analysis of stable clones

RN46A cells were co-transfected with 5 µg of NUDR-pcDNA3 expression plasmid and 0.25 µg of PGK-puro (gift of M. McBurney, OHRI) using Pfx-7 lipid mixture (Invitrogen). Cells were selected for puromycin resistance (3 µg/ml) for 3-4 weeks and antibiotic-resistant clones were picked, expanded and tested for the expression of NUDR using Northern (not shown) and Western blot analysis. For ligand binding studies, cell membranes from parental or NUDR-transfected RN46A cells were prepared as described

(Albert et al., 1990) and stored at -80°C. Aliquots of thawed and dissociated membrane preparations (100 µg/tube) were added to triplicate tubes containing 200 µl TME and 10 nM [³H]-8-OH-DPAT (Amersham, Pharmacia Biotech) without or with 10 µM 5-HT to determine total vs. non-specific binding at room temperature (30 min). Reactions were terminated by filtration through GF/C (Whatman) filters, washing with 3 ml of cold buffer (50mM Tris-HCl, pH 7.4) and 5 ml of scintillation fluid added to filters to quantify radioactivity by liquid scintillation counting. For semi-quantitative RT-PCR, total RNA from rat RN46A cells was isolated using Trizol reagent (Invitrogen) and genomic DNA was removed by DNase treatment (confirmed by PCR analysis). RT-PCR was done using SuperScript One-step reverse transcriptase-PCR (RT-PCR) with Platinum Taq (Invitrogen), using specific primers for rat 5-HT1A and GAPDH: 5-HT1A (360-bp), 5'-GCCATCGCGCTAGACAGGTA-3' (sense) and 5'-GCGGTGCCGACGAAGT-3' (antisense); rat GAPDH (120-bp), 5'-CATGGCCTTCCGTGTTCTACCC-3' (sense) and 5'-CCTCGGCCGCCTGCTTCA-3' (antisense). Amplified cDNA fragments were run on a 1.2% agarose gel containing ethidium bromide and quantified using UN-SCAN-IT software version 4.3 (Silk Scientific Corporation). All values are normalized to GAPDH.

4.4 Results

4.4.1 Association of the C(-1019)G polymorphism with major depression and suicide

Genomic DNA extracted from blood samples of 129 depressed patients and 134 age- and ethnicity-matched controls was amplified by PCR using primers directed at the repressor region of the 5-HT1A receptor gene and sequenced. The C(-1019)G polymorphism was the only alteration detected within this region (Fig. IV.1). There was a two-fold increase in

the frequency of the homozygous G/G allele in severely depressed patients versus controls (Table IV-1). The genotype ($P = 0.0017$) and allele frequencies ($P = 0.0006$) were significantly different between patients with major depression and control subjects, indicating an association between the C(-1019)G polymorphism and major depression. To assess its possible association with suicide, genotype and allele frequencies of the C(-1019)G polymorphism were investigated in an independent sample comprising 102 suicide completers and 116 normal controls of similar ethnic background (French-Canadian, Table IV-2). The suicide cases carried the G(-1019) variant more frequently than controls (genotypes: $P = 0.002$, alleles: $P = 0.00008$). Importantly, the homozygous G/G genotype was four times more frequent among suicide completers. Thus the G(-1019) allele was associated with completed suicide and with major depression in two separate populations.

4.4.2 Specific protein-DNA interaction at an imperfect palindrome containing C(-1019)

We addressed whether the C(-1019)G polymorphism of the 5-HT_{1A} receptor gene might affect 5-HT_{1A} receptor expression. The C(-1019) polymorphism (in italics) is incorporated within an imperfect palindrome (bold): 5'-AACGAAGAC ACACTCG **GTCTTCTT**-3' and we first addressed whether proteins bind to this DNA sequence. Electrophoretic mobility shift assay (EMSA) was done using a 26-bp probe incorporating the palindrome in the absence or presence of nuclear extract from RN46A cells, a serotonergic raphe neuronal cell line that expresses 5-HT_{1A} receptors (Eaton et al., 1995; Storrington et al., 1999). A single major protein-DNA complex was detected using the

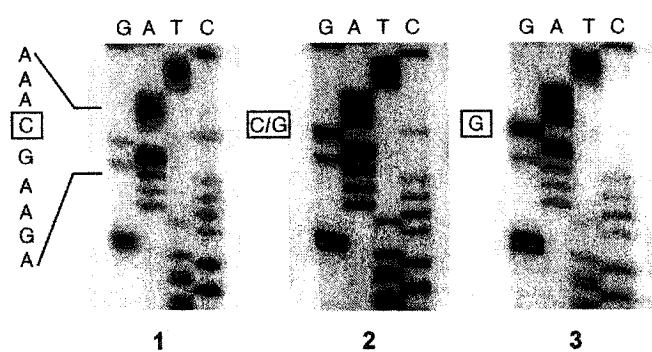


Figure IV.1 – A C(-1019)G polymorphism of the human 5-HT1A receptor promoter.

A 716-bp fragment in the repressor region of the human 5-HT1A promoter was amplified by PCR and sequenced. Shown is partial DNA sequence from PCR products of three different depressed patients that revealed: **1**, the homozygous C(-1019) sequence; **2**, the heterozygous sequence with both C and G at the (-1019) site; and **3**, the homozygous G(-1019) sequence.

Table IV-1 – Distribution of the 5-HT1A Receptor G(-1019) Gene Polymorphism in Depressed Patients and Control subjects from the Ontario cohort.

Subjects	N	Genotypes			Alleles	
		C/C	C/G	G/G	C	G
Controls	134	50	68	16	168	100
Major depression	129	30	63	36	123	135

Control subjects versus depressed patients: genotype, $\chi^2 = 12.79$, $df = 2$, $P = 0.0017^{**}$; allele, $P = 0.0006^{***}$. Presence of any C allele vs. absence of any C allele $P = 0.0018^{**}$. Presence of any G allele vs. absence of any G allele $P = 0.0158^*$.

Table IV-2 – Distribution of the 5-HT1A Receptor G(-1019) Gene Polymorphism in Suicide Completers and Normal Controls from the Quebec cohort.

Subjects	N	Genotypes			Alleles	
		C/C	C/G	G/G	C	G
Controls	116	85	26	5	196	36
Suicide	102	55	30	17	140	64

Control subjects versus suicide cases: genotype, $\chi^2 = 12.41$, $df = 2$, $P = 0.002^{**}$; allele, $P = 0.00008^{***}$. Presence of any C allele vs. absence of any C allele $P = 0.003^{**}$. Presence of any G allele vs. absence of any G allele $P = 0.0045^{**}$.

C(-1019) allele (26bp-C), which was competed by unlabelled 26-bp oligonucleotides but not by unrelated E2F oligonucleotides, indicating a specific interaction (Fig. IV.2A). Importantly, the G(-1019) allele displayed considerably reduced protein-DNA complex formation with an approximate 2-fold decrease in affinity (Fig. IV.2B). A specific protein-DNA complex of the same mobility as in RN46A raphe cells was also demonstrated *in vivo*, using extracts from adult rat brain regions including the midbrain, which contains the raphe nuclei (Fig. IV.2C).

In order to identify specific trans-acting proteins, six copies of the 26-bp polymorphic element were placed upstream of various selectable markers and 2×10^6 independent clones of a human brain cDNA library were screened by the yeast one-hybrid approach. Two clones (76D, 18C) trans-activated the C(-1019) allele robustly, and one (33B) weakly trans-activated (Fig. IV.2D). The C(-1019)G mutation reduced trans-activation in a clone-specific manner. Clones 76D (70-fold reduction in activity to detection limit) and 18C (15-fold reduction) were sensitive to the C(-1019)G change while clone 33B was relatively insensitive and was not examined further.

Clone 76D was identical to human NUDR/suppressin/DEAF-1 (Gross and McGinnis, 1996; Huggenvik et al., 1998; LeBoeuf et al., 1998; Sugihara et al., 1998), a homologue of the *Drosophila* helix-loop-helix transcription factor, Deformed epidermal autoregulatory factor (DEAF-1). NUDR (nuclear DEAF-1-related protein) is a novel transcription factor containing a SAND domain that binds to the minimal consensus TTCG site (Huggenvik et al., 1998; Bottomley et al., 2001). The imperfect palindrome of the 5-HT1A receptor contains an inverted TTCG and the C-G change destroys the NUDR element. Clone 18C had 91% nucleotide homology with mouse Hairy/Enhancer-of-split-5

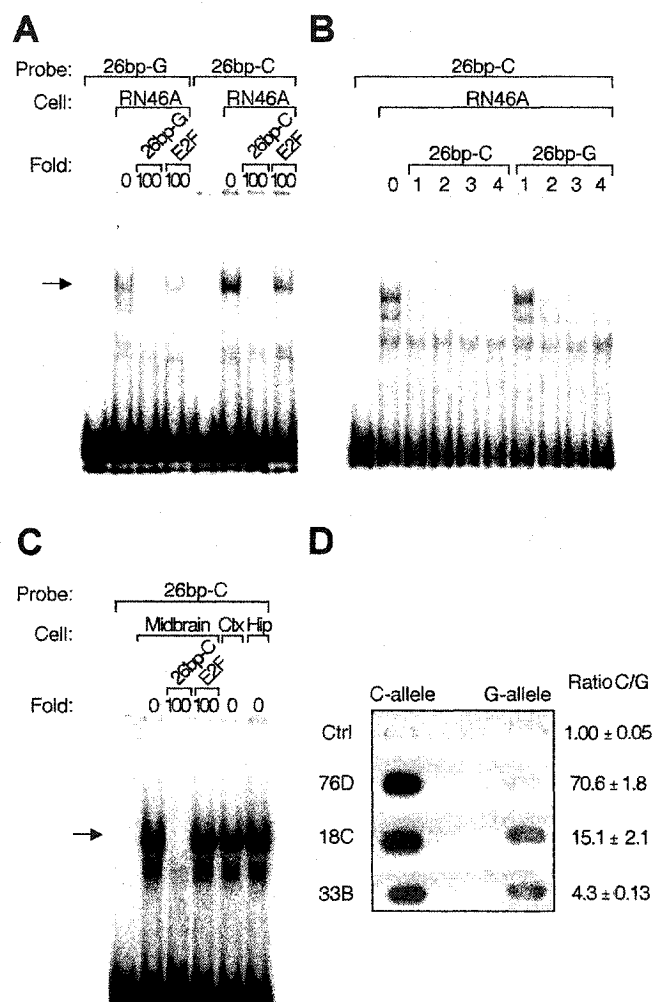


Figure IV.2 – Allele-specific association of a nuclear protein complex with the C(-1019) palindrome of the 5-HT1A receptor gene.

EMSA was done using a 26-bp probe that includes the C(-1019)G polymorphism of the human 5-HT1A receptor gene associated with depression. Labeled 26-bp oligonucleotide probes were present in samples as the C(-1019) or the G(-1019) allele as indicated. Unlabelled 26-bp oligonucleotides (26bp-C or 26bp-G) or unrelated E2F oligonucleotides (E2F) were added at 100-fold or 1-, 2-, 3- or 4-fold molar concentration to the incubation as indicated. (A) Using RN46A nuclear extracts, a single major specific complex was observed (arrowhead) that showed preferential binding to the 26bp-C oligonucleotide. (B) Decreased competition was observed with the 26bp-G oligonucleotide, which required a two-fold molar excess to start competing with the 26bp-C probe for RN46A nuclear extracts. (C) Nuclear extracts from rat brain tissues, including raphe/midbrain, cortex (Ctx) and hippocampus (Hip) showed specific binding to the labeled 26bp-C oligonucleotide (arrowhead). (D) Yeast one-hybrid cloning of binding proteins specific for the C(-1019) palindrome allele. Six copies of the C(-1019) or G(-1019) alleles of the 26-bp element of the 5-HT1A receptor gene were integrated in the yeast genome 5' to the *LacZ* gene. The two yeast strains generated were non-transformed (Ctrl) or transformed with plasmid DNA from the indicated cDNA clones (76D, 18C, or 33B). Trans-activation was measured by β -galactosidase activity in a plate assay (left), and by quantitative spectrophotometry to calculate the activity ratio of C/G (right), expressed as mean $\pm$ SD (n=2).

(*Drosophila*), Hes5. This helix-loop-helix transcription factor binds to a consensus N box (CACNAG) (Akazawa et al., 1992) and a similar sequence (CACNCG) is located near the C-G polymorphism. Both NUDR and Hes5 function as transcriptional repressors (Akazawa et al., 1992; Michelson et al., 1999), and their ability to regulate the 5-HT1A receptor gene was examined in 5-HT1A receptor-positive (RN46A) or receptor-negative (HEK 293) cells.

4.4.3 Repressor activity of the C(-1019) palindrome sequence

The repressor activity of NUDR or Hes5 at the human 5-HT1A promoter (Parks and Shenk, 1996) was examined by cotransfecting expression plasmids with a luciferase reporter construct containing 1.128 kb of promoter sequence comparing the C(-1019) or G(-1019) alleles denoted 5-HT1A(C) and 5-HT1A(G), respectively (Figs IV.3A-B). In vector-transfected cells, the basal luciferase activity of the two alleles of this promoter construct was not significantly different, hence the role of the polymorphism in basal 5-HT1A transcription was unclear. The -1128-luc 5-HT1A construct contains multiple interacting elements that could mask the effect of the C to G transition on basal transcription. Co-transfection of NUDR or Hes5 inhibited the transcriptional activity of the 5-HT1A(C) construct ($P < 0.0001$) in HEK 293 and RN46A cells. By contrast, NUDR did not repress the 5-HT1A(G) construct while Hes5 partially inhibited transcription of the G(-1019) allele in HEK 293 cells, but failed to repress the 5-HT1A promoter in RN46A cells. Thus, repression of the 5-HT1A promoter by NUDR and Hes5 was specific for the C(-1019) allele, although Hes5 had weak activity at the G(-1019) site.

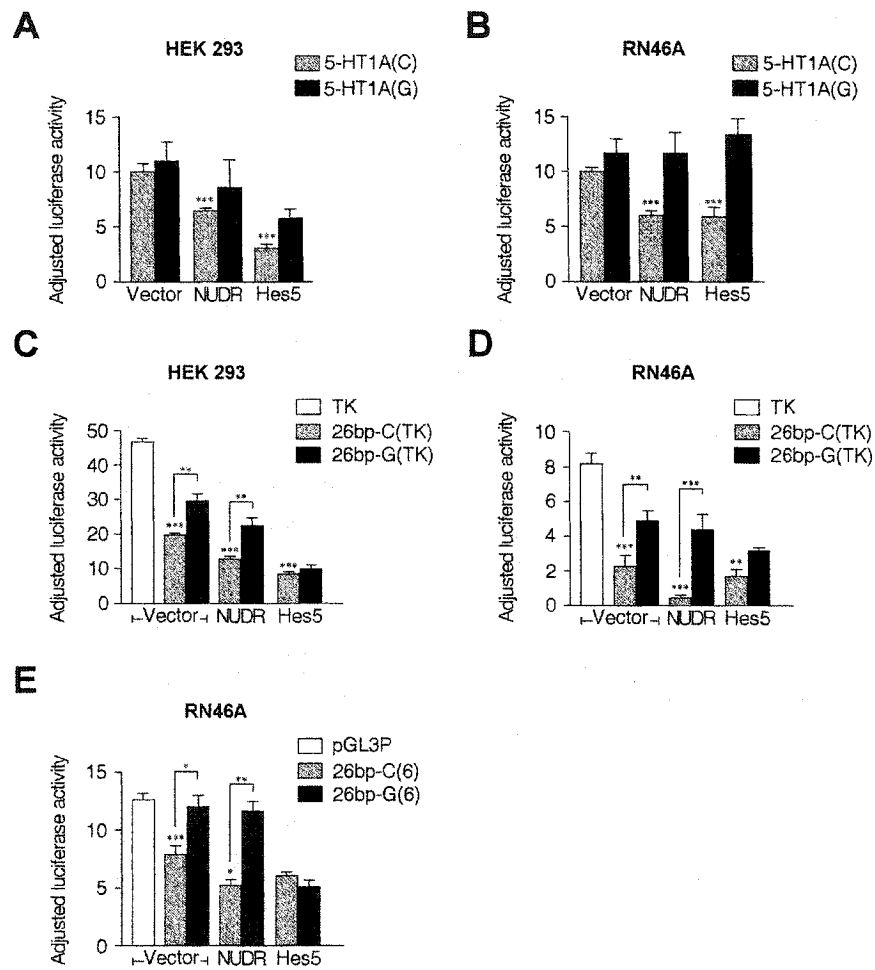


Figure IV.3 – Trans-repression at the palindrome of the 5-HT1A receptor gene by NUDR and Hes5: differential sensitivity to the C(-1019)G polymorphism.

Human 5-HT1A receptor-negative HEK 293 (A, C) or receptor-positive raphe RN46A cells (B, D, E) were cotransfected with the indicated luciferase reporter constructs and vector (pcDNA3), NUDR or Hes5 expression plasmids, as indicated. Data are expressed as adjusted luciferase activity corrected for transfection efficiency by determining the ratio of Luciferase activity/ β -galactosidase activity as described (Ou et al., 2000), and are presented as mean $\pm$ SD of triplicate samples from experiments that were repeated at least twice. Statistical significance compared to vector control (for NUDR and Hes5) or promoter control (for vector) was determined by one way ANOVA; significant differences between C and G alleles were evaluated by unpaired t-test with two-tailed *P* values: **P*<0.05, ***P*<0.005, ****P*<0.0005. (A, B) Repression of the human 5-HT1A promoter (-1128-bp to the initiation ATG) by NUDR and Hes5 in HEK 293 (A) and RN46A cells (B). The C(-1019) or G(-1019) allele of the -1128-bp 5-HT1A promoter-luciferase reporter construct (5-HT1A(C) and 5-HT1A(G), respectively) was cotransfected with vector, NUDR or Hes5. Compared to vector, NUDR and Hes5 repressed 5-HT1A(C), but lacked significant activity at the G(-1019) allele, 5-HT1A(G). (C, D) The 26-bp 5-HT1A palindrome mediates repression of TK promoter by NUDR and Hes5. Reporter constructs containing TK promoter alone (TK, open bars), or the 26-bp 5-HT1A palindrome C or G allele placed upstream (5') of TK (26bp-C(TK) or 26bp-G(TK), respectively) were cotransfected with vector, NUDR, or Hes5 plasmids in HEK 293 (C) or RN46A cells (D). NUDR and Hes5 mediated repression via the 26-bp DNA elements, but only NUDR was entirely specific for the C allele. (E) Repression of SV40 promoter (pGL3P, open bar) at a hexamer of 26-bp

elements in RN46A cells. Six copies of the 26-bp element (26bp-C(6) or 26bp-G(6)) were placed upstream of the SV40 promoter in the pGL3P plasmid. The C-G change blocked basal repression and repression augmented by NUDR but not Hes5.

To address more directly whether the 26-bp 5-HT1A palindrome sequence functions as a repressor element it was placed adjacent to heterologous promoters, TK and SV40. Since the -1128-luc 5-HT1A promoter construct contains multiple DNA elements, repression by NUDR or Hes5 could be mediated by indirect mechanisms such as antagonism of enhancer proteins that act at other elements. The repressor activity of NUDR and Hes5 at the C- or G-allele (26bp-C(TK) or 26bp-G(TK)) of the 26-bp 5-HT1A palindrome sequence placed 5' to the TK promoter was examined in HEK 293 and RN46A cells (Figs IV.3C-D). In vector (pcDNA3) transfected cells, the basal activity 26bp-C(TK) construct was reduced to 45 and 25% of the control (TK) reporter activity in HEK 293 and RN46A cells, respectively, suggesting the presence of endogenous repressors in these cells. Repression of basal transcription activity of the TK promoter was significantly attenuated for the 26bp-G(TK) construct in both cell lines, although its activity was significantly less than for TK promoter alone. This indicates that the 26-bp 5-HT1A palindrome confers basal repression that is sensitive, but not completely inhibited by the C-G polymorphism. Compared to vector, cotransfection with NUDR further repressed TK promoter activity of the 26bp-C(TK) construct ($P < 0.001$) by 35% in HEK 293 cells and 80% in RN46A (Figs IV.3C-D). Transfection with Hes5 significantly reduced the activity of 26bp-C(TK) by 60% in HEK 293 and by only 20% in RN46A cells. Importantly, in both cell lines NUDR failed to repress the 26bp-G(TK) construct compared to vector control, while Hes5-induced repression was not significantly different for C- or G-alleles in either cell line. Thus, both NUDR and Hes5 mediated repression via DNA elements present on the 5-HT1A palindrome sequence, but only NUDR was entirely specific for the C allele.

To further validate the repressor activity of the 26-bp 5-HT1A palindrome at different heterologous promoter and to provide a more sensitive assay, six copies of the palindrome were placed 5' to the viral SV40 promoter (C-allele, 26bp-C(6); G-allele, 26bp-G(6)). To stringently address the specificity of NUDR or Hes5 at the C- or G-allele of the palindrome, RN46A raphe cells were cotransfected with these SV40 reporter constructs and vector, NUDR or Hes5 expression constructs (Fig. IV.3E). In vector-transfected cells, basal transcriptional activity of the 26bp-C(6) construct was reduced by 40% compared to the SV40 promoter control (pGL3P), while the G-allele (26bp-G(6)) lacked basal repressor activity (Fig. IV.3E). Both NUDR and Hes5 repressed transcription of 26bp-C(6) by a further 25-35%, but NUDR failed to repress the 26bp-G(6) construct, whereas Hes5 repressed the G-allele to the same extent as the C allele. The ability of Hes5 to repress at the G allele is consistent with the detectable activity of Hes5 at the G-allele in the yeast one-hybrid assay (Fig. IV.2D). Taken together these results indicate that the C(-1019) allele of the palindrome sequence mediated repression of the 5-HT1A or heterologous (SV40 or TK) promoters, while the G allele was inactive or weakly active. Moreover, both NUDR and Hes5 function as repressors at the 26-bp palindrome of the 5-HT1A receptor, but NUDR is exclusively selective for the C allele.

4.4.4 Detection of NUDR in a protein-DNA complex from raphe nuclear extracts

Since NUDR specifically repressed the C(-1019) allele of the 5-HT1A promoter, we assessed the importance of recombinant or endogenous NUDR in raphe RN46A cells or raphe/midbrain nuclear extracts to recognize the 26-bp palindrome C(-1019) allele (26bp-C) using EMSA and DNase I protection analysis (Fig. IV.4). Incubation of *in vitro*-

transcribed and translated recombinant NUDR bound to labeled 26bp-C and this was competed with 100-fold unlabeled 26bp-C but not the unrelated E2F DNA element (Fig. IV.4A). By contrast, binding of recombinant Hes5 to 26bp-C was detectable but weak (data not shown). Secondly, nuclear extracts from RN46A cells were incubated with labeled 26bp-C and a major protein-DNA complex was competed using unlabeled 26bp-C but not E2F. NUDR was detected in protein-DNA complexes using a specific antibody generated against NUDR (see Methods) that specifically recognizes NUDR on Western blot (see Fig. IV.5A). Incubation of nuclear extracts with anti-NUDR retarded the migration of the major protein-DNA complex (Fig. IV.4B), while rabbit pre-immune serum did not affect the migration (Fig. IV.4C). Incubation of 26bp-C with anti-NUDR alone did not result in non-specific background, indicating that the super-shifted complex contains NUDR. A second faster migrating complex was competed by 26bp-C (Fig. IV.4B), but was not consistently observed (Fig. IV.4C) and was not shifted by NUDR antibody. These results indicate that NUDR is a component of the major protein-DNA complex formed with the 5-HT1A palindrome from raphe RN46A cell nuclear extracts.

To confirm specific binding of NUDR to the 26-bp element in the 5-HT1A promoter, a DNase I protection assay was conducted using an end-labeled DNA fragment spanning the region from -723 to -1128 bp of the 5-HT1A receptor gene (Fig. IV.4D). With increasing concentrations of DNase I, raphe/midbrain extracts protected a progressively restricted region centered between -1016/-1019 bp, as observed for recombinant NUDR protein (R). This region corresponds to the inverted NUDR binding site (TTCG), the last residue of which is altered by the polymorphism to the inactive TTCC

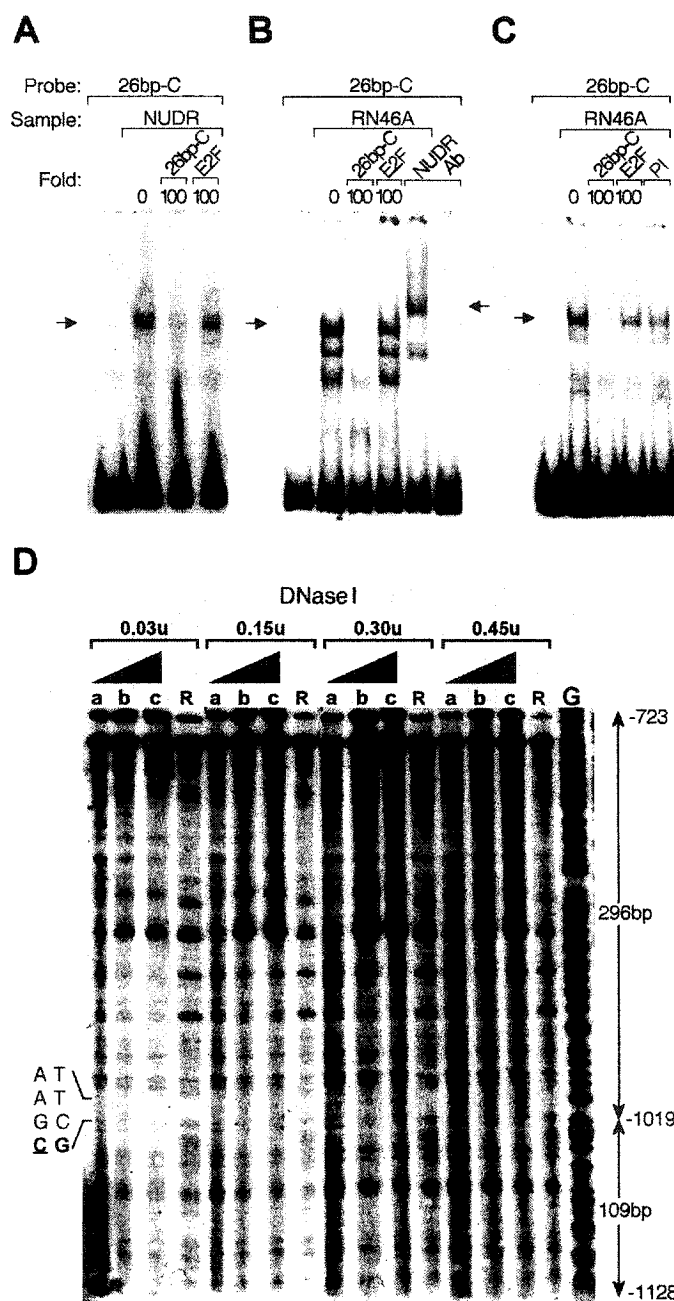


Figure IV.4 – Presence of NUDR bound to the C(-1019) allele in RN46A nuclear extracts.

EMSA was done with the 26bp-C probe using recombinant NUDR or RN46A nuclear extracts as indicated. Unlabelled 26-bp oligonucleotides (26bp-C) or unrelated E2F oligonucleotides (E2F) were added at 100-fold molar excess to the incubation as indicated. (A) *In vitro*-transcribed and -translated recombinant NUDR bound specifically to the 26bp-C probe. (B) Binding of endogenous NUDR to the C(-1019) allele in RN46A nuclear extracts. A major specific complex was observed (left arrowhead) that was supershifted (right arrowhead) upon incubation with anti-NUDR antibody; anti-NUDR alone did not bind to probe. (C) Negative control experiment showing that no supershift of the protein-DNA complex in RN46A cells was induced using rabbit pre-immune serum (PI). (D) Localization of NUDR binding site within the proximal 5-HT1A promoter region by DNase I protection assay. A 405-bp DNA probe spanning the region between -723 and -1128 was treated with the indicated units of DNase I in the absence (a) or presence of 10 µg (b) or 20 µg (c) of raphe/midbrain nuclear extracts, or in the presence of recombinant NUDR protein (R). Midbrain extracts and NUDR protected a region centered between -1016 and -1019, corresponding to the TTCG NUDR recognition sequence and the polymorphic site (C(-1019)G). The nucleotide position within the 5-HT1A 5'-flanking sequence is marked adjacent to the sequencing reaction (G).

sequence. These data indicate that a nuclear protein complex containing NUDR is present in the midbrain and RN46A cells and recognizes specifically the NUDR DNA element that is present within the palindrome sequence of the 5-HT1A receptor gene. The presence of NUDR in RN46A or adult midbrain extracts and its binding pattern suggests that NUDR is the predominant factor bound to the 5-HT1A palindrome sequence in these tissues.

4.4.5 Down-regulation of 5-HT1A receptor protein and binding by NUDR in raphe cells

To further elucidate the role of NUDR in regulation of the 5-HT1A receptor, we used the anti-NUDR antibody to examine the tissue and cellular expression of NUDR protein (Fig. IV.5A). By Western blot NUDR was identified as a single species of 59 kDa, corresponding to its predicted molecular mass (Huggenvik et al., 1998). Anti-Hes5 detected a major 28 kDa protein corresponding to Hes5 (Fig. IV.5B). Both NUDR and Hes5 proteins were expressed in embryonal RN46A cells, but only NUDR was expressed in nuclear extracts from adult brain tissues including midbrain/raphe, hippocampus and cortex. Consistent with this, Hes5 RNA is strongly expressed in embryonic nervous system but is weakly expressed in adult brain (Akazawa et al., 1992). Thus, NUDR functions as a repressor at the 5-HT1A palindrome and is strongly expressed in adult raphe cells.

Having identified the presence of NUDR protein in RN46A cells and midbrain nuclear extracts and its functional importance to regulate 5-HT1A gene transcription (above), we determined whether NUDR regulated the level of functional 5-HT1A receptors in raphe cells. Transcriptional assays indicated that NUDR represses the rat 5-HT1A

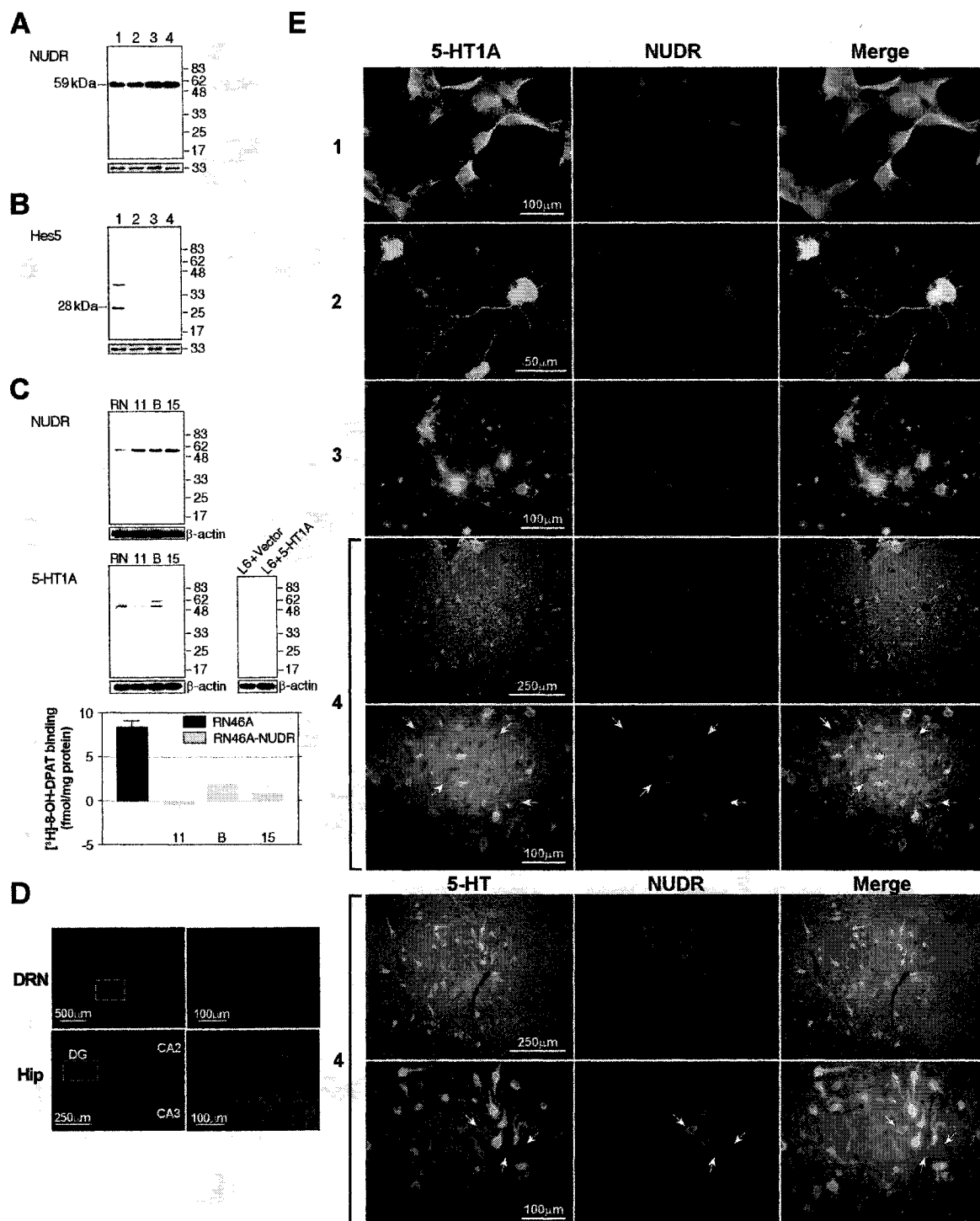


Figure IV.5 – NUDR protein is expressed in 5-HT1A receptor-positive cells and brain regions and regulates 5-HT1A protein expression.

(A, B) Western blot analysis of nuclear extracts from RN46A cells (1), adult rat raphe/midbrain (2), cortex (3) and hippocampus (4) using anti-NUDR (A) or anti-Hes5 (B) antibodies. A common 33-kDa band on Coomassie-stained gel is shown as a loading control. NUDR was expressed in nuclear extracts from RN46A cells and rat brain tissues while Hes5 expression was restricted to RN46A cells. (C) Western blot analysis and [³H]-8-OH-DPAT binding for RN46A cells (RN) stably expressing NUDR (clones 11, B, and 15). Left panels: NUDR reduced 5-HT1A binding and 5-HT1A protein expression. β -actin immunoreactivity was tested to confirm equal loading. Right panel: Western blot analysis showing specific 5-HT1A receptor immunoreactivity in myoblast L6 cells transfected with the rat 5-HT1A expression vector but not with pcDNA3 vector, as indicated. (D) Strong NUDR immunostaining was detected in the dorsal raphe nucleus (DRN) and the CA2, CA3 and dentate gyrus (DG) of the hippocampus (Hip). (E) Colocalization (in yellow) of NUDR (in red) and 5-HT1A receptor or 5-HT (in green) in RN46A cells (1), primary cultures of embryonic hippocampal (2) and cortical (3) cells and dorsal raphe nucleus (4). In the dorsal raphe nuclei, some cells (indicated by an arrow) that stained for NUDR displayed weak 5-HT1A receptor or 5-HT staining.

promoter (data not shown). Thus, rat RN46A cells were stably transfected with NUDR and assayed for 5-HT1A receptor protein and binding activity (Fig. IV.5C). Three clones transfected with NUDR displayed a 4-5 fold increase in NUDR protein levels compared to non-transfected cells. Expression of 5-HT1A protein was detected by immunoblot and was reduced by over ten-fold in clones 11 and 15, but less markedly in clone B. The specificity of the 5-HT1A antibody was verified by absence of signal in receptor-negative rat L6 myoblasts, but presence of the 65 kDa species in L6 cells transfected with a rat 5-HT1A receptor expression plasmid (Fig. IV.5C, right panel). The 55 kDa species detected in raphe RN46A cells likely represents an additional glycosylated form of the 5-HT1A receptor previously described in rat brain (Verdot et al., 1995). The level of specific 5-HT1A binding sites was also reduced (clone B) or abolished (clones 11 and 15) consistent with the relative protein expression in these clones. Furthermore, the level of 5-HT1A RNA detected by semi-quantitative RT-PCR analysis (see Methods) was reduced by $84 \pm 5\%$, $47 \pm 8\%$ or $50 \pm 12\%$ in clones B, 11, and 15 compared to RN46A cells (mean $\pm$ S.E., $n=3$). Thus transcriptional repression by NUDR results in a marked decrease in endogenous 5-HT1A RNA, protein and binding sites in RN46A raphe cells.

4.4.6 Colocalization of NUDR and 5-HT1A receptors *in vivo*.

To provide further evidence supporting a role of NUDR in regulation of 5-HT1A receptors *in vivo*, the precise localization of NUDR was determined by immunofluorescence of brain sections and cells (Figs IV.5D, E). NUDR protein was most abundant in rat brain structures that express 5-HT1A receptors including the dorsal raphe nucleus, frontal cortex (not shown) and pyramidal cells of hippocampal areas CA1 (not shown), CA2, CA3 and

dentate gyrus (Fig. IV.5D). In order to address whether NUDR is expressed in the same cells as 5-HT_{1A} receptors, dual immunofluorescence studies were done. NUDR protein was colocalized with 5-HT_{1A} receptor protein in raphe RN46A cells, and in embryonic (E18) hippocampal and cortical primary cultures (Figs IV.5E, panels 1-3). NUDR immunoreactivity observed in raphe RN46A, hippocampal and especially cortical cells was localized primarily to nuclear or perinuclear areas, whereas 5-HT_{1A} receptors were also present in the cell body and neuronal processes. In both primary cortical and hippocampal cultures and tissue sections over 95% of cells expressing 5-HT_{1A} receptors also showed NUDR immunoreactivity. The presence of NUDR in the nuclei of these 5-HT_{1A}-positive hippocampal and cortical cells is consistent with its role to regulate 5-HT_{1A} receptor expression.

In tissue sections, NUDR and 5-HT_{1A} receptors were colocalized in the majority of cells of the dorsal raphe nucleus (Fig. IV.5E, panel 4). NUDR and 5-HT_{1A} receptor proteins were also colocalized in the hippocampus and cortex (data not shown). In the raphe nuclei, NUDR was also expressed in 5-HT-containing cells. Since 5-HT_{1A} autoreceptors are present on the majority of serotonin neurons, colocalization of NUDR with these markers supports its role to regulate somatodendritic 5-HT_{1A} autoreceptor expression in raphe neurons *in vivo*. NUDR was also present in a small proportion of 5-HT_{1A}- or 5-HT-negative cells in the raphe (Fig. IV.5E, arrows) and other brain regions, suggesting additional roles for NUDR.

We determined whether the NUDR-positive cortical and hippocampal cells represent neurons using dual immunofluorescence for both NUDR and neuronal markers MAP2, the major microtubule associated protein or β 3 tubulin (TuJ1), an early neuronal marker (Fig. IV.6A).

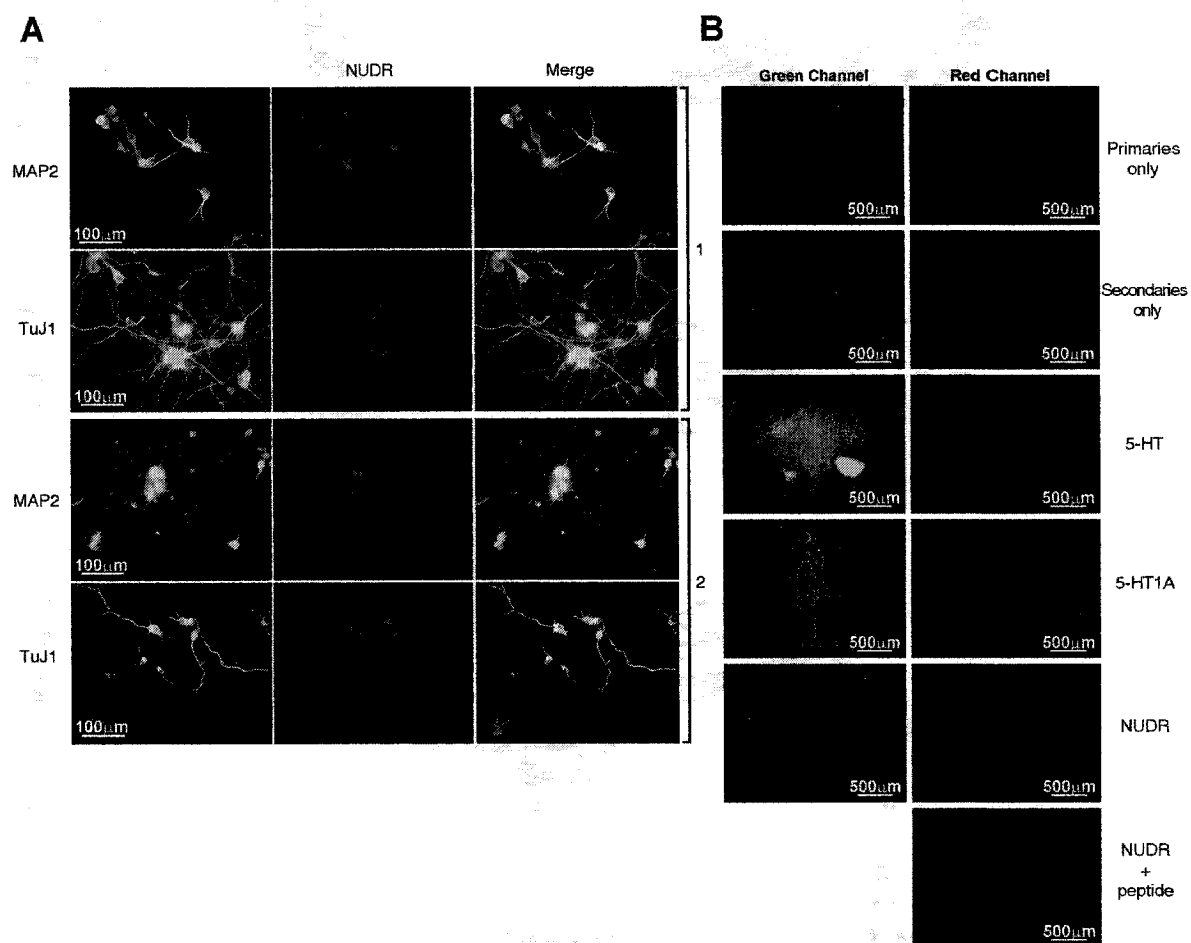


Figure IV.6 – Co-staining of NUDR with neuronal markers and specificity of immunohistochemical staining.

Primary cultures of cortical (1) and hippocampal (2) cells were co-stained with antibodies to NUDR and neuronal markers MAP2 and TuJ1, demonstrating the presence of NUDR in neurons. (B) Specificity of 5-HT, 5-HT1A and NUDR staining in raphe nuclei. Immunofluorescence was visualized through the green and red channels as indicated using primary 5-HT1A and NUDR antibodies in the absence of secondary antibodies, no primary antibodies in the presence of both secondary antibodies as indicated. Specific immunoreactivity through the green but not the red channel using anti-5-HT or anti-5-HT1A antibodies in the presence of both secondary antibodies. NUDR immunoreactivity observed through the red channel was absent in the green channel using both secondary antibodies. NUDR immunofluorescence in the presence of hNUDR (36-51) blocking peptide (100 µg) is greatly reduced.

As expected, MAP2 was localized primarily in the cell body and proximal processes, whereas TuJ1 extended to distal processes of stained cells. Although NUDR was largely co-localized with both neuronal markers, a small proportion of cells stained for NUDR or a neuronal marker only. Thus the majority of NUDR-positive cells in hippocampus and cortical cultures were neurons.

To address concerns of background staining and antibody specificity several control experiments were done using sections of the raphe nuclei (Fig. IV.6B). Fixed sections or cells that were incubated with primary or secondary antibody alone, or pre-incubated with NUDR peptide antigen displayed only background immunoreactivity. Furthermore, no bleed-through of fluorescence between red and green channels was detected. Taken together, these results indicate that NUDR protein is present in serotonergic neurons and is co-expressed with the 5-HT_{1A} receptor in RN46A cells, raphe nuclei, hippocampus and other brain regions corresponding to the reported distribution of 5-HT_{1A} receptors (Albert et al., 1990; Chalmers and Watson, 1991; Pompeiano et al., 1992). The pattern of NUDR expression in brain and its co-expression with 5-HT_{1A} receptors supports a role in transcriptional regulation of the 5-HT_{1A} receptor gene *in vivo*.

4.5 Discussion

These findings constitute the first evidence for a functional genetic association of a 5-HT receptor polymorphism with major depression and suicide. Depressed patients were about twice as likely as controls to have the homozygous G(-1019) genotype, whereas suicide victims were four times as likely to carry the same genotype. Although the results observed in depressed patients are consistent with those observed among suicide

completers, they may not be regarded as independent replications. Accordingly, while major depression is one of the most important predictors of suicide, not all depressed patients commit suicide and not all suicide completers have major depression before their death. In this regard, an interesting question is whether or not the possible effect of the C(-1019)G polymorphism on suicide is conditional on depression. The design of this study, however, does not allow us to address this question and further studies are needed. Additional studies are also needed to validate our findings in independent samples and by means of complementary designs because of the inherent limitations of case-controlled association studies. The C(-1019)G polymorphism of the 5-HT1A receptor gene could be one of several serotonin gene regulatory polymorphisms predisposing to depression and to suicide. For example, altered transcriptional regulation of the 5-HT transporter gene is associated with specific alleles implicated in mental illness (Lesch et al., 1996; MacKenzie and Quinn, 1999). Our results are consistent with postulated roles of the 5-HT1A receptor and dysregulation of the serotonergic system in depression and suicide (Blier and de Montigny, 1994; Albert et al., 1996; Artigas et al., 1996; Mann, 1999; Mann et al., 2001) and represent the first evidence associating specific transcription factors (NUDR, Hes5) with major depression and completed suicide.

These results suggest a molecular mechanism by which the single nucleotide C(-1019)G polymorphism may regulate 5-HT1A gene expression *in vivo* by de-repression of the 5-HT1A promoter in pre-synaptic raphe neurons leading to reduced serotonergic neurotransmission (Fig. IV.7). The C(-1019)G change dramatically impaired transcriptional repression of the 5-HT1A receptor gene by the identified trans-acting proteins NUDR and Hes5 (Fig. IV.3). Although both NUDR and Hes5 repressed the

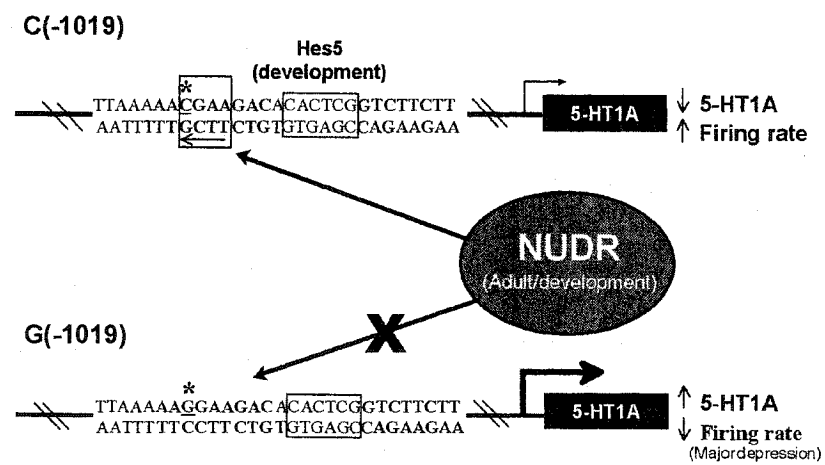


Figure IV.7 - Functional model for derepression at the 5-HT1A promoter by the C(-1019)G polymorphism.

The C(-1019)G polymorphism prevents binding of the transcriptional repressor NUDR resulting in enhanced 5-HT1A receptor expression in raphe neurons that may occur in development to adulthood. Increased expression of 5-HT1A somatodendritic autoreceptors would be expected to reduce serotonergic tone, which is associated with major depression. The C(-1019)G polymorphism also impairs repression by Hes5 which may play a role as a developmental regulator of 5-HT1A gene expression.

5-HT1A receptor gene, the repressor activity of NUDR was the most sensitive to the C(-1019)G polymorphism associated with depression and NUDR was the major factor bound to the C(-1019) site in nuclear extracts from serotonergic raphe neurons. In rat RN46A raphe cells, NUDR repressed both human and rat (data not shown) 5-HT1A promoter-luciferase constructs. Importantly, stable expression of NUDR greatly reduced the expression and binding of endogenous 5-HT1A receptors and RNA indicating that NUDR negatively regulates both 5-HT1A gene transcription and receptor expression. In the dorsal raphe nucleus and RN46A cells, NUDR protein is co-expressed with the 5-HT1A receptor and binds to the 5-HT1A promoter at the polymorphic TTCG site, implicating NUDR in 5-HT1A regulation *in vivo* as well as in RN46A cells. Since RN46A cells were originally derived from e13 raphe neurons (White et al., 1994) and NUDR is also expressed in adult brain (LeBoeuf et al., 1998), NUDR may repress the 5-HT1A receptor gene throughout development and into adulthood. Because NUDR immunoreactivity is also present post-synaptically in 5-HT1A-expressing hippocampal and cortical neurons, de-repression of the 5-HT1A gene should be induced by the G(-1019) allele in these cells. However NUDR can act as a repressor or an enhancer, depending on cell type and promoter sequence (Huggenvik et al., 1998; Bottomley et al., 2001). Interestingly, our preliminary results suggest that NUDR enhances, rather than represses 5-HT1A transcriptional activity in various hippocampal and septal cells (data not shown). Thus the G(-1019) allele de-represses 5-HT1A transcription presynaptically, but may have the opposite effect to reduce NUDR-enhanced 5-HT1A transcription in post-synaptic cells. The net effect of these changes would be a reduction in serotonergic neurotransmission.

The role of Hes5 to regulate 5-HT1A receptor expression *in vivo* is less clear. Hes5 may be involved in developmental regulation of 5-HT1A receptor expression, as Hes5 RNA is strongly expressed in the embryonic nervous system and declines in the adult (Akazawa et al., 1992). Interestingly, in early post-natal development Hes5 RNA is expressed in a sub-population of cells of the hippocampal dentate gyrus and subventricular zone, and in the cortex (Stump et al., 2002). As an important mediator of Notch signaling (Kageyama and Ohtsuka, 1999), Hes5-induced repression could be important for the actions of Notch1 to inhibit dendritic elongation and promote branching (Sestan et al., 1999; Redmond et al., 2000). Hes5-mediated repression of 5-HT1A expression would be expected to reduce inhibitory somatodendritic signaling of the 5-HT1A receptor.

To assess more directly its role in the regulation of the 5-HT1A receptor *in vivo*, raphe-specific transgenic expression or gene knockout of NUDR will be necessary. Since the C(-1019)G polymorphism is not present in rat or mouse genes, direct assessment of its function in animals will be difficult. Ultimately, it will be important to correlate this polymorphism with the level of 5-HT1A receptor expression in human patients. To date, it has been difficult to address whether gene regulatory polymorphisms result in altered expression of the protein product in human tissue. For example, although the 5-HT transporter promoter polymorphism was identified as functional in transformed human lymphoblasts (Lesch et al., 1996), a significant correlation between the polymorphism and 5-HT transporter protein expression in human postmortem tissues has not been reported (Mann et al., 2000). This undoubtedly reflects in part the complex nature of gene regulation *in vivo* (via multiple enhancers, repressors, and hormone response elements) and the presence of compensatory mechanisms (e.g., desensitization) that are recruited to

normalize expression. However, the recent demonstration that people with this 5-HT transporter polymorphism show enhanced amygdala metabolism in response to aversive stimuli (Hariri et al., 2002) highlights the significant neurological consequences that can result from small promoter variations.

The level of expression of the 5-HT_{1A} receptor has been implicated in mental illnesses (Blier and de Montigny, 1994; Albert et al., 1996; Artigas et al., 1996). Reduction of forebrain 5-HT_{1A} receptor expression is correlated with anxiety, as seen in 5-HT_{1A} gene knockout mice that display anxiety-like behaviours (Heisler et al., 1998; Parks et al., 1998; Ramboz et al., 1998). Inducible expression of 5-HT_{1A} receptors in the forebrain of 5-HT_{1A}^{-/-} mice between post-natal days 5-21 was sufficient to restore anxiety-like behaviour to normal (Gross et al., 2002), indicating the importance of developmental regulation of 5-HT_{1A} receptors. Conversely, a specific increase in midbrain raphe 5-HT_{1A} autoreceptors is correlated with depression and suicide (Stockmeier et al., 1998). Furthermore, down-regulation or antagonism of the 5-HT_{1A} autoreceptor by co-administration of pindolol enhances the clinical efficacy of antidepressant treatments (Artigas et al., 2001). However, recent PET studies using the 5-HT_{1A} antagonist ¹¹C-WAY100,635 suggest that 5-HT_{1A} receptors may be reduced in patients with bipolar rather than unipolar depression. Technical issues of image acquisition, data analysis and study design have contributed to some of the apparent inconsistencies across studies (Mann et al., 1996; Lowther et al., 1997; Mann, 1999; Drevets et al., 2000; Sargent et al., 2000). Another recent study reported a 43% decrease in 5-HT_{1A} autoreceptor number in the midbrain of depressed suicide victims compared to controls (Arango et al., 2001). However this decrease was possibly due to a reduction in raphe cell number, which would

decrease serotonergic neurotransmission. These disparate findings in depressed patients almost certainly reflect the heterogeneity of the disorder and the fact that increase in 5-HT_{1A} autoreceptors is only one of multiple mechanisms that can contribute to a decrease in serotonergic transmission that is associated with depression. Our results are consistent with a model that predicts that incorporation of the C(-1019)G change would result in derepression of the 5-HT_{1A} receptor gene in serotonergic raphe cells, leading to overexpression of the 5-HT_{1A} autoreceptor to reduce serotonergic neurotransmission (Albert et al., 1996) (Fig. IV.7). The association of the G(-1019) allele with major depression and suicide suggests that impaired repression of the 5-HT_{1A} receptor could contribute to a predisposition towards unipolar depression and its most severe outcome. However, further studies are needed to better characterize the association of the G(-1019) polymorphism with additional mental illnesses or behavioural phenotypes and its correlation with altered expression of the 5-HT_{1A} receptor *in vivo*, or in post-mortem tissues from depressed patients.

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CHAPTER V – DISCUSSION

Based on the importance of 5-HT_{1A} receptor expression in the serotonergic system and mood disorders, I hypothesized that transcriptional regulatory mechanisms involved in basal expression of this gene may play a significant role in the etiology of major depression and related disorders. Furthermore, the chronic time course required for clinical efficacy of antidepressant treatments and their effect on 5-HT_{1A} receptor expression suggested that transcriptional modulation of this gene may contribute to antidepressant effectiveness. Thus, the main focus of my thesis was to elucidate the mechanisms involved in transcriptional regulation of the human 5-HT_{1A} receptor gene, and how these mechanisms might be altered in depression, leading to elevated levels of 5-HT_{1A} autoreceptors and reduced serotonergic activity in depressed patients.

I first characterized the regions most important for basal regulation of the transcription of the 5-HT_{1A} receptor. The results in Chapter II identified a powerful repressor region consisting of three repressor elements, two copies homologous to the novel rat DRE (Ou et al., 2000) and a consensus RE-1 (Schoenherr et al., 1996), confined to a region between -1624 and -1550 bp that control the expression of the human 5-HT_{1A} receptor gene. I investigated the relative contribution of each of these elements to gene repression in neuronal cells endogenously expressing the 5-HT_{1A} receptor, and non-neuronal cells, which do not express the receptor. Under basal conditions, all three elements maintain strong repression at the 5-HT_{1A} receptor gene independently of cell type. However, I showed that neuronal and non-neuronal cells utilize different mechanisms to repress 5-HT_{1A} expression. In neuronal cells, the repressor protein

Freud-1 mediates repression via the DRE repressor element in a manner that is independent of histone deacetylation of the gene. By contrast, in non-neuronal cells, where Freud-1-mediated repression appears to be dispensable (Ou et al., 2000), REST and one or more repressor(s) recruit HDAC activity to silence 5-HT1A receptor gene expression. Thus, I postulate that recruitment of distinct, cell-specific co-repressor complexes involved in chromatin remodeling provides a greater level of regulation that may allow for a highly refined determination of cell specificity. The presence of two similar copies of the DRE and one of the RE-1 in a restricted 74-bp region, and their conservation throughout evolution, almost certainly reflects the importance of a tight negative regulation in this area. I have screened this region for polymorphisms that could associate with mood disorders, but have identified none thus far, although a rare polymorphism cannot be excluded. Given the complexity of the processes implicated in the notably strong repression at this site, I predict that genetic alterations in this region would have too severe consequences to be detected in humans. However, recent findings emerging from the Albert laboratory suggest that the dopamine D2 receptor gene, which also contains a DRE site in the second intron that appears to be regulated by Freud-1, also contains two functional polymorphisms in adjacent regions to the DRE. The proximal polymorphism appears to impair Freud-1 binding to the DRE and reduces its repressor activity at the D2-DRE. The presence of the D2 polymorphism could contribute to elevated levels of D2 receptors observed in schizophrenia (Rogaeva et al., 2002). By analogy, a 5-HT1A promoter polymorphism even outside of the DRE element may weaken repression by Freud-1 and predispose to depression in humans.

Secondly, in Chapter III I described the cloning by yeast one-hybrid screening, and the characterization of Freud-1 as a new DNA binding protein that strongly represses the 5-HT1A receptor promoter in neurons. Freud-1 and 5-HT1A RNA and protein are coexpressed in the raphe nuclei but lower levels of 5-HT1A receptors in raphe cells are detected in the presence of Freud-1, consistent with its role in reducing 5-HT1A receptor gene expression. Importantly, Freud-1 repressor activity is modulated by CAM kinases which represents a novel mechanism for regulation of gene expression by calcium signalling.

Finally in Chapter IV, I have characterized a novel functional C(-1019)G 5-HT1A receptor polymorphism located in a region with significant silencing activity (see Appendix A), and described an increase in frequency of the homozygous G(-1019) allele in both depressed and suicide victims versus normal controls. I provided evidence that the polymorphism is located within a binding site for the transcription factors NUDR and Hes5 that appear to function as repressors in raphe cells. The presence of the homozygous G(-1019) allele in the experimental population is predicted to lead to decreased binding and transcriptional repression by NUDR and Hes5, suggesting that these populations might exhibit derepression of the 5-HT1A receptor in tissues which express these transcriptional regulators. NUDR function is more sensitive to the polymorphism, and NUDR but not Hes5 is expressed in the adult brain nuclear extracts that I examined. Others have previously shown that Hes5 RNA is weakly expressed but detectable in whole brain tissue (Akazawa et al., 1992) (hence its presence in the cDNA library that we screened), although protein expression has not been shown in adult. Therefore I focused mainly on the role of NUDR to regulate 5-HT1A receptor expression. My results demonstrate that NUDR

markedly decreases 5-HT1A receptor RNA (see Appendix B), protein and binding sites in raphe RN46A cells and that *in vivo* raphe cells strongly express NUDR colocalized with 5-HT1A receptor. Thus, the data convincingly support the role of NUDR as a sequence-specific transcriptional repressor at the polymorphic site of the 5-HT1A receptor gene. My data indicates the 5-HT1A DREs play a greater role in the regulation of basal 5-HT1A receptor transcription since deletion of one or the other DRE dis-inhibited basal transcription in raphe cells whereas the polymorphism had little effect on basal transcription of the 5-HT1A promoter. By contrast, repression of a 209-bp segment was impaired by the polymorphism G-allele (Appendix A). This could be due to the presence of other strong regulators in the larger promoter region (Parks and Shenk, 1996) that may mask the effect of the C to G transition on basal transcription. Alternately, the polymorphism may incompletely block the activity of repressors such as Hes5, which is expressed in RN46A cells used for this assay. Further mutation of this element, and examination in adult raphe-derived cells that lack Hes5 will be necessary to fully address the importance of the element in 5-HT1A autoreceptor expression.

It should be noted that the experimental design of this thesis was in some ways limited by the unavailability of well-characterized human 5-HT1A-expressing cell lines. Therefore, because human gene segments were studied in rat cells, and because cell lines rather than real neurons were used, some aspects of cell-specific expression may not have been detected in these assays. However the key aspects I described that include the arrangement of promoter, DRE/RE-1 elements and their regulation of 5-HT1A, and the regulation of 5-HT1A by NUDR are conserved.

The work that I presented has significant and interesting implications to understand the mechanisms involved in the etiology and the treatment of major depression. Increased 5-HT_{1A} autoreceptor expression is associated with depression and suicide (Stockmeier et al., 1998), and down-regulation of this receptor is postulated to permit the action of antidepressants to enhance serotonergic neurotransmission (Albert et al., 1996; Pineyro and Blier, 1999). 5-HT_{1A} receptor partial agonists such as buspirone are effective anti-anxiety agents (Charney et al., 1990). Oppositely, gene knockout of the 5-HT_{1A} receptor consistently generates mice with an anxiety phenotype (Heisler et al., 1998; Parks et al., 1998; Ramboz et al., 1998). This phenotype can be rescued by early post-natal expression of the receptor in hippocampal/prefrontal regions (Gross et al., 2001). Thus regulation of the basal level of expression of the 5-HT_{1A} receptor may play a role in neurodevelopmental wiring of anxiety or depression pathways, in addition to regulating the level of 5-HT_{1A} receptor expression and serotonergic activity in the adult.

Freud-1 expression in embryonal-derived RN46A and E18 primary cultures of cortical and hippocampal neurons suggests that it may be involved in developmental regulation of gene transcription in the brain. Freud-1 RNA is also detected in cortical progenitors and stem cells (Appendix C). NUDR and Hes5 are both expressed in embryonal RN46A cells and during development (Gross and McGinnis, 1996; Huggenvik et al., 1998; Ohtsuka et al., 2001), hence either repressor could also be a key regulator of 5-HT_{1A} receptor expression during development. NUDR has been shown to interact with LMO-Clim complexes, which are transcription factors known to be involved in development and patterning in several systems (Sugihara et al., 1998; Bulchand et al., 2003). Hes5 is expressed in a sub-population of cells in the hippocampal dentate gyrus and

subventricular zone, and in the cortex in early post-natal development (Stump et al., 2002). As an important mediator of Notch signalling, Hes5-induced repression could be important for the actions of Notch1 to inhibit dendritic elongation and promote branching (Ohtsuka et al., 1999; Sestan et al., 1999; Redmond et al., 2000). Hes5-mediated repression of 5-HT1A expression at earlier stages in development when Hes5 RNA and protein are abundantly expressed would be expected to reduce inhibitory somatodendritic signalling of the 5-HT1A receptor. The C(-1019)G functional polymorphism I have identified might be more important to regulate 5-HT1A expression during pre- or post-natal development leading to behavioural changes in the adult. As reported by Gross et al., 2002, inducible forebrain expression of the 5-HT1A receptor up to P15, but not in the adult 5-HT1A knockout mouse, rescues the behavioural phenotype to reduce anxiety related behaviours in these mice. By analogy, expression of the 5-HT1A autoreceptor during early post-natal development could be a critical determinant of predisposition to depression, regardless of receptor levels in adulthood.

Alternatively, adult brain NUDR may also be important to regulate 5-HT1A receptor gene expression later in life, and I suggest a model whereby the presence of the homozygous G(-1019) allele prevents binding and repression by NUDR and leads to decreased serotonergic neurotransmission and increased risk of depression and suicide. However, because NUDR is expressed ubiquitously in the brain, one could think that if the thesis is correct, the data would suggest an up-regulation of 5-HT1A receptors throughout the brain. Therefore, an important concern regards NUDR activity at post-synaptic 5-HT1A receptors and the possibility that NUDR-mediated repression at these sites may counteract the modest change observed in presynaptic sensitivity. Interestingly, I obtained

data indicating that NUDR acts as an *enhancer* of 5-HT1A receptor promoter activity in post-synaptic (non-serotonergic) 5-HT1A expressing cell lines such as septal SN-48, NG108-15 and hippocampal H19-7 cells (Appendix D and data not shown). Enhancer as well as repressor activities of NUDR have been described (Huggenvik et al., 1998; Michelson et al., 1999). The reasons for this are still not clearly understood, but appear to depend on the cellular context. Differences in post-translational modifications of NUDR, such as the extent of basal phosphorylation, might also contribute to varied roles of NUDR in different cells and tissues. Along with a SAND domain that is involved in DNA binding and chromatin-associated transcriptional regulation (Bottomley et al., 2001), NUDR also contains an HLH motif that is responsible for dimerization. Heterodimerization between NUDR and other SAND-containing proteins that may have differential pre- and post-synaptic expression may account for the ability of NUDR to either repress or activate in different cell types. Intriguingly, in SN-48 cells NUDR is found exclusively in the nucleus (Appendix D). Oppositely, in raphe cells, NUDR is mainly perinuclear. Subcellular localization of NUDR may therefore play a role in the extent of repressor/enhancer activities in pre/post-synaptic densities respectively. Although these data will need to be confirmed and extended, they suggest that while the C(-1019)G polymorphism de-represses the 5-HT1A receptor presynaptically, it may actually decrease 5-HT1A receptor expression postsynaptically to further decrease 5-HT neurotransmission in subjects with the homozygous G-allele.

Although still speculative at this point, a mechanistic explanation for antidepressant treatment may emerge from the results presented in this thesis. The CAMK-dependent regulation of Freud-1 suggests that calcium signalling may activate transcription of the

5-HT_{1A} receptor *in vivo*. Conversely, activation of 5-HT_{1A} autoreceptors by antidepressant treatment could utilize calcium- and Freud-dependent mechanisms to desensitize the 5-HT_{1A} autoreceptor (Fanelli and McMonagle-Strucko, 1992; Hervas et al., 2001). Activation of 5-HT_{1A} autoreceptors, such as seen following acute antidepressant treatment, decreases $[Ca^{2+}]_i$ by G protein-mediated inhibition of N- and P/Q-type calcium channels, and indirectly by hyperpolarization due to opening of inwardly rectifying potassium channels (Chen and Penington, 1996; Bayliss et al., 1997a, b). These 5-HT_{1A}-mediated actions to decrease $[Ca^{2+}]_i$ would reduce basal CAMK-mediated inhibition of Freud-1. Unopposed Freud-1 activity in turn may repress raphe 5-HT_{1A} receptor gene expression, leading to downregulation of the receptor and restoration of serotonergic neurotransmission. On the other hand, it has been demonstrated that chronic treatment with SSRIs can stimulate the activity of CAMKII in the hippocampus (Popoli et al., 1995). A straightforward prediction of this would be that inhibition of Freud-1 by CAMKII for example, may increase 5-HT_{1A} receptor expression in post-synaptic areas such as the hippocampus, which would have net effect of increasing serotonergic neurotransmission. Further experiments will be needed to test these hypotheses.

Based on the observation that NUDR is mainly cytoplasmic or perinuclear in raphe cells, and because nuclear localization, presumably, is required for NUDR-mediated transcriptional repression (Michelson et al., 1999), I hypothesised that antidepressants may decrease 5-HT_{1A} autoreceptor expression by facilitating the translocation of NUDR to the nucleus. To test this hypothesis, I treated RN46A cells with 8-OH-DPAT to activate 5-HT_{1A} receptors, mimicking antidepressant treatment. Some of my very preliminary results, that will require confirmation, indicate that NUDR may translocate to the nucleus

following 5-HT_{1A} receptor agonist treatment (see Appendix E). Recent studies have demonstrated that chronic antidepressant therapy upregulates the cAMP cascade in hippocampus and cerebral cortex, including increased particulate levels of PKA, and upregulating the function and expression of CREB. Thus, select changes in gene expression (e.g., CREB-regulated genes) may take place during antidepressant treatment and may account for the therapeutic effects of drugs (Popoli et al., 2001). Similarly, inhibition of cAMP and PKA signalling following activation of 5-HT_{1A} receptors in raphe cells may lead to cellular changes in NUDR-interacting proteins or NUDR itself that result in its translocation to the nucleus to modulate 5-HT_{1A} gene transcription. Further investigations using PKA or PKC activators and inhibitors will be necessary to address this possibility.

If true, this observation would not be the first to suggest that activation of 5-HT_{1A} receptors is a critical component in the mechanism of action of antidepressants. A recent study has demonstrated that disruption of antidepressant-induced neurogenesis in the hippocampus blocks the behavioural response to drug therapy (Santarelli et al., 2003). Furthermore, since 5-HT_{1A} knockout mice are insensitive to the neurogenic and behavioural effects of fluoxetine, it is proposed that SSRIs efficacy is dependent upon 5-HT_{1A} activation. By contrast, 5-HT_{1A} null mice respond to TCAs suggesting that these drugs may act through 5-HT_{1A}-independent pathways. Analogously, various drug regimens may have different effects on NUDR activity/translocation, which may help explain together with the antidepressant-mediated neurogenesis study, why some patients respond better to particular drugs and not others.

Despite the fact that antidepressants will generally offer a relatively high degree of efficacy in a majority of depressed patients, an appreciable proportion still will not respond to treatment under seemingly similar conditions. Pharmacogenetic studies are demonstrating a significant influence of genetic mechanisms on the efficacy of clinically prescribed drugs (Mancama and Kerwin, 2003). The C(-1019)G polymorphism may help enlighten the basis for inter-individual variation in treatment response. Effectively, even if NUDR translocates to the nucleus following antidepressant administration, I have shown that binding and repression by NUDR at the homozygous G(-1019) allele are impaired in raphe cells. Therefore, the C(-1019)G polymorphism may serve as an indicator of responsiveness to antidepressants. To address this, I have grouped 119 clinically depressed patients as either “responsive” or “non-responsive” to pharmacotherapy and according to their genotype. While most depressed patients analysed were responsive to chronic antidepressant treatment (Appendix F), in the “responsive group” less patients carried the homozygous G(-1019) genotype, and there were twice as many patients with this genotype compared to the homozygous C(-1019) genotype in the “non-responsive group”. This suggests that depressed patients with the homozygous G(-1019) genotype are less likely to respond to antidepressant medication than those homozygous for the C allele. I further subdivided these patients according to the drug regimen received, and analysed drug efficacy to decrease the HAM-D 17 item score following chronic administration (Appendix G). A trend towards a higher HAM-D score was observed in patients homozygous for the G allele. However, when all patients were grouped together to increase the sample size, the HAM-D score became significantly higher in patients carrying the homozygous G(-1019) genotype. Given the small sample size for each drug treatment, a statistically significant

association of drug-specific response with genotype at the -1019 polymorphic site was not observed, although several treatments had a trend towards lower response in homozygous G(-1019) allele. The polymorphism may associate with the severity of the disease but I did not detect an association in this restricted population sample (Appendix H). To keep in consideration, is the fact that all patients analysed were originally selected as severely depressed which may have masked the effect of the polymorphism on the severity of the disease. Therefore, this study will need to be extended and replicated. As data accumulates, it may be possible to correlate the C(-1019)G polymorphism with properties such as severity or drug treatment response.

The possible diagnostic or prognostic implications of the polymorphism for depression or antidepressant treatments is particularly important, since many patients respond inadequately to pharmacological intervention while others react adversely. Clinically, this often results in a trial and error approach to find the best treatment regimen, which can extend the period of depression and is also often correlated with increased suicide risk. It is estimated that 16% of the normal population is susceptible to at least one episode of major depression in their lifetime. This prevalence is probably an underestimate, since many episodes of major depression may go unreported due to the social stigma attached to mental illness. Therefore, the C(-1019)G polymorphism may provide a useful genetic marker for a predisposition to major depression. A sizable proportion of normal individuals (4-12%) display the homozygous G(-1019) genotype. A genetic test that is predictive of depressive tendencies in "normal" non-depressed subjects may allow for prophylactic treatment to avoid episodes of depression. These measures could include educating patients about potential risk factors for them to privilege a

psychological stress-free environment, counselling, or early treatment prior to development of an episode of major depression. The understanding of how 5-HT_{1A} receptor gene expression may be altered by impaired repressor protein interaction at the C(-1019)G polymorphism could lead to new or better/individualized therapeutic avenues for the treatment of major depression some of which may target those repressors directly. Finally, antidepressant compounds that regulate the 5-HT_{1A} receptor, are effective (in addition to major depression) in the treatment of generalized anxiety disorder, panic disorder, obsessive compulsive disorder, premenstrual dysphoric disorder (PMDD) and eating disorders which may be associated with this polymorphic marker. The C(-1019)G polymorphism could hence be used as a possible diagnostic or prognostic marker for mental illness and behavioural disorders, as well as a predictive marker of behavioural traits.

The possible implication of the C(-1019)G polymorphism in other serotonergic-related mental illnesses will need to be investigated in future studies. For example, a similar genetic study design as for major depression and suicide could be used to investigate the role this polymorphism may play in PMDD, which affects 3-8% of women in reproductive age. SSRIs are effective in the treatment of PMDD which supports the hypothesis that PMDD is associated with a dysregulation of the serotonergic system (Steiner and Born, 2000; Carr and Ensom, 2002). A search for other polymorphisms within the 5-HT_{1A} receptor regulatory region may also prove to be useful in identifying new genetic markers that may associate with major depression or related illnesses. In the depressed or suicide population samples that we have analysed, a considerable proportion of individual were homozygous for the C(-1019) allele. This is not surprising given that

depression is a complex disorder that most certainly involves multiple environmental and genetic factors (Champoux et al., 2002; Caspi et al., 2003). Thus, other polymorphisms within the 5-HT1A receptor gene, or other genes that regulate the serotonin, noradrenergic or other neurotransmitter systems (e.g., other receptors, transporters, or biosynthetic genes) may be mutated and lead to decreased levels of serotonin that are associated with major depression. The T(-102)C polymorphism in the 5-HT2A receptor has been associated with suicidal ideation in patients with major depression (Du et al., 2000). The short allele of the long polymorphic repeat of the SERT gene has been associated with reduced transcriptional activity and anxiety-related behaviour in human (Lesch et al., 1996), whereas the long allele is associated with obsessive-compulsive (Bengel et al., 1999) disorder and completed suicide (Du et al., 1999). More recently, this same polymorphism was also shown to moderate the influence of stressful life events on depression (Caspi et al., 2003). Association studies between the C(-1019)G polymorphism and these or other psychological parameters linked to mood disorders will be of great interest to help evaluate the predisposition of an individual to major depression.

The human genetic findings that led to association of the C(-1019)G polymorphism with major depression and suicide came from two independent populations by two independent research groups, substantiating the importance of this polymorphism in mood disorders. However, because of the limitations of association studies, our results will require replication in other population samples. An example of such limitation is the finding (by Arias et al., 2002) of a lack of association between this C(-1019)G polymorphism and major depression in a sample of individuals collected in Spain. In this study, control and MDD subjects were only matched on the basis of geographic location of

subject's grand parents, therefore ethnicity and/or sex could have influenced the results and could account for the disparate findings between this study and ours. Differences in the control groups between the two studies are noted and may reflect differences in the prevalence of the alleles in different populations. It has commonly been documented that differences in ethnicity can affect the relative abundance of some alleles (Liu et al., 1998; Padyukov et al., 2001b; Padyukov et al., 2001a). It is also possible that in some populations significant effects of the polymorphism in depression cannot be detected because of its higher prevalence in the general population. Moreover, while most subjects were males in our suicide cohort (with the highest prevalence for the G(-1019) allele), there were more subjects (75% of patients) in the Arias (2002) study who were females, raising the possibility that the polymorphism may affect differently men and women for depression risk. It will therefore be interesting to look at the baseline prevalence of the C(-1019)G polymorphism in different ethnicity and gender groups.

I demonstrated that in rat raphe RN46A cells stably transfected with NUDR, 5-HT1A RNA is diminished and there is a substantial reduction in both 5-HT1A protein and binding, further demonstrating that NUDR regulates protein expression, not only transcription activity of the 5-HT1A receptor. This suggests that NUDR represses both 5-HT1A transcriptional activity and protein levels and that the polymorphism would thus lead to increased proteins in raphe cells that express NUDR abundantly. We have yet to show directly that the polymorphism affects 5-HT1A receptor levels *in vivo*. Future studies will be necessary to address whether changes in receptor or transporter proteins in human brain (*in vivo* or postmortem) can be associated with promoter polymorphisms. To date, efforts to demonstrate this association have not been successful due to the number of

transcriptional and post-transcriptional factors that regulate levels of receptor or transporter *in vivo*. An informative precedent is set by the well known 5-HT transporter polymorphism which was associated with an anxious behavioural phenotype, but for which *in vivo* studies have offered conflicting results. Although the 5-HT transporter polymorphism was identified as functional in human transformed lymphoblast cell lines (Lesch et al., 1996), studies attempting to show a correlation between the polymorphism and SERT protein expression in human postmortem tissues have not yielded significant correlation. This undoubtedly reflects the complex nature of gene regulation *in vivo*, and the presence of compensatory mechanisms that are recruited to normalize expression.

PET studies have been performed in a very limited number of individuals, but do not fully support the predictions of the current study. In those studies, reduced 5-HT_{1A} ligand binding was demonstrated in depressed patients in different regions of the brain, but most prominently in the midbrain raphe. This may be because the ¹¹C-WAY 100635 used to measure 5-HT_{1A} binding has a halftime of 20 min., so equilibrium binding may not be achieved. In addition, binding is not modified by treatment with SSRI and it is not clear which receptor sites are measured (e.g., internalized, glial) using this technique (Drevets et al., 1999; Drevets et al., 2000). Moreover, most patients in these studies were suffering from bipolar disorder rather than major depression. This may have contributed to the disparate findings in 5-HT_{1A} receptor levels in depressed individuals (Stockmeier, 1997; Drevets et al., 1999; Drevets et al., 2000). Finally, to keep in mind is the possibility that decrease in 5-HT_{1A} receptors observed in these studies may be due to a reduction of 5-HT neurons in the raphe nuclei of depressed individuals (Arango et al., 2001). It will be interesting to repeat these experiments in a greater number of patients suffering strictly

from major depression, and to correlate the results with regards to 5-HT1A receptor levels with the genotype carried by these patients at the polymorphic -1019 site.

Although the C/G polymorphism observed in the human 5-HT1A promoter does not occur naturally in the rat 5-HT1A promoter, the NUDR/Hes5 responsive element is present in rat. In fact, by transient transfection using the rat -1519 bp luciferase reporter construct, I have been able to demonstrate that both NUDR and Hes5 mediate repression at the rat 5-HT1A promoter to a comparable extent ($\cong 40\%$) as what seen with the human 5-HT1A promoter (Appendix I). This finding validates the use of rodent-derived animal models to study the mechanism of NUDR and Hes5 action. However, since the mouse 5-HT1A gene does not have the polymorphism, it will be necessary to introduce a large segment of the human gene to “humanize” the mouse 5-HT1A receptor gene. This is an ambitious project that remains premature, since it is not clear how large a fragment will be required to direct correct tissue-specific expression of the 5-HT1A gene. Ultimately, since appropriate animal models of the human gene are not readily available, the correlation between depression, the polymorphism, and receptor expression will require long-term collection of human postmortem brains of depressed victims for which strict clinical and medication (ideally drug-free) status will have been established.

NUDR and Freud-1 knockout mice showing impaired transrepression of 5-HT1A gene expression and hence increased receptor levels would greatly strengthen our hypothesis. However, both NUDR and Freud-1 are also expressed in peripheral tissues (Appendix J and Fig. III.5, respectively) as well as in 5-HT1A and 5-HT negative cell line suggesting other roles for these proteins, and therefore the gene knockouts may have other phenotypic abnormalities that could complicate the analysis. I have detected very high

levels of NUDR immunoreactivity in the subcommissural organ (SCO) (Appendix K). The SCO receives neural inputs from different neurotransmitter systems including dopamine, noradrenaline, and different neuropeptides such as vasopressin, vasotocin, oxytocin, angiotensin, substance P, somatostatin, and galanin (Jimenez et al., 2001). Interestingly, the most important input to the SCO is a serotonergic innervation from the DRN that appears to inhibit its activity (Sakumoto et al., 1984). The SCO is an enigmatic secretory gland of the brain, which is believed to be derived from ependymal (glial) precursor cells and is thought to be involved in CSF regulation (Mikkelsen et al., 1997) and clearance of brain monoamines (Rodriguez and Caprile, 2001). Thus far, the presence of 5-HT_{1A} receptors in this brain structure has not been investigated, but it will be interesting to test whether NUDR regulates 5-HT_{1A} receptor gene expression in the SCO, its relation with serotonin and the physiological consequence, if any, of such interaction. Evidence suggests that signal transduction cascades operating in this organ may exert important impact on gene expression. For instance, CREB is now considered a SCO transcription factor that can be activated by the second messengers cAMP and calcium. Substance P and ATP stimulate the phosphorylation of CREB apparently via a calcium-dependent mechanism and are thus involved in the control of gene expression in the bovine SCO (Schoniger et al., 2002). Similarly, NUDR may exert transcriptional control in the SCO that may be dependent upon serotonergic signalling-dependant cascades that may involve the 5-HT_{1A} or other 5-HT receptors.

The fact that it is still not clear what sort of changes if any are seen in 5-HT_{1A} receptor levels in depressed patients carrying the homozygous G(-1019) genotype does not detract from the conclusion of our study because the NUDR-mediated repression that is

occurring *in vivo* could well be critical at earlier stages in development, regardless of receptor levels in adulthood. This thesis focused on the polymorphism in the 5-HT1A receptor and its association with mental illness. While changes in NUDR (and Freud-1) expression may also be associated with mental illness, we have not directly addressed this potential mechanism in the current study. Long-term collection of brain samples from depressed victims will be necessary before we can address this interesting possibility.

Having identified Freud-1, NUDR and Hes5 as important transcriptional regulators of 5-HT1A receptor gene, a candidate gene for major depression and anxiety, we need to identify the molecular mechanisms of transcriptional regulation by these proteins. Yeast two-hybrid or affinity purification approaches could be used to identify tissue-specific proteins interacting with these regulators. NUDR for instance may interact with different co-activators or co-repressors that may explain its enhancer and repressor abilities in pre- and post-synaptic 5-HT1A expressing sites. Unlike NUDR and Hes5, putative involvement of Freud-1 in major depression awaits to be investigated. The search for other Freud-1 regulated genes is also of great interest.

Also, additional transcription factors that remain to be identified and characterized may regulate the 5-HT1A repressor elements I have studied. In screening by yeast one hybrid approach a human brain cDNA library using the 26 bp polymorphic element, I identified two different clones with 100% homology to the human Ku autoantigen p70 subunit protein (Reeves and Sthoeger, 1989) that were shown to transactivate the palindrome C(-1019) allele. Because transactivation by this clone (clone 33B, Fig. IV.2D) was weaker than transactivation by NUDR and Hes5, and because the G(-1019) allele did not impair greatly transactivation, I decided to focus on Hes5 and NUDR. However, this

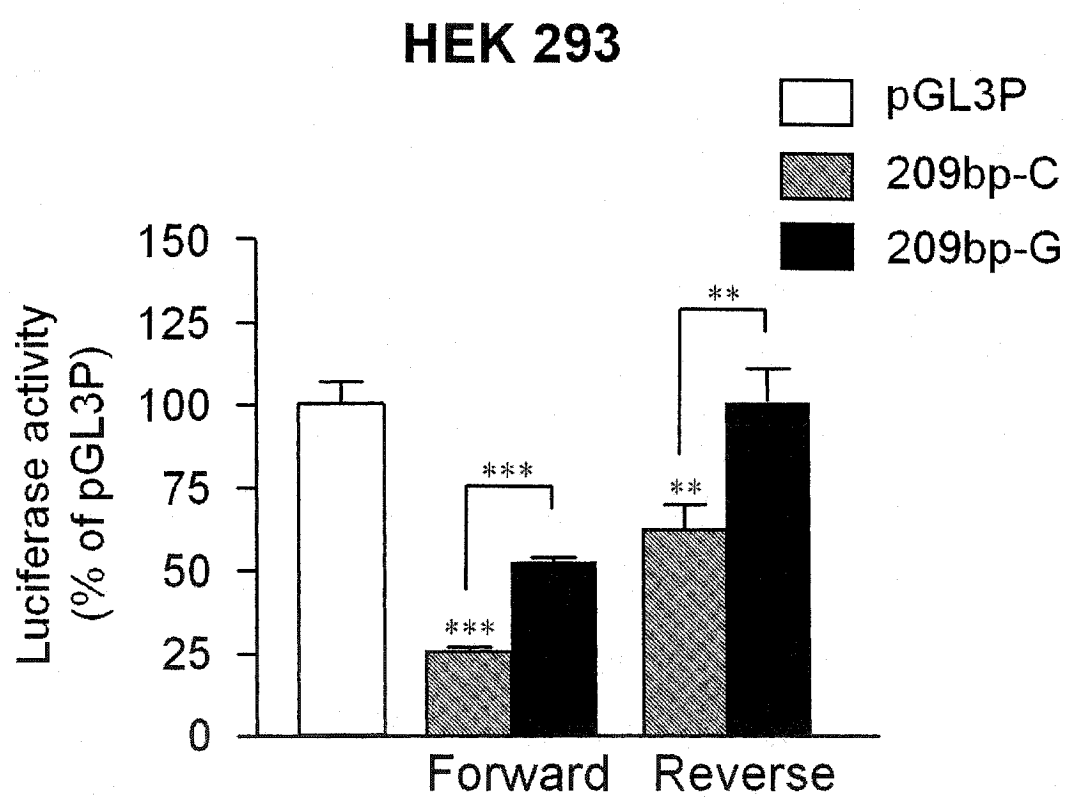
protein could still be important in the regulation of the human 5-HT_{1A} receptor gene and merits investigation. The human Ku autoantigen is a heterodimeric (p70/p80) nuclear protein of the leucine zipper transcription factor family that was first detected using autoantibodies from sera of patients with systemic autoimmune diseases (Mimori et al., 1981). Later, it was shown to associate with a DNA-dependent protein kinase (DNA-PK) that phosphorylates chromatin-bound proteins *in vitro* (Anderson and Lees-Miller, 1992) and is involved in double-stranded DNA break repair as well as telomeric length maintenance and silencing (Boulton and Jackson, 1998). DNA-PK is a nuclear serine/threonine protein kinase that is targeted to the DNA via the Ku protein and that phosphorylates DNA-binding proteins, including several transcription factors such as p53 and Sp1 (Mayo et al., 1997). Because DNA-PK may modulate transcriptional activity of various transcription factors, we can speculate that NUDR and/or Hes5 that interact at the same DNA binding site might be targets for Ku/DNA-PK-mediated phosphorylation. Interestingly, differential DNA-binding activity of Ku can be observed depending on the brain region analysed with highest levels in the pituitary, cerebellum and hypothalamus and evidence suggest that Ku may be important during development (Bakalkin et al., 1998). The tissue-specific activity of Ku and hence DNA-PK phosphorylation activity could contribute to the differential subcellular localization and/or enhancer/repressor activity of NUDR.

In conclusion, major depression is a complex and heterogeneous disorder that involves many different genes, brain regions, neurotransmitters, signalling pathways and environmental triggers. An increasing body of evidence from animal studies suggests that antidepressants may act on specific transcription factors and target genes that regulate

processes such as neurotransmitter release or neuroprotection and neuronal survival. While these and other results provide evidence for the monoamine and neurogenic hypotheses for depression, the pathophysiology of the disease is still poorly understood. The transcriptional mechanisms that I have identified (summarized in Appendix L) provide new models that could account for alteration in serotonergic neurotransmission implicated in depression and suicide. The results presented in this thesis are potentially very interesting for both the clinical and basic science communities. The strength and originality of this work resides in the demonstration that the C(-1019)G polymorphism is a functional polymorphism with clinical association to major depression and suicide and that the derepression mechanism I propose could provide a mechanism for predisposition to these disorders. The fact that so few polymorphisms have been convincingly associated with depression increases the potential importance of this study. This work also represents the first report of a functional gene polymorphism associated with both major depression and completed suicide. It also constitutes the first evidence associating NUDR with mental illness. The extent of characterization of this functional polymorphism, its associated DNA element and binding proteins, and their roles in regulation of the 5-HT_{1A} receptor represents the most extensive understanding of any polymorphism that has been associated with mental illness thus far. Finally, I believe that these pioneering studies, along with studies of the SERT promoter, highlight the general importance of transcriptional regulatory mechanisms in the predisposition to mental illness and will stimulate research into analysis of these mechanisms in other candidate genes relevant to mental illness. The field of psychiatric genetics is just beginning to address the role of promoter polymorphisms. I believe that this work will serve as a new paradigm in the field for

identifying polymorphism associated with mental illness that have functional significance in terms of regulating candidate genes, such as the 5-HT_{1A} receptor. It is hoped that, with time, the identification of more biological markers involved in the pathophysiology of depression and other mental illnesses may guide decisions about specific treatments for particular patients.

APPENDICES

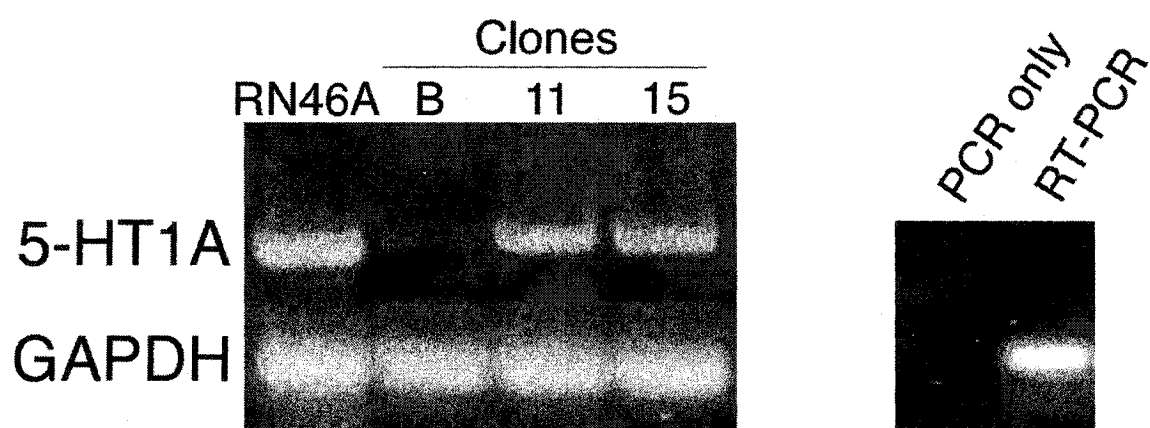


APPENDIX A

A- Figure VI.1 – C(-1019)G sensitive repressor activity of the polymorphic region at the SV40 promoter.

A 209-bp fragment spanning the C(-1019)G polymorphism was placed upstream of the SV40 promoter in pGL3P (209bp-C or 209bp-G) in the forward or reverse orientation. The C-allele of the 209-bp fragment displayed significant repressor activity, which was reduced in the G-allele.

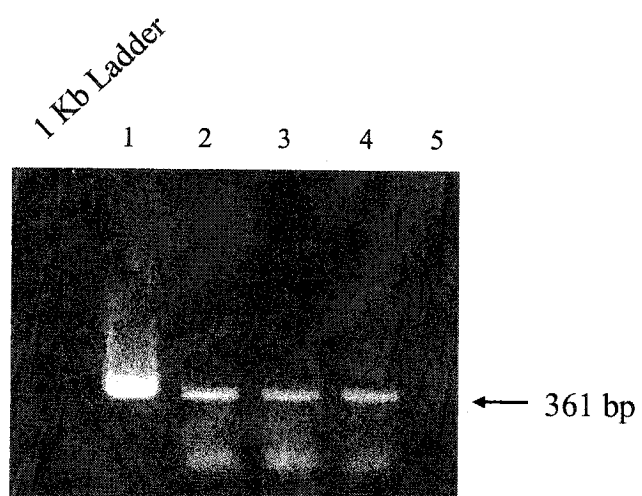
METHODS: Luciferase plasmids (209bp-C) and (209bp-G) were obtained from DNA samples homozygous for C or G alleles, respectively, by PCR amplification of the -876/-1085-bp region of the human 5-HT1A gene and subcloning into the *Sma*I site of pGL3-Promoter (Promega) in both orientations. HEK 293 cells were transfected as described (Ou et al., 2000). Luciferase activity was assessed and corrected for transfection efficiency by determining the ratio of Luciferase activity/ β galactosidase as described (Ou et al., 2000), and is presented as percent of control (pGL3P). Data is presented as mean $\pm$ SD of triplicate samples from three independent experiments. Significance compared to control or as indicated * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$.



APPENDIX B**B- Figure VI.2 – Reduced 5-HT1A receptor RNA in RN46A clones expressing NUDR.**

One-step RT-PCR was done for both 5-HT1A and GAPDH using RNA prepared from RN46A cells and NUDR-transfected clones. The data are normalized to the levels of GAPDH present in each cell line from triplicate replications. Clones B, 11, and 15 had $16.4 \pm 5.4\%$, $53.4 \pm 8.1\%$ and $49.6 \pm 11.9\%$ of 5-HT1A mRNA of control RN46A cells (mean $\pm$ S.E., n=3). PCR of RNA samples gave no specific bands, ruling out a contribution from genomic DNA.

METHODS: Isolation of total RNA from rat RN46A cells was carried out using Trizol reagent, and the procedure described by the manufacturer (Invitrogen). Genomic DNA were removed from the samples by DNase treatment. SuperScript One-step reverse transcriptase-PCR (RT-PCR) with Platinum Taq procedures, as described by the manufacturer (Invitrogen), were followed using specific primer sequences for rat 5-HT1A and GAPDH. The 360 nucleotide (nt) rat 5-HT1A cDNA fragment was amplified using the following oligonucleotides as primers: 5' GCCATCGCGCTAGACAGGTA 3' (upstream) and 5' GCGGTGCCGACGAAGT 3' (downstream). A 112 nt cDNA fragment specific to rat GAPDH was synthesized using 5' CATGGCCTTCCGTGTTCTACCC 3' (upstream) and 5' CCTCGGCCGCGCTGCTTCA 3' (downstream) oligonucleotides as primers. Amplified cDNA fragments were run on a 1.2% agarose gel containing ethidium bromide. Band intensities were measured using UN-SCAN-IT software version 4.3 (Silk Scientific Corporation). All values are normalized to the housekeeping gene GAPDH. The absence of genomic DNA was confirmed by replacing the RT/Platinum Taq with Platinum Taq along with the GAPDH oligonucleotides in the one-step RT-PCR reaction.



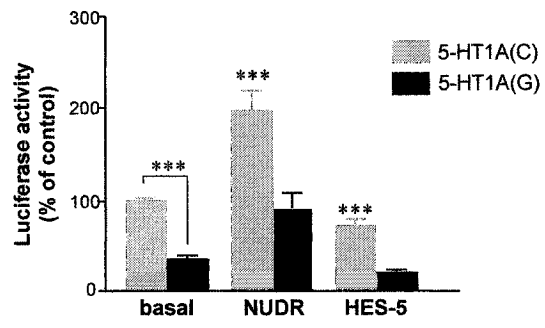
1. pcDNA3-Freud-1
2. Stem cells
3. E15-16 cortical progenitors
4. Cortical neurons
5. Negative control

APPENDIX C

C- Figure VI.3 – Freud-1 RNA in cortical progenitors and stem cells.

PCR amplification of a 361 bp portion of Freud-1 coding sequence in 1) pcDNA3 vector containing full length Freud-1(positive control) or cDNA prepared from total RNA of 2) mouse stem cells, 3) E15-16 mouse cortical progenitor cells and 4) mouse cortical neurons. No amplification was obtained using empty pcDNA3 vector (negative control).

**SN-48
(n=6)**



5-HT1A



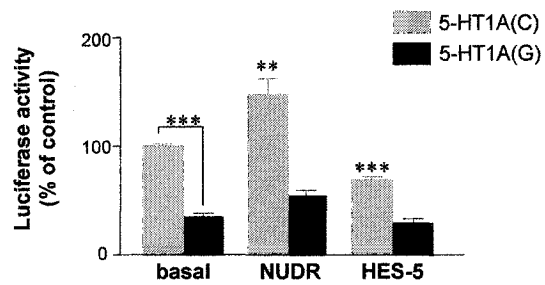
NUDR



Overlay



**NG108-15
(n=4)**



5-HT1A



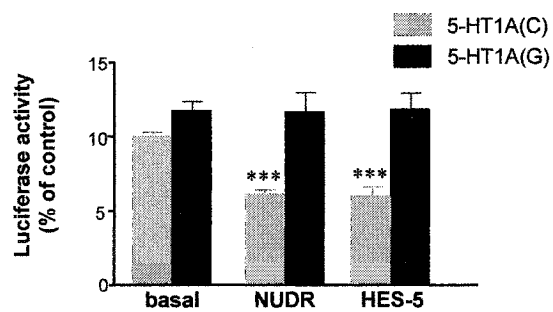
NUDR



Overlay



**RN46A
(n=5)**



5-HT1A



NUDR



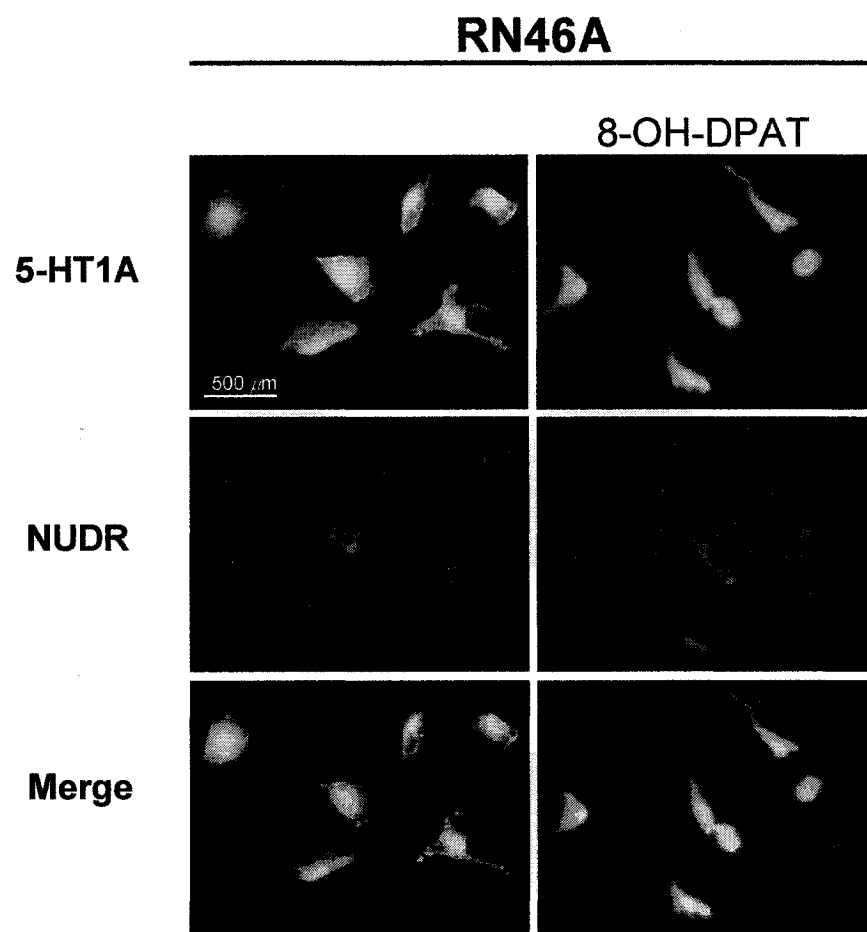
Overlay



APPENDIX D

D- Figure VI.4 – Enhancer properties of NUDR and repressor activity of Hes5 in a post-synaptic model of 5-HT1A expressing cell line.

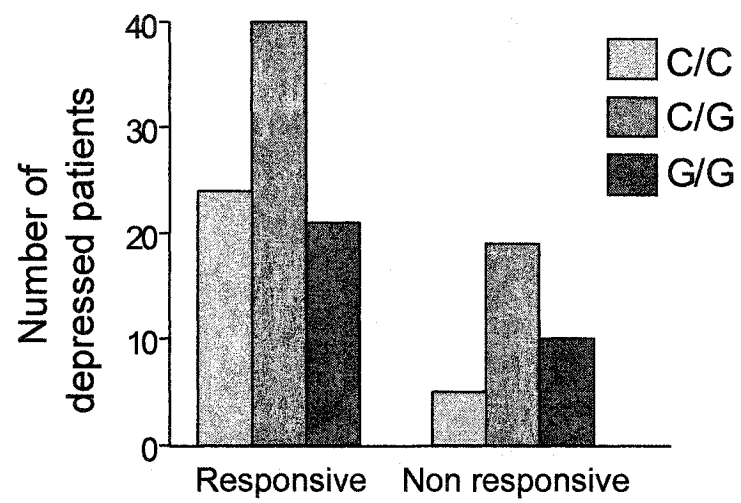
The human 5-HT1A promoter (-1128-bp to ATG) luciferase reporter constructs with the C(-1019) or G(-1019) allele, denoted 5-HT1A(C) and 5-HT1A (G) respectively, were transfected into mouse septal SN-48 and neuroblastoma-glioma NG108-15 cells, models of post-synaptic 5-HT1A expressing cell lines. Cells were cotransfected with vector (pcDNA3), NUDR or Hes5 expression plasmids as indicated. Luciferase activity was corrected for transfection efficiency by calculating the ratio of luciferase activity/ β -galactosidase activity and normalized to control (pcDNA3) transfections. NUDR enhances basal 5-HT1A promoter activity at the C(-1019) allele, but its activity is greatly impaired at the G(-1019) allele. Hes5 weakly represses basal activity of the C(-1019) allele, but lacks significant repressor activity at the G(-1019) allele. The greater basal activity of 5-HT1A(C) compared to 5-HT1A(G) and the further increase in activity upon NUDR transfection suggest that NUDR possesses strong enhancer properties postsynaptically that are reduced at the G allele. In postsynaptic SN-48 cells, NUDR immunoreactivity (in red) was colocalized with 5-HT1A receptor (in green) but was restrained to the nucleus. Repressor activity of NUDR and Hes5 and the perinuclear localization of NUDR in raphe RN46A cells (chapter IV) are shown for comparison. Data is presented as mean $\pm$ SD of triplicate samples collected from at least four independent experiments as indicated. Statistical analysis compared to vector control (for NUDR and Hes5) was determined by one way ANOVA; significant differences between C and G alleles were evaluated by unpaired t-test with two-tailed *P* values: ***P*≤0.005, ****P*≤0.0005.



APPENDIX E

E- Figure VI.5 – Nuclear translocation of NUDR following 5-HT1A receptor agonist treatment.

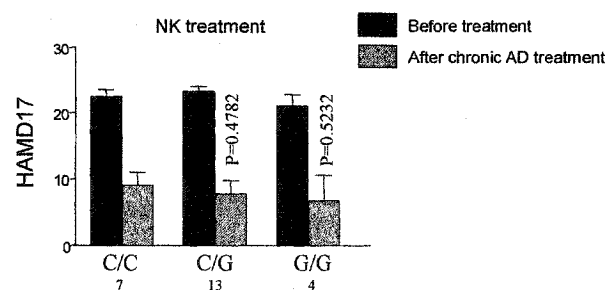
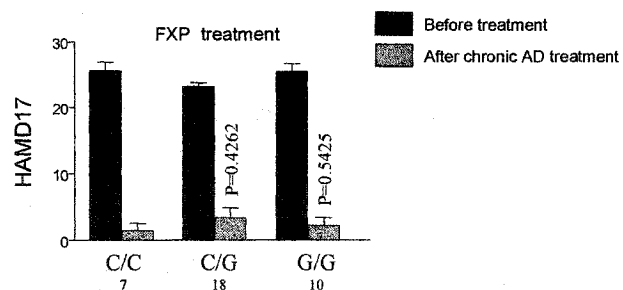
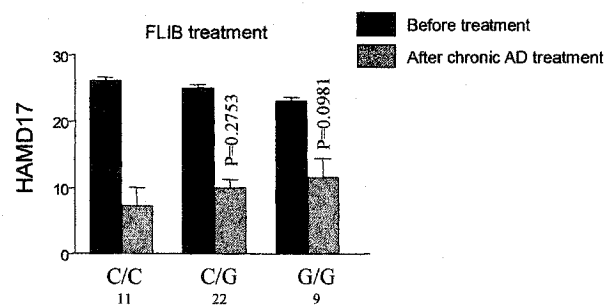
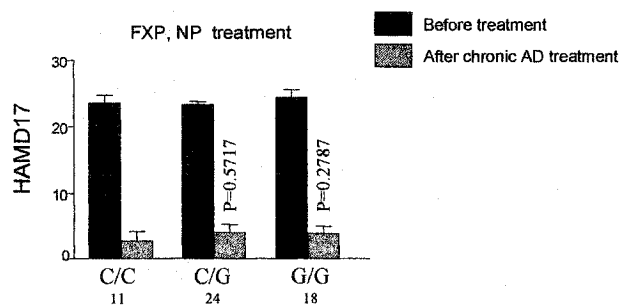
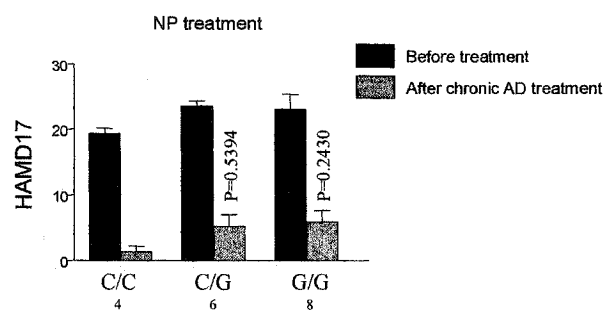
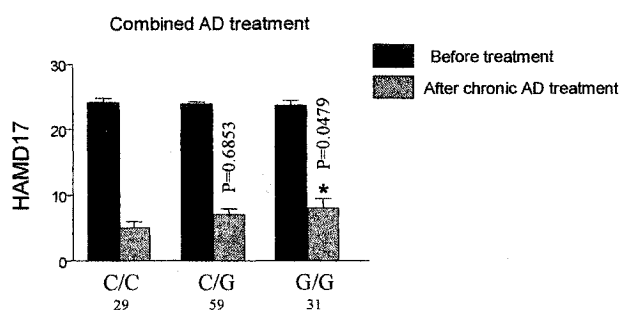
In raphe RN46A cells expressing 5-HT1A receptors, subcellular localization of NUDR was cytoplasmic and concentrated around the perinuclear area. Upon treatment for 16 hours with 10 μ M 8-OH-DPAT, NUDR translocated to the nucleus. Immunostaining and cell culture were performed as described in the material and methods of chapter IV.



	Total patients	C/C	C/G	G/G	Allele C	Allele G
Responsive	85	24 (28%)	40 (47%)	21 (25%)	88 (52%)	82 (48%)
Non-responsive	34	5 (15%)	19 (56%)	10 (29%)	29 (45%)	39 (57%)

APPENDIX F**F- Figure VI.6 – C(-1019)G genotype-dependant responsiveness to antidepressant medication.**

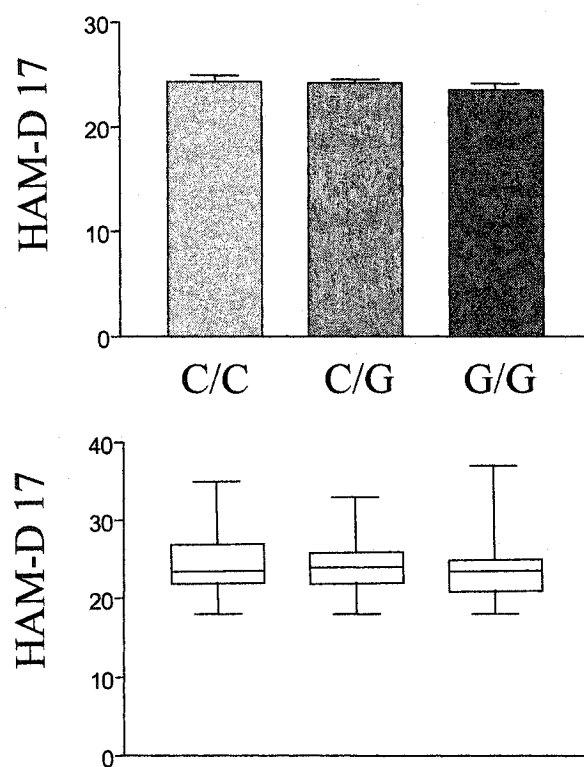
Of the 129 depressed patients genotyped in chapter IV, 10 discontinued antidepressant therapy and the remaining 119 patients were grouped as either “responsive” if HAM-D 17 score decreased by at least half of initial value following chronic antidepressant therapy, or “non-responsive”. Most depressed patients were responsive to chronic antidepressant treatment. However, in the responsive group less patients carried the homozygous G(-1019) genotype, and in the non-responsive group there were twice as many patients with the homozygous G(-1019) genotype compared to the homozygous C(-1019) genotype. Responsive versus non-responsive patients: genotype, $\chi^2 = 2.412$, $df = 2$, $P = 0.2994$; allele, $P = 0.2510$. Genotype G/G versus non-G/G: $P = 0.6466$. Genotype C/C versus non-C/C: $P = 0.1578$.



APPENDIX G

G- Figure VI.7 – Decreased response to antidepressant treatment in homozygous G(-1019) depressed patients.

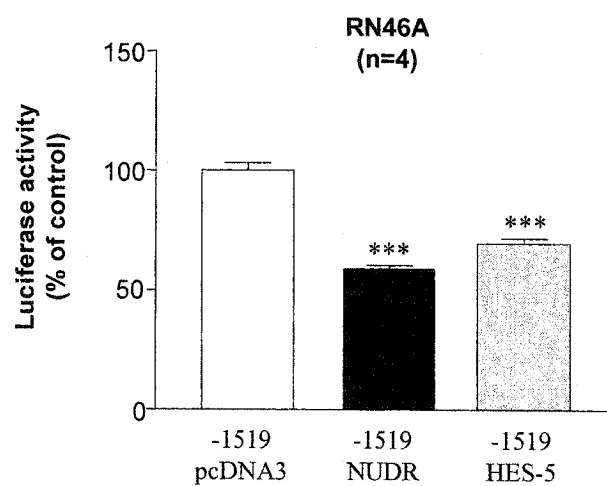
The efficacy of various antidepressant drugs in the treatment of major depressive disorder was examined in 119 patients who completed the drug trial study. Depressed individuals were administered one of the following daily oral doses: fluoxetine-FX (selective serotonin reuptake inhibitor, 75-150 mg); nefazodone/pindolol-NP (weak serotonin-noradrenalin reuptake blocker, potent 5-HT₂ receptor antagonist/5-HT_{1A} antagonist, (3x 2.5 mg /20-40 mg); fluoxetine/pindolol-FXP (10-20 mg/4-16 mg); NKP608A-NK (Novartis NK₁ receptor antagonist, 0.55 or 25 mg); flibanserin-FLIB (5-HT_{1A} agonist and 5-HT_{2A} antagonist, twice 20-50 mg or once 20-100 mg daily), for a period of at least 3 weeks. Although no difference in the severity of depression was noted across genotypes before treatment according to the HAM-D 17 score, there was a trend ($P= 0.6853$) towards a reduced effectiveness of antidepressants amongst heterozygous and a significant decrease in responsiveness amongst individual carrying the G(-1019)G genotype ($P= 0.0479$) compared to depressed patients carrying the C(-1019)C genotype. The numbers of patients in each genotype groups are as indicated. Statistical comparison of C/G or G/G allele to C/C allele is shown for each treatment with P values as indicated.



APPENDIX H

H- Figure VI.8 – Severity of depression according to genotype at the C(-1019)G polymorphic site.

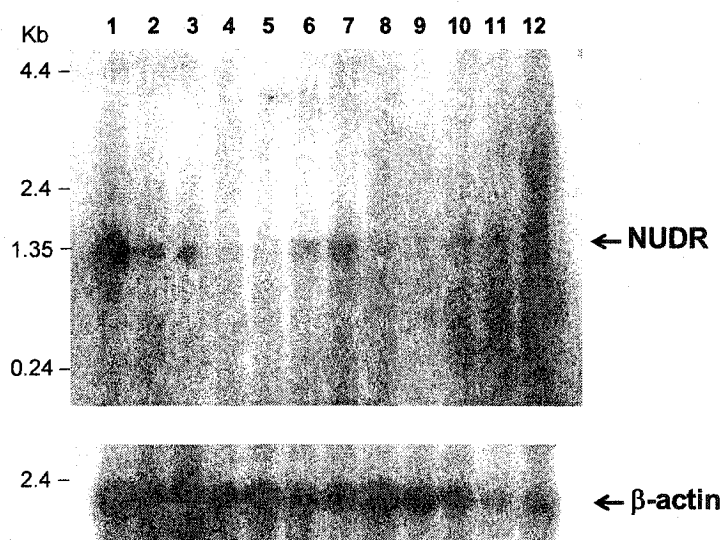
The average HAM-D 17 score of the 129 depressed patients characterized for their genotype at the -1019 polymorphic site (chapter IV) was analyzed to determine the severity of depression in these patients. In this restricted population sample of severely depressed patients, the polymorphism does not associate with the severity of the disease.



APPENDIX I

I- Figure VI.9 – NUDR and Hes5 -mediated repression at the rat 5-HT1A promoter.

RN46A cells were transiently transfected with the rat luciferase reporter construct -1519 (Ou et al., 2000). Cells were cotransfected with vector (pcDNA3), NUDR or Hes5 expression plasmids as indicated. Luciferase activity was corrected for transfection efficiency by measuring the ratio of luciferase activity/ β -galactosidase activity as described (Ou et al., 2000) and normalized to control (pcDNA3) transfections as indicated. Data is presented as mean $\pm$ SD of triplicate samples from four independent experiments. Statistical significance was determined by one way ANOVA *** $P \leq 0.0005$.

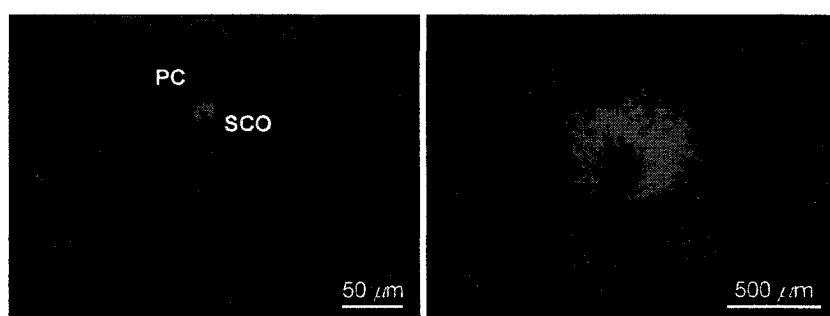


1. Brain
2. Heart
3. Skeletal muscle
4. Colon (no mucosa)
5. Thymus
6. Spleen
7. Kidney
8. Liver
9. Small intestine
10. Placenta
11. Lung
12. Peripheral blood leucocytes

APPENDIX J

J- Figure VI.10 – Tissue distribution of NUDR RNA expression.

NUDR mRNA expression in human tissues with highest levels in the brain. The Human 12-lane MTN® Blot (Clontech) was used for Northern blot analysis according to manufacturer's recommendations using the full length NUDR as a probe. RNA size marker bands are indicated on the right margin of the blot. Hybridization to NUDR probe is shown. Blot was stripped following manufacturer's protocol, and blot reprobed with Human β -Actin cDNA Control Probe (Clontech).

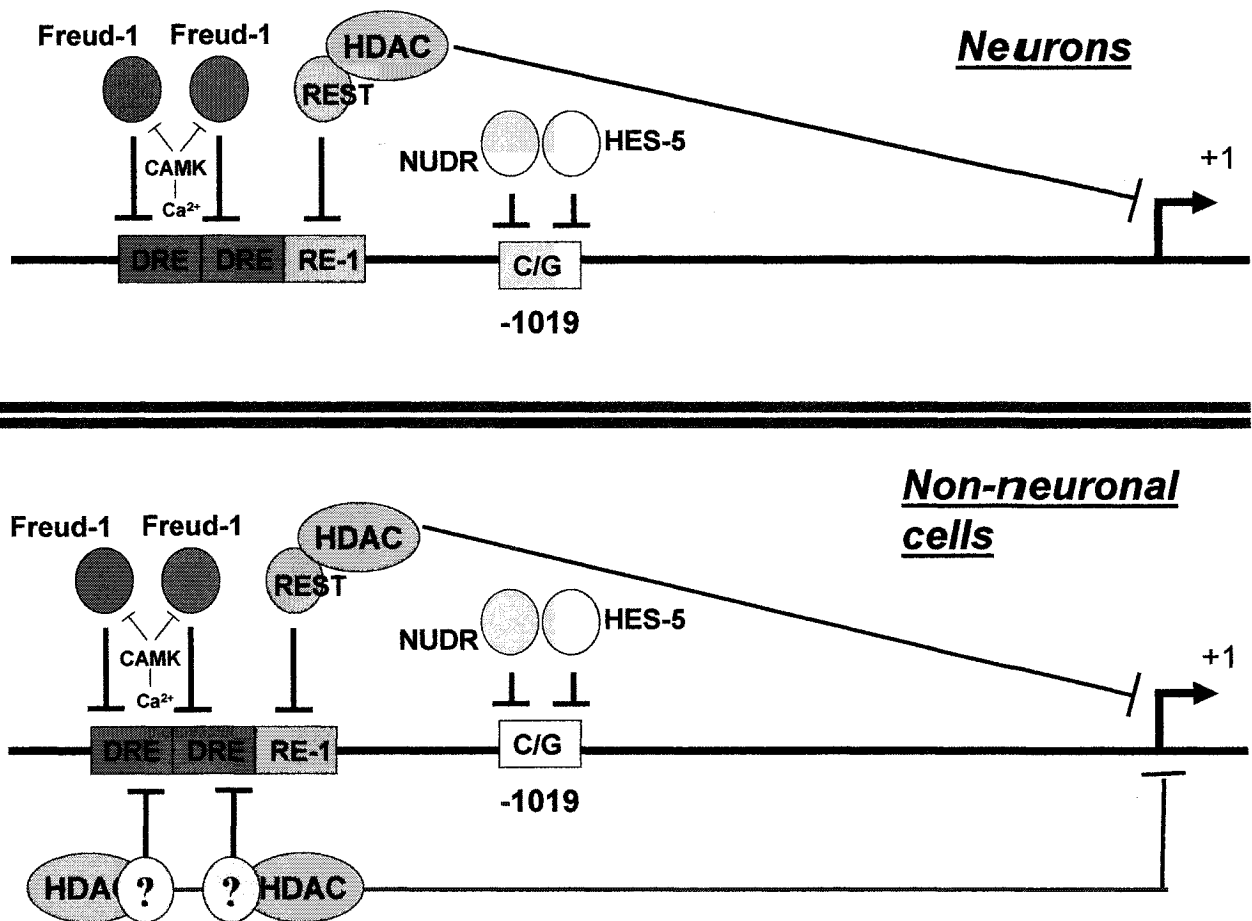


APPENDIX K

K- Figure VI.11 – NUDR protein is expressed in the subcommissural organ (SCO).

Intense NUDR immunoreactivity was detected in the SCO of the rat brain. This organ is located below the posterior commissure (PC) and above the fourth ventricle and receives considerable serotonergic innervation from the dorsal raphe nucleus.

METHODS: Immunostaining using NUDR antibody and 12 μm adult rat brain sections was performed as described in the material and methods of chapter IV under immunofluorescence.



APPENDIX L

L- Figure VI.12 – Schematic representation of the human 5-HT1A receptor promoter and key regulators that have been identified in this thesis project.

A region between -1624 and -1550 bp with strong repressor activity contains a consensus RE-1 element that confers REST-inducible repression and two copies of the previously described dual repressor element (DRE) (Ou et al., 2000). The novel calcium-regulated Freud-1 repressor binds to the DREs to reduce basal 5-HT1A receptor expression. Freud-1 mediates HDAC-independent repression in neuronal 5-HT1A positive cells, while REST or other DRE binding proteins recruit HDAC-dependant mechanisms to silence the receptor in non-neuronal 5-HT1A negative cells. A functional C(-1019)G polymorphism in the human 5-HT1A promoter associates with major depression and suicide. The occurrence of the G allele at -1019 bp prevents binding and repression by specific transcription factors NUDR and Hes5 and results in de-repression of the 5-HT1A receptor gene and hence, may contribute to the susceptibility to depression.

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