uOttawa

L'Université canadienne
Canada's university

FACULTÉ DES ÉTUDES SUPÉRIEURES ET POSTOCTORALES

uOttawa

L'Université canadienne
Canada's university

FACULTY OF GRADUATE AND POSDOCTORAL STUDIES

Margaret Czesak

AUTEUR DE LA THÈSE / AUTHOR OF THESIS

Ph.D. (Cellular and Molecular Medicine)

GRADE / DEGREE

Faculty of Medicine – Department of Cellular and Molecular Medicine

FACULTÉ, ÉCOLE, DÉPARTEMENT / FACULTY, SCHOOL, DEPARTMENT

Transcriptional Regulation of the 5-HT1A Receptor Gene by Deformed Autosomal Regulatory Factor 1

TITRE DE LA THÈSE / TITLE OF THESIS

Paul Albert

DIRECTEUR (DIRECTRICE) DE LA THÈSE / THESIS SUPERVISOR

CO-DIRECTEUR (CO-DIRECTRICE) DE LA THÈSE / THESIS CO-SUPERVISOR

Antonio Colavita

Diane Lagacé

David Leonardo
(Columbia Univeristy)

Laura Trinkle-Mulcahy

Gary W. Slater

Le Doyen de la Faculte des etudes supérieures et postdoctorales / Dean of the Faculty of Graduate and Postdoctoral Studies

TRANSCRIPTIONAL REGULATION OF THE 5-HT1A RECEPTOR GENE BY DEFORMED AUTOSOMAL REGULATORY FACTOR 1

By: Margaret Czesak

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

Department of Cellular and Molecular Medicine

Faculty of Medicine, University of Ottawa

Your file Votre référence
ISBN: 978-0-494-73924-2
Our file Notre référence
ISBN: 978-0-494-73924-2

DEDICATION

I would like to dedicate this thesis to my parents, Ewa and Zbigniew. Your unconditional love and support has given me the strength to be the person I am today. Thank you for your patience and understanding, for your motivation and kind words, for having faith in my abilities and for never giving up. You have provided me with every opportunity to appreciate life and accomplish my dreams and for that I will be forever in your dept. I can only hope to follow in your footsteps and someday be as kind and generous to my children as you have been to me. I love you both with all my heart.

ACKNOWLEDGEMENTS

I would like to take this opportunity to express my sincere appreciation to everyone who has contributed to this thesis.

First and foremost, I would like to thank my supervisor, Dr. Paul Albert, for believing in my abilities and for taking a leap of faith and giving me the opportunity to become a graduate student in his lab. His solid guidance, patience and words of encouragement in times of doubt have given me the strength to believe in myself, my technical skills and my knowledge capacity needed to complete this thesis. Dr. Albert is a remarkable scientist and an inspiring teacher, I will always be grateful for all that I have learned because of him and look forward to applying this knowledge in my future career endeavors.

I would like to extend my appreciation to the members of my advisory committee: Drs. David Park, Antonio Colavita and Oudette Leuneville, for their constructive advise and project guidance over the years. I would also like to thank our collaborators: Dr. Mark Austin for conducting experiments on human tissues as well as Dr. Jane Visvader for generating and sharing Deaf-1 knockout mice.

I would like to thank past and present members of Paul Albert's laboratory, mainly Dr. Sylvie Lemonde for providing the foundation of my research through her inspiring thesis data, Dr. Chris Bown for teaching me how to culture primary neurons, Mireille Daigle for her technical assistance, Mariam Deria for being a wonderful honors student and project assistant, Federico Remes Lenicov for his

help in optimizing Real-Time PCR and Dr. Brice Lefrancois for expert troubleshooting advise and animal care assistance.

A heartfelt thank you goes out to three graduate students who have been my pillars in times of need and became dear friends over the years. I would like to thank Dr. Kelly McClellan, who has volunteered her time and expertise in teaching me in situ hybridizations, who has reviewed my scholarship applications and provided valuable suggestions for their improvement, and who has patiently brainstormed countless ideas with me to troubleshoot research roadblocks. She has been an inspiring example of an ideal graduate student who's drive and perseverance both academically and politically, has increased my self-determination to become a better student. I would also like to thank Matthew Mount, a fellow graduate student for his countless assistance in conducting animal perfusions and Dr. Jason Middleton for being a great listener during my times of academic stress.

Last, but certainly not least, I would like to express my deepest gratitude to my husband Adrian Jean, the love of my life, whose kind words, unconditional love and respect, and moral support over the years have given me the strength in my times of doubt. I will be forever grateful for his patience and help with the numerous computer related challenges during my years as a graduate student.

The research presented in this thesis was supported by the Canada Doctoral Studentship Award from the Canadian Institutes of Health Research.

ABSTRACT

Major Depressive Disorder (MDD) is the most commonly diagnosed psychiatric illness, occurring in 15-20% of the population and is characterized by prolonged mood quality impairment. It is a multifactorial disease and both genetic and environmental factors contribute to its etiology. Evidence suggests that impaired serotonin (5-HT) neurotransmission is an important underlying cause of depressive disorder. The 5-HT1A receptor is a powerful regulator of both pre and post-synaptic 5-HT neurotransmission, and becomes compromised in depression. Evidence suggests that alterations in 5-HT1A gene expression observed in depression result from compromised transcriptional regulation of the 5-HT1A receptor gene. As such, genetic variations and factors involved in the transcriptional machinery that drives 5-HT1A receptor expression are expected to play a key role in establishing normal behavioural phenotypes.

I initially characterized the regulatory properties of two transcription factors, Deaf-1 and Hes-5, which have previously been shown to bind the 5-HT1A promoter region spanning a 26-bp palindrome sequence that encompasses a C(-1019)G polymorphism. Using reporter assays, I established Deaf-1 as a cell type specific regulator of the 5-HT1A receptor gene. Deaf-1 was shown to act as transcriptional repressor of the 5-HT1A promoter in pre-synaptic raphe nuclei cell lines, while it activated 5-HT1A promoter expression in post-synaptic cells. Hes-5 on the other hand, remained a transcriptional repressor in all cell types. The activity of both transcription factors was impaired in the presence of the C(-1019)G

allele. Moreover, Deaf-1 mediated regulation of the 5-HT1A receptor appeared to be HDAC-dependent.

Additionally, I utilized mice lacking a functional Deaf-1 protein, to study the *in vivo* effects of Deaf-1 on 5-HT1A receptor gene expression. Deaf-1 was shown to bind the mouse 5-HT1A promoter sequence and mediate cell-type specific transcriptional repression or enhancement of 5-HT1A receptor gene expression, similar to that observed using *in vitro* cell lines. As a result, Deaf-1 knockout mice exhibited upregulation of pre-synaptic 5-HT1A receptor gene, which correlated decreased 5-HT neurotransmission.

In conclusion, I have identified a unique mechanism of regulating the 5-HT1A gene expression by Deaf-1, both *in vitro* and *in vivo*. I established the importance of the C(1019)G polymorphism in regulating 5-HT1A gene and characterized a novel mouse model for studying the effects of Deaf-1 on the serotonin system.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

-/-	knockout
Δ	deletion
[^{3}H]	tritiated
[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-*n*-propylamino)tetralin
A	adenine
aa	amino acids
AC	adenylyl cyclase
Acc.	accession
ACTH	adrenocorticotropin hormone
AP-1	activator protein-1
Asp	aspartate
Asn	asparagine
ATP	adenosine-5'-triphosphate
βARK	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
CNS	central nervous system
CO_2	carbon dioxide
Con	consensus
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
DG	dentate gyrus
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
DNase I	deoxyribonuclease I
dNTP	deoxynucleotide triphosphate
DRE	Dual Repressor Element
DTT	dithiothreitol
DRN	dorsal raphe nucleus
E	embryonic day

ECT	electroconvulsive therapy
EDTA	ethylenediaminetetraacetic acid
EGTA acid	ethylene glycol-bis (aminoethyl ether) N, N, N', N'-tetraacetic
EMSA	electrophoretic mobitity shift assay
FBS	fetal bovine serum
FRE	Five-prime repressor element
Freud-1 protein-1	Five-prime Repressor Element Under Dual Repression binding
g	gramme
G	guanine
Gα	alpha subunit of G protein
Gβγ	beta gamma subunit of G protein
GAL4-AD	GAL4 activation domain
GAL4-DBD	GAL4 DNA binding domain
GABA	γ-aminobutyric acid
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
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
i_1	first intracellular loop
i_2	second intracellular loop
i_3	third intracellular loop
Ile	isoleucine
Iono	ionomycin
IP_3	inositol trisphosphate
Inr	initiator
K	lysine
kb	kilobases
KCl	potassium chloride
kDa	kilodalton
Leu	leucine
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 phosphate	nitro blue tetrazolium chloride/ 5-bromo-4-chloro-3-indolyl
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 *Pyrococcus furiosus*
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PMDD	premenstrual dysphoric disorder
PMSF	phenylmethylsulfonyl fluoride
PVN	paraventricular nucleus
PTX	pertussis toxin
Py	pyrimidine
r	rat
R	arginine
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
Real-Time PCR	real-time-polymerase chain reaction
S	serine
SAND	Sp100, AIRE-1, NucP41/75, DEAF-1

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
SNC	substantia nigra pars compacta
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 *Thermococcus aquaticus*
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 domaine
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
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-Gal	5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside

THESIS FORMAT

This thesis is presented as a collection of manuscripts. Chapter I is an overall introduction to the serotonin system, including both historical and theoretical background. Chapter II is a paper entitled "Cell-specific repressor or enhancer activities of Deaf-1 at a serotonin 1A receptor gene polymorphism". This manuscript has been published in Journal of Neuroscience. Chapter III consists of a second paper entitled: Characterization of rat rostral raphe primary cultures: multiplex quantification of serotonergic markers". This paper has been published in Journal of Neuroscience Methods. Chapter IV is a paper entitled " Altered 5-HT1A gene expression in mice lacking the functional Deaf-1 protein". This paper has been submitted to Journal of Neuroscience and is currently being revised. Chapter V is a general discussion of the thesis work, how it relates to the actual knowledge in the field of serotonin research and its overall relevance to the field of neuroscience with respect to mood disorders.

CHAPTER 1 — INTRODUCTION

The following sections from this chapter:

Section 1.3.3 p.35

Section 1.4.3–1.5.3 p. 49-59

Have been modified with permission from:

Le François B, Czesak M, Steubl D, Albert PR. (2008) Transcriptional regulation at a HTR1A polymorphism associated with mental illness. Neuropharmacology.Nov;55(6):977-85. Epub 2008 Jun 29. Review

See Appendix A for permission

1.1 Serotonin System

"Serotonin is a drug that makes you feel good. It's what all the pharmaceutical companies pump into those wonderful little anti-depressants. It's also a drug God decided to pump through our brains when we do things we like. It's like a little reward for good behaviour. So when you do something kind for someone else, the person you're helping has serotonin released in their brain and feels happier...and so do you. Good news, two more serotonin-induced happier people in the world!"
Wayne Dyer, 2004

1.1.1 Historical Overview of the Serotonin System

The discovery of serotonin stems from the detection of a novel amine substance responsible for gut motility, extracted from the gastric mucosa of the stomach. Further research traced its origin to the enterochromaffin cells of the gastrointestinal tract, hence its original name 'enteramine' (Vialli and Erspamer, 1933). Simultaneously, an endogenous vasoconstrictor substance in blood was identified, eventually leading to the isolation of serotonin (Rapport et al., 1948) (Rapport, 1949). The name 'serotonin' was given as an obvious derivative from the combination of two Latin words: *serum* meaning blood and; *tonus* meaning to stretch. Its chemical and crystal structure was established shortly afterwards (Rapport, 1949) and eventually synthesized into 5-hydroxytryptamine (5-HT), which opened a new avenue for research worldwide (Hamlin and Fisher, 1951).

In the early fifties, high concentrations of 5-HT were found in the mammalian brain (Twarog and Page, 1953) followed by the discovery of its heterogeneous distribution in the central nervous system (CNS) (Amin et al., 1954). In 1956, 5-HT was finally identified as a neurotransmitter in the CNS (Bogdansky et al., 1956). The role of 5-HT in mental disorders such as depression was first established through pharmacological studies using the tranquilizing drug reserpine, found to lower concentrations of 5-HT in the brain, leading to depression (Wooley and Shaw, 1954). In 1964, Dahlstrom and Fuxe discovered the existence of serotonergic neurons in the CNS, furthering the advancement of knowledge regarding the role of 5-HT in brain neurotransmission (Dahlstrom and Fuxe, 1964).

Initially, receptors responsible for the specific binding of 5-HT on cells were discovered and classified by Gaddum and Picarelli (Gaddum and Picarelli, 1957). Their simple system of classification categorized 5-HT receptor into two classes based on their location and agonist specificity. 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. Further agonist research, followed by the discovery of radioligands, suggested other subtypes of receptors may also be involved in regulating 5-HT neurotransmission (Saxena et al., 1971), (Saxena, 1972), (Bennett and Aghajanian, 1974). In fact, both 5-HT1 and 5-HT2 receptor subtypes were identified in the rat cortical tissues based on their high binding affinity radioligands [^{3}H]5-HT and [^{3}H]spiperone, respectively (Peroutka and Snyder, 1979). In order to further characterize the 5-HT1 receptor, two additional subtypes were later identified on the basis of their high and low

affinity for spiperone, respectively (Pedigo et al., 1981). Eventually, an international committee established specific criteria for classifying 5-HT receptors in a more uniform fashion as either "5-HT1-like" (corresponding to a few "D" or 5-HT1), 5-HT2-like" (analogous to most "D" or 5-HT2) and 5-HT3 (equivalent to "M") (Bradley et al., 1986).

To date, a total of 18 mammalian receptor subtypes have been identified (Le Francois et al., 2008). With the rapid advancements in molecular biology techniques, including cloning of genes, additional receptors may be identified and implicated as crucial regulators of the 5-HT system in the future.

1.1.2 Serotonin Metabolism

Serotonin belongs to a class of monoamine neurotransmitters that contain one amino group that is connected to an aromatic ring by a two-carbon chain. Synthesis of 5-HT in the body is dependent on the uptake of an essential amino acid L-tryptophan (corresponding to the codon UGG). Tryptophan belongs to a group of aromatic amino acids called indoles, and is primarily derived from one's dietary intake. Some of the foods enriched in this amino acid include chocolate, oats, mangoes, diary products, chickpeas, various seeds and peanuts. Although the human body on average contains 10mg of serotonin, only about 2% is localized to the brain. The highest concentration is found in the enterochromaffin cells of the gastrointestinal tract making up 90% of total 5-HT uptake, while approximately 8% is localized to the circulating platelets, where it plays a key role in haemostasis and coagulation (Gershon 1985).

Due to the restrictive properties of the blood-brain barrier that prevent entry of peripheral 5-HT into the brain, serotonergic neurons must synthesize their own 5-HT. The initial step in 5-HT synthesis involves the hydroxylation of tryptophan by the rate-limiting enzyme tryptophan hydroxylase (TPH) to form 5-hydroxytryptophan (5-HTP). It is important to note that two isoforms of this enzyme exist in the body, tryptophan hydroxylase-1 and -2 (TPH1 and TPH2). TPH2 is the primary enzyme for 5-HT synthesis in the brain, while TPH1 is found in the pineal gland as well as in the peripheral tissues where it constitutes the most abundant isoform (Dumas et al., 1989; Walther et al., 2003). The next step in 5-HT synthesis is the decarboxylation of 5-HTP, mediated by the L-aromatic amino acid decarboxylase, which produces 5-HT. In the pineal gland, an additional series of enzymes metabolizes 5-HT into melatonin, responsible for regulating the circadian rhythm (Figure 1.1).

Once synthesized, the cytoplasmic 5-HT is transported into secretory vesicles by the vesicular monoamine transporter protein (VMAT) where it accumulates (Liu and Edwards, 1997). The release of 5-HT is regulated by a calcium-dependent process called exocytosis, where synaptic vesicles fuse with the plasma membrane (containing the neurotransmitter), and deliver 5-HT to the synaptic cleft. This entire mechanism depends on cell depolarization, which triggers the opening of voltage sensitive calcium channels by increasing intracellular Calcium (Ca^{2+}). The increase in calcium levels within the neuron leads to Ca^{2+}-calmodulin/cAMP-dependent phosphorylation of vesicle membrane protein called Synapsin I that is responsible for fixing the secretory granules to the cytoskeleton network. The uncoupling of

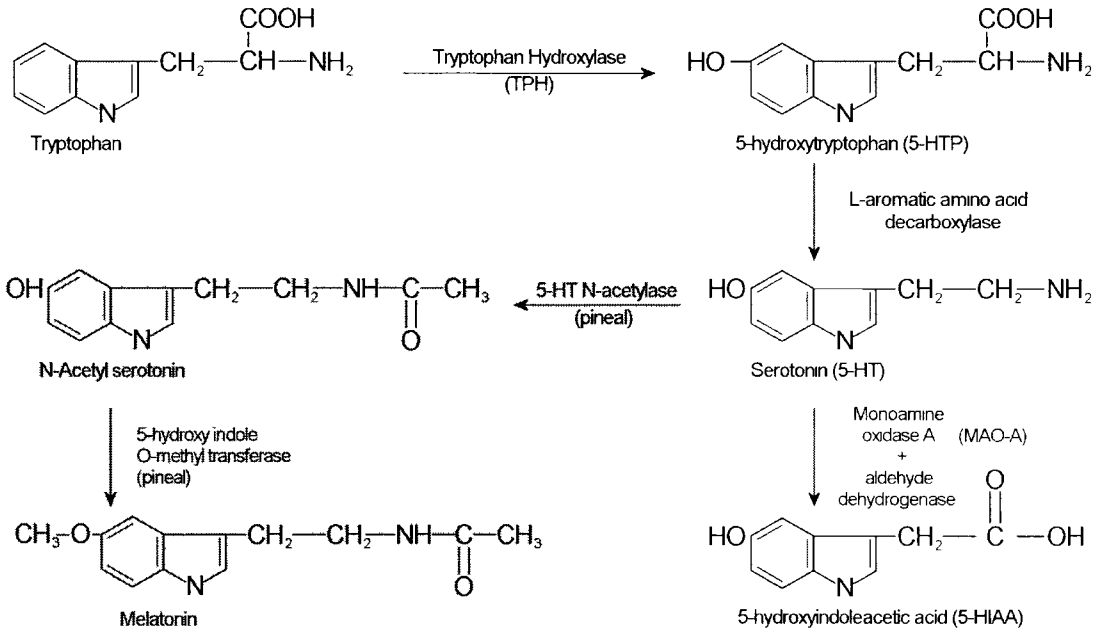


Figure 1.1: Metabolic pathways of Serotonin Synthesis and Degradation

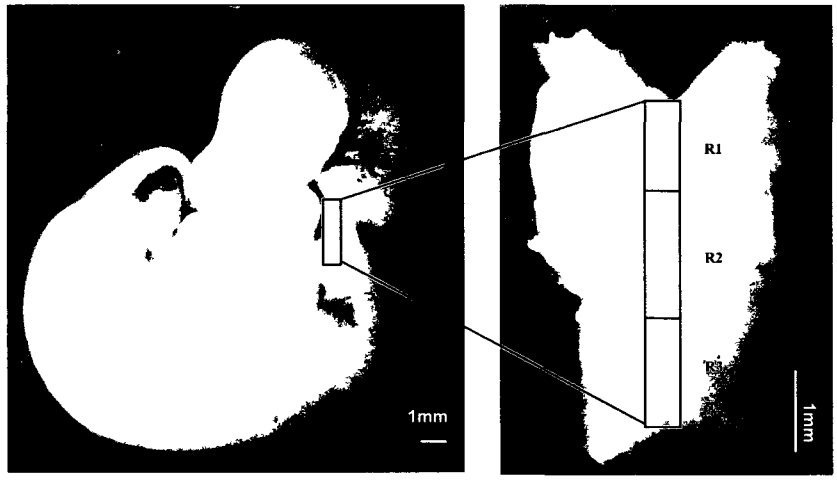
Serotonin is synthesized from an amino acid (tryptophan) by the rate limiting enzyme tryptophan hydroxylase, which converts tryptophan to 5-hydroxytryptophan. This is then converted to 5-hydroxytryptamine (or 5-HT) by L-aromatic amino acid decarboxylase (AADC). Copyright © Sylvie Lemonde, PhD.

synapsin causes release of vesicles from the cytoskeleton to allow for easy migration to the presynaptic membrane where they are docked for future release of 5-HT (Pineyro and Blier, 1999). The metabolic breakdown of 5-HT is mediated by the mitochondrial enzyme monoamine oxidase A (MAO-A) which converts serotonin to its major metabolite 5-hydroxyindoleacetic acid (5-HIAA), which is excreted into the cerebrospinal fluid (Figure 1.1) (Youdim and Finberg, 1991).

1.1.3 Brain Serotonin System

The serotonin system is localized primarily within the midline raphe nuclei of the brainstem (Tork, 1990). Originally, the raphe nuclei have been divided into nine groups labelled B1 through B9 (as shown in Figure 1.2) (Dahlstrom and Fuxe, 1964), which today correspond to the following areas: raphe pallidus nucleus (Rpa, B1), raphe obscurus nucleus (Rob, B2 and B4), raphe magnus (RMg, B3), the median raphe nucleus (MRN, B5 and B8), the dorsal raphe nucleus (DRN, B6 and B7) and the caudal linear nucleus (Cli, B8). Additional regions outside of the raphe nuclei have also been identified to have subpopulations of 5-HT containing cell bodies. These include the supralemniscular region (corresponding to B9) localized adjacent to the medial lemniscus, the ventrolaterial medulla (the B3 cluster), central gray area of the medulla oblongata (B4) and in the nucleus pontis oralis (B8 and B9) (Tork, 1990). Finally, the raphe nuclei can be further divided in two larger subpopulations: the rostral group (Cli, DRN, MRN, B9) located in the midbrain and pons and the caudal group (RMg, Rpa, Rob) found in the medulla oblongata of the brainstem.

A.



B.

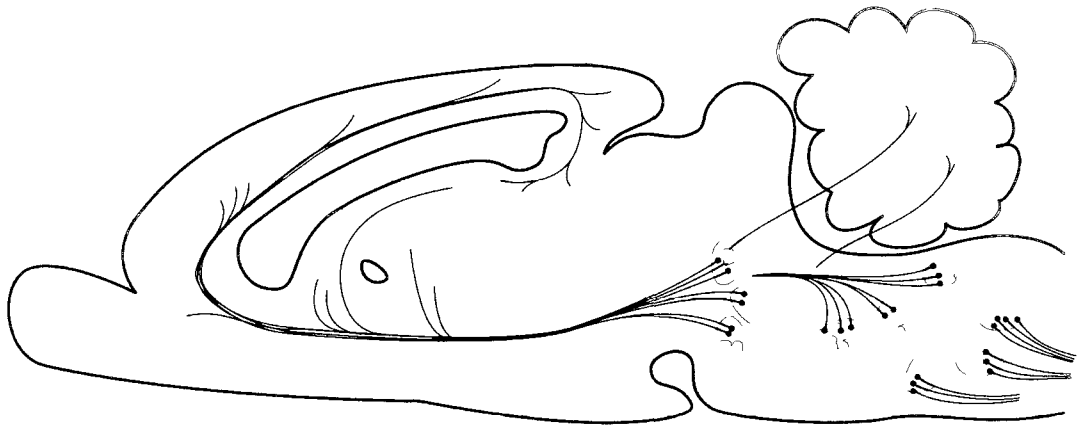


Figure 1.2: Localization of the Raphe Nuclei

A) Sample dissection showing the brainstem raphe nuclei of a E12 5 mouse The rostral raphe region, including rhombencephalon 1,2 and 3 is dissected out and displayed as a saggital section (this is unpublished data) B) Saggital diagram depicting the localization of the brainstem raphe nuclei, including the dorsal (B7-9), median (B4-6) and caudal (B1-3) regions Adapted and modified from Kandel et al , Fourth Edition, 2000

The neural circuitry that stems from 5-HT neurons of the raphe nuclei and surrounding regions allows all levels of the CNS to receive serotonergic innervation through the extensive branching of 5-HT axonal projections. The rostral raphe nuclei (composed of DRN and MRN) sends 5-HT projections to the majority of the limbic system including the amygdala, thalamus, hypothalamus, olfactory bulb, hippocampal formation, basal ganglia and the cingulated gyrus (Figure 1.3). Passing through some of these limbic structures, 5-HT neurons also project to the prefrontal cortex (Baker et al., 1990; Jacobs and Azmitia, 1992). In total, the dorsal raphe nucleus accounts for approximately 50-60% of the 5-HT producing neurons in the human brain, while the median raphe nucleus contains the second largest cluster of 5-HT neurons. Together, these regions are responsible for all the ascending projections in the brain (Baker et al., 1990). Although axons from the DRN and MRN differ in structure, both co-exist in most brain areas, with the exception of the striatum, which receives mainly axons from the dorsal raphe (D-fibers) while the dentate gyrus receives mainly median raphe axons (M-fibers) (Brown and Molliver, 2000). Oppositely, the caudal raphe nuclei are responsible for descending projections to the spinal cord and the cerebellum. It is through both the dorsal and caudal connections that serotonin mediates its effects on a wide range of functions including perception, thermoregulation, control of appetite, sleep, learning and memory, mood, behaviour, 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).

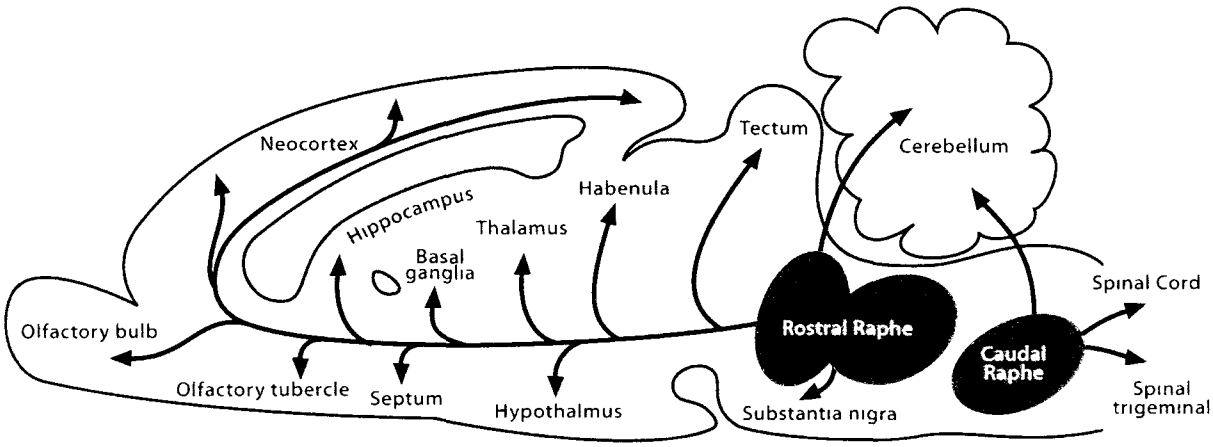


Figure 1.3: Summary of Serotonin Projections in the Brain

Saggital representation of the 5-HT neuronal pathways in the brain 5-HT neurons originate from the rostral (dorsal and median) or the caudal raphe nuclei and send both descending projections in the spinal cord as well as ascending projections into the cerebellum, forebrain and throughout the CNS Adapted and modified from Kandel et al , Fourth Edition, 2000

A characteristic morphological trait of the DRN is its "butterfly" shape in cross-section, and its striking bilateral symmetry. This shape is divided into three main subregions. The "body" portion of the butterfly is referred to the area located just below the cerebral aqueduct and is called the dorsomedial region while the area surrounding the medial longitudinal fasciculus (MLF) is called the ventromedial region (Tork, 1990). The "wings" of the DRN are composed of the dorsolateral and ventrolateral regions while the "tail" part is referred to as the caudal component. The MRN is located along the midline of the ponds and medulla (ventral to the DRN) in a mid-saggital plane, with additional cells scattered outside of this midline region in no particular orientation (Baker et al., 1990; Pineyro and Blier, 1999).

In total, the rostral raphe nuclei contain 85% of all serotonergic neurons in the mammalian brain (Hornung, 2003). The remaining cells constitute non-serotonergic neurons that express a variety of neurotransmitters and neuropeptides including dopamine (DA), noradrenaline (NA), γ-aminobutyric acid (GABA), glutamate, enkephalin, substance P, thyrotrophin-releasing hormone (TRH) and others (Jacobs and Azmitia, 1992). Such neuronal heterogeneity contributes to the diverse morphological characteristics of the raphe neurons, affecting their shape and size as well as their pharmacological responses (Marinelli et al., 2004; Baker et al., 1990).

1.1.4 Development of the Serotonin System

Although the development of serotonin neurons has been studied in humans, primates and rodents, its characterization remains incomplete. Serotonergic neurons arise from neuroepithelial progenitors located near the floor plate of the neural tube(Goridis and Rohrer, 2002; Drevets et al., 2007). In humans, serotonin neurons first appear in the raphe nuclei at embryonic week 5 and are fully defined by week 15 (Whitaker-Azmitia, 2001). In the mouse, 5-HT immunoreactivity can be seen in the rostral group at embryonic day 12, while 5-HT neurons in the caudal group differentiate two days later (Jacobs and Azmitia, 1992). Various developmental triggers are required to ensure that proper serotonergic differentiation takes place. The entire process is guided by developmental growth factors and other signaling molecules, responsible for regulating cell division. The intersection of Sonic hedgehog (Shh) production in the notochord, fibroblast growth factor 4 (FGF4) in the primitive streak and fibroblast growth factor 8 (FGF8) in the midbrain-hindbrain organizing center (MHO) forms a three dimensional Cartesian space that directs development of 5-HT neurons caudal to the MHO (Gaspar et al., 2003) (as shown in Figure 1.4A). These molecules diffuse to form a concentration gradient responsible for inducing a complex genetic cascade as a result.

The Hairy Enhancer of Split (Hes) family of developmental proteins also plays an important role in ensuring timely serotonergic differentiation. Of these, Hes1, Hes3 and Hes5 are involved in the maintenance of neuronal progenitors while Hes6 promotes neuronal differentiation by inhibiting the other Hes proteins (Fior and Henrique, 2005). Hes1, 3 and 5 are expressed exclusively in neural

progenitors while Hes6 is expressed during embryogenesis and maintained into adulthood (Bae et al., 2000). Hes proteins belong to a family of basic helix-loop-helix (bHLH) domain proteins and have the ability to recruit additional transcriptional co-repressors. Hes protein expression is regulated through activation of the Notch signaling pathway in which one of the four Notch receptors (Notch 1-4) becomes activated by one of its four ligands (Jagged-1, Jagged-3 and delta-like-1 and 3) (Jarriault et al., 1998; Mizutani et al., 2001; Ohtsuka et al., 2001). Upon ligand binding, the Notch intracellular domain becomes cleaved, translocates to the nucleus and interacts with the DNA binding protein RBP-JK to activate transcription of Hes proteins, which in turn leads to transcriptional repression of the differentiation factor called Mash1 (Mammalian Achaete-Shute Homolog 1)(Akazawa et al., 1992; Hirata et al., 2001). Thus, Notch-induced lateral inhibition ensures that cell differentiate to distinct fates from an initially homogeneous cell population (Jarriault et al., 1998).

As the neuroepithelial progenitors differentiate into serotonergic cells, levels of Hes1 and Hes5 decrease, while the homeobox gene Nkx2.2 is induced by Shh (Sonic hedgehog) (Briscoe et al., 1999). In addition, the pro-neuronal Mash1 protein is also essential for the differentiation of 5-HT neurons at this stage (Pattyn et al., 2004). Mash1 is required for the generation of postmitotic precursors after the shutdown of Phox2b and acts together with Nkx2 to activate downstream effectors of this pathway. Nkx2.2 functions with the related Nkx6.1 protein to activate Gata-2 and Gata-3, which are two zinc-finger proteins responsible for the specification of the rostral and caudal raphe groups of 5-HT neurons, respectively(van Doorninck et

al., 1999). The next downstream factor required for 5-HT neuronal differentiation is the LIM homeobox transcription factor 1 β (Lmx1b), which is involved in the commitment and maintenance of 5-HT neurons and is a transcriptional activator of the transcription factor called Pet-1 (pheochromocytoma 12 Ets E26 transformation-specific factor; also knows as Fifth Ewing Variant or FEV in humans) (Hendricks et al., 1999; Cheng et al., 2003). However a recent study has suggested that activation of Lmx1b, Pet1 and Gata2/3 occurs in parallel rather than downstream from one another (as shown in Figure 1.4B) (Pattyn et al., 2004).

Pet-1 in turn is essential for the expression of a number of serotonergic genes including tryptophan hydroxylase (TPH), aromatic L-amino acid decarboxylase (AADC) and the serotonin transporter (5-HTT), each playing a crucial role in establishing a functional serotonergic system. Interestingly enough, disrupting the function of any of these transcriptional regulators involved in the specification of 5-HT neurons results in substantial deficits in 5-HT neuron differentiation, migration and signalling, however, few of the gene disruptions studied to date result in a complete deficit in serotonergic development (see Table 1 for complete list of animal phenotypes). This implies that other regulators of differentiation and development of serotonergic phenotype are almost certain to be involved.

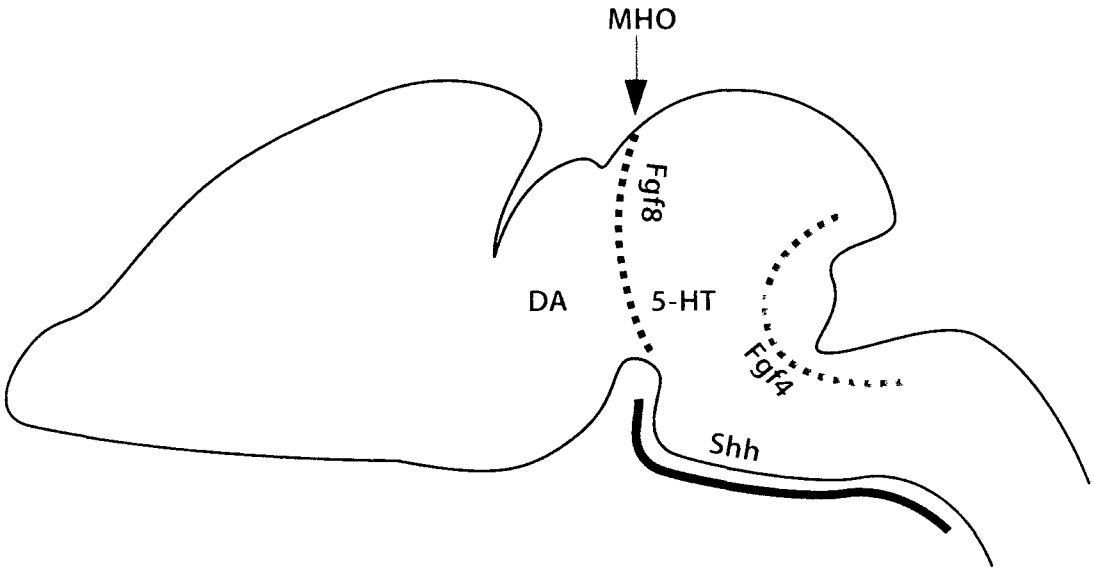


Figure 1.4-A: Developmental Regulation of Serotonin Neurons

Specification of 5-HT precursors in the neural tube is a number of determinants, including FGF8 (fibroblast growth factor-8) along the Midbrain-Hindbrain Organizing Centre (MHO) and Sonic Hedgehog (Shh) in the ventral line of the notochord. Shh and FGF8 drive Dopamine (DA) expression rostral to the MHO. Expression of the FGF4 (fibroblast growth factor 4) along the primitive streak caudal to the MHO drives 5-HT neuron specification. Adapted and modified from Gaspar et al., 2003.

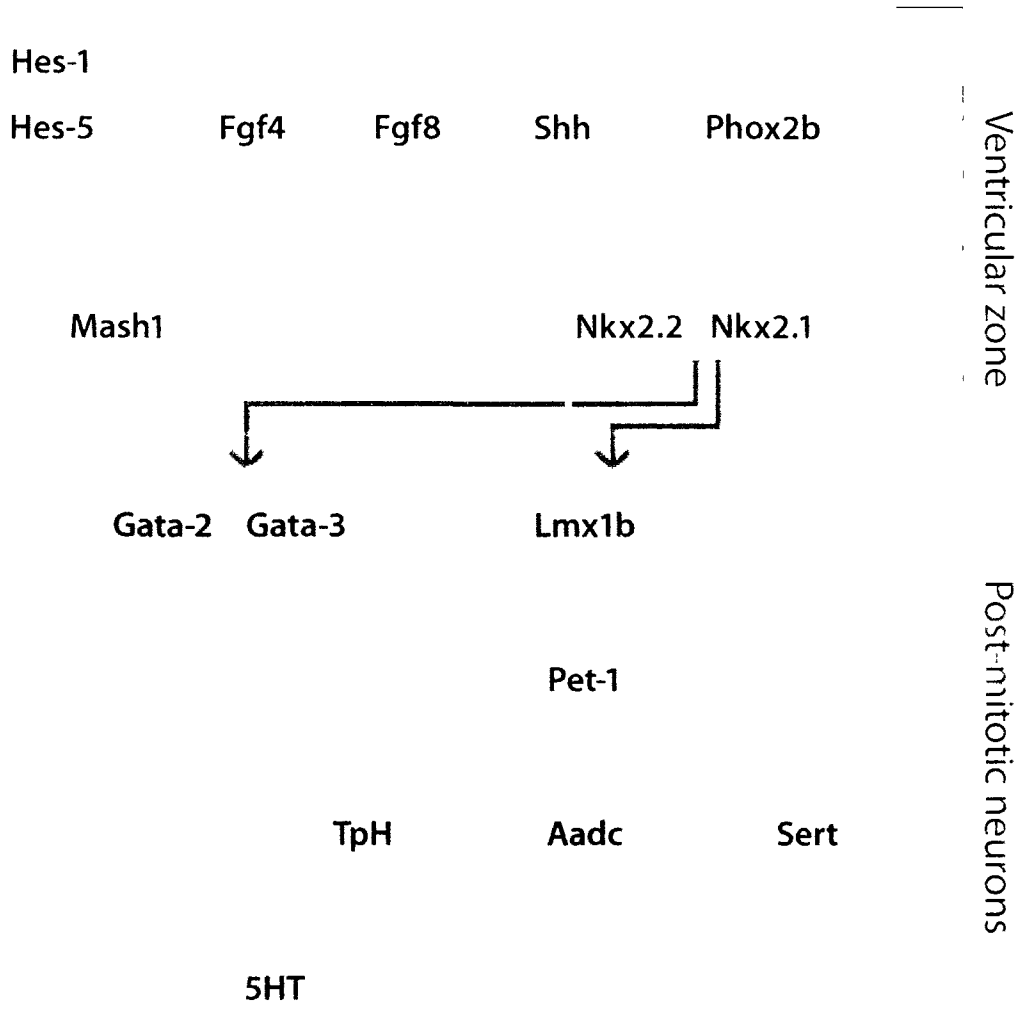


Figure 1.4-B: Developmental Regulation of Serotonin Neurons

Pathway of 5-HT differentiation shows that FGF8, FGF4 and Shh are necessary for initial cell specification in the ventricular zone, inducing Mash1 and Nkx developmental regulators which in turn drive the expression of post-mitotic neurons by activating Gata-2 and -3 along with Lmx1b, which eventually leads to 5-HT neuronal expression. Adapted and modified from Gaspar et al., 2003.

Table 1.1: Mouse Models of 5-HT System Development

Gene	Reference (s)	Knockout Phenotype(s)
Nkx2.2	(Briscoe et al., 1999; Cheng et al., 2003)	Developmental switch from ventral to dorsal cell orientation and development of motor neurons in place of interneurons
Gata-2	(van Doorninck et al., 1999; Craven et al., 2004)	Loss of rostral 5-HT neuronal development
Gata-3	(van Doorninck et al., 1999) Craven et al., 2004	Loss of caudal 5-HT neuronal development
Lmx1b Lmx1b (conditional KO in raphe nuclei)	(Chen et al., 1998; Smidt et al., 2000; Ding et al., 2003; Zhao et al., 2006; Zhao, 2007)	Gross defects in Dopamine, 5-HT, kidney and liver development Loss of 5-HT neuronal differentiation
Pet-1	(Hendricks et al., 2003)	Loss of 20-30% of 5-HT neurons Behavioural phenotypes: Increased anxiety and aggression
TPH1	(Cote et al., 2003; Cote et al., 2007)	90% decrease in 5-HT, no gross anatomical abnormalities but mice develop cardiac insufficiency in adulthood
TPH2	(Gutknecht et al., 2008)	Lack of 5-HT synthesis with no obvious alteration in morphology or serotonergic neuron formation
SERT	(Bengel et al., 1998; Fabre et al., 2000; Gobbi et al., 2001)	anxiety and depression, sleep disturbances, increase in 5-HT tone, decrease in 5-HT1A density
MAO-A	(Cases et al., 1995; Shih and Chen, 1999)	Increased aggression, increased 5-HT, Norepinephrine and dopamine levels, decreased 5-HT1A binding and density
Hes-1	(Ishibashi et al., 1995; Tomita et al., 1996; Ohtsuka et al., 2001)	severe neural tube defects
Hes-5	(Ishibashi et al., 1995)	premature neural differentiation
Hes-1 and Hes-5	(Ohtsuka et al., 1999)	enhanced severity of premature differentiation
Hes-1 -3 and -5	(Kageyama et al., 2005; Hatakeyama et al., 2006)	neuroepithelial cells prematurely differentiate into neurons at E8.5, all glial cells prematurely differentiate at E10

1.1.5 Characterization of Serotonin Receptors

Serotonin neurotransmission acts through a wide variety of membrane-bound receptors. Initially characterized by its pharmacological properties, today this family of receptors is differentiated based on their structural (amino acid sequences) and biochemical (post-receptors mechanisms of signal transduction) properties, in addition to their agonist/antagonist selectivity. According to these properties, 5-HT receptors have been classified into seven families (5HT1-5HT7) and at least 15 different subtypes (see Table 1.2) (Filip and Bader, 2009). With the exception of one, all 5-HT receptor subtypes are made up of seven transmembrane domains, belong to the metabotropic receptor family and are referred to as G-protein coupled receptors (GPCR). Oppositely, 5-HT3 receptors are members of an ionotropic family and are composed of four transmembrane domains. This family of receptors is characterized as cation-selective ligand-gated ion channel receptors and are assembled as a pentamer of receptor subunits. 5-HT3 receptors consist of multiple isoforms with similar physiological and pharmacological profiles and require a heterotrimeric combination of 5-HT3A and 5-HT3B subunits in order to be functional (Hoyer et al., 1994).

GPCR receptors mediate a variety of cellular responses through their downstream signaling pathways, including regulating intracellular levels of cAMP through adenyl cyclase, activation of phospholipase C (PLC), modulation of K^+ and Ca^{++} channel activity and stimulating gene expression (Barnes and Sharp, 1999). As the name implies, 5-HT receptor subtypes couple to heterotrimeric guanine

nucleotide-exchange proteins (G proteins), which are composed of two subunits: Gα GTPase and Gβγ. The Gα subunit is further categorized in five subfamilies based on their signalling transduction pathways, and includes the G_s, $G_{i/o}$, G_z, G_q/G_{11} and G_{12}/G_{13} subfamilies (Dohlman et al., 1991).

The 5-HT1 family receptors exhibit between 40-63% amino acid sequence identity and couple preferentially to G_i/G_o proteins to inhibit adenylyl cyclase (AC) activity thus, decreasing the production of cAMP (Table 1.1). It is the only family of receptors that is expressed pre-synaptically and plays an important role in autoregulation of serotonergic neurotransmission. The 5-HT2 receptors exhibit a 46-50% overall sequence identity and belong to $G_{q/11}$-coupled receptors. They mediate their effects through coupling to phospholipase C-induced phosphoinositol hydrolysis to stimulate diacylglycerol and inositol 1,4,5-triphosphate accumulation, resulting in protein kinase C activation and intracellular Ca^{++} release, respectively (Filip and Bader, 2009). 5-HT4 receptors couple positively to AC and enhance cAMP production via activation of the G_s subunit. 5-HT5 receptor subtypes bear 70-88% overall sequence homology and couple negatively to AC, inducing a preferential inhibition of forskolin-stimulated cAMP production. 5-HT6 family of receptors is composed of two splice variants with no pharmacological differences, which positively couple to AC via G_s proteins to induce cAMP formation. 5-HT7 receptors are positively coupled to AC via G_s proteins to elevate cAMP levels. Taken together, the 5-HT system is a complex network of receptors and signaling pathways, often working together to control various physiological events. In addition, animal knockout models have been utilized to help understand the physiological roles of

Table 1.2: Classification of serotonin receptors

Receptor	Expression	Selective Agonist (s)	Selective Antagonist (s)	Effector(s)	Knockout Phenotype
5-HT1A	widespread in brain, but mainly: hippocampus, cortex and raphe nuclei	8-OH-DPAT flesinoxan, LY 228729, osemozotan, PRX-00023, U 92016A	WAY-100635, lecozotan, p-MPPI, robalzotan,	G_i/G_o ↓ AC	Increased anxiety, decreased exploration, reduced antidepressant-like phenotype, cognitive impairment
5-HT1B	widespread in brain but mainly in basal ganglia, cortex and terminals of neurons in raphe nuclei	CGS 12066B, almotriptan, eletriptan, GR 46611, L 694247, LY 310762, PNU 109291	GR 127935 cyanopindolol, AR-A00002, GR 55562, NAS-181, SB 216641, SB 224289	G_i/G_o ↓ AC	Increased aggression; increased exploration, decreased anxiety, increased response to cocaine
5-HT1D	basal ganglia, hippocampus, cortex	sumatriptan PNU 109291 GR 46611	BRL 15572	G_i/G_o ↓ AC	Unknown
5-HT1E	cortex, caudate putamen, claustrum	BRL 54443	methiothepin	G_i/G_o ↓ AC	Not found in mice
5-HT1F	dorsal raphe nuclei, hippocampus, coretex, claustrum, caudate nucleus, brainstem	LY344864, LY344370, BRL 54443, naratriptan, rizatriptan, sumatriptan	methiothepin	G_i/G_o ↓ AC	Unknown
5-HT2A	cortex, claustrum, hippocampus, hypothalamus, basal ganglia	MDL100907 DOI, DOB, DOM, PNU 22394, TCB-2	ketanserin, 4F 4PP, cinanserin, M11939, PNU 96415E	G_q/G_{11} ↑PLC	Decreased anxiety, decreased response to hallucinogens, enteric nervous system reactivity
5-HT2B	cerebellum, septum, hypothalamus, amygdala	αMe5-HT, BW 723C86, 5-methoxy-tryptamine	LY53857 S13200646 SB204741	G_q/G_{11} ↑PLC	Dilated cardiomyopathy, lethal phenotype due to heart defects

5-HT2C	choroids plexus, cortex, hippocampus, amygdala, striatum, substantia nigra	1methylpsilocin, ALEPH-2, CP 809101, lorcaserin, mCCP, MK212, WAY 629	RS 102221, SB 242084, SDZ SER-082	G_q/G_{11} ↑PLC	Altered feeding behaviour; obesity; audiogenic seizures; enhanced cocaine response, cognitive impairment
5-HT3	widespread in brain, but mainly in nucleus tractus solitarius, dorsal vagal complex, limbic structures	SR57227 2Me5-HT 1-phenyl-biguanide, m-chlorophenynl-biquanide	ondansetron, bemesetron, dolasetron, Y 25130, zatosetron	Ligand-gated cation channel	Reduced pain perception
5-HT4	basal ganglia, cortex, septum, hippocampus	Cisapride BIMU8 LS 650155, ML 10302, mosapride, RS 67506, TD-5108	GR113808 SB204070, LY 353433, RS 23597-109 RDZ 205557, SB 203186	Gs ↑AC	Decreased stress response, hypersensitivity to seizures
5-HT5A	hippocampus, hypothalamus, olfactory bulb, cortex, thalamus, striatum, pons	Unknown	SB 699551	G_i/G_o ↓ AC	Increased exploration, altered LSD response
5-HT5B	habenula, raphe nuclei, hippocampus (in rodents)	Unknown	SB 699551	Unknown	Unknown
5-HT6	widespread in brain, but mainly in striatum, amygdala, hippocampus, cortex	E-6801, EMD 386088, R-13c, WAY 466, BCG20-761, BVT 74316, LY 483518, MS-245	RO630563, SAM-531, SB 258510A, SB 271046, SB 742457, SGS-518, SUVN-502, SYN-114	Gs ↑AC	Altered response to alcohol
5-HT7	thalamus, hippocampus, cortex, amygdala, suprachiasmatic nucleus	AS 19, LP 12, LP 44	SB258719, DR 4004, DR 4365, SB258741, SB 2656104-A	Gs ↑AC	Antidepressant-like phenotype, disturbed circadian rhythms, abnormal thermoregulation

Full review of receptor characterization in Filip and Bader, 2009

many of these receptor subtypes, shedding light on their molecular expression patterns and behavioural phenotypes (Table 1.1) (Filip and Bader, 2009). To this day, deciphering the process of 5-HT mediated neurotransmission and its targeting receptors is an ongoing endeavor that is crucial in the development of novel receptor-specific drugs with higher efficacy and fewer side effects.

1.2 The 5-HT1A Receptor

1.2.1 The 5-HT1A receptor as a regulator of the serotonin system

The 5-HT1A receptor is one of the most abundantly expressed 5-HT receptor subtypes in the brain (Peroutka, 1993). Over the years, the human 5-HT1A receptor has become very well characterized, as it was the first 5-HT receptor to be cloned and fully sequenced (Kobilka et al., 1987b; Kobilka et al., 1987a, Fargin et al., 1988). Concurrently, the rat 5-HT1A was cloned using a probe to the coding region of the hamster β_2-adrenergic receptor (Albert et al., 1990) followed by the identification of the murine 5-HT1A receptor, which was isolated from a mouse brain cDNA library (Charest et al., 1993). The 5-HT1A receptor has a widespread pattern of expression in the brain. Presynaptically, it is localized on the cell bodies of serotonergic cells of the raphe nuclei where it acts as an autoreceptor, while post-synaptically it can be found on a large number of pyramidal cells and interneurons of the cortex, the hippocampus as well as the amygdala, the hypothalamus and the septum (Sotelo et al., 1990; Burnet et al., 1995).

The human 5-HT1A gene is an intronless gene, found on chromosome 5, locus 5q11.2-q13 (Kobilka et al., 1987). The 5-HT1A receptor consists of a single

421 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 (TM_1-TM_7) linked by three intracellular (i_1, i_2, i_3) and three extracellular loops(Chanda et al., 1993; Martin et al., 1998). In addition, four consensus sites for phosphorylation by protein kinase C (PKC) (Thr^{229}, Ser^{253}, Thr^{343} in the i_3 loop and Thr^{149} in the i_2 loop) have been implicated in receptor desensitization. Interaction of the receptor with a G protein occurs at the level of the intracellular loops and the internal C-terminal tail. On the other hand, sites for ligand binding appear to vary depending on the receptor and the ligand. It is believed that binding of ligands (such as 5-HT) occurs via the negatively charged Aspartic acid (Asp) residue in the TM_3, which forms a salt bridge with the protonated NH_2 group of monoamines. In addition, the hydrophobic and hydrogen bonding interactions between a ligand and the residues in $TM_{6/7}$ are also believed to play a role in binding affinity (Ostrowski et al., 1992; Albert et al., 1998).

The 5-HT1A receptor is an inhibitory receptor that couples mainly via the pertussis toxin (PTX)-sensitive G_i/G_o. Upon agonist binding, the 5-HT1A receptor undergoes a conformational change and activates the G protein complex. When the G protein is in its inactive state, it consists of a Gα (GDP-bound), Gβ and Gγ subunit. Once activated, GDP is exchanged for GTP, which results in dissociation of the GTP-bound α subunit from the βγ dimmer (Gilman, 1987; Bourne et al., 1991), followed by subsequent activation of various effector molecules. More specifically, the Gαi subunit inactivates adenylyl cyclase activity, thus reducing cAMP accumulation (Figure 1.5). On the other hand, the Gβγ subunit activates its own signaling

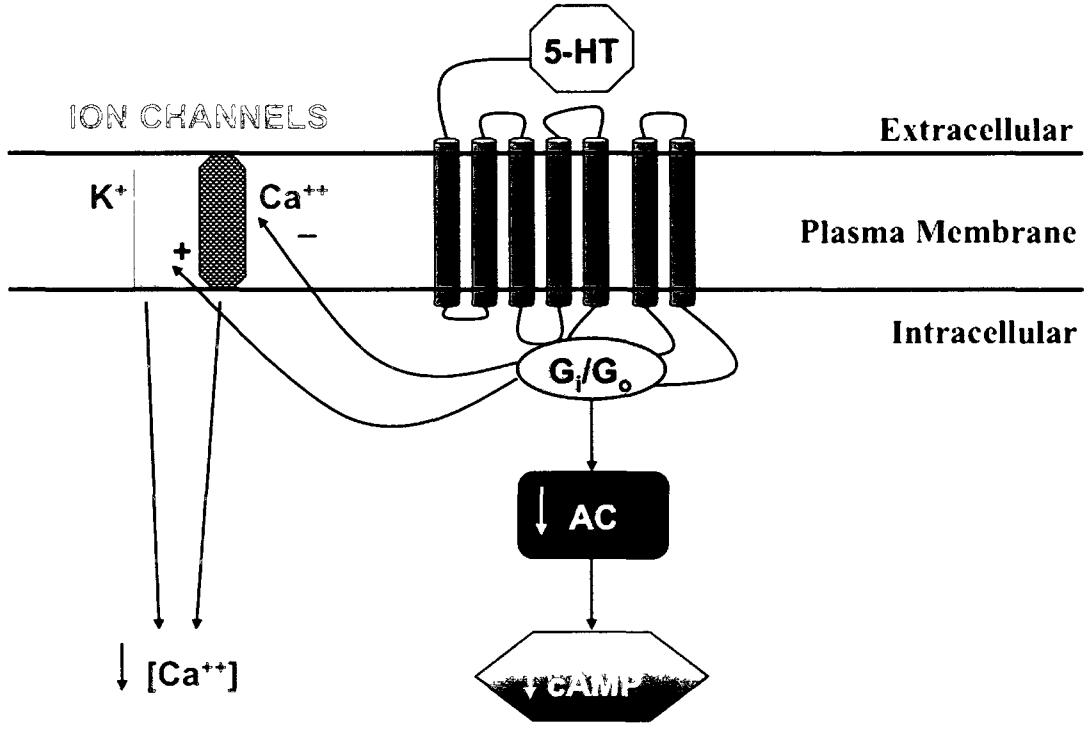


Figure 1.5: 5-HT1A receptor Activation and Signaling

The 5-HT1A receptor signals via Gi/Go proteins to mediate inhibitory actions in neurons. These include 1) inhibition of adenylyl cyclase (AC) which decreases cyclic AMP (cAMP) levels; 2) opening of potassium (K^+) channels to hyperpolarize the membrane potential and reduce the firing rate, and 3) inhibition of N-type or L-type calcium channels to decrease intracellular calcium concentration (Ca^{++}). Adapted and modified from Albert and Lemonde, 2004.

pathways 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) β2 and β3 isoforms (Exton, 1996), adenylyl cyclase type II and IV (Sunahara et al., 1996) and phosphatidyl-inositol-3-kinase-dependent and tyrosine kinase-dependent activation of the MAPK cascade (van Biesen et al., 1996). The hydrolysis of GTP to GDP by the Gα subunit allows for all of the G protein subunits to re-associate themselves back to an inactive heterotrimeric complex.

In addition to its complex signaling pathways, the 5-HT1A receptor plays a crucial role in the control of serotonergic activity and neurotransmission. The presynaptic 5-HT1A 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). This is accomplished through the coupling of 5-HT1A autoreceptors to two different ion channels: the potassium channel and the calcium channel (Figure 1.5). When the 5-HT1A receptor becomes activated, it 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). As a result, 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 (varicosities of axonal 5-HT fibers). As part of a negative feedback mechanism, serotonin in turn activates 5-HT1A autoreceptors to suppress the discharge of serotonergic neurons and reduce the

release of serotonin. Apart from its active role in regulation of 5-HT neurotransmission, the 5-HT1A receptor also influences regulation of other neurotransmitters. Stimulation of 5-HT1A receptors facilitates acetylcholine (ACh) and noradrenaline (NA) release in the brain, as well as increase in blood levels of corticotrophin-releasing hormone (CRH) and adrenocorticotropic hormone (ACTH) (Fink and Gothert, 2007; Jorgensen, 2007; Filip and Bader, 2009).

In addition to 5-HT1A autoreceptors, both 5-HT1B and 5-HT1D (and to lesser extend 1E and 1F) receptor subtypes are also present presynaptically in the DRN, and help in regulating negative feedback inhibition of the firing rate of serotonin out of the raphe. More specifically, the 5-HT1B/1D receptors are found primarily at sites of serotonin release (i.e., synaptic terminals or axonal varicosities) and activation of these receptors 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). These autoreceptors are only activated when an excess of serotonin is present in the extracellular space, and therefore "fine tune" the release of 5-HT (Adell et al., 2002). It is important to note that both 1B and 1D also function as heteroreceptors on gamma-aminobutyric acid (GABA), ACh and glutamate neurons to control the release of their corresponding neurotransmitters (Lanfumey and Hamon, 2004).

Besides the direct pathway of negative feedback inhibition by pre-synaptic 5-HT1A autoreceptors, an indirect feedback mechanism also exists. This negative feedback pathway regulates serotonergic activity through the involvement of postsynaptic 5-HT1A receptors located in the median prefrontal cortex (mPFC).

Disruption of mPFC projections to the raphe prevents the inhibitory actions of 5-HT1A agonist on raphe firing (Ceci et al., 1994; Hajos et al., 1999) and inhibition of mPFC 5-HT1A receptors augments glutamatergic stimulation of raphe neurons (Celada et al., 2001). Thus, both pre- and postsynaptic 5-HT1A receptors constitute pivotal players in the regulation of the serotonergic neurotransmission and thus represent attractive targets for the treatment of mood disorders.

1.2.2 Acute Desensitization of the 5-HT1A Receptor

The initial step in the process of acute receptor desensitization involves agonist-induced activation of the 5-HT1A receptor, and results in the uncoupling of G-proteins from the receptor. This process is very rapid (seconds to minutes) and occurs as a result of receptor phosphorylation by second-messenger regulated kinases (PKA and PKC) and by G protein-coupled receptor kinases (GRKs) (Ferguson, 2001). In general, both PKA and PKC, which are activated by cAMP 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 (including the 5-HT1A receptor) in a "heterologous", or "non-agonist-specific" manner (not dependent on ligand activation) (Lembo and Albert, 1995; Wu et al., 2002). On the other hand, "agonist specific" desensitization of the 5-HT1A receptor is dependent on GRK-induced phosphorylation of activated (agonist-occupied) receptors (Benovic et al., 1986; Hausdorff et al., 1990). GRKs belong to a family of serine/threonine kinases comprising seven members (GRK1-GRK-7). In order to phosphorylate the 5-HT1A 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 (GRK1, GRK4 and GRK6) while the others are not (although GRK2 and GRK3 contain Gbeta-gamma-interacting domains). Ultimately, GRKs phosphorylate the 5-HT1A receptor, which results in receptor uncoupling, similar to that induced by PKC/PKA activity. The uncoupling process generates high affinity sites for the binding of β-arrestin proteins and ensures that 5-HT1A desensitization occurs by preventing further coupling to G proteins.

The next step in acute receptor desensitization involves agonist-promoted endocytosis, or internalization. This mechanism is also quite rapid and occurs within minutes leading to the removal of receptor from the plasma membrane into clathrin coated vesicles via the recruitment of β-arrestins and the assembly of clathrin-coated pits (Ferguson et al., 1996). This step can be rapidly reversible depending of the affinity of the phosphorylated receptor for β-actin (Ferguson, 2001). Interestingly, the internalized receptor can still initiate new signaling pathways such as coupling to mitogen-activated protein kinase (MAPK) rendering it an acute initiator of various adaptive changes, hence, not completely inactive.

The final step in acute receptor desensitization involves targeting the receptor for degradation or recycling. This process involves prolonged agonist-stimulation, which takes hours or several days to complete and may involve monoubiquitinylation of the receptor (or its associated proteins) to target vesicles for fusion with lysosomes resulting in receptor degradation (Shenoy and Lefkowitz, 2003). Unfortunately, the processes involved in acute receptor degradation are still

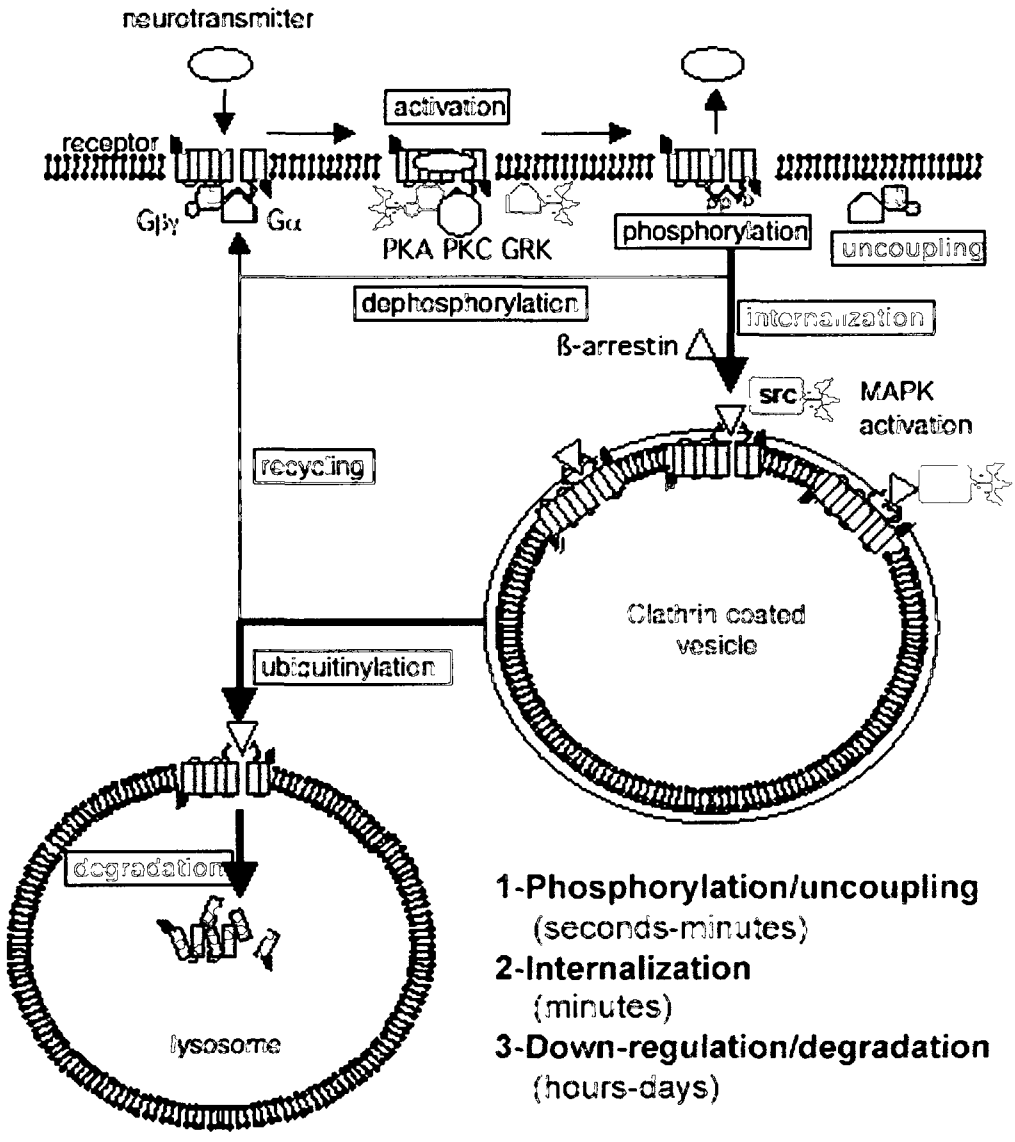


FIGURE 1.6: Acute Desensitization of the 5-HT1A Receptor

Mechanisms of receptor desensitization include: 1) receptor activation which leads to uncoupling, 2) receptor internalization mediated via clathrin-coated vesicles and 3) receptor down-regulation and/or degradation by fusing with lysozomes. Copyright © Albert and Lemonde, 2004, Sage Publications.

incompletely understood. Moreover, long-term (chronic) desensitization of the 5-HT1A receptor, rather than its acute internalization, is believed to be involved in regulating depression.

1.3 Major Depressive Disorder

1.3.1 Overview of Major Depression

According to the World Health Organization (WHO), major depressive disorder (MDD, also known as unipolar depression) effects at least 16% of the population, and is predicted to rise from fourth to second leading cause of disability by the year 2020 (Doris et al., 1999). Depression remains one of the most under-recognized and under-treated disorders, with less than 10% of patients receiving adequate treatment, and is still considered a "privileged disorder" (Keller and Boland, 1998). It is characterized by a wide variety of symptoms that can include: prolonged depressed mood, diminished interest or pleasure (anhedonia), sleep disturbances, changes in appetite and weight, decreased sex drive, fatigue or loss of energy, general avoidance, psychomotor agitation, feelings of worthlessness, difficulty in concentrating and recurrent thoughts of death or suicide (Williams, 2001); American Psychiatric Association, 1994). In addition, MDD is a major predisposing factor for suicide, which accounts for 24% of the deaths among depressed aged 15-24 years old, and 16% among those aged 25-44 (Doris et al., 1999; Fava and Kendler, 2000). Chronic MDD (which effects at least one third of all patients) is diagnosed by a prolonged episode of one of more symptoms that last longer than two years. In addition, a patient who has two or more episodes of MDD symptoms

has a 60-90% recurrence rate of this disorder (Keller, 2002). Women are about twice as likely as men to develop the disease, which may be attributed in part, to hormonal variations (Weissman et al., 1993; Lehtinen and Joukamaa, 1994; Fava and Kendler, 2000). The onset of MDD symptoms in women is often more prevalent after puberty, which corresponds to the time of major change in the neuroendocrine reproductive axis, and may be influenced by factors such as: menstrual cycle, pregnancy, miscarriage, postpartum time-period and menopause.

Major depression is a heterogeneous disorder, bearing both genetic and environmental components, where approximately 40%-50% of the risk for depression is of genetic origin (Fava and Kendler, 2000). Although MDD is a highly heritable disorder, correlating genes with this illness has thus far been challenging. This is most likely due to its multigenetic factors that combine various gene mutations to create a synergistic effect, which manifests itself as predisposition to the illness. Thus, no single genetic variation has a major risk factor associated with MDD directly, which makes the genetics of this disorder very difficult to characterize. In addition, monozygotic twin studies reveal that vulnerability to depression is only partially genetic, and in fact, multiple risk genes must interact in conjunction with non-genetic/environmental factors to elicit the disease (Hyman, 2000). Most common environmental stressors that contribute to the development of this illness include psychological stressors related to the loss of a job, income or loved one as well as humiliation and abuse (both sexual or physical) (Kessler, 1997; Fava and Kendler, 2000). Certain personality traits such as neuroticism, which reflect the predisposition to develop emotional upset under stress, can increase the

risk of developing MDD (Fava and Kendler, 2000). However, it is important to note, that not all stressful life experiences result in, or contribute to depression.

Oppositely, genetic predisposition can also influence ones ability to respond to stress, which in turn can trigger the onset of the disorder. Pharmacological evidence suggests that impaired serotonin neurotransmission contributes to the underlying cause of depression. As such, an important study has shown that a functional polymorphism in the promoter region of the 5-HT transporter gene (responsible for 5-HT reuptake into neurons) can effect how stressful life events influence the onset of depression (Caspi et al., 2003). Individuals with at least one copy of the short allele exhibit more severe symptoms of depression and suicidability in relation to stressful life events than those homozygous for the long allele, emphasizing the importance of genes in response to environmental stimuli and behavioural phenotype.

1.3.2 Treatment of Major Depressive Disorder

Over the years, various approaches to successful treatment of depression have been administered including psychotherapy, electroconvulsive therapy (ECT), as well as treatment with oral antidepressant medications, some proving more effective than others. The first generation of oral antidepressants was composed of Tricyclic antidepressants (TCAs) and Monoamine Oxidase Inhibitors (MAOIs) but their clinical use over the years has become limited due to their wide range of side effects, including serious cardiovascular complications, sedation, weight gain, hypotension and lethality when overdosed (Fava and Kendler, 2000). TCAs (e.g., imipramine and

clomipramine, desipramine) at therapeutic doses are likely to inhibit noradrenergic and, to a lesser extent, serotonergic reuptake and to antagonize several neurotransmitter receptors (Richelson, 1991). TCAs can mediate at least five different activities, functioning as: (1) a serotonin reuptake inhibitor (increase post-synaptic 5-HT1A receptor sensitivity, without altering autoreceptor sensitivity (de Montigny and Aghajanian, 1978; Chaput et al., 1991), (2) a noradrenaline reuptake blocker; (3) an anticholinergic-antimuscarinic drug; (4) an α_1-adrenergic agonist; and (5) an antihistamine (Stahl, 1998). As an alternative to TCA treatments, patients resistant to treatment are sometimes prescribed MAOI therapy instead. MAO inhibitors function by inhibiting the activity of monoamine oxidase, an enzyme responsible for deaminating various neurotransmitters (Krishnan, 2007). Since MAOIs are very powerful, they display potential lethal drug interactions, and are thus reserved as a last line of defense (Lopez-Munoz and Alamo, 2009). MAOIs are comprised of both the classical, irreversible as well as non-selective inhibitors of monoamine oxidase (e.g., tranylcypromine and phenelzine). Recently, more selective MAOIs, specific for MAO-A (deaminates 5-HT, melatonin, epinephrine and norepinephrine) or MAO-B (deaminates phenylethylamine and dopamine) subtypes of the enzyme have been designed with reversible properties (e.g., moclobemide). In addition, a patch form of this inhibitor (selegiline) has recently been developed with timed-release technology (Culpepper and Kovalick, 2008).

Instead, a new generation of antidepressants has been discovered with fewer side effects and higher efficiency, which includes selective serotonin reuptake inhibitors (SSRI). Due to their structural similarity to monoamines and 5-HT in

particular, SSRIs block the pre-synaptic reuptake sites for serotonin, thus increasing 5-HT levels in the synaptic cleft while prolonging activation of post-synaptic receptors (Sharp et al., 1997). Few examples of well-known SSRIs include: fluvoxamine-Luvox®, paroxetine-Paxil®, citalopram-Celexa®, sertaline-Zoloft® and fluoxetine-Prozac®. In addition, SSRI help alleviate other disorders associated with serotonergic dysfunction such as anxiety, panic disorder, obsessive-compulsive disorder (OCD) eating disorder and posttraumatic stress disorder (PTSD), making them that much more attractive in treatment (Raap and Van de Kar, 1999). More recently, a new line of antidepressant treatments has been developed that target both, the serotonin and norepinephrine reuptake inhibitors (SNRIs) (Deecher et al., 2006). Some more popular SNRIs include: venlafaxine-Effector®, milnacipran-Ixel®, desvanlafaxine-Pristiq® and Duloxetine-Cymbalta®. A class of antidepressants with triple reuptake inhibitor properties has also been invented, targeting the serotonin-norepinephrine-dopamine reuptake systems (SNDRI) by blocking the serotonin transporter (SERT), the norepinephrine transporter (NET) and dopamine transporter (DAT), respectively. Fairly new and still in clinical trials, this group of compounds has one available NDRI called bupropion-Zyban® (Zisook et al., 2006). Finally, partial agonists such buspirone, ipsapirone and gepirone are also sometimes used in treatment, with binding efficacy for the 5-HT1A receptor, and function to compete with 5-HT for receptor occupancy (Fanelli et al., 1990). In each case, the ultimate goals are greater efficacy, fewer side-effects and quicker onset of action.

1.3.3 The Role of 5-HT1A Receptors in Anxiety and Depression

Given the central role of the 5–HT1A receptor in regulation of the serotonin system and its post-synaptic targets, many studies have examined the involvement of 5-HT1A receptors in the etiology of depression and anxiety-like behaviours. There is now accumulating evidence that dysregulation of 5-HT1A receptor levels occurs in patients suffering from depression and related mood disorders. Analysis of postmortem brainstem samples from depressed suicides highlights a significant increase in presynaptic levels of the 5-HT1A autoreceptor compared to normal individuals, particularly in rostral divisions of the dorsal raphe nuclei which project to the prefrontal cortex (Stockmeier et al., 1998; Boldrini et al., 2008). In contrast, positron emission tomography (PET) analyses have found that 5-HT1A binding potential is decreased in cortical and raphe regions of patients suffering from depression compared to control individuals (Meltzer et al., 2004; Drevets et al., 2007). However, a number of variables in depressed subjects, including antidepressant treatment, chronic cortisol elevation, or decreased numbers of 5-HT neurons could decrease 5-HT1A receptor expression compared to controls, contributing to decreased 5-HT1A binding potential. In post-mortem tissue from depressed suicide victims, the number of serotonergic neurons appears to be reduced in the dorsal raphe (Arango et al., 2001). The reduction of 5-HT neurons is accompanied by a decrease in the number of serotonergic projections to the cortex of depressed suicide victims (Austin et al., 2002). Therefore, it remains unclear whether 5-HT1A autoreceptor levels are increased in the raphe of depressed individuals, since such changes may be hidden by compensatory, developmental or

hormonal changes. Postsynaptically, 5-HT1A receptor mRNA levels are decreased in the hippocampus of patients suffering from major depression (Lopez-Figueroa et al., 2004). Similarly, a decrease in 5-HT1A signaling is also observed in post-mortem cortical tissue from suicide victims(Hsiung et al., 2003), implying impaired coupling of the receptor to downstream effectors. PET imaging studies have confirmed lower levels of 5-HT1A receptors in the dorsolateral prefrontal cortex of human subjects with major, bipolar or post-partum depression (Drevets et al., 2000; Sargent et al., 2000; Drevets et al., 2007; Hasler et al., 2007; Moses-Kolko et al., 2008), as well as in patients suffering from panic disorder (Neumeister et al., 2004). Altogether, these studies demonstrate that alterations in the 5-HT1A are observed in patients suffering from major depression, bipolar depression and anxiety disorders. Overall, increased presynaptic 5-HT1A autoreceptors, decreased postsynaptic 5-HT1A levels, or decreased 5-HT-positive neurons are all expected to cause a decrease in serotonergic neurotransmission, which is a hallmark of depression.

Despite limitations in their ability to model human mental illness, animal models further support a role for the 5-HT1A receptor in anxiety/depression-like phenotypes (Gordon and Hen, 2004). Knockout mice lacking the 5-HT1A receptor (everywhere, throughout life) show increased anxiety-like behaviour in a variety of tests, however, they exhibit decreased behavioural despair in response to stress (Heisler et al., 1998; Parks et al., 1998; Ramboz et al., 1998). Until recently, these mixed phenotypes of anxiety and lack of stress-induced despair associated with depression have yielded confusing interpretations, mainly since these studies did not distinguish between the role of 5-HT1A auto and hetero-receptors in regulating

behaviour. Interestingly enough, two new mouse models have recently been developed that specifically target either the pre- or the post-synaptic 5-HT1A receptor expression. In the pre-synaptic model, increasing levels of 5-HT1A autoreceptors in adulthood is sufficient to impact reactivity to stress and depression-related behaviour without affecting anxiety measures observed in previous knockout studies (Richardson-Jones et al.). Oppositely, the post-synaptic mouse model demonstrates that mice overexpressing 5-HT1A heteroreceptors in the early postnatal period show reduced anxiety behaviours (Kusserow et al., 2004). In addition, early postnatal restoration of 5-HT1A receptor expression in the forebrain structures is sufficient to completely rescue the anxiety phenotype of the 5-HT1A adult knock-out mice (Gross et al., 2002). Therefore, pre-synaptic 5-HT1A expression is crucial in modulating normal serotonergic tone and anti-depressive behaviour while 5-HT1A receptor expression in postsynaptic regions plays a key role in ensuring proper serotonergic development and establishing a normal anxiety phenotype (Alexandre et al., 2006; Mehta et al., 2007a).

1.3.4 The Role of 5-HT1A receptors in Antidepressant Treatment

Strong evidence suggests that the reduction in serotonergic activity observed in depressed patients is due to increased levels of 5-HT1A autoreceptors. Unfortunately, pharmacological agents used to treat major depression such as MAOIs, TCAs and particularly SSRIs are only effective after chronic drug administration ranging between 2 to 6 weeks, which correlates to the time course of clinical improvement (Montgomery, 1997, Blier and de Montigny, 1994). This

implies that long-term adaptive changes with respect to serotonergic regulation must be taking place.

Previous studies have shown that the presence of somatodendritic autoreceptors on 5-HT neurons provides a sensitive mechanism of self-regulation, whereby 5-HT released from axon collaterals reaches 5-HT1A autoreceptors and decreases neuronal firing rates (Blier and de Montigny, 1985). It is this negative feedback autoregulatory mechanism that is believed to account for the delay in efficacy of SSRIs and explains the failure of acute antidepressant therapy which resides in the fact that the rapid increase in synaptic 5-HT levels seen following SSRI blockade of the SERT, is compensated by the reduction in firing frequency due to recurrent activation of 5-HT1A autoreceptors (Pineyro and Blier, 1999). However, the sustained upregulation of 5-HT leads to a selective and progressive desensitization of the 5-HT1A autoreceptors, thereby disinhibiting serotonergic neurotransmission and restoring firing frequency to normal (Figure 1.7). It is the homologous down-regulation of 5-HT1A autoreceptors, which accounts for the late therapeutic effects of antidepressant treatments (Albert and Lemonde, 2004; Blier and de Montigny, 1994). Understanding mechanisms involved in long-term transcriptional regulation of the 5-HT1A receptor are thus crucial in improving drug efficacy for quicker alleviation of depressive symptoms.

Naturally, improving the onset of SSRI treatments and thus bypassing the period of time required to downregulate 5-HT1A autoreceptors, has been investigated (Artigas et al., 1996; Trillat et al., 1998; Kinney et al., 2000). Co-administration of SSRIs with the mixed β-adrenoreceptor/5-HT1A receptor

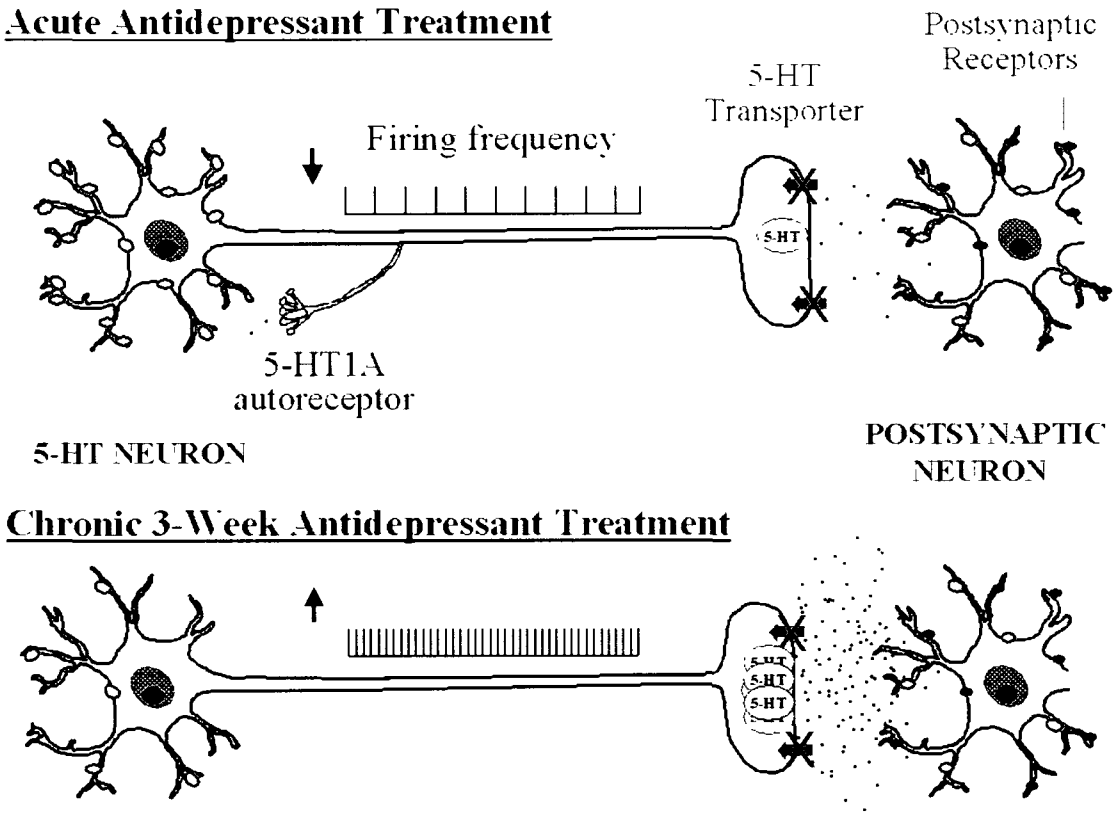


Figure 1.7: SSRI-Mediated Desensitization of the 5-HT1A Receptor

Prior to SSRI treatment, activation of 5-HT1A autoreceptors is mediated by the continuous 5-HT release from axon collaterals or through diffusion from the synaptic cleft. Upon acute SSRI treatment, the blockage of 5-HT transporter increases free 5-HT, as well as 5-HT1A autoreceptor inhibition, resulting in an overall decreased firing rate. Upon chronic SSRI treatment, homologous desensitization of 5-HT1A receptors disinhibits serotonergic firing, thus increasing 5-HT release. Adapted and modified from Albert and Lemonde, 2004.

antagonist pindolol or the selective 5-HT1A antagonist WAY 100635 produces a greater elevation of extracellular brain 5-HT levels compared with SSRI treatment alone (Hjorth et al., 1996) and accelerates the onset of antidepressant action in humans from three weeks to one (Artigas et al., 1994; Blier and Bergeron, 1995). In addition to 5-HT1A agonists, recent findings have discovered a putative new class of antidepressants with a rapid onset of action that specifically target 5-HT4 receptors. Animal studies using 5-HT4 agonists such as RS673333 show that acute treatment of only 3 days results in 5-HT1A receptor desensitization, similar to that observed with classical antidepressant responses seen only after 2-3 weeks (Lucas et al., 2007). Moreover, 5-HT4 agonists reduce behavioural patterns characteristic of a depressive phenotype, thus furthering their antidepressant potential.

Oppositely, post-synaptic 5-HT1A receptors localized to target regions appear to be resistant to the desensitization process and in addition, appear slightly upregulated following antidepressant treatment (Blier and de Montigny, 1990; Hensler, 2003). Thus, forebrain areas highly innervated by the raphe nuclei projections (such as the hippocampus) result in elevated 5-HT levels. To further verify this mechanism, studies using SERT knockout mice lacking the functional 5-HT transporter, show significantly reduced levels of 5-HT1A receptor mRNA and protein in the raphe nuclei. Oppositely, levels of 5-HT1A expression are increased in the hippocampus region of these mice, thus mimicking chronic SSRI treatment outcome and further implicating 5-HT1A desensitization as a result of abnormal serotonin regulation (Fabre et al., 2000). In addition to behavioural improvement, SSRI treatment can also result in improved neurogenesis (generation of new

neurons) in the adult hippocampus (Malberg et al., 2000). Chronic fluoxetine treatment promotes not only the proliferation of progenitors cells in the dentate gyrus, but also the maturation of newborn granule cells which then integrate into local circuitry (Encinas et al., 2006). As such, ablation of hippocampal neurogenesis by X-irradiation has been shown to completely prevent the behavioural improvement following chronic antidepressant treatment (Santarelli et al., 2003). In addition, enhancement of neurogenesis by chronic SSRI treatment is completely blocked in the 5-HT1A knockout mice, thus implicating 5-HT1A receptors in SSRI-mediated neuro-regeneration (Santarelli et al., 2003). It is likely that increasing 5-HT levels in the hippocampus, activates hippocampal 5-HT1A receptors which triggers a signaling cascade that facilitates proliferation and maturation of granule cells (Patel and Zhou, 2005; Mehta et al., 2007b). In fact, a recent study suggests that 5-HT1A receptors may directly mediate proliferation of neurospheres derived from adult hippocampal neural stem cells (Benninghoff et al.). However, the exact mechanism of 5-HT1A receptor action in SSRI-induced neurogenesis remains largely unknown.

1.4 Regulation of 5-HT1A Gene Expression

Regulation of gene expression utilizes complex transcriptional machinery, which controls level of DNA transcription and its subsequent translation intro protein. It is important to note that the process of transcriptional downregulation leads to reduction in the total number of receptors present in a cell or tissue and therefore

differs from acute receptor internalization, which can be viewed as a rapid physical redistribution of the receptor without any detectable change in receptor number.

1.4.1 Overview of Transcriptional Regulation

In eukaryotes, DNA is wound around a complex of histones (H2A, H2B, H3 and H4) forming an octameric structure called the nucleosome (Lewin, 1997). An additional linker histone (H1) locks the DNA in place around the nucleosome and allows for higher order structures to form. The interaction between nucleosomes enables the formation of the secondary chromatin structure, which can be highly condensed into transcriptionally inactive (or silenced) heterochromatin or decondensed, forming transcriptionally active euchromatin (Elgin and Grewal, 2003). Various types of post-translational modifications help regulate the secondary chromatin structure by modifying both the core and linker histone tails, which protrude from the nucleosome (Latham and Dent, 2007). The main epigenetic processes, which contribute to the regulation of gene expression without sequence modification include: methylation, acetylation, phosphorylation, ubiquitination, SUMOylation and ADP-ribosylation (Kuo and Allis, 1998; Ito, 2007; Oki et al., 2007; Swaminathan et al., 2007; Zhao, 2007). For instance, chromatin remodeling can be accomplished by histone acetylation of lysine residues via histone acetyltransferanses (HATs). HAT transfers the acetyl moiety from acetyl coenzyme A to the amino group of lysine residues, which neutralizes the positive charges and reduces the affinity of histones for the negatively charged DNA (Ito, 2007; Yang and Seto, 2007). This induces a more open DNA structure, providing transcription factors with DNA access to

enhance gene expression (Utley et al., 1998, Latchman, 2005). Oppositely, histones are deacetylated by histone deacetylases (HDACs), which catalyzes the removal of an acetyl group and tightens (or supercoils) the chromatin structure to repress gene transcription by preventing the accessibility of other transcription factors to DNA (Kuo and Allis, 1998). DNA methylation of cytosine residues within CpG sites (cytosine-phosphate-guanosine) is catalyzed by DNA methyltransferases (DNMTs) and also results in transcriptional repression of genes.

The transcription of one DNA strand into its complementary RNA strand is mediated by three types of RNA polymerases: RNA polymerase I (large rRNA), RNA polymerase II (mRNA) and RNA polymerase III (tRNA and other small RNAs). Transcription occurs in three phases (initiation, elongation and termination), during which the 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 which is then translated into a protein in a set of complex reactions that occur on the ribosome (Letchman, 2005). An important region involved in the initiation process of DNA transcription is the proximal promoter region, located within the first 100-200 bp immediately upstream of the initiation start site, and is defined as the minimal DNA sequence capable of promoting and initiating transcription. Within this region are various initiation sequences, which function to recruit regulatory proteins involved in constitutive expression of the gene.

Located approximately 30 nucleotides upstream of the transcription initiation site, the TATA box consists of an AT-rich sequence (consensus TATA(A/T)A(A/T)) which recruits the TATA-binding protein (TBP). Once bound to

the promoter sequence, the TPB recruits TBP-associated factors (TAFs), collectively referred to as the transcription factor IID (TFIID) (Latchman, 2005). Binding of TFIID is stabilized by TFIIA and TFIIB thus forming a large molecular complex. TFIIB then recruits RNA polymerase II, which enters the initiation complex with TFIIF, followed by TFIIE (Zawel et al., 1993). TFIIH is a large complex of proteins and is the last factor to adhere to the complex. Its helicase activity unwinds the DNA and ensures that the appropriate strand is transcribed to initiate transcription, while its kinase properties mediate phosphorylation of RNA polymerase II in its C-terminal domain to relax the preinitiation complex and commence elongation (Ogbourne and Antalis, 1998).

A recent study of over 1000 gene promoter sequences found that only 34% contain a consensus TATA box sequence while the remaining genes have other initiation motifs (Suzuki et al., 2001). Amongst them is the Initiator (Inr) motif, located within a few bases from the initiation start site (Smale, 1997). In the absence of the TATA box, Inr facilitates the binding of TFIID and determines the precise location of the initiation start-point. In the case of a promoter that is both TATA-less and initiator-less, initiation can occur in the presence of GC rich regions (GC boxes) (Pedersen et al., 1999), which are recognized by transcription factors such as Sp1 and Myc-associated zinc finger protein (MAZ). The CCAAT box is another motif found in a wide variety of promoters (Latchman, 2005). Its sequence binds a family of C/EBP (CCAAT/enhancer binding protein) proteins as well as the CTF/NF1 (CCAAT transcription factor/ nuclear factor 1) transcriptional complex, stimulating transcription and DNA replication respectively. Finally, some TATA-less promoters

contain ETS motifs and the pyrimidine-rich initiator motifs that also have the ability to recruit transcriptional initiators (Parks and Shenk, 1996; Smale, 1997), which in turn recruit specific cofactors such as TAFs and TFIID, ultimately leading to the recruitment of RNA polymerase II (Azizkhan et al., 1993; Bouwman and Philipsen, 2002).

Although the core proximal promoter, located within the first 200 nucleotides upstream of the start codon is crucial for the initiation of transcription, sequences located more distal also play an important role in gene regulation. Enhancer elements which bind gene activators and repressor elements which bind gene repressors, can often be located several Kb upstream of the initiation start site (Goodbourn, 1990). These regulators are tissue specific and often form complexes through their protein-protein interactions, thus enabling recruitment of the transcriptional machinery to the promoter. They can function in an orientation-independent manner, which in some cases allows for potential looping of these elements onto the promoter to interact with components of the transcription initiation complex. The activity of enhancer or repressor elements can vary between promoters and may depend on the proximity and overall spatial arrangement of other elements within a particular gene. Gene expression can be effected by the activity of an inducible system, in which various response elements respond to external signals and "induce" the rate of gene expression. Some of the more common inducible elements include the heat-shock element (HSE), cAMP-responsive element (CRE) and steroid-thyroid hormone responsive elements (HRE), which bind HSF, CREB and nuclear receptors, respectively (Tanguay, 1988; Montminy et al., 1990).

These transcription factors often recognize palindrome sequences and bind as homodimers or heterodimers.

Although regulatory mechanisms controlling the initiation of transcription are crucial in regulating gene expression, virtually every step between regulation of genomic DNA structure to generation and metabolism of protein is closely regulated. Following transcriptional initiation, the RNA undergoes a process of elongation followed by RNA maturation, which includes addition of the 5'cap, splicing out introns, and polyadenylation (Latchman, 2005). Finally the mature RNA is transported to the cytoplasm where it undergoes stages of translation, which includes initiation, elongation and termination. The synthesized protein can undergo a wide variety of post-translational modifications as mentioned earlier. Due to the number of steps involved in proper gene expression, it becomes extremely difficult to study and predict the outcome of every regulatory cascade involved or altered. Thus, an important step in understanding tissue- and disease-specific transcriptional regulation of a target gene is the identification of gene-specific transcriptional regulators that play a role in regulating its transcription.

1.4.2 5-HT1A transcriptional regulation

The 5-HT1A gene is composed of a proximal promoter containing highly conserved DNA elements for the binding of zinc-finger binding proteins Sp1/MAZ1 and NFκB within the initial 1-kb of the 5'-sequence (Parks and Shenk, 1996). These elements drive transcriptional initiation via multiple TATA-less sites (for human and mouse 5-HT1A genes) or a TATA-containing site (in the rat 5-HT1A gene) (Storring et al.,

1999). In addition, a few consensus NFκB sites are present in the rat and human promoters, but the contribution of each to 5-HT1A gene regulation still needs to be determined (Cowen et al., 1997). Upstream of the 1-kb promoter/enhancer, a powerful 31-nucelotide dual repressor element (DRE, located between -1519/-1590 bp) was identified to be essential for silencing of the 5-HT1A receptor gene. The DRE is composed of a 14-bp 5'-portion (FRE) that is recognized by a transcriptional repressor called Freud-1 (Five-prime Repressor Element under dual repression -1) (Ou et al., 2000) (Figure 1.8). It mediates gene repression in neuronal cells expressing the 5-HT1A receptor and has also recently been linked to non-syndromal mental retardation (Basel-Vanagaite et al., 2006; Rogaeva et al., 2007; Noor et al., 2008). In addition, a novel repressor has recently been identified called Freud-2 (Five-prime Repressor Element under dual repression -2), which binds to the 5-HT1A DRE (at either 5' or 3' end) and represses the human 5-HT1A receptor gene expression in non-serotonergic cells and neurons (Hadjighassem et al., 2009). A novel GRE (glucocorticoid responsive element) half site repeat (5'-TGTCCT) separated by six nucleotides (at -1169 bp) confers negative regulation of the rat 5-HT1A receptor promoter via interaction with GR/MR (glucocorticoid/ mineralocorticoid) heterodimers. This element is also conserved in the mouse and human but functionality has only been investigated for the rat nGRE (Ou et al., 2001).

A consensus 21-bp Repressor Element-1/Neuron Restrictive Silencing Element (RE-1/NRSE) was identified in the 5-HT1A promoter sequence, which binds an important transcriptional repressor known as the Repressor Element-1

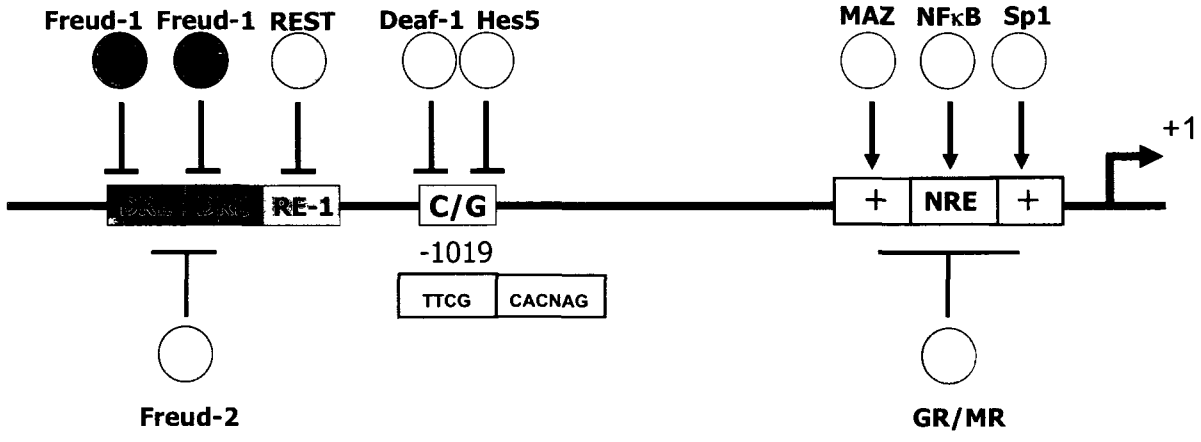


Figure 1.8: 5-HT1A Receptor Promoter Region

Transcriptional regulatory elements of the human 5-HT1A gene show the enhancer region upstream with its corresponding domains for the transcriptional activators MAZ, NFkB and Sp1. The site for GR/MR binding is also located within the enhancer region of the promoter. The repressor region is shown further upstream and it includes biding sides for transcriptional repressors Freud-1/Freud-2 and REST. Located approximately 1kb upstream of the initiation start site is the 26-bp palindrome which includes the C/G polymorphism and binds two transcriptional regulators, Deaf-1 and Hes5. Adapted and modified from Albert and Lemonde, 2004.

Silencing Transcription factor (REST, also known as Neuron-Restrictive Silencing Factor, or NRSF) (Lunyak and Rosenfeld, 2005; Shimojo and Hersh, 2006). REST is a tissue-specific silencer that suppresses gene expression outside of the nervous system. 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. It is not surprising that the transcription factor Pet-1, responsible for regulating serotonin system development as described earlier, contains binding sites within the promoter region of several genes that are markers of serotonin neurons including the serotonin transporter, tryptophan hydroxylase, L-amino acid decarboxylase as well as the 5-HT1A receptor gene. In the 5-HT1A receptor gene, Pet-1 ETS domain interacts at the AGCAGGAAGTT enhancer sequence from -137 to -127 bp of the 5-HT1A receptor where it is thought to activate its transcription in serotonergic neurons (Hendricks et al., 1999). Finally, the human 5-HT1A promoter contains a 26-bp palindrome sequence that encompasses binding sites for two important transcription factors involved in 5-HT1A gene regulation, known as Deaf-1 and Hes-5 (Figure 1.8).

1.4.3 5-HT1A transcriptional regulation at the C(-1019)G polymorphism

In recent years, genetic variations in the 5-HT1A receptor gene have been implicated in the pathology of mood disorders. Many single nucleotide polymorphisms (SNP) have been discovered in the 5-HT1A gene, although only 23 thus far have been formally validated (Drago et al., 2007). Several of these genetic

variants have been studied in more detail, and those include Ile28Val, Arg219Leu and Gly22Ser, but each was found to be quite rare and have very low prevalence in the normal population (Bruss et al., 1995; Rotondo et al., 1997; Bruss et al., 2005). However, in 1999, Wu and Comings reported a common C(-1019)G polymorphism in the human 5-HT1A receptor promoter region. In subsequent studies it was shown that this polymorphism (bold) is located in an imperfect 26-bp palindrome region with the following sequence: 5'-AA**C**GAAGACACACTCGGTCTTCTT-3' (Lemonde et al., 2003). This palindromic region is recognized by two transcription factors, Deaf-1 and Hes5, in an allele-specific manner, where the C-allele binds to these proteins while the G-allele does not.

Deaf-1, which stands for Deformed epidermal autoregulatory factor-1, was first identified in *Drosophila* as a non-homeodomain cofactor for the embryonic gene called Deformed that plays a role in proper development of segmentation patterns (Gross and McGinnis, 1996; Veraksa et al., 2002). Mammalian Deaf-1 was cloned as a binding protein for a retinoic acid response element and shown to have enhancer activity at the proenkephalin promoter, but not thymidine kinase promoter (Huggenvik et al., 1998). Oppositely, Deaf-1 has the ability to act as a repressor at the heterogeneous nuclear ribonucleoprotein A2/B1 promoter (Michelson et al., 1999), making it a factor with both enhancer and repressor abilities. Atypical expression of Deaf-1 also plays an important role in the development of cancer where its loss of function mutations produce early embryonic arrest in *drosophila* (Veraksa et al., 2002) while its overexpression enhances proliferation in human breast epithelial cells (Barker et al., 2008).

Deaf-1 knockout mice exhibit neural tube closure defects and 80% of these knockouts display exencephaly during early embryonic development (Hahm et al., 2004). Deaf-1 has been shown to interact with several co-regulators including Lim Domain Only-4 (LMO4), a calcium-regulated transcription factor that plays a crucial role in development (Sugihara et al., 1998; Kashani et al., 2006). Interestingly, LMO4 knockout mice display a similar, but more severe, exencephaly phenotype to that observed in Deaf-1 knockouts (Hahm et al., 2004; Tse et al., 2004; Lee et al., 2005) thus predicting potential overlapping transcriptional actions of these factors.

The mammalian homologue of Deaf-1 is often referred to as NUDR (Nuclear Deaf-1-Related Protein), which is a 59-kDa transcription factor sharing 46% amino acid similarity with the *Drosophila* protein (Sugihara et al., 1998). Deaf-1 is a transcription factor containing a Helix-loop-helix (HLH) motif consisting of two amphipathic alpha helices, connected by a flexible loop responsible for mediating both DNA binding and dimerization (Lewin, 1997). Deaf-1 also contains a conserved ~80-sequence motif called SAND (for Sp100, Aire-1, NucP41/75 and DEAF-1) responsible for DNA binding (Christensen et al., 1999; Bottomley et al., 2001; Huggenvik et al., 1998). The SAND domain structure represents a novel compact $\alpha_{(4)}/\beta_{(5)}$ fold in which a conserved KDWK sequence motif is found within an alpha-helical, positively charged surface patch. Deaf-1 contains a highly conserved carboxyl-terminal zinc-finger motif called the MYND (named for MYeloid translocation protein 8, Nervy, Deaf-1) domain which is also involved in mediating protein-protein interactions (Gross and McGinnis, 1996; Spadaccini et al., 2006). In addition, Deaf-1 contains two self-interacting domains, suggesting the potential for dimerization (Huggenvik et al.,

1998) as well as a nuclear localization signal (NLS) and a nuclear export signal (NES), which provides a basis for the control of its subcellular localization of function.

Deaf-1 was initially identified as a transcriptional regulator of 5-HT1A gene expression using the yeast-two-hybrid approach and its functional interaction with the C-allele of the 26-bp palindrome sequence was confirmed through reporter assays (Lemonde et al., 2003; Czesak et al., 2006). Deaf-1 co-localization with 5-HT1A was shown in the dorsal raphe nucleus (DRN) as well as primary hippocampal and cortical cells (Lemonde et al., 2003). In the DRN, a negative correlation was observed between Deaf-1 and 5-HT1A staining, implying it has repressive abilities. Interestingly enough, Deaf-1 is known to bind with strong affinity to one or more copies of the TTCG motif (Gross and McGinnis, 1996; Huggenvik et al., 1998; Bottomley et al., 2001), which is found in the 5-HT1A palindrome sequence at the site of the C(-1019)G polymorphism. The C to G alters the Deaf-1 consensus site substantially enough to prevent its binding (Lemonde et al., 2003). As a result, Deaf-1 represses the C(-1019) allele of the 5-HT1A promoter (in certain cell types), but not the G-allele.

Hes proteins are the mammalian homologues of *Drosophila Hairy* and *Enhancer of split* proteins that belong to the basic helix-loop-helix (bHLH) class of transcription factors and control the proliferation and differentiation of neural stem cells during embryogenesis (Kageyama et al., 2005). Disruption of Hes1, Hes3 and Hes5 genes leads to premature activation of proneuronal bHLH gene Mash1, accelerated neurogenesis and major defects in the formation of brain structures

(Ishibashi et al., 1995; Hatakeyama et al., 2006). Hes proteins have been shown to inhibit neurogenesis by two distinct mechanisms: 1) they activate transcriptional repression following direct binding to DNA motifs called the N-Box (CACNAG) and recruit Groucho/TLE transcriptional co-repressors (McLarren et al., 2001) or; 2) they inhibit the activity of proneuronal basic-helix-loop-helix (bHLH) proteins (Kageyama and Ohtsuka, 1999).

A putative N-box element was recently identified within the 26-bp palindrome region of the 5-HT1A promoter (proximal to the C(-1019)G polymorphism) using the yeast-two hybrid approach (Lemonde et al., 2003; Czesak et al., 2006). Not surprisingly, Hes5 was shown to bind and repress the 5-HT1A receptor gene in a variety of reporter assays(Lemonde et al., 2003; Jacobsen et al., 2008). Interestingly, Hes1 repressor activity at the N-box was stronger than Hes5, and largely insensitive to the C-G polymorphism(Jacobsen et al., 2008). Since repression by Hes5 is sensitive to the C(-1019)G 5-HT1A polymorphism, it is possible that the G-allele could be associated with increased expression of the 5-HT1A receptor in embryonic 5-HT neurons and could contribute to alterations in the serotonin system that favor the development of depression. For example, proliferation of raphe neurons is enhanced four-fold in embryonic cultures from 5-HT1A knockout mice compared to control mice (Rumajogee et al., 2004), suggesting that an upregulation of embryonic 5-HT1A autoreceptors may reduce 5-HT neuron number as seen in depressed suicides (Arango et al., 2001). Thus, alteration in 5-HT1A receptor expression by the C(-1019)G in embryonic or early life stages may be more important in determining adult predisposition to depression and anxiety than

the absolute level of 5-HT1A receptors observed in the adult (Gross et al., 2002; Kusserow et al., 2004; Alexandre et al., 2006).

1.5 The 5-HT1A C(-1019)G Polymorphism

1.5.1 Association of the 5-HT1A C(-1019)G Polymorphism with Mental Illness

The 5-HT1A C(-1018)G was initially reported as a common polymorphism in Caucasian subjects (Wu and Comings, 1999) and was later re-designated as the 5-HT1A C(-1019)G rs6295 polymorphism. A few years later, this polymorphism was shown to be a functional element in which the G-allele and G/G genotype was associated with major depression and completed suicide in two different cohorts (Lemonde et al., 2003). Shortly thereafter, the association of the 5-HT1A G(-1019) allele and the G/G genotype with major depression was replicated by other studies (Parsey et al., 2006b; Anttila et al., 2007; Kraus et al., 2007; Neff et al., 2009) (Table 1.3).

However, some studies have not observed clear association of the G(-1019) allele with depression (Arias et al., 2002; Huang et al., 2004). This discrepancy may be due in part to the frequency of the risk allele in the population studied where the frequency of the G/G genotype can be as high as 25%. Thus, the odds ratios are decreased and much larger populations are needed to observe association. By contrast the G/G frequency in Chinese populations is much lower, and associations have been observed with treatment response. One strategy to overcome the high frequency of the G/G genotype has been to examine discrete subsets of depression.

Table 1.3 : C(-1019)G Association studies

Condition	RISK-fold	Ethnicity
Major Depressive Disoder	GG-2.25	Caucassian (Ontario)
Suicide	GG-3.4	Caucassian (Quebec)
Neo-Neurotic	G-1.09	Caucassian (German)
Panic with Agoraphobia	G-1.7	Caucassian (German)
Schizophrenia	GG-1.7	70% Caucassian
Substance Abuse	GG-1.7	Unknown
Panik Attack	GG-1.6	Unknown
Neo-Neurotic	GG-1.12	Caucassian (German Alcoholic)
Self-percieved stress	GG-3	Caucassian (diagnosed with Lupus)
Major Depressive Disorder (plus treatment resistance)	GG-3.2	Caucassian (with BDNF 196A polymoprhism)
Decreased phisical activity	GG-4.8	Caucasian (Migraineurs)
Premenstrual Dysphoric Disorder (PMDD)	GG-3.65	Caucassian (English)
Major Depressive Disorder	GG-1.34	Caucassian (Utah)

**For a full review see (LeFrancois et al, 2008)*

For example, interferon-alpha induced depression has been associated with the G/G genotype in a German population (Kraus et al., 2007) while an association with major depression has not been reported. Another strategy to enhance the power of associations with common polymorphisms such as the 5-HT1A C(-1019)G is to examine genetic epistasis among multiple functional risk alleles. For example, risk alleles of the 5-HT transporter and 5-HT1A receptor genes have in combination been associated with reduced response to antidepressants (Arias et al., 2005a). Association has also been observed between 5-HT1A C(-1019)G polymorphism and Catechol-O-Methyl Tranferase (COMT-Val158Met) or Brain-Derived Neurotrophic Factor (BDNF-Val66Met) risk alleles with panic disorder or major depression, respectively (Freitag et al., 2006; Anttila et al., 2007). As such, gene-gene interactions may explain why some studies do not observe an association with 5-HT1A G(-1019) allele alone. Thus, ethnicity, disease and genetic heterogeneity among subjects may obscure associations of mental illness with common polymorphisms.

In addition to an association with depression and suicide, the 5-HT1A C(-1019)G polymorphism has been associated with several related traits or disorders involving the serotonin system (Table 1.3) (Mann, 2003). For example, the C(-1019)G polymorphism is also associated with panic disorder (with agoraphobia in women) (Strobel et al., 2003), panic attack (Huang et al., 2004), as well as with personality traits, such as neuroticism in normal or alcoholic subjects (Strobel et al., 2003; Koller et al., 2006), variation in self-perceived stress in Lupus patients (Birmingham et al., 2006) and avoidance of physical activity in migraine subjects

(Marziniak et al., 2007). On the other hand, association of this polymorphism with other serotonin-related conditions such as bipolar, obsessive compulsive, generalized anxiety and eating disorders have not been reported. It is important to note that often association studies may be influenced by various environmental factors, thus, impairing the clarity of their outcomes. For instance, the G-allele has been associated with suicide attempt in alcoholic subjects (Koller et al., 2006) and suicidal behaviour (Sawiniec et al., 2007) but not with suicide attempt in a group of subjects with heterogeneous mental conditions (such as depression, borderline personality disorders or schizophrenia, alcoholism or other drug abuse conditions (Serretti et al., 2007). In addition, interaction between the 5-HT1A genotype and stressful life conditions may also enhance the results of some studies. In summary, further studies of the 5-HT1A C(-1019)G polymorphism using more carefully defined and homogeneous populations may be necessary in order to observe significant associations.

1.5.2 Association of the C(-1019)G Polymorphism with Antidepressant Treatment

Recently, several studies have indicated an association of the 5-HT1A C(-1019)G polymorphism with response to antidepressant treatment. Initial studies in Caucasian subjects with major depression suggested that the G(-1019) allele is associated with weaker response to SSRI/pindolol or flesinoxan, a 5-HT1A receptor partial agonist (Lemonde et al., 2004). Several reports have supported this association of the G(-1019) allele or G/G genotype with reduced response to SSRI antidepressants, although one recent study did not (Levin et al., 2007). The G/G

genotype was also associated with reduced treatment response in combination with the 5-HT transporter gene polymorphism (5-HTTLPR, "SS" short-allele genotype) (Arias et al., 2005b). Since the G(-1019) allele predicts increased levels of 5-HT1A autoreceptors, a reduction in raphe firing and an increased resistance to SSRI antidepressants may be observed and thus, a greater extent of desensitization of 5-HT1A autoreceptors would be required (Blier and Ward, 2003). In summary, several studies suggest an association of the C/C genotype with greater response to SSRI antidepressants. However, it remains to be determined whether the C(-1019)G genotype associates with placebo or other antidepressant treatments that target additional systems such as norepinephrine or dopamine.

1.5.3 Association of the C(-1019)G 5-HT1A polymorphism with Molecular Phenotypes in the Human Brain

Evidence suggests that presence of the C(-1019)G polymorphism (that is associated with depression) correlates with an altered serotonergic system (Meyer-Lindenberg and Weinberger, 2006). One important phenotype marker that is predicted to be associated with the 5-HT1A C(-1019)G polymorphism is the level of 5-HT1A binding potential. Two PET studies have addressed this issue using positron emission tomography with 5-HT1A-specific antagonist [11C]WAY 100635 as ligand. In normal subjects having the G/G genotype, a trend for increase in 5-HT1A binding potential was observed in the raphe/midbrain region of (David et al., 2005). However, the association was significant and stronger in depressed or bipolar depressed individuals (Parsey et al., 2006a), suggesting that a depressed patient is

less able to compensate for the 5-HT1A autoreceptor dys-regulation due to the G-allele, leading to a more robust over-expression of 5-HT1A autoreceptors.

The 5-HT1A G(-1019) allele has also been associated with abnormalities in amygdala structure and activity. Amygdala reactivity to happy, sad, or fearful faces provides a cue to emotional predisposition, and subjects with the 5-HTT polymorphism (S/S genotype) display increased amygdala reactivity to fearful faces as detected by functional magnetic resonance imaging (Hariri et al., 2002). Increased levels of raphe 5-HT1A receptors are associated with increased amygdala reactivity (Fisher et al., 2006), suggesting that over-expression of 5-HT1A autoreceptors and reduced 5-HT tone leads to hyper-activity of the amygdala. Consistent with this, one study reported an association of the G/G allele with reduced amygdala activation in response to fearful faces, in normal subjects (Fakra et al., 2009). In patients with panic disorder, the 5-HT1A G(-1019) and 5-HTT S/S genotypes were both associated with increased amygdala reactivity to happy faces (Domschke et al., 2006; Dannlowski et al., 2007), suggesting that the 5-HT1A G/G genotype can enhance both disease- and region-specific phenotypes. Interestingly, the G/G genotype is also associated with reduced amygdala volume in patients with borderline personality disorder and major depression (Zetzsche et al., 2008).

In summary, the 5-HT1A G(-1019) genotype is associated with alterations in 5-HT1A autoreceptor levels, amygdala reactivity and amygdala volume that are most pronounced in individuals with other risk alleles (such as the 5-HTT S/S genotype) and who have mental illness such as borderline personality disorder, depression, or panic attack. Interestingly, the pattern of receptor and activity

alteration also appears to depend on the disease outcome. These studies implicate dys-regulation of 5-HT1A receptor expression at the G(-1019) site results in altered 5-HT1A receptor expression, neural activity, and structural development of brain regions implicated in mood and emotion.

1.6 Thesis Rationale, Goals and Objectives

Serotonin neurotransmission is believed to play a major role in the control of mood and behaviour. As such, various mood disorders including depression appear to be partially the result of decreased serotonergic activity. Long-term adaptive changes in 5-HT1A receptor gene expression have been implicated in the delayed therapeutic response to antidepressant treatment. In fact, it is the transcriptional desensitization of the 5-HT1A receptor, which allows for an increase in 5-HT neurotransmission, that triggers the antidepressant response. However, there still remains a large variability in inter-individual pharmacological response patterns, most likely due to the heterogeneity of the illness, genetic predisposition as well as variable degrees of sensitivity in those effected.

Since the 5-HT1A receptor is a key element in mediating serotonergic tone, the regulatory mechanisms involved in its expression patterns, as well as the genetic variations responsible for its abnormal expression, could contribute to the etiology of major depressive disorder and the variable response rates to treatments. Thus, knowledge of the biological factors that may predispose an individual to depression and its mechanism of action will help develop novel detection tools for earlier

diagnosis of the disorder as well as help in developing new treatments with faster onset of action, greater efficacy and fewer side effects.

As discussed above, transcriptional regulation of the 5-HT1A receptor gene is implicated in predisposition to mental illnesses which involve abnormal regulation of the serotonin system, such as major depression. Since the C(-1019)G 5-HT1A polymorphism is associated with depression and impaired response to antidepressant treatment and prevents the proper binding of Deaf-1 to the promoter, I hypothesized that regulation of the 5-HT1A receptor by Deaf-1 is critical for maintaining normal 5-HT1A receptor expression. Specifically, I addressed the following:

1. 5-HT1A receptor gene expression is regulated by Deaf-1 in a cell-type dependent manner
2. Deaf-1 regulates 5-HT1A receptor expression in vivo
3. Loss of Deaf-1 expression leads to altered 5-HT1A receptor gene expression in the mouse brain, that is similar to that observed in depressed patients

The major aim of my thesis is to examine transcriptional regulation of the 5-HT1A receptor gene by the transcription factor Deaf-1, both *in vitro* and *in vivo*. In Chapter 2, I have characterized the transcriptional regulation of the 5-HT1A gene expression in neuronal and non-neuronal cells in culture by focusing on the role of Deaf-1 and Hes5 in regulating 5-HT1A promoter activity at the site of the C(-1019)G polymorphism. In Chapter 3, I characterized a novel technique of micro-dissecting

and culturing primary pre-synaptic raphe neurons that express a high level of markers involved in regulating the 5-HT system, which also serves as a new model for studying the serotonergic system *in vivo*. In Chapter 4, I identified a Deaf-1 binding site in the mouse 5-HT1A promoter and established a role for Deaf-1 in regulating the 5-HT1A receptor expression in various brain regions in mice lacking a functional Deaf-1 protein.

More importantly, I have established that transcriptional regulation of the 5-HT1A receptor gene by Deaf-1 is altered in the presence of the promoter polymorphism, in a tissue-specific manner that is consistent with the altered 5-HT1A receptor expression pattern observed in depression. These studies identify Deaf-1 as an important potential therapeutic target needed to normalize 5-HT1A receptor expression patterns in depression.

CHAPTER 2

The following chapter has been reproduced in its entirety with from:

Czesak M, Lemonde S, Peterson EA, Rogaeva A, Albert PR. (2006) Cell-specific repressor or enhancer activities of Deaf-1 at a serotonin 1A receptor gene polymorphism. J Neurosci Feb 8;26(6):1864-71.

See Appendix A for permission

CELL-SPECIFIC REPRESSOR OR ENHANCER ACTIVITIES OF NUDR/DEAF-1 AT A 5-HT1A RECEPTOR GENE POLYMORPHISM

Margaret Czesak*, Sylvie Lemonde*, Erica A. Peterson, Anastasia Rogaeva, Paul R. Albert

Ottawa Health Research Institute (Neuroscience)
Department of Cellular and Molecular Medicine
University of Ottawa
Ottawa, Canada, K1H 8M5

Abbreviated Title: Cell-specific regulation at a 5-HT1A polymorphism

Keywords: 5-HT1A receptor, transcription factor, epigenetic, raphe, polymorphism, anxiety, major depressive disorder; Theme: serotonin receptors.

Author Contribution: *Margaret Czesak and Sylvie Lemonde contributed equally to this work. Margaret Czesak conducted the preparation of the manuscript and all its associated revisions.

Acknowledgments: This research was supported by a grant from Canadian Institutes of Health Research (CIHR) (P.R.A.). M.C. received a Scholarship from the Ontario Graduate Scholarships in Science and Technology, S.L. was supported by a Studentship from CIHR, E.A.P. was partly supported by a summer internship award from the University of Ottawa Faculty of Medicine, A.R. received a K. M. Hunter/CIHR Studentship, and P.R.A. holds the Novartis/CIHR Michael Smith Chair in Neuroscience.

2.1 Abstract

The serotonin-1A (5-HT1A) receptor is the primary somatodendritic autoreceptor that inhibits the activity of serotonergic raphe neurons, and is also expressed in non-serotonergic cortical and limbic neurons. Alterations in 5-HT1A receptor levels are implicated in mood disorders and a functional C(-1019)G 5-HT1A promoter polymorphism has been associated with depression, suicide, and panic disorder. We examined the cell-specific activity of identified transcription factors human NUDR/Deaf-1 and Hes5 at the 5-HT1A C(-1019) site. In serotonergic raphe RN46A cells, Deaf-1 and Hes5 repressed the 5-HT1A receptor gene at the C(-1019)-allele, but not the G(-1019)-allele. However, in non-serotonergic cells that express 5-HT1A receptors (septal SN48, neuroblastoma SKN-SH and neuroblastoma/glioma NG108-15 cells), Deaf-1 enhanced 5-HT1A promoter activity at the C(-1019)-allele but not the G-allele, while Hes5 repressed in all cell types. The enhancer activity of Deaf-1 was orientation-independent and competed out Hes5 repression. To test whether Deaf-1 activity is intrinsic, the activity of a Gal4DBD (DNA binding domain)-Deaf-1 fusion protein at a heterologous Gal4 DNA element was examined. Gal4DBD-Deaf-1 repressed transcription in RN46A cells, but enhanced transcription in SN48 cells indicating that these opposite activities are intrinsic to Deaf-1. Repressor or enhancer activities of Deaf-1 or Gal4DBD-Deaf-1 were blocked by histone deacetylase inhibitor trichostatin A. Thus the intrinsic activity of Deaf-1 at the 5-HT1A promoter is opposite in pre- vs. post-synaptic neuronal cells and requires deacetylation. Cell-specific regulation by Deaf-1 could underlie region-specific alterations in 5-HT1A receptor expression in different mood disorders.

2.2 Introduction

The serotonin system originates from neurons of the raphe nuclei that are negatively regulated by pre-synaptic 5-HT1A autoreceptors (Penington et al., 1993; Bayliss et al., 1997). Post-synaptic 5-HT1A receptors are also strongly expressed in brain regions that regulate mood and emotion (Törk, 1990; Jacobs and Azmitia, 1992). Anxiety, depression, and suicide appear to result from reduced serotonergic activity and antidepressants such as 5-HT-selective reuptake inhibitors (SSRIs) are thought to enhance serotonergic activity in part by desensitization of 5-HT1A autoreceptors. Oppositely, in midbrain tissue from depressed suicides, elevated levels of presynaptic 5-HT1A receptors were observed (Stockmeier et al., 1998), an alteration that would reduce serotonergic activity.

We postulated that up-regulation of 5-HT1A autoreceptors could be due to genetic alterations such as the 5-HT1A C(-1019)G functional promoter polymorphism. Several studies have associated the 5-HT1A G(-1019) allele with major depression, suicide, panic disorder or decreased response to antidepressants (Lemonde et al., 2003; Strobel et al., 2003; Lemonde et al., 2004b; Serretti et al., 2004). The 5-HT1A G(-1019) allele fails to bind identified repressors Deaf-1 and Hes5, leading to upregulation of autoreceptor expression. Consistent with a role of this site in 5-HT1A autoreceptor regulation, increases in 5-HT1A autoreceptor expression in individuals with the G/G genotype has been observed (David et al., 2005; Parsey et al., 2005). Conversely, positron emission tomography imaging studies of depressed patients indicate decreased levels of post-synaptic 5-HT1A receptors in the cortex and hippocampus (Drevets et al., 1999; Sargent et al., 2000;

Albert and Lemonde, 2004). Furthermore, 5-HT1A-null mice display increased anxiety-related behaviour which is rescued by early post-natal expression of the post-synaptic 5-HT1A receptor in the forebrain (Gross et al., 2002). We therefore addressed whether the C(-1019)G site might mediate differential regulation of the 5-HT1A receptor in pre- versus post-synaptic cells.

In this study, the activity of Deaf-1 at the 5-HT1A C(-1019) site was compared in serotonergic raphe cells and non-serotonergic neuronal or glial cells that express similar levels of endogenous 5-HT1A receptors including: SN48 rat septal x mouse neuroblastoma cells, NG108-15 rat glioma x mouse neuroblastoma cells, and SKN-SH human neuroblastoma cells (Charest et al., 1993; Ansorge et al., 2004; Fricker et al., 2005). Cell lines with intrinsic expression of 5-HT1A receptors were chosen to ensure the presence of relevant transcription factors necessary for 5-HT1A receptor expression. We show that Deaf-1 enhances 5-HT1A promoter activity in the post-synaptic/glial cell models and that this enhancement is attenuated in the presence of the C(-1019)G polymorphism. In contrast, Hes5 represses 5-HT1A gene transcription in all cell types examined. The cell-specific activity of Deaf-1 in 5-HT1A gene regulation could account for region-specific decreases in post-synaptic 5-HT1A receptor levels reported in depressed patients.

2.3 Materials & Methods

2.3.1 Reporter constructs

The luciferase plasmids pGL3P-RE-1, pcDNA1-REST, 5-HT1A(C) (-1128 to ATG) and 26bp-C(6)F, Gal4-DBD fusion constructs and X2G2P reporter constructs have been previously described (Lemonde et al., 2003; Lemonde et al., 2004a). Briefly, the luciferase plasmid 5-HT1A(C) was obtained by insertion of a -1128-bp *Kpn*I–*BssH*II fragment of the human 5-HT1A receptor gene into pGL3-Basic (Promega) digested with *Kpn*I and *Mlu*I. The 5-HT1A(G) construct was generated by unique site elimination mutagenesis (Amersham Biosciences) of C(-1019)G in 5-HT1A(C). 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 *Sma*I-cut pGL3-Promoter to generate 26bp-C(6) and 26bp-G(6). Full-length Deaf-1 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) before subcloning in the *EcoR*1 site of pcDNA3 (Invitrogen). The 26bp-C(6)R was generated by ligation of a *Nhe*I/*Xho*I blunted fragment, obtained from 26bp-C(6)F construct into pGL3B (Promega) digested by *Sma*I. All plasmids were purified by CsCl equilibrium gradient centrifugation, quantified spectrophotometrically, and verified by dideoxynucleotide DNA sequencing.

2.3.2 Cell Culture and transfection

Septal SN48, NG108-15 and SKN-SH neuroblastoma cells were grown in Dulbecco's modified Eagle's medium (Gibco BRL, Rockville, MD, USA) supplemented with 10% v/v heat-inactivated fetal bovine serum at 37°C in 5% CO_2. Binding studies of crude membranes from these cells using [^{3}H]-DPAT revealed (mean±range, n=2) 3.0±0.5, 8.3±1.6, 2.7±1.0 fmol/mg protein of 5-HT1A receptor sites, respectively. RN46A cells (2.4±0.6 fmol/mg 5-HT1A sites (Kushwaha and Albert, 2005)) were grown on Primaria six-well plates (Falcon, Franklin Lakes, NJ) in Neurobasal medium (Gibco BRL) supplemented with 10% v/v heat-inactivated fetal bovine serum, 0.1% PenStrep and 0.04% of L-Glutamine, at 33°C in 5% CO_2 (Lemonde et al., 2003; Lemonde et al., 2004a). Media were changed 16-24 h prior to transfection. Except for the RN46A and SKN-SH cells, transfections were done using calcium phosphate co-precipitation as described previously (Charest et al., 1993) with 20 µg luciferase construct and 5 µg pCMVβgal per 10-cm plate or with 10 µg of luciferase-expressing vector, indicated amounts of pcDNA expression constructs or empty vector and 0.5 µg pCMVβgal to normalize transfection efficiency, maintaining the total DNA transfected at 25 µg. Raphe RN46A cells and SKN-SH cells were transfected with 1:2 and 1:1.5 ratios, respectively, of plasmid:Lipofectamine2000 reagent (Invitrogen Inc), using 10 µg/plate of luciferase plasmid and equal amount of protein-expression constructs or empty vector and 2 µg/plate of pCMVβgal. In some experiments, 200 nM trichostatin A (TSA) or vehicle (0.75% ethanol) was applied to the culture medium at time of transfection.

2.3.3 Reporter assays

Luciferase and β-galactosidase assays were performed as previously described (Ou et al., 2000; Lemonde et al., 2003). Activities were obtained from at least three independent experiments in which triplicate transfections were performed and corrected for transfection efficiency by calculating the ratio of luciferase/β-galactosidase activity, and normalizing to vector-transfected extracts. Data are presented as mean ± *SD*. Statistical significance was evaluated using two-tailed unpaired t-test and 95% confidence intervals with GraphPad Prism software (San Diego, CA) unless indicated otherwise.

2.3.4 Nuclear and cytosolic fractionation

Fractionation was adapted from previously-described methods (Lemonde et al., 2003; Subramanian and Chinnadurai, 2003; Zuccato et al., 2003). Cells (10^7) were trypsinized and collected in 14 mL of HBBS+EDTA buffer (118 mM NaCl, 4.6 mM KCl, 10 mM D-glucose, 5 mM EDTA, 20 mM Hepes pH 7.2), pelleted at 4°C for 4 min at 500g and resuspended in 1 mL of resuspension buffer (10 mM KCl, 10 mM Na-HEPES pH 7.6, 1.5 mM $MgCl_2$) and allowed to swell on ice for 10 min. The resulting cells were collected by spinning for 10 min at 4 °C at 500g and the pellets lysed using 50 μL extraction buffer per 2-3X10^6 cells (10 mM KCl, 10 mM Na-HEPES pH 7.6, 1.5 mM $MgCl_2$, 0.1 % NP-40, 0.5 mM DTT, 0.5 mM spermidine, 0.15 mM spermine, 1 mM PMSF, 1X Protease Inhibitor Cocktail (Roche Diagnostics)). Nuclear pellets were collected by centrifugation at 1,000g for 2 min at 4 °C and washed three times using 1 mL of wash buffer (50 mM NaCl, 20 mM Na-HEPES pH 7.6, 25 %

Glycerin, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM spermidine, 0.15 mM spermine, 1 mM PMSF, 1X Protease Inhibitor Cocktail) and lysed using 50 μL nuclear extraction buffer per 2-3X10^6 cells (500 mM NaCl, 20 mM Na-HEPES pH 7.6, 25 % Glycerin, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM spermidine, 0.15 mM spermine, 1 mM PMSF, 1X Protease Inhibitor Cocktail) and rotated for 30 min at 4 °C. The nuclear proteins were collected from the supernatant following centrifugation for 15 min at 16,000g at 4 °C to clear cell debris.

2.3.5 Western blot analysis

Nuclear or cytosolic proteins (50 μg/reaction) were separated by SDS-PAGE on a 12% gel, transferred to nitrocellulose membrane and blocked using TBS containing 5% dry milk and 0.02% Tween 20. Immunoreactive proteins were detected with rabbit anti-Deaf-1 antibody (Lemonde et al., 2003) at a dilution of 1:5000 followed by a 1:2000 dilution of the secondary horseradish peroxidase (HRP)-linked anti-rabbit antibody. Anti-histone 1 (nuclear marker) antibody (Upstate) was used at 1:1,000 (Zuccato et al., 2003). Anti-c-Raf (cytosolic marker) antibody (BD Transduction Laboratories) was used at 1:3000 dilution (Brown et al., 2004).

2.3.6 Electrophoretic mobility shift assay

Sense and antisense oligonucleotides of the 26-bp Deaf-1 element (C-allele) with CC/GG 3'-overhangs were hybridized and labeled with [α-^{32}P]-dCTP using Klenow fragment DNA polymerase (Ou et al., 2000). Nuclear extract (5 μg/reaction) was preincubated with or without competitor DNA in a 20-μl reaction containing gel-shift DNA binding buffer (20 mM Hepes, 0.2 mM EDTA, 0.2 mM EGTA, 100 mM KCl, 5%

glycerol and 2mM DTT, PH=7.9), 250 ng herring sperm DNA, at room temperature for 20 min. Unlabeled doubled-stranded PEA3 sequences (5-GGGATCCAGGAAGTGA-3) were used as non-specific competitor and were electrophoresed on a non-denaturing 5% acrylamide/Tris-glycine gel at 4°C to resolve protein-DNA complexes. For SKN-SH extracts, the probe was trapped in poorly migrating non-specific protein/DNA aggregate that was not competed by specific oligonucleotide.

2.4 Results

2.4.1 Cell- and allele-specific activity of Deaf-1 at the 5-HT1A promoter

The transcriptional activity of Deaf-1 and Hes5 was compared in serotonergic (RN46A, Figure 2.1A) and non-serotonergic (SN48, NG108-15, and SKN-SH; Figure 1B) 5-HT1A receptor-positive neuronal cells co-transfected with C(-1019) or G(-1019) human 5-HT1A promoter-luciferase reporter constructs (5-HT1A(C) or (G), respectively). Cell lines with intrinsic expression of 5-HT1A receptors were chosen to ensure the presence of relevant transcription factors necessary for 5-HT1A receptor expression. As previously observed in RN46A raphe cells, both Deaf-1 and Hes5 repressed the activity of the 5-HT1A(C) construct by 50% but had no effect at the 5-HT1A(G) construct, but there was no significant difference in basal activity between the two alleles. By contrast, in SN48 and NG108-15 there was a 70% reduction in basal activity of 5-HT1A(G) compared to the 5-HT1A(C) construct, with a smaller but significant reduction in SKN-SH cells (Figure 2.1B). Furthermore, cotransfection of Deaf-1 significantly increased the activity of 5-HT1A(C) construct,

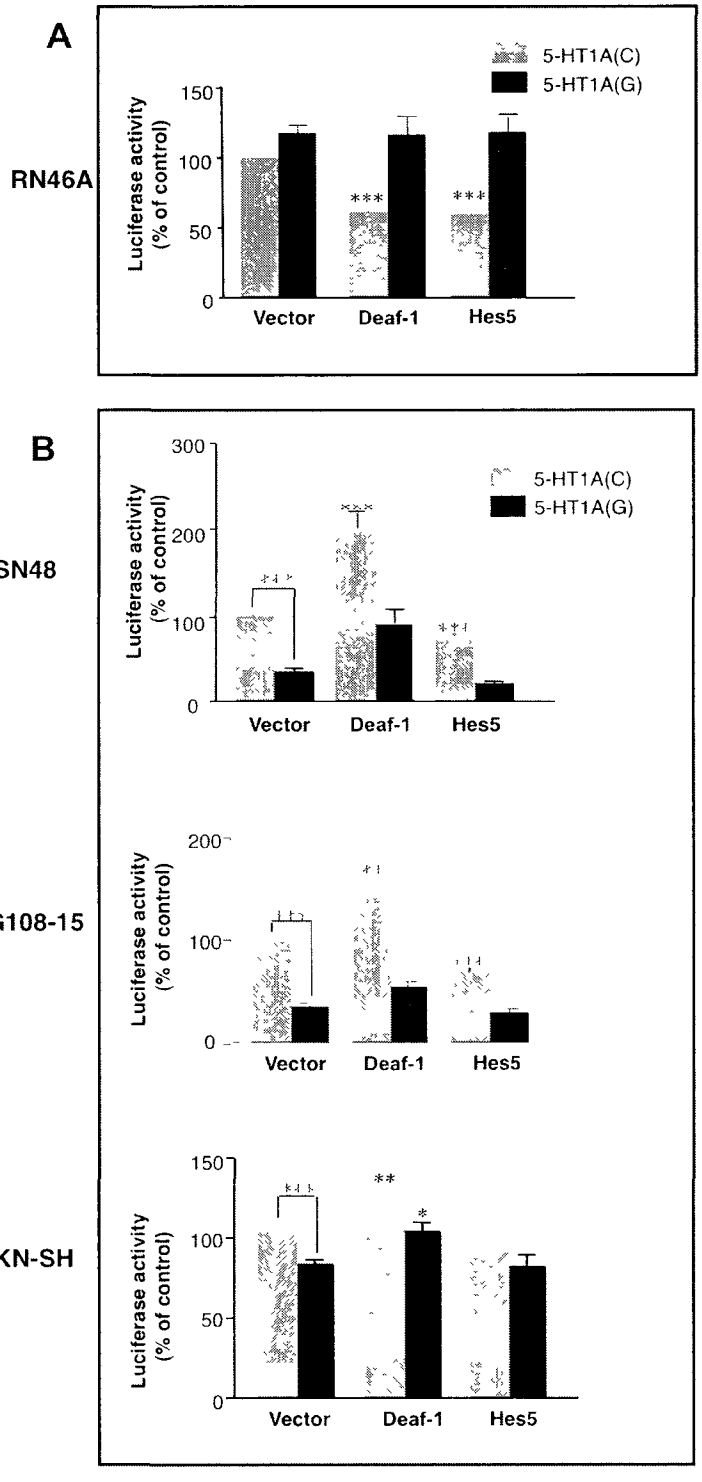


Figure 2.1: Cell-type dependent activity of Deaf-1 at the 5-HT1A C(-1019) site.

Constructs containing the C(-1019) or G(-1019) allele of the human 5-HT1A promoter (-1128-bp to ATG) fused to luciferase (5-HT1A(C) and 5-HT1A(G), respectively) were co-transfected with vector (pcDNA3), Deaf-1 or Hes5 expression plasmids as indicated in 5-HT1A-expressing neuronal cell lines: rat raphe RN46A (A), mouse septal SN-48, neuroblastoma-glioma NG108-15 or human SKN-SH neuroblastoma cells (B). Adjusted luciferase activity was corrected for transfection efficiency by calculating the ratio of luciferase activity/β-galactosidase activity and normalized to control (pcDNA3) transfections. Data are presented as mean ±SD of triplicate samples collected form at least three independent experiments as indicated. Statistical analysis compared to vector control (for Deaf-1 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: $^{**}P \leq 0.01$, $^{***}P \leq 0.001$.

but not the 5-HT1A(G) construct. Deaf-1 also enhanced 5-HT1A promoter activity in 5-HT1A-expressing H19-7 rat hippocampal cells (data not shown). By contrast, Hes5 mediated a significant repression of the 5-HT1A(C) but not G-allele in SN48 and NG108-15 cells. Thus, in several models of post-synaptic or glial 5-HT1A gene expression, Deaf-1 enhanced 5-HT1A transcriptional activity at the C(-1019) site, while it repressed 5-HT1A transcription in serotonergic raphe cells.

2.4.2 Deaf-1 expression and localization

Since endogenous Deaf-1 could influence basal 5-HT1A transcription, Deaf-1 protein was detected in nuclear and cytosolic extracts by Western blot using a specific antibody which recognized a single 60-kDa species (Lemonde et al., 2003) (Figure 2.2A). Levels of Deaf-1 were highest in nuclear extracts from RN46A cells, with low or undetectable levels in SN48, SKN-SH and NG108-15 nuclear fractions. By contrast Deaf-1 was abundantly expressed in cytosolic extracts from these cell types. Markers specific for nuclear (H1-histone) or cytosolic (c-Raf protein) fractions indicated undetectable cross-contamination between fractions and similar protein loading among cell types. The DNA binding activity of Deaf-1 in nuclear fractions was determined (Figure 2.2B). A specific Deaf-1/DNA complex with labeled human Deaf-1 element (C-allele) was observed that was completely competed with excess unlabelled Deaf-1 element (Sp), but weakly competed by unrelated PEA-3 site primers (NSp). Deaf-1 binding activity paralleled the nuclear protein content identified by Western blot (Figure 2.2A), greatest in RN46A cells and weak but detectable in SN48, SKN-SH, and NG108-15 cells. Thus endogenous Deaf-1 from rat

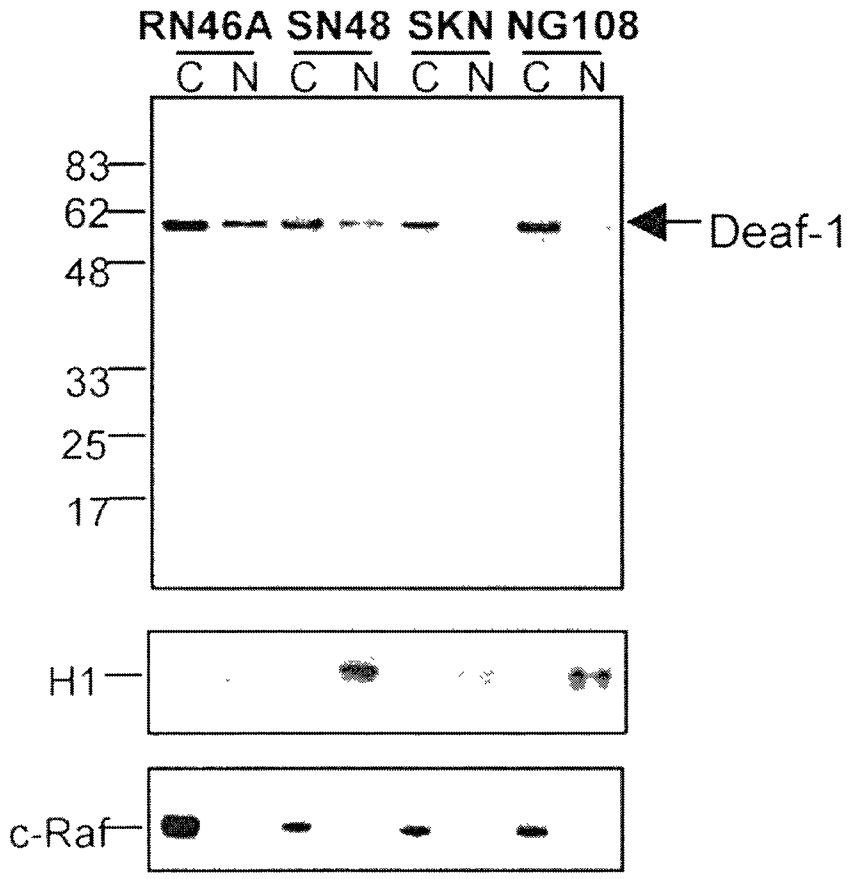


Figure 2.2-A: Endogenous Deaf-1 protein expression and DNA binding activity.

Deaf-1 localization. Nuclear (N) and cytosolic (C) extracts (50 µg/lane) from RN46A, SN48, SKN-SH and NG108-15 cells were subjected to Western blot analysis using specific anti-Deaf-1 antibody. The blot was reprobed using antibodies for markers of nuclear (histone-H1) and cytosolic (c-Raf) to assess loading and purity of extracts. Molecular weight markers (kDa) are as indicated.

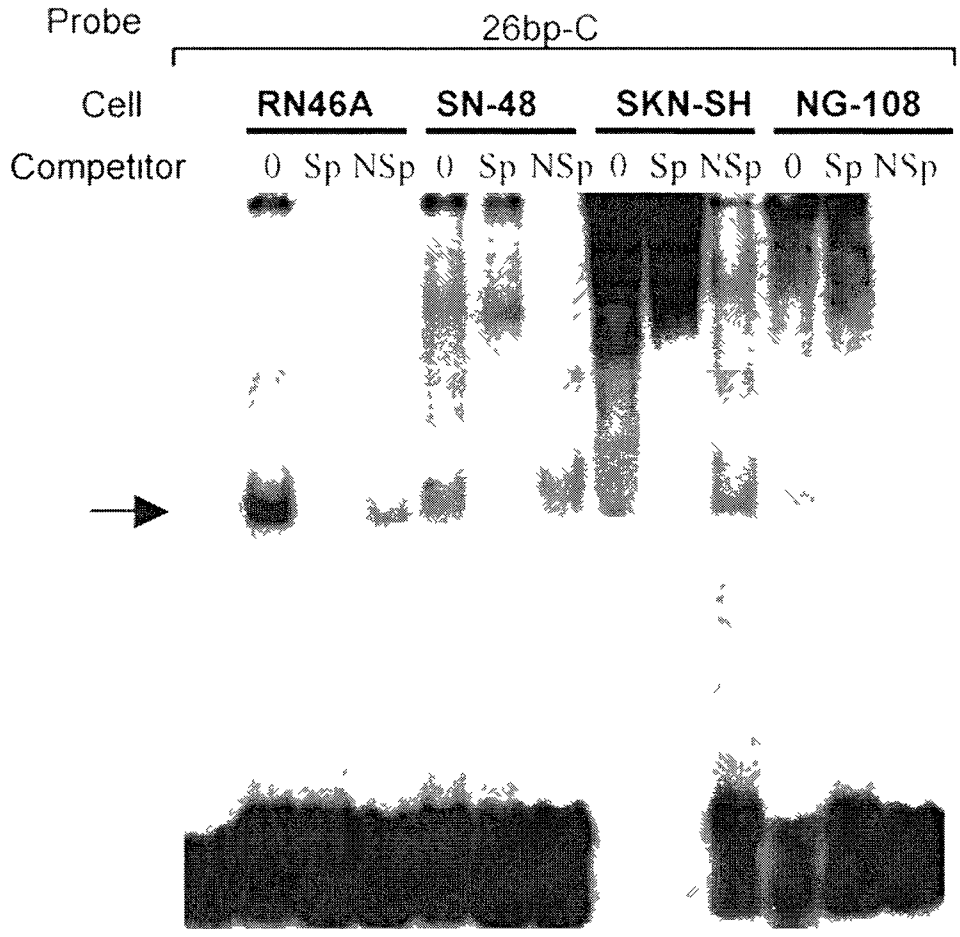


Figure 2.2-B: Endogenous Deaf-1 protein expression and DNA binding activity.

Deaf-1 DNA binding activity Electrophoretic mobility shift assay was done using nuclear extracts from RN46A, SN48 or NG108-15 cells that were incubated with labeled 26-bp(C) probe in the presence of 100-fold molar excess of unlabeled 26 bp(C) (Sp) or unrelated PEA3 (NSp) oligonucleotides, as indicated A major specific Deaf-1 complex was observed (left arrowhead)

or human nuclei binds to the 5-HT1A site in accordance with its protein abundance. The abundance of nuclear Deaf-1 in RN46A cells suggests that Deaf-1 may repress endogenous 5-HT1A receptor expression.

2.4.3 Deaf-1 enhancer/repressor activity at the C(-1019) Deaf-1 site

To determine whether the 26-bp Deaf-1 element is sufficient to confer cell-specific activity of Deaf-1, the activity of Deaf-1 at a reporter construct containing the Deaf-1 element (in six copies) placed upstream of the SV40 promoter (26bp-C(6)) was compared in RN46A and SN48 cells (Figure 2.3). In RN46A cells, Deaf-1 almost completely inhibited transcriptional activity, while in SN48 cells Deaf-1 mediated enhancer activity. Repressor or enhancer activity of Deaf-1 was observed in forward or reverse orientations of Deaf-1 sites, as expected for orientation-independent enhancer or repressor activity. In cells transfected with a control vector lacking Deaf-1 sites, Deaf-1 had no effect (data not shown). Thus, the 26-bp Deaf-1 element is sufficient to confer cell-specific Deaf-1-mediated repressor or enhancer activity.

Regulation of the 5-HT1A promoter could involve interactions between Deaf-1 and Hes5 at the 26-bp 5-HT1A palindrome. To address this, Deaf-1 and Hes5 were cotransfected in RN46A or SN48 cells and transcriptional activity at the 26-bp palindrome measured (Figure 2.4). In RN46A cells, both Deaf-1 and Hes5 repressed transcriptional activity, and together Deaf-1 and Hes5 induced a similar repression (Figure 2.4A). In SN48 cells, Deaf-1 enhanced activity of the 26bp-C(6) construct, while Hes5 repressed its activity (Figure 2.4B). Interestingly, when cotransfected

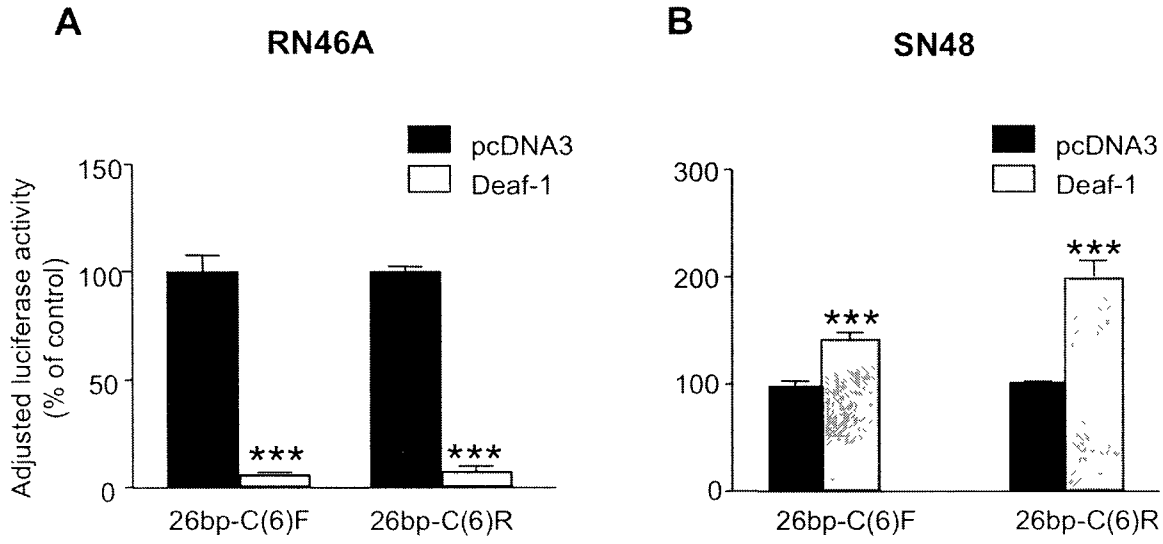


Figure 2.3: Orientation-independent repressor and enhancer activities of Deaf-1 at the 5-HT1A palindrome.

In RN46A (A) or SN48 (B) cells, luciferase reporter constructs containing 6 copies of the 26-bp 5-HT1A palindrome in the forward (26bp-C(6)F) or reverse orientation (26bp-C(6)R) relative to the SV40 promoter were co-transfected with vector (pcDNA3) or Deaf-1 expression plasmid. Luciferase activity was normalized to vector control, and data are presented as mean ±SD of triplicate samples collected from four independent experiments. Statistical analysis was performed by unpaired t-test with two-tailed *P* values: $***P \leq 0.001$.

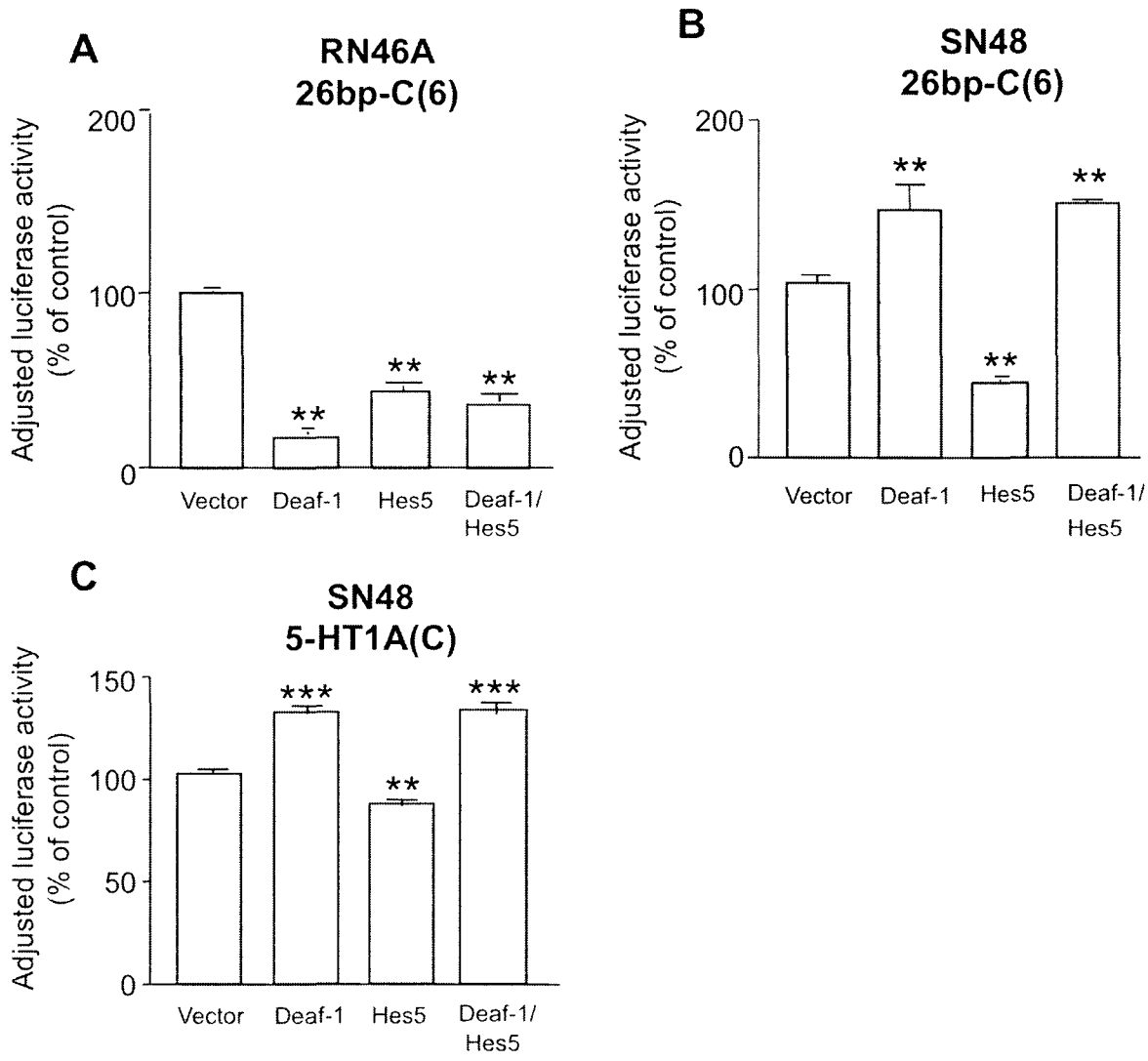


Figure 2.4: Deaf-1 competes with Hes5 to enhance 5-HT1A promoter activity.

The 26bp-C(6) reporter construct (containing 6 copies of the C(-1019) allele of the 26-bp 5-HT1A palindrome) or the 5-HT1A(C) promoter construct was co-transfected with vector (pcDNA3), Deaf-1 or Hes5 plasmids in RN46A cells (A) or SN48 cells (B, C). Luciferase activity was normalized to control transfections. Data are presented as mean ± SEM of triplicate samples from three separate experiments. Significance compared to vector control as indicated: *P<0.05, **P<0.01, ***P<0.001.

with Hes5, Deaf-1-mediated enhancement was the dominant effect. A similar pattern was observed using the 5-HT1A promoter-luciferase construct, with Deaf-1 enhancer activity dominant over Hes5 repression (Figure 2.4C). Thus, the enhancer activity of Deaf-1 could result from a competition with endogenous repressors such as Hes5.

2.4.4 HDAC-dependent repressor/enhancer activity of Deaf-1

Since recruitment of histone deacetylase (HDAC) is implicated in gene repression (Ayer, 1999), we tested the effect of HDAC inhibitor trichostatin A (TSA) on Deaf-1-mediated repressor or enhancer activity at the 5-HT1A promoter (Figure 2.5). We also tested the 5-HT1A RE-1 element placed upstream of the SV40 promoter in the pGL3P vector (pGL3P-RE1), the target of the prototypic repressor REST/NRSF (REST) that mediates TSA-sensitive repression (Lemonde et al., 2004). In RN46A cells, Deaf-1 reduced 5-HT1A promoter activity and this action was attenuated by TSA treatment. Similarly, the silencer activity of REST at RE-1 was also partially reversed by TSA treatment in RN46A cells. In SN48 cells, Deaf-1-induced enhancer activity was also inhibited by TSA, indicating that HDAC activity is also required for Deaf-1-mediated enhancer activity. In SN48 cells, TSA treatment reversed REST-mediated repression to enhanced activity compared to vector (pGL3P), consistent with the finding that REST can recruit silencer or enhancer activities (Jepsen et al., 2000). Thus Deaf-1-mediated recruitment of HDAC can either repress or enhance 5-HT1A promoter activity, depending on the cellular context.

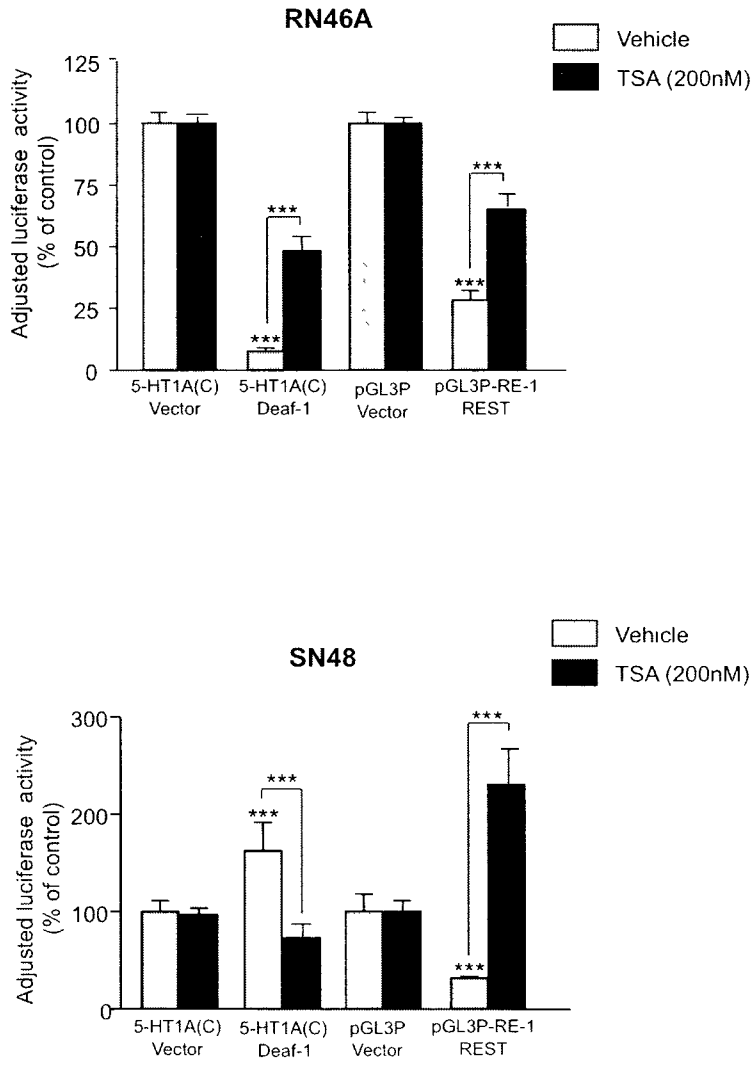


Figure 2.5: Repressor and enhancer activities of Deaf-1 at the 5-HT1A promoter are TSA-sensitive.

The -1128bp 5-HT1A-luciferase construct (5-HT1A(C)) was cotransfected with vector (pcDNA3) or Deaf-1 plasmids in rat raphe RN46A and mouse septal SN-48 cells. As a positive control for TSA, a luciferase construct containing human 5-HT1A RE-1 placed 5' to SV40-promoter was cotransfected with vector (pcDNA1) or REST plasmids. Cells were treated with vehicle or TSA (200 nM) for 24 h, as indicated. Luciferase activity/β-galactosidase activity was normalized to control (vector) transfections. Data are presented as mean ±SD of triplicate samples collected form at least three independent experiments as indicated. Statistical analysis was determined by unpaired t-test with two-tailed *P* values:***$P \leq 0.001$.

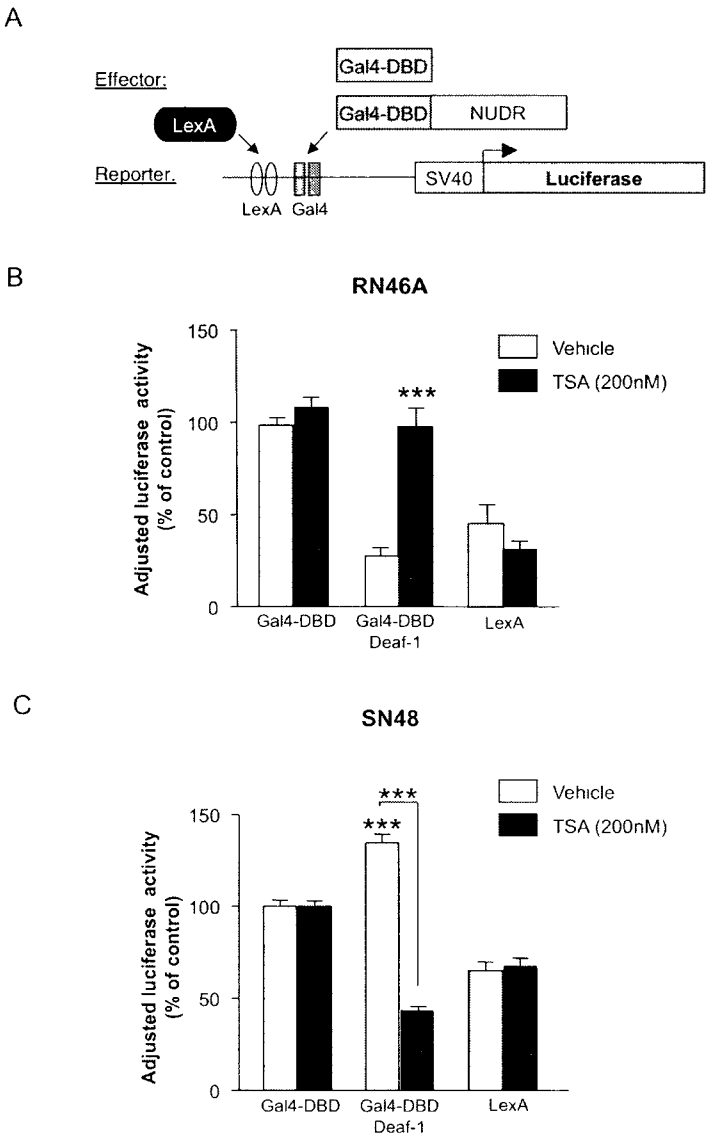


Figure 2.6: Cell-specific and TSA-sensitive repressor and enhancer activities are conferred by Deaf-1 at a heterologous DNA element.

A. A model for the action of effectors Gal4-DBD (Gal4 vector), Gal4DBD-Deaf-1 fusion protein, or LexA (positive control) at the X2G2P reporter construct containing two LexA and Gal4 sites upstream of SV40 promoter-luciferase. These constructs were cotransfected in 5-HT1A-expressing rat RN46A (B) or mouse SN48 cells (C), which were treated for 24 h with vehicle or TSA (200 nM). Luciferase activity was normalized to Gal4 vector (100%). Data are presented as mean ±SD of triplicate samples collected form at least two independent experiments as indicated. Statistical analysis was by unpaired t-test with two-tailed *P* values: ***$P \leq 0.001$.

2.4.5 Intrinsic repressor/enhancer activity of Deaf-1

Deaf-1-mediated repressor/enhancer activity could result from competition with other factors (e.g., Hes5) or represent an intrinsic property of Deaf-1. To test the latter possibility, a completely heterologous hybrid system was used: full-length Deaf-1 was fused to the Gal4 DNA binding domain (DBD) and its activity tested at a reporter construct containing Gal4 elements (Figure 2.5A). Full-length Deaf-1 was used to preserve protein-protein interactions. Compared to the vector (Gal4DBD), Gal4DBD-Deaf-1 repressed the SV40 promoter in RN46A cells (Figure 2.6), indicating that Deaf-1 confers repression when recruited to a heterologous element and promoter. This effect was entirely blocked by TSA, indicating that Deaf-1 repression is dependent on HDAC activation; whereas LexA-mediated repression was not reversed by TSA, as previously observed (Lemonde et al., 2004). When transfected in SN48 cells, Gal4-Deaf-1 induced a small but significant enhancement of transcription that was inhibited to below basal activity by TSA, while LexA induced HDAC-independent repression (Figure 2.6C). The effect of TSA indicates a primary role for HDAC in Deaf-1 enhancer activity and reveals a possible HDAC-independent Deaf-1 repressor activity. Thus Deaf-1-induced enhancer activity in SN48 cells is HDAC-dependent and is conferred by Deaf-1 itself and is not due to displacement of the binding of another repressor to the 26-bp palindrome (such as Hes5).

2.5 Discussion

Deaf-1, a cell-specific repressor and enhancer: In this study, we show that Deaf-1 binds to the C(-1019) site to repress 5-HT1A receptor gene transcription in a presynaptic serotonergic cell line (RN46A) while Deaf-1 enhanced transcription in post-synaptic neuronal or glial cells (SN48, NG108-15, SKN-SH), as shown in the model (Supplementary Figure 2.1). The finding that the G(-1019) allele blocked both enhancer and repressor actions of Deaf-1 argues for a direct effect of Deaf-1 at the 5-HT1A promoter. Hes5 mediated repression in all cell lines, which was attenuated in post-synaptic models by the presence of Deaf-1-mediated enhancer activity. This suggests that Deaf-1 and Hes5 compete for binding to the 26-bp palindrome, but that Deaf-1 may have higher affinity for binding than Hes5. However, Deaf-1-induced enhancement did not result from competition with an endogenous stronger repressor of the 26-bp 5-HT1A palindrome element since cell-specific Deaf-1 enhancer activity was present in the heterologous GAL4-DBD system. The possibility that differences in activator or repressor activity result from competition with binding to endogenous promoters is unlikely, since the number of genomic sites for Deaf-1 is at least 100-fold lower that of transfected reporter construct, based on 10% transfection efficiency. We postulate that Deaf-1 recruits cell-specific protein complexes to the DNA to either repress or enhance transcription.

2.5.1 Multiple trans-acting functions of Deaf-1

Deaf-1 has previously been shown to have either repressor or enhancer function depending on the gene analyzed. Deaf-1 is the human homologue of Deaf-1, a DNA-

binding protein that was first shown to bind the autoregulatory element of the *deformed* gene in *Drosophila melanogaster* (Gross and McGinnis, 1996). In these studies, Deaf-1 synergistically activated deformed protein autoregulation of the 120-bp *deformed* response element, thus displaying enhancer activity. Human Deaf-1 trans-activated preproenkephalin promoter activity by 30-fold, but this effect appeared to be independent of DNA binding to the promoter (Huggenvik et al., 1998). However in monkey kidney CV-1 cells, Deaf-1 repressed the hnRNP A2/B1 and Deaf-1 promoters via sites located downstream from transcriptional initiation (Michelson et al., 1999). In contrast to these studies in different genes, depending on the cell type Deaf-1 repressed or enhanced gene transcription at the same C(-1019) site of the 5-HT1A receptor gene. This Deaf-1 site conferred cell-specific regulation at a heterologous promoter, implying that Deaf-1 recruits cell-specific regulatory factors to repress or enhance transcription.

2.5.2 Different functional domains within Deaf-1 could mediate its enhancer or repressor activities

The structure of Deaf-1 includes the conserved DNA-binding SAND domain (Sp100, AIRE-1, NucP41/75, Deaf-1) and a C-terminal MYND (myeloid, nervy, and Deaf-1) domain (Gross and McGinnis, 1996). Deaf-1 also contains two protein-interaction domains for its interaction with both Lmo4 and NLI/CLIM transcriptional regulators, which mediate embryonic pattern formation and cell fate (Sum et al., 2002). Deaf-1-null mice display phenotypic abnormalities including exencephaly and transformation of cervical segments that resemble those of Lmo4-null mice, further suggesting functional overlap between Deaf-1 and LMO4 (Hahm et al., 2004;

Tse et al., 2004; Lee et al., 2005). LMO4 has been shown to recruit various NLI proteins that lead to transcriptional activation or repression (Bach et al., 1997; Jurata and Gill, 1997). Thus, recruitment by Deaf-1 of different protein complexes could direct enhancement or repression of gene transcription.

Deaf-1 also contains both nuclear import (Jensik et al., 2004) and export sites (Huggenvik et al., 1998) to regulate trafficking. The finding that nuclear Deaf-1 content is greatest in RN46A cells (Figure 2.2A) suggests that Deaf-1 may be stabilized in the nucleus (perhaps in heterochromatin) to exert its repressor activity. We observed little effect of the C(-1019)G mutation on basal activity of 5-HT1A reporter constructs in RN46A cells (Figure 2.1A) where endogenous Deaf-1 may be bound to the endogenous 5-HT1A gene and inaccessible to the reporter construct. Yet, there was a clear effect of the C(-1019)G change on basal activity in post-synaptic cells where Deaf-1 is largely cytosolic and may be free to translocate to the reporter construct. At reporter constructs, transfected Deaf-1 displayed greater repressor activity than enhancer activity (e.g., Figure 2.3, 2.4), since the background activity of endogenous Deaf-1 to repress is lower than basal enhancer activity (Figure 2.1A vs. 2.1B). Thus the preferential localization of Deaf-1 in the nucleus of RN46A cells appears to correlate with its greater activity as a repressor.

2.5.3 HDAC dependence of Deaf-1 function

Both repressor and enhancer activities of Deaf-1 were blocked by inhibition of HDAC using the HDAC-specific (class I and II) inhibitor TSA (Yoshida et al., 1990), implicating HDAC in these activities of Deaf-1. Histone deacetylases (HDACs)

regulate the acetylation of histone and non-histone proteins, as well as the recruitment of transcriptional regulatory protein complexes to chromatin (Yang, 2004). Besides their roles in transcriptional repression, deacetylases can also positively regulate gene expression by deacetylation of transcription factors (Yang and Gregoire, 2005). For example, recruitment of HDAC by STAT5 proteins leads to deacetylation of C/EBPbeta, resulting in transcriptional activation of the Id-1 repressor gene (Xu et al., 2003). Thus, TSA-mediated inhibition of HDAC activity can block repression as well as enhancer activity.

Deaf-1 provides an unusual example of a transcription factor that upon directly binding to the same DNA regulatory element can recruit HDAC-dependent mechanisms to either repress or enhance gene expression. Transcription factors, such as nuclear receptors or myocyte enhancer factor-2 (MEF2), can function as repressor or enhancer but repressor activity involves recruitment of HDAC, while enhancer activity requires recruitment of histone acetylase activity and the absence of HDAC (Glass and Rosenfeld, 2000; McKinsey et al., 2001). Alternately, enhancer activity can be mediated through indirect protein-protein interactions. For example REST can both repress corticotrophin releasing hormone gene expression by binding to its RE-1 element, and enhance its transcription via a mechanism independent of RE-1 in NG108-15 cells (Seth and Majzoub, 2001). Generally, REST represses most target genes by association with co-repressors including Sin3A, nuclear receptor corepressor (NCoR) or CoREST. However, the co-repressor NCoR can mediate HDAC-dependent gene activation (Jepsen et al., 2000), a mechanism

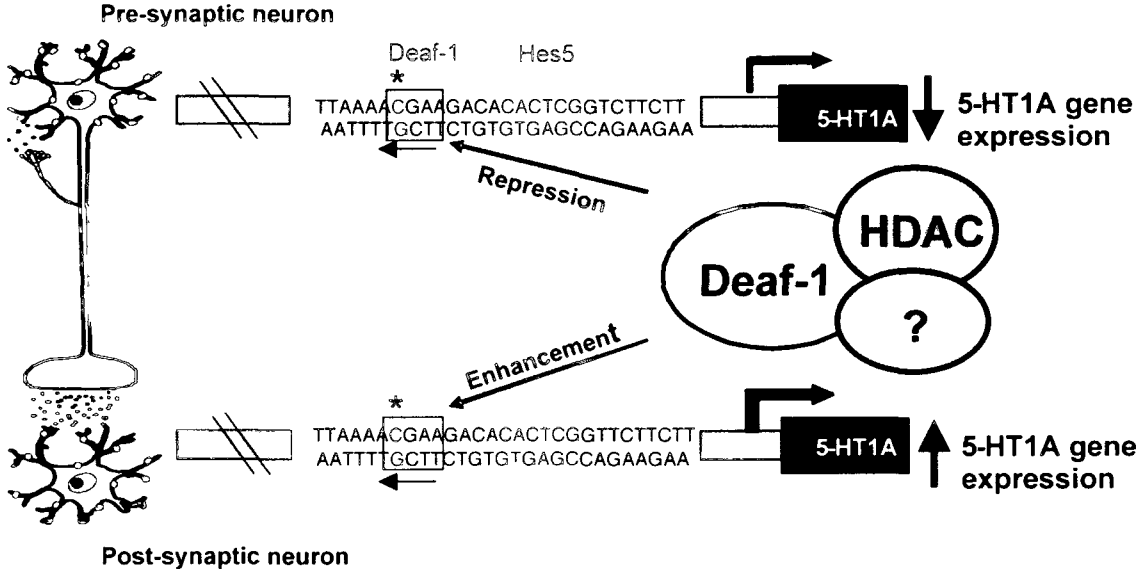
that could account for the HDAC dependent activation of the 5-HT1A gene in post-synaptic cells by Deaf-1.

2.5.4 Implications for depression

Identifying mechanisms that control 5-HT1A receptor expression has strong clinical relevance as the 5-HT1A receptor is a major direct or indirect target of antidepressant drugs (Pineyro and Blier, 1999; Blier and Ward, 2003; Albert and Lemonde, 2004). Increase in 5-HT1A autoreceptor expression has been associated with major depression (Stockmeier et al., 1998). In normal subjects and especially unmedicated depressed subjects, vivo imaging studies reveal increased 5-HT1A autoreceptors in the raphe area in G/G vs. non-G/G genotype, consistent with de-repression of Deaf-1 at the 5-HT1A G-allele (David et al., 2005; Parsey et al., 2005). Post-synaptically, however, subjects with major depressive or panic disorder have a significant decrease in 5-HT1A mRNA (Lopez-Figueroa et al., 2004) and decreased 5-HT1A receptor levels in hippocampus and cortex (Sargent et al., 2000; Bhagwagar et al., 2004; Neumeister et al., 2004). Furthermore, upon chronic antidepressant treatment, 5-HT1A autoreceptors are preferentially desensitized, while post-synaptic 5-HT1A receptors are not (Pineyro and Blier, 1999). The cell-specific function of Deaf-1 could underlie differential regulation of pre- and post-synaptic 5HT1A receptor expression *in vivo* (Supplementary Figure 2.1). The G/G genotype could confer de-repression of the 5-HT1A gene in the raphe, but reduced Deaf-1 enhancer activity in post-synaptic neurons. The extent of Deaf-1 enhancer activity varied depending on the cell type, with strongest activity in septal SN48 cells. By

analogy the contribution of Deaf-1 to 5-HT1A receptor expression in various brain regions, and the influence of the C(-1019)G genotype will differ depending on the neuronal cell type.

In summary, we have identified a novel HDAC-dependent regulatory mechanism by which Deaf-1 mediates cell-specific enhancer or repressor activity to regulate the 5-HT1A receptor gene. These divergent regulatory mechanisms by Deaf-1 may confer differential regulation of the 5-HT1A receptor in pre- vs. post-synaptic brain regions *in vivo*.



Supplemental Figure 2.1: Model of Deaf-1-mediated transcriptional regulation of the 5-HT1A promoter

Deaf-1 binds to a minimal consensus TTCG Deaf-1 site to repress 5-HT1A receptor gene transcription in pre-synaptic raphe cells, while it enhances transcription in post-synaptic 5-HT1A-expressing cells. Hes5 acts as a transcriptional repressor in a cell-independent manner. Cell-specific transcriptional repression and enhancement of the 5-HT1A promoter are intrinsic activities of Deaf-1 that require histone deacetylase (HDAC) activation and are abolished when the C(-1019)G polymorphism is present.

2.6 REFERENCES:

Albert PR, Lemonde S (2004) 5-HT1A Receptors, Gene Repression, and Depression: Guilt by Association. Neuroscientist 10:575-593.

Ansorge M, Tanneberger C, Davies B, Theuring F, Kusserow H (2004) Analysis of the murine 5-HT receptor gene promoter in vitro and in vivo. Eur J Neurosci 20:363-374.

Ayer DE (1999) Histone deacetylases: transcriptional repression with SINers and NuRDs. Trends Cell Biol 9:193-198.

Bach I, Carriere C, Ostendorff HP, Andersen B, Rosenfeld MG (1997) A family of LIM domain-associated cofactors confer transcriptional synergism between LIM and Otx homeodomain proteins. Genes Dev 11:1370-1380.

Bayliss DA, Li YW, Talley EM (1997) Effects of serotonin on caudal raphe neurons: activation of an inwardly rectifying potassium conductance. J Neurophysiol 77:1349-1361.

Bhagwagar Z, Rabiner EA, Sargent PA, Grasby PM, Cowen PJ (2004) Persistent reduction in brain serotonin1A receptor binding in recovered depressed men measured by positron emission tomography with [11C]WAY-100635. Mol Psychiatry 9:386-392.

Blier P, Ward NM (2003) Is there a role for 5-HT(1A) agonists in the treatment of depression? Biol Psychiatry 53:193-203.

Brown KA, Roberts RL, Arteaga CL, Law BK (2004) Transforming growth factor-beta induces Cdk2 relocalization to the cytoplasm coincident with dephosphorylation of retinoblastoma tumor suppressor protein. Breast Cancer Res 6:R130-139.

Charest A, Wainer BH, Albert PR (1993) Cloning and differentiation-induced expression of a murine serotonin1A receptor in a septal cell line. J Neurosci 13:5164-5171.

David SP, Murthy NV, Rabiner EA, Munafo MR, Johnstone EC, Jacob R, Walton RT, Grasby PM (2005) A functional genetic variation of the serotonin (5-HT) transporter affects 5-HT1A receptor binding in humans. J Neurosci 25:2586-2590.

Drevets WC, Frank E, Price JC, Kupfer DJ, Holt D, Greer PJ, Huang Y, Gautier C, Mathis C (1999) PET imaging of serotonin 1A receptor binding in depression. Biol Psychiatry 46:1375-1387.

Fricker AD, Rios C, Devi LA, Gomes I (2005) Serotonin receptor activation leads to neurite outgrowth and neuronal survival. Brain Res Mol Brain Res 138:228-235.

Glass CK, Rosenfeld MG (2000) The coregulator exchange in transcriptional functions of nuclear receptors. Genes Dev 14:121-141.

Gross C, Zhuang X, Stark K, Ramboz S, Oosting R, Kirby L, Santarelli L, Beck S, Hen R (2002) Serotonin1A receptor acts during development to establish normal anxiety-like behaviour in the adult. Nature 416:396-400.

Gross CT, McGinnis W (1996) DEAF-1, a novel protein that binds an essential region in a Deformed response element. EMBO J 15:1961-1970.

Hahm K, Sum EY, Fujiwara Y, Lindeman GJ, Visvader JE, Orkin SH (2004) Defective Neural Tube Closure and Anteroposterior Patterning in Mice Lacking the LIM Protein LMO4 or Its Interacting Partner Deaf-1. Mol Cell Biol 24:2074-2082.

Huggenvik JI, Michelson RJ, Collard MW, Ziemba AJ, Gurley P, Mowen KA (1998) Characterization of a nuclear deformed epidermal autoregulatory factor- 1 (DEAF-1)-related (NUDR) transcriptional regulator protein. Mol Endocrinol 12:1619-1639.

Jacobs BL, Azmitia EC (1992) Structure and function of the brain serotonin system. Physiol Rev 72:165-229.

Jensik PJ, Huggenvik JI, Collard MW (2004) Identification of a Nuclear Export Signal and Protein Interaction Domains in Deformed Epidermal Autoregulatory Factor-1 (DEAF-1). J Biol Chem 279:32692-32699.

Jepsen K, Hermanson O, Onami TM, Gleiberman AS, Lunyak V, McEvilly RJ, Kurokawa R, Kumar V, Liu F, Seto E, Hedrick SM, Mandel G, Glass CK, Rose DW, Rosenfeld MG (2000) Combinatorial roles of the nuclear receptor corepressor in transcription and development. Cell 102:753-763.

Jurata LW, Gill GN (1997) Functional analysis of the nuclear LIM domain interactor NLI. Mol Cell Biol 17:5688-5698.

Kushwaha N, Albert PR (2005) Coupling of 5-HT1A autoreceptors to inhibition of mitogen-activated protein kinase activation via Gbetagamma subunit signaling. Eur J Neurosci 21:721-732.

Lee SK, Jurata LW, Nowak R, Lettieri K, Kenny DA, Pfaff SL, Gill GN (2005) The LIM domain-only protein LMO4 is required for neural tube closure. Mol Cell Neurosci 28:205-214.

Lemonde S, Rogaeva A, Albert PR (2004a) Cell type-dependent recruitment of trichostatin A-sensitive repression of the human 5-HT1A receptor gene. J Neurochem 88:857-868.

Lemonde S, Du L, Bakish D, Hrdina P, Albert PR (2004b) Association of the C(-1019)G 5-HT1A functional promoter polymorphism with antidepressant response. Int J Neuropsychopharmacol 7:501-506.

Lemonde S, Turecki G, Bakish D, Du L, Hrdina PD, Bown CD, Sequeira A, Kushwaha N, Morris SJ, Basak A, Ou XM, Albert PR (2003) Impaired repression at a 5-hydroxytryptamine 1A receptor gene polymorphism associated with major depression and suicide. J Neuroscience 23:8788-8799.

Lopez-Figueroa AL, Norton CS, Lopez-Figueroa MO, Armellini-Dodel D, Burke S, Akil H, Lopez JF, Watson SJ (2004) Serotonin 5-HT1A, 5-HT1B, and 5-HT2A receptor mRNA expression in subjects with major depression, bipolar disorder, and schizophrenia. Biol Psychiatry 55:225-233.

McKinsey TA, Zhang CL, Olson EN (2001) Control of muscle development by dueling HATs and HDACs. Curr Opin Genet Dev 11:497-504.

Michelson RJ, Collard MW, Ziemba AJ, Persinger J, Bartholomew B, Huggenvik JI (1999) Nuclear DEAF-1-related (NUDR) protein contains a novel DNA binding

domain and represses transcription of the heterogeneous nuclear ribonucleoprotein A2/B1 promoter. J Biol Chem 274:30510-30519.

Neumeister A, Bain E, Nugent AC, Carson RE, Bonne O, Luckenbaugh DA, Eckelman W, Herscovitch P, Charney DS, Drevets WC (2004) Reduced serotonin type 1A receptor binding in panic disorder. J Neurosci 24:589-591.

Ou XM, Jafar-Nejad H, Storring JM, Meng JH, Lemonde S, Albert PR (2000) Novel dual repressor elements for neuronal cell-specific transcription of the rat 5-HT1A receptor gene. J Biol Chem 275:8161-8168.

Parsey RV, Oquendo MA, Ogden RT, Olvet DM, Simpson N, Huang YY, Van Heertum RL, Arango V, Mann JJ (2005) Altered Serotonin 1A Binding in Major Depression: A [carbonyl-C-11]WAY100635 Positron Emission Tomography Study. Biol Psychiatry.

Penington NJ, Kelly JS, Fox AP (1993) Whole-cell recordings of inwardly rectifying K+ currents activated by 5-HT1A receptors on dorsal raphe neurones of the adult rat. J Physiol 469:387-405.

Pineyro G, Blier P (1999) Autoregulation of serotonin neurons: role in antidepressant drug action. Pharmacol Rev 51:533-591.

Sargent PA, Kjaer KH, Bench CJ, Rabiner EA, Messa C, Meyer J, Gunn RN, Grasby PM, Cowen PJ (2000) Brain serotonin1A receptor binding measured by positron emission tomography with [11C]WAY-100635: effects of depression and antidepressant treatment. Arch Gen Psychiatry 57:174-180.

Serretti A, Artioli P, Lorenzi C, Pirovano A, Tubazio V, Zanardi R (2004) The C(-1019)G polymorphism of the 5-HT1A gene promoter and antidepressant response in mood disorders: preliminary findings. Int J Neuropsychopharmacol 7:453-460.

Seth KA, Majzoub JA (2001) Repressor element silencing transcription factor/neuron-restrictive silencing factor (REST/NRSF) can act as an enhancer as well as a repressor of corticotropin-releasing hormone gene transcription. J Biol Chem 276:13917-13923.

Stockmeier CA, Shapiro LA, Dilley GE, Kolli TN, Friedman L, Rajkowska G (1998) Increase in serotonin-1A autoreceptors in the midbrain of suicide victims with major depression-postmortem evidence for decreased serotonin activity. J Neurosci 18:7394-7401.

Strobel A, Gutknecht L, Rothe C, Reif A, Mossner R, Zeng Y, Brocke B, Lesch KP (2003) Allelic variation in 5-HT(1A) receptor expression is associated with anxiety- and depression-related personality traits. J Neural Transm 110:1445-1453.

Subramanian T, Chinnadurai G (2003) Association of class I histone deacetylases with transcriptional corepressor CtBP. FEBS Lett 540:255-258.

Sum EY, Peng B, Yu X, Chen J, Byrne J, Lindeman GJ, Visvader JE (2002) The LIM domain protein LMO4 interacts with the cofactor CtIP and the tumor suppressor BRCA1 and inhibits BRCA1 activity. J Biol Chem 277:7849-7856.

Törk I (1990) Anatomy of the serotonergic system. Ann N Y Acad Sci 600:9-35.

Tse E, Smith AJ, Hunt S, Lavenir I, Forster A, Warren AJ, Grutz G, Foroni L, Carlton MB, Colledge WH, Boehm T, Rabbitts TH (2004) Null mutation of the Lmo4 gene or a combined null mutation of the Lmo1/Lmo3 genes causes perinatal lethality, and Lmo4 controls neural tube development in mice. Mol Cell Biol 24:2063-2073.

Xu M, Nie L, Kim SH, Sun XH (2003) STAT5-induced Id-1 transcription involves recruitment of HDAC1 and deacetylation of C/EBPbeta. EMBO J 22:893-904.

Yang XJ (2004) Lysine acetylation and the bromodomain: a new partnership for signaling. BioEssays 26:1076-1087.

Yang XJ, Gregoire S (2005) Class II histone deacetylases: from sequence to function, regulation, and clinical implication. Mol Cell Biol 25:2873-2884.

Yoshida M, Kijima M, Akita M, Beppu T (1990) Potent and specific inhibition of mammalian histone deacetylase both in vivo and in vitro by trichostatin A. J Biol Chem 265:17174-17179.

Zuccato C, Tartari M, Crotti A, Goffredo D, Valenza M, Conti L, Cataudella T, Leavitt BR, Hayden MR, Timmusk T, Rigamonti D, Cattaneo E (2003) Huntingtin interacts with REST/NRSF to modulate the transcription of NRSE-controlled neuronal genes. Nat Genet 35:76-83.

CHAPTER 3

The following chapter has been reproduced in its entirety from:

Czesak M, Burns AM, Lenicov FR, Albert PR. (2007) Characterization of rat rostral raphe primary cultures: multiplex quantification of serotonergic markers

J Neurosci Methods. Aug 15;164(1):59-67. Epub 2007 Apr 7.

See Appendix A for permission

CHARACTERIZATION OF RAT ROSTRAL RAPHE PRIMARY CULTURES: MULTIPLEX QUANTIFICATION OF SEROTONERGIC MARKERS

Margaret Czesak, Ariel M. Burns, Federico Remes Lenicov and Paul R. Albert*

Ottawa Health Research Institute (Neuroscience)
Department of Cellular and Molecular Medicine
University of Ottawa
Ottawa, Canada, K1H 8M5

Abbreviated Title: Quantification of serotonergic markers in raphe cells

Keywords: 5-HT1A receptor, transcription factor, real-time PCR, repressor, differentiation, raphe, serotonin.

Acknowledgements

We thank Dr. Slack for providing us with samples of the Tuj1 primary antibody, Dr. Park for the TH primary antibody and Dr. Bennett for the NeuN antibody. We also thank Mireille Daigle (OHRI Institute) for excellent technical guidance throughout the revision process. M.C. holds the Canada Graduate Studentship from CIHR and P.R.A. holds the Novartis/CIHR Michael Smith Chair in Neuroscience.

3.1 Abstract

Previous reports establishing raphe cultures typically yield less than 1% serotonin (5-HT)-positive neurons and are impractical for transcriptional studies. In this study, we have established primary cultures enriched in 5-HT neurons and quantified the proportion of cells expressing serotonergic and non-serotonergic markers. We have also shown the feasibility of using the multiplex real-time PCR technique to measure the relative amounts of RNA for some of these markers. Rostral raphe cells derived from E13-15 rat embryos were cultured for 7 days and analyzed by quantitative immunofluorescence and western blot analysis. In these cultures, ~8% of neurons were immunopositive for serotonergic markers (5-HT or tryptophan hydroxylase (TPH)). The % of cells labeled for GFAP (glial marker), tyrosine hydroxylase (catecholaminergic), and GAD65/67 (GABAergic) was 5, 1, and 54%, respectively. Transcription factors REST/NRSF and Deaf-1 were present in 9 and 98% of cells, respectively. Multiplex quantitative RT-PCR (Q-PCR) analysis was done for TPH2, 5-HT1A receptor or Deaf-1 RNAs paired with GAPDH RNA as control. Using this approach, standard curves for each RNA were obtained over 200-fold concentration range of dilution with r^2 values > 0.99. The relative abundances determined by Q-PCR are consistent with the expression of TPH2>Deaf-1>5-HT1A receptor RNA in serotonergic raphe cells. The standard error of TPH2 RNA levels between cultures was <20%, indicating a consistent purity of 5-HT neurons. Thus, we have generated a highly consistent and reproducible model system that is enriched in 5-HT neurons and that will be valuable in future investigation of serotonergic regulation.

3.2 Introduction

The brainstem raphe nuclei are the primary site of serotonin (5-HT) synthesis in the mammalian brain (Dahlstrom and Fuxe, 1964). These neurons are characterized into subpopulations based on their regional differences in morphologic features and projection patterns. The dorsal raphe nuclei (DRN) contain the largest collection of 5-HT neurons (Tork, 1990). It has been estimated that the number of 5-HT neurons within the human DRN is approximately 235,000 (Baker et al., 1990), while only 20,000 5-HT neurons are present in the rat (Jacobs and Azmitia, 1992). Although low in number, 5-HT neurons densely innervate the brain through their extensive and diffuse axonal collaterals. The rostral division of 5-HT neurons projects to the forebrain, including the cerebral cortex, limbic system, basal ganglia and diencephalon. Caudal 5-HT neurons project primarily to the spinal cord, while both groups innervate the brainstem and cerebellum (Steinbusch, 1981; Kohler and Steinbusch, 1982; Tork, 1990; Vertes, 1991). It is through these extensive projections that 5-HT regulates a wide range of physiological and behavioural functions such as learning, memory, emotion and behaviour (Jacobs and Azmitia, 1992; Barnes and Sharp, 1999).

In the rat, the first 5-HT neurons are detected at embryonic day 12 (E12) by 5-HT immunoreactivity. By E14, three groups of serotonergic neurons (rostral, medial and caudal) can be defined; subgroups emerge from these and enlarge until E19 (Aitken and Tork, 1988; Konig et al., 1988). The synthesis of 5-HT is regulated by the levels of its precursor tryptophan and by the activity of tryptophan hydroxylase (TPH). A second isoform of TPH, termed TPH2, has been identified as

the rate-limiting enzyme in raphe neurons (Walther et al., 2003). Among the 14 known mammalian 5-HT receptor genes, the most abundant subtype expressed on serotonergic raphe neurons is the somatodendritic 5-HT1A autoreceptor (Sotelo et al., 1990; Riad et al., 2000). Activation of the 5-HT1A autoreceptor inhibits adenylyl cyclase and calcium currents and activates potassium channels (Raymond et al., 1999). Thus, 5-HT1A autoreceptors decrease cell firing rates and 5-HT release to mediate negative feedback inhibition of serotonergic neurons. Hence, the 5-HT1A autoreceptor is a powerful regulator of both pre- and post-synaptic serotonergic neurotransmission.

Transcriptional regulation of the 5-HT1A gene affects autoreceptor levels and thus is believed to modulate 5-HT neurotransmission (Albert and Lemonde, 2004). An important transcription factor involved in controlling 5-HT1A gene expression is Deformed Epidermal Autoregulatory Factor-1 (Deaf-1/NUDR/Suppressin) (Lemonde et al., 2003). It acts as a repressor of 5-HT1A gene transcription in RN46A cells, which are a model of pre-synaptic rat raphe nuclei cells. In addition, it has been proposed that dys-regulation of Deaf-1 may play an important role in major depressive disorder (MDD) through de-repression of raphe 5-HT1A gene transcription. This would increase autoreceptor levels and decrease 5-HT firing, which may contribute to the pathobiology of MDD (Lemonde et al., 2003; Czesak et al., 2006).

Previous studies have had variable success in establishing raphe cultures enriched in 5-HT neurons, and the presence of other neurotransmitter subtypes or transcription factors was not extensively characterized (Galter and Unsicker, 1999,

2000; Lautenschlager et al., 2000; Rumajogee et al., 2002). One objective of this study was to establish rat primary rostral raphe cultures enriched in 5-HT neurons and to quantify the proportion of cells expressing key serotonergic and non-serotonergic markers. Moreover, we utilized the real-time PCR technique to consistently measure relative amounts of RNA for some of these markers including: the rate-limiting enzyme for 5-HT synthesis, TPH2; the 5-HT1A autoreceptor; and the transcriptional factor Deaf-1, which regulates 5-HT1A receptor gene expression. Furthermore, we have assessed the levels of several other neuronal and non-neuronal markers to more fully characterize these cultures, and find a low frequency of glial and catecholaminergic markers. These cultures were consistently enriched in 5-HT neurons and allowed for reproducible multiplex quantification of RNAs that will permit transcriptional studies of 5-HT neurons.

3.3 Materials and Methods

3.3.1 Primary Cultures

Pregnant Sprague-Dawley rats (estimated E13-E15) were obtained from Charles River Inc. Rats were euthanized according to the experimental protocol approved by the University of Ottawa animal care facility (protocol # NSI-53). Briefly, rats were placed into CO_2/O_2 chamber until loss of consciousness, followed by cervical dislocation. Embryos were removed via c-section and placed in Hank's Balanced Salt Solution (HBSS) without Ca^{2+} (Wisent Inc, St. Bruno, QC, Canada). Their stage of development was assessed by morphological criteria in accordance to the Carnegie stages of human development (O'Rahilly and Muller, 1994). Embryos younger than

E13 or older than E15 were discarded to ensure consistency of cultures. Rostral raphe neurons were dissected from the midbrain according to the parameters outlined in Lautenschlager et al. 2000. Briefly, heads were removed from the embryos under a dissecting microscope (Zeiss KL 1500 LCD), meninges were removed and the midbrain/brainstem gently dissociated. The neural tube was opened ventrally and flattened in a petri dish containing HBSS without Ca^{2+} (Wisent Inc.). A strip of tissue of approximately 0.5 mm in width was dissected at the midline of the rostral rhombencephalon (Konig et al., 1988). Further dissection of the region between B4 and B9 raphe nuclei was done to enrich for rostral 5-HT neurons.

Raphe tissue was resuspended in 5 mL of HBSS without Ca^{2+} and triturated ten times, the homogenate was strained (BD Biosciences, Mississauga, ON, Canada) to remove debris and an equal amount of HBSS with Ca^{2+} added. Cells were centrifuged (500 g, 5 min) and the pellet was resuspended in 5 mL of Neurobasal media (Invitrogen, Burlington, ON, Canada) containing B27 supplement (Invitrogen), 1% PenStrep (Wisent Inc.) and 0.4% L-Glutamine (Wisent Inc.). 6-well plates (Sarstedt Inc. Montreal, QC, Canada) were coated with poly-D-lysine (Sigma Inc. St. Louis, MO, USA) and pre-incubated with neurobasal media (1 mL per well) overnight in the 37°C incubator. Average yield was 12 embryos/rat from which two 6-well plates of cells plated at 3×10^6 cells/well were obtained. Two days after plating, 1 mL of medium from each well was replaced with fresh medium. Cells were cultured for 7 days in vitro (7 DIV).

3.3.2 Immunohistochemistry

Cells were fixed using Lana's Fixative (4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer, pH 7.1). Cells were rinsed three times in 10 mM phosphate-buffered saline (PBS). All cells treated with primary antibodies were incubated at 4°C overnight on top of a vibrating device to enhance antibody binding (Jacobsen and Staines, 2004). To detect glial cells, polyclonal rabbit anti-glial fibrillary acidic protein (GFAP) antibody was used at a dilution of 1:500 (Dako Inc. Mississauga, ON, Canada). For early neuronal differentiation, mouse neuron specific β III Tubulin antibody-1 (Tuj-1) was used as marker, at a dilution of 1:25 (kind gift from Dr. Slack, originally from Dr. David Brown) while mouse Neuronal Nuclei (NeuN) was used as a marker of mature neurons, at a dilution of 1:50 (kind gift from Dr. Bennett, originally from Chemicon Inc.). Mouse monoclonal anti-tyrosine hydroxylase (TH) antibody was used at 1:1000 dilution (kind gift from Dr. Park, originally from Immunostar, Hudson, WI, USA) to distinguish catecholaminergic neurons. 5-HT positive neurons were identified using rat anti-serotonin monoclonal antibody (Chemicon Inc.) at a dilution of 1:100; additionally, polyclonal sheep anti-TPH antibody (Chemicon Inc.) was used at dilution of 1:200. For the transcription factor Deaf-1, rabbit anti-Deaf-1 polyclonal antibody was generated using a Deaf-1 multiple-antigenic peptide sequence EAEEPVLSRDEDSEED corresponding to amino acids 36-51 (Cedarlane, Hornby, ON, Canada) (Lemonde et al., 2003) and used at a dilution of 1:200. For γ-aminobutyric acid (GABAergic) neurons, monoclonal mouse anti-glutamic acid decarboxylate-65 antibody (BD PharMingen, Mississauga, ON, Canada) was used at 1:1000 dilution, and polyclonal rabbit anti-glutamic

decarboxylase-65/67 antibody (Chemicon Inc.) was used at a dilution of 1 1000 A goat polyclonal antibody dilution of 1 200 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used to detect the Repressor Element Silencing Transcription Factor/Neuron-restrictive Silencing Factor (REST/NRSF) Each primary antibody was washed off 3 times with PBS and a secondary antibody against mouse, rat or rabbit was applied at a dilution of 1 1000 accordingly. Secondary antibodies used were Alexa Fluor 488 donkey anti-rabbit IgG, Alexa Fluor 594 goat anti-rabbit IgG, Alexa Fluor 594 rabbit anti-mouse IgG, Alexa Fluor 488 goat anti-rat IgG, Alexa Fluor 594 donkey anti-goat IgG and Alexa Fluor donkey anti-sheep IgG (Molecular Probes, Eugene, OR, USA) Hoechst staining (Sigma Inc.) was applied at 1 2000 dilution for 10 min and cells were washed 3 times with PBS Cells that stained positive for each of the markers where counted as % total live cells (20X resolution using Axiovert S100 Zeiss microscope, Northern Eclipse 6.0 software) which were indicated by positive Hoechst staining of nuclear DNA

3.3.3 Western blot analysis

Primary raphe cultures were rinsed three times with PBS and the cells collected. Cells were centrifuged at 500 g for 2 min and pellet was resuspended in 200-400 μL of 1% Nonidet P-40 solution containing protease inhibitors (Sigma Inc) Whole-cell extracts were sonicated (Sonic Dismembrator-60, Fisher Scientific, Nepean, ON, Canada) 3 times for 10 sec at setting 3 RMS Proteins where denatured by boiling for 5 min in 1X SDS buffer and placed on ice. 50 μg of total cell lysate was loaded onto a 12% electrophoresis gel and run according to previously described protocol

(Lemonde et al., 2003). Antibodies used where the same as described in the above immunohistochemistry section. Unless otherwise stated, same antibody dilutions were used for immunohistochemistry as for Western blots. In the case of anti-mouse NeuN antibody, a dilution of 1:1000 was used instead. Anti-rabbit Deaf-1 antibody was used at a dilution of 1:2000. Mouse GAD65 was used at a dilution of 1:1000 (BD PharMingen). Sheep TPH antibody was used at a dilution of 1:1000. Monoclonal mouse β-actin antibody (Sigma Aldrich) was used as loading control at a dilution of 1:5000. Secondary antibodies used at a dilution of 1:2000 were as follows: goat anti-rabbit HRP-linked antibody (Cell Signaling, Beverly, MA, USA), goat anti-mouse HRP-conjugated antibody and rabbit anti-goat HRP-conjugated antibody (Jackson Labs, Soham, Cambridgeshire, UK). Proteins were visualized on Kodak BioMax MR film using BM Chemiluminescence blotting substrate (Roche Diagnostics, Laval, QC, Canada).

3.3.4 Quantitative Real-Time RT-PCR (Q-PCR)

Total RNA was obtained from single wells of a 6-well plate. Wells were rinsed twice with ice-cold PBS and cells were lysed with 200 μL of TRIzol (Invitrogen) for 5 min and extracted at room temperature using 200 μL chloroform for 2 min. Lysates were centrifuged (10,000 g, 15 min, 4ºC) and the aqueous phase precipitated in isopropanol overnight at -20ºC. The pellet was rinsed with 70% ethanol and resuspended in 25 μL of DEPC-treated water to 0.3 μg/μL to 1 μg/μL. Samples were treated with DNAse using Turbo DNA-free kit (Ambion, Foster City, CA, USA) according to manufacturer's protocol. Briefly, 1 μL of DNAse was applied to 5 μg of

RNA for 30 min at 37ºC. After inactivation and pelleting of the enzyme, RNA was quantified by spectrophotometry at 260 nm. Concentrations ranged from 0.07 to 0.10 μg/μL and 260/280 ratios were >1.8.

Reverse transcription was carried out on 0.5 μg of DNAse-treated RNA in 20 μL reactions containing 2 μl of random decamer oligonucleotide primers (Ambion) and 2 mM dNTP mix. Reactions were heated for 3 min at 70ºC. After annealing on ice, first-strand buffer (Ambion), 25 U of M-MLV reverse transcriptase (Ambion, USA) and 10 U of RNAse inhibitor (Promega, Madison, WI, USA) were added for 60 min at 42ºC, and reactions terminated by 10 min treatment at 95ºC. Parallel reactions lacking M-MLV enzyme served as no reverse transcription controls. Newly synthesized cDNA was stored at -20ºC.

Q-PCR was performed using TaqMan chemistry. Probes and primers for the four genes studied were purchased as Gene Expression kits (Applied Biosystems, Foster City, CA, USA). Product numbers are as follows: TPH2, Rn00598017_m1; Deaf-1, Rn00582534_m1; 5-HT1AR, Rn00561409_s1 and GAPDH, 4352338E. TPH2, Deaf-1 and 5-HT1A probes are FAM-labelled probes, while GAPDH is a VIC-labelled probe. The expected size and location of each product is as follows: GAPDH is not specified, TPH2 is 64 bp spanning exon boundary 8-9, 5-HT1A is 68 bp (starting at 866 bp) and there is no exon boundary since the gene is intronless, and Deaf-1 is 68 bp spanning exon boundary 11-12 (starting at 1555 bp in the ORF). The identity of the TaqMan PCR products was verified by direct sequencing (5-HT1A, Deaf-1) or Southern blot analysis using TPH2 cDNA as probe (for TPH2). GAPDH was used as internal control and its probes were included in every multiplex reaction. Reactions

were carried out in 10 μL volume. Each reaction contained 5 μL of TaqMan Universal PCR Mastermix (Applied Biosystems), 0.5 μL of FAM-labelled probe, 0.5 μL of VIC-labelled probe, 3 μL of nuclease-free water prepared as a master-mix, and 1 μL of sample. Standard curves for each gene were prepared by serial dilution of the most concentrated cDNA sample measured in duplicate or triplicate such that the standard curves spanned the range of values of the duplicate test samples. Simultaneously, control samples without template DNA (no template controls) were run for each gene. In addition, at least one point of each standard curve was run in duplicate along with the samples as a positive control. Reactions were carried out in a Rotor Gene 3000 cycler (Corbett Research, Sydney, Australia). The cycling program used was: 95°C or 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 60 sec.

Data were analysed using commercially available Rotor Gene v6.0 software. The software found the best fit for the four standard curves. Threshold was defined as 10 times the baseline. Relative amounts of each target gene to GAPDH were obtained as $10^{(CtT-CtG)}$, where CtT is the threshold cycle for the target gene and CtG is the threshold cycle for GAPDH in the same tube (Livak and Schmittgen, 2001). Duplicates were averaged and the resulting values were used to calculate SEM. Outliers (validated by Q test with at least 90% confidence) were discarded.

3.4 Results

3.4.1 Characterization of midbrain cultures by immunostaining

Midbrain cultures enriched in raphe cells were characterized by immunofluorescence to determine the frequency of cells stained with various neuronal and glial markers (Figure 3.1). The specificity of the antibodies used was further tested by Western blot analysis (Figure 3.2). By 7 DIV, many of the cells had extended processes as can be seen with phase-contrast microscopy (Figure 3.1A).

3.4.2 Proportion of 5-HT neurons

In order to assess the proportion of serotonergic neurons present, rat rostral raphe cultures were stained with antibodies to 5-HT or TPH and co-stained with Hoechst to visualize the nuclei of all living cells present (Figure 3.1A and 3.1C). After 7 DIV, primary rat rostral raphe cultures, which include both the dorsal and median raphe nucleus, had 7.9% of 5-HT positive cells (Figure 3.1D). The proportion of 5-HT-positive neurons remained consistent between cultures. Similarly, TPH, the rate-limiting enzyme for 5-HT synthesis, was present in 7.2% of cells (Figure 3.1C and 3.1D). The slightly lower proportion of TPH- vs. 5-HT-positive cells may reflect different antibody sensitivity or low TPH expression in some 5-HT neurons (Hendricks et al., 2003). Western blot analysis detected a single TPH species at the expected size of 55 kDa (Figure 3.2). Both 5-HT- and TPH-positive cells displayed a differentiated neuronal appearance with extensive neuronal processes. Thus, the proportion of 5-HT neurons in these cultures was about 8%.

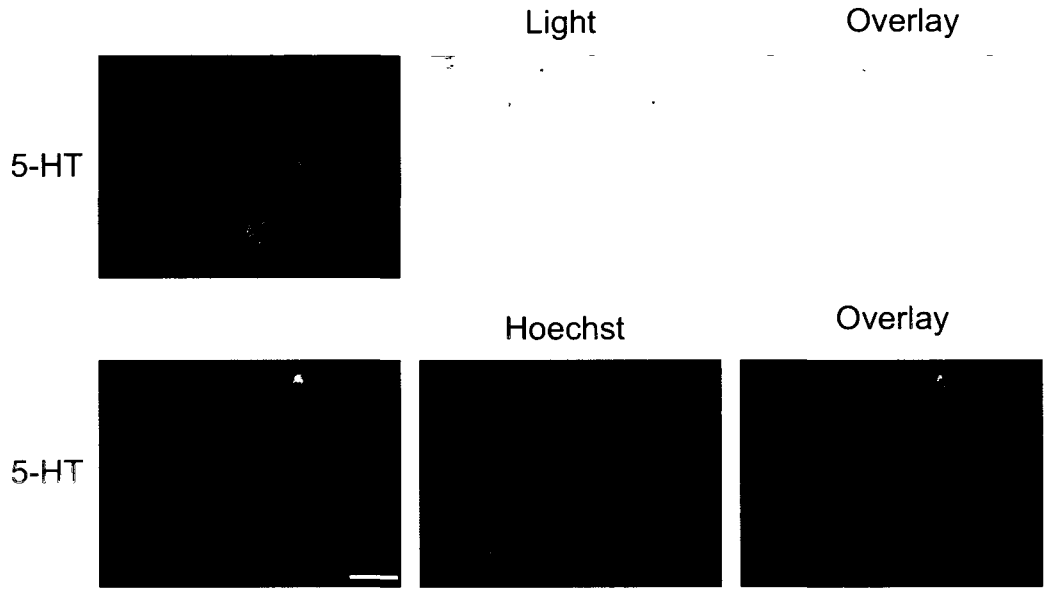


Figure 3.1-A: Quantification of marker frequency in rat rostral raphe cultures using immunofluorescent staining.

Raphe cultures at 7 DIV were fixed and stained for various neuronal and non-neuronal markers. A-C show representative images shown at 20X magnification (scale bar represents 50 microns). A) Immunofluorescent staining for the presence of 5-HT in raphe cultures, compared to total cell number (view by light microscopy) or total nuclei (Hoechst staining).

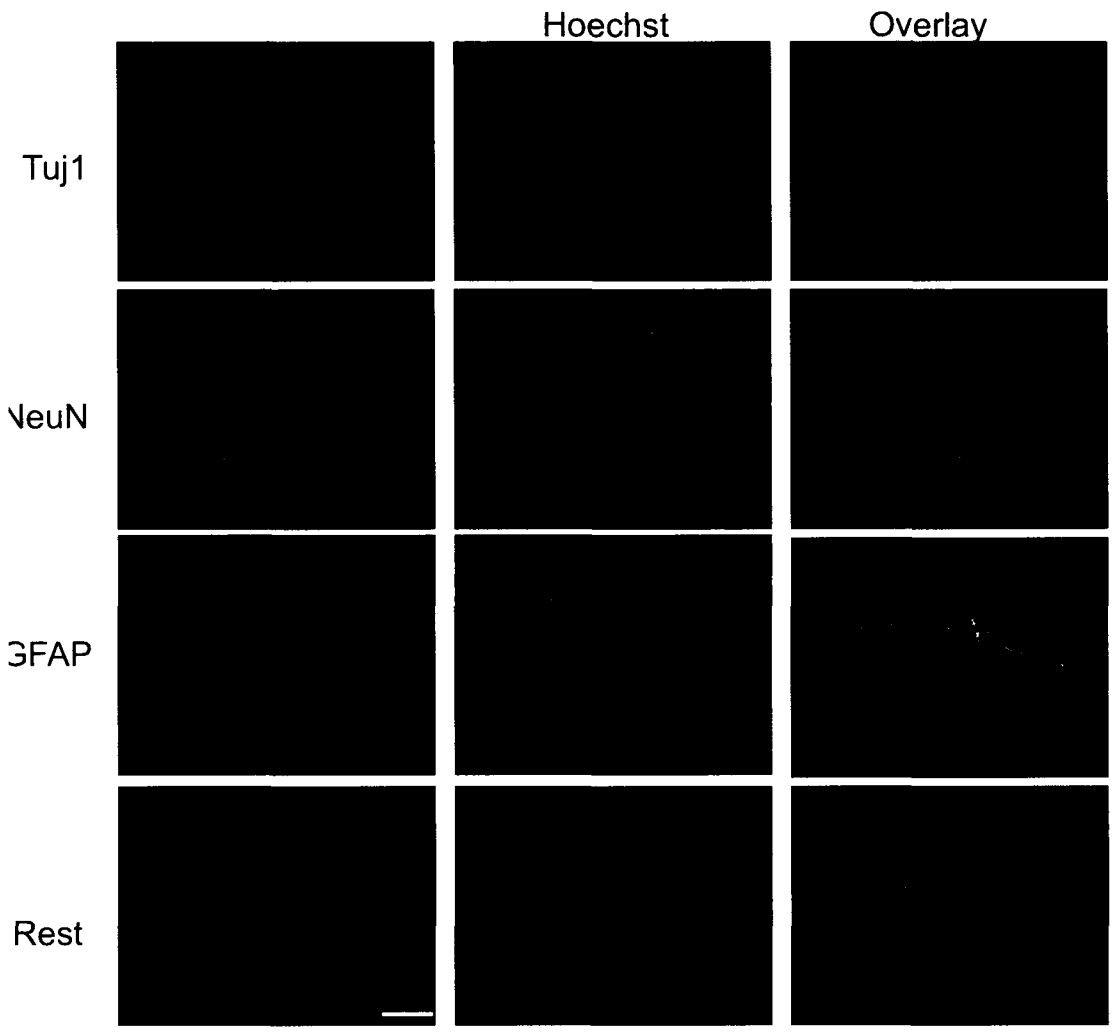


Figure 3.1-B: Quantification of marker frequency in rat rostral raphe cultures using immunofluorescent staining.

Raphe cultures at 7 DIV were fixed and stained. Representative images are shown at 20X magnification (scale bar represents 50 microns). Immunoflourescent staining of neuronal and non-neuronal cell markers compared to Hoechst stained nuclei.

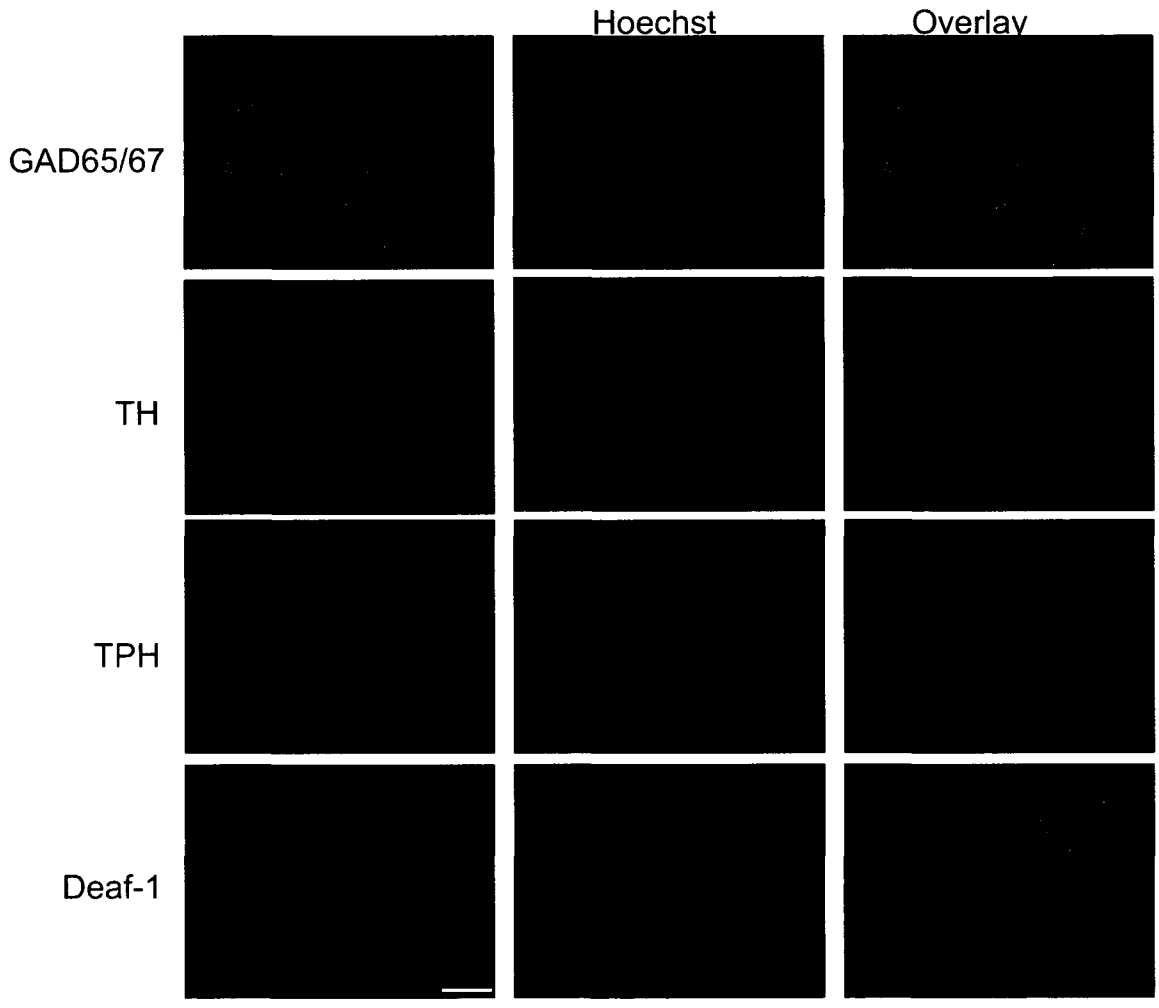


Figure 3.1-C: Quantification of marker frequency in rat rostral raphe cultures using immunofluorescent staining.

Raphe cultures at 7 DIV were fixed and stained Representative images are shown at 20X magnification (scale bar represents 50 microns) Staining of various neurotransmitter synthesis markers as well as Deaf-1, each compared to Hoechst stained nuclei

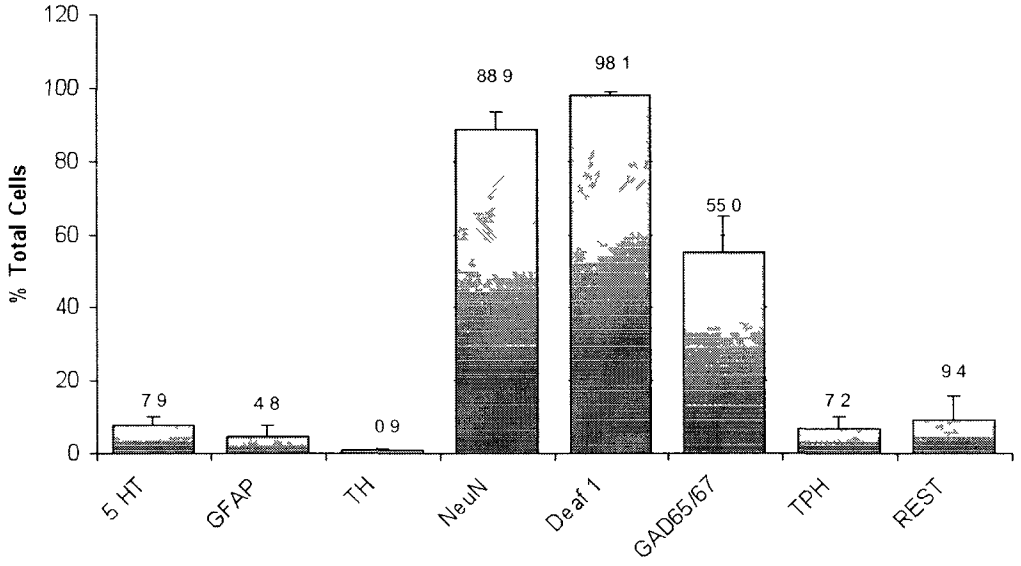


Figure 3.1-D: Quantification of marker frequency in rat rostral raphe cultures using immunofluorescent staining.

Quantification of cells stained positive for various markers Cells were counted as the percent of total nuclei (from viable cells) stained positive for Hoechst At least 6 different cultures were used to obtain the counts with at least 3 random fields of view counted per well stained Data are shown as mean % of total cells ± SEM

3.4.3 Proportion of neurons

The proportion of neuronal cells was tested using neuron specific Tuj-1 and NeuN antibodies, which are early and late neuronal markers, with 50 kDa and 46-48 kDa molecular weights, respectively (Figure 3.2). The majority of neurons exhibited positive Tuj-1 (Figure 3.1B), while 90.7% of neurons were NeuN positive (Figure 3.1B, 3.1D). Tuj-1-positive cells displayed extended processes, as evidenced by the tubulin staining in Figure 1B. To estimate glial cell number, cultures were stained for GFAP (Figure 3.1B). The proportion of non-neuronal, GFAP-positive cells in these raphe cultures was 4.8% (Figure 3.1D), and GFAP-positive cells displayed multiple processes typical of activated astrocytes (Figure 3.1B). GFAP was also expressed in the raphe protein extract, at the expected molecular weight of 52-55 kDa (Figure 3.2). Proliferation of glial cells in these cultures may have been attenuated by use of Neurobasal medium with B27 supplement, which inhibits the growth of non-neuronal cell types (Brewer at al., 1993). Thus these midbrain cultures contained at least 90% neuronal cells. Furthermore, cultures stained positive for REST/NRSF, which is expressed in progenitor or non-neuronal cells where it functions to inhibit the differentiation of neuron-specific genes. REST/NRSF was present in 9.4% of cells (Figure 3.1D), consistent with a large proportion of differentiated neurons.

3.4.4 Markers of neuronal subtypes

In addition to 5-HT-positive cells, we characterized the presence of other neuronal subtypes in these cultures using a variety of markers. Initially the presence of GABA-

positive cells was observed using anti-GABA staining (data not shown). This was confirmed by using antibodies for glutamic acid decarboxylase (GAD) 65 and 67, enzymes responsible for synthesizing GABA (Figure 3.1C). Western blot analysis further confirmed the abundance of these enzymes at the expected molecular weight of 65-67 kDa (Figure 3.2). As expected, the antibody specific for both subtypes of the enzyme (GAD65/67) stained more intensely than when GAD 65-specific antibody was used alone (Figure 3.2). Furthermore, cell counts of raphe cultures revealed that approximately 54.9% of these cells were GAD65/67 positive (Figure 3.1C, 3.1D), constituting the largest population of neurons in these cultures. To identify dopamine- or noradrenaline-positive neurons, we stained for tyrosine hydroxylase (TH) (Figure 3.1C, 3.1D), the rate-limiting enzyme in dopamine synthesis. TH was expressed in only 0.9% of neurons, implying that the proportion of catecholaminergic neurons is about 12% of the number of 5-HT neurons present in the cultures.

3.4.5 Deaf-1: a pleiotropic marker

Deaf-1 is a transcriptional regulator of 5-HT1A gene expression (Lemonde et al., 2003), hence the expression of Deaf-1 was examined by immunohistochemistry and Western blotting. Deaf-1 was expressed in the majority of cell types, 98.1% (Figure 3.1C and Figure 3.1D) and the anti-Deaf-1 antibody recognized a single species with the expected molecular weight of 59 kDa (Figure 3.2). The abundance of Deaf-1 is consistent with previous results that demonstrate that Deaf-1 is present in both serotonergic and non-serotonergic cells in sections of raphe nuclei, and hence may

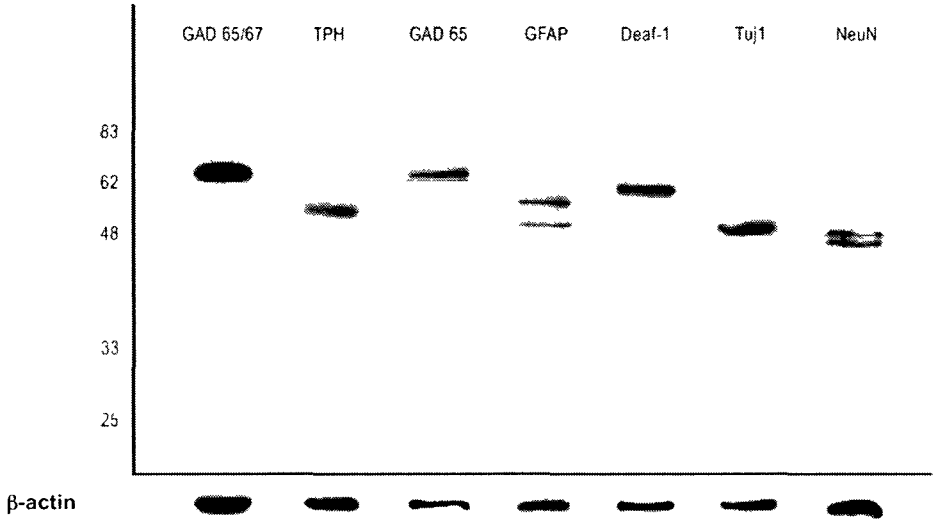


Figure 3.2: Western blot analysis of whole cell lysates for markers.

Cells from rostral midbrain cultures were collected after 7 DIV and protein extracts from whole cell lysates (50 µg/lane) were assessed for different proteins present in cultures by Western blot using the indicated antibodies. β-actin was used as loading control. Migration of molecular weight markers (kDa) is indicated on the ordinate.

regulate multiple genes (Lemonde et al., 2003). As observed previously by cell fractionation studies, Deaf-1 immunostaining was present in cytoplasm and nuclei of stained cells (Czesak et al., 2006).

3.4.6 Multiplex Q-PCR

The level of 5-HT1A, TPH2 and Deaf-1 RNA was quantified using Q-PCR. Multiplex analysis of raphe cultures was done for TPH2, 5-HT1A receptor or Deaf-1 RNAs paired with GAPDH RNA as control. Standard curves for each gene were produced using RNA extracted from raphe cultures and diluted over 200 times, demonstrating the linearity of the response over the range of values studied (r^2=0.992 for 5-HT1A, r^2=0.991 for TPH2, r^2=0.997 for NUDR and r^2=0.993 for GAPDH) (Figure 3.3A). The relative concentration of each RNA (R) was normalized to the concentration of GAPDH RNA using the formula: $R = 10^{(CtT-CtG)}$, where CtT is the target gene Ct and CtG is that of GAPDH (Livak and Schmittgen, 2001). The Ct (Threshold Cycle) values were always within the range of the standard curve for each gene analyzed as shown by representative sample curves (Figure 3.3B). "No reverse" transcription and "No template" controls did not amplify within this range. The reproducibility was greater amongst values obtained from same-day cultures, as opposed to cultures harvested on different occasions. Assuming equivalent reverse transcription efficiencies, Deaf-1 RNA was the most abundant, in agreement with its widespread expression, while for 5-HT specific markers, TPH2 RNA was intermediate and 5-HT1A RNA was least abundant (Figure 3.3C).

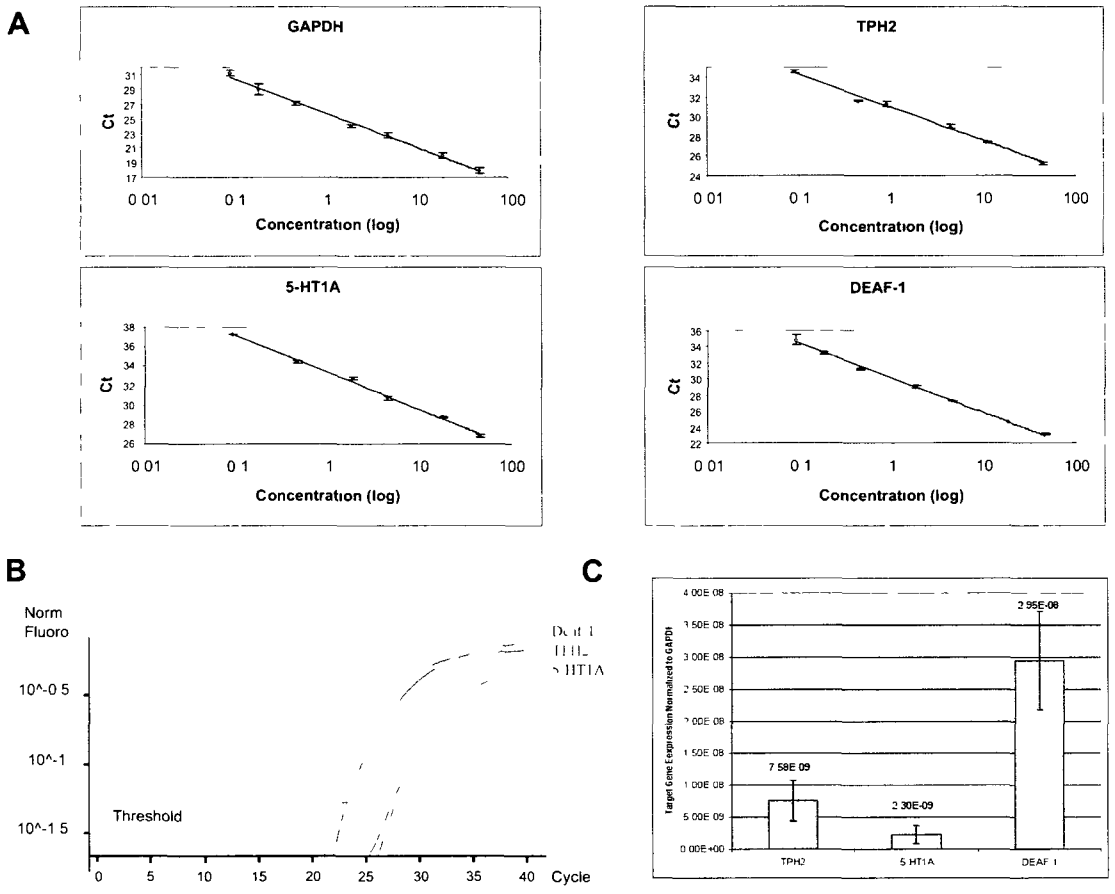


Figure 3.3: Multiplex Q-PCR for quantification of 5-HT1A receptor, Deaf-1 and TPH2 RNAs in raphe primary cultures.

A) Standard curves of serial dilutions of cell extracts were generated and analyzed by regression for each marker RNA, including GAPDH used as internal control. Correlation for GAPDH is Ct= -4.775*log(conc) + 25.710 (R^2 = 0.98795) and efficiency = 0.62. The correlation for 5-HT1A is Ct= -3.809*log(conc) + 33.331 (R^2 = 0.99208) and efficiency = 0.83. The correlation for Deaf-1 is Ct= -4.264*log(conc) + 30.033 (R^2 = 0.99339) and efficiency = 0.72. The correlation for TPH2 is Ct= -3.371*log(conc) + 30.942 (R^2 = 0.99071) and efficiency = 0.98. B) Sample curves showing representative amplification of each of the marker RNAs. C) Average relative abundance of marker RNAs. Graph showing relative amounts compared to GAPDH of TPH2, 5-HT1A and Deaf-1 RNAs from multiple independent cultures. Data are shown as mean ± SEM of at least three independent experiments.

3.5 Discussion

3.5.1 Characterization of raphe cultures

We have established midbrain cultures from E13-15 rat embryos that are enriched in serotonergic cells. These cultures were derived from the tissue rostral to the pontine flexure, which separates the B4-B9 raphe nuclei (including dorsal and median nuclei) from the caudal raphe nuclei (Konig et al., 1988). The dorsal raphe projects specifically to the prefrontal cortex, lateral septum and amygdala, while the medial raphe projects to the medial septum, cingulate and dorsal hippocampus (Beck et al., 2004), as well as limbic areas involved in depression. Thus, these cultures provide a model for understanding regulatory changes implicated in depression (Adell et al., 2002). Typically, embryonic midbrain cultures have yielded less than 1% 5-HT positive neurons, a limitation for mechanistic studies. For example, only 200/75,000 cells (0.3%) were 5-HT positive in a recent study (Rumajogee et al., 2002). Galter and Unsicker (1999) reported ~1% TPH positive neurons in rostral rhombencephalon cultures from E14 rat embryos after 24h IV; the percentage of 5-HT positive neurons declined until 6 DIV and then stabilized (Galter and Unsicker, 1999). They also reported less than 1% TH positive neurons, and virtually no GFAP positive astroglia. Lautenschlager et al. (2000) obtained about 4% overall, and as high as 7.5% 5-HT positive neurons in a small subgroup of their rostral cultures from E14 rat embryos at 14 DIV (Lautenschlager et al., 2000). In their studies they did fine dissection of the rostral raphe region to optimize the yield, using trypsin/EDTA to dissociate the cells, a treatment that we found reduced the neuronal viability of our cultures and was omitted. In our approach, gentle

mechanical dissociation of cells prior to plating ensured a higher rate of survival than with enzymatic treatment, especially if cells were already undergoing differentiation upon harvesting and were prone to injury. Thus we routinely obtained a high yield of approximately 8% 5-HT positive neurons from the entire rostral raphe, from rat embryos spanning a two-day range of development (E13-E15), an improvement over previous preparations. As with any primary culture system, the accuracy with which the appropriate region of the midbrain is harvested is critical. Thus, the level of precision with which these dissections were conducted could account in part for the increased yield of serotonergic neurons in comparison to other findings. It should be noted that treatment of these cultures with exogenous growth factors, such as TGF-β and BDNF, has been shown to significantly induce expression of serotonergic markers (Galter and Unsicker, 1999, 2000). These treatments were not applied to our culture system in order to maintain as physiological a baseline condition as possible since these cultures serve as a model for future regulatory studies of the serotonergic system.

The raphe cultures we have characterized had approximately 5% GFAP-positive astrocytes, with the remainder being predominantly neuronal cell types based on Tuj1 and NeuN staining. Other groups (Hery et al., 2000) indicate that their serum-free rostral raphe cultures contain <5% glial cells, while Lautenschlager et al. (2000) obtained 9% glial cells. Glial proliferation is suppressed under serum-free culture conditions using B27/Neurobasal medium (Brewer et al., 1993). In our study, further treatment of cultures with cytosine arabinoside to inhibit glial proliferation had toxic effects on cell survival, even at very low concentrations

(concentration range tested was between 2 μM and 10 μM) and was not pursued. However, the presence of glia appears to be beneficial to 5-HT neuronal survival through the secretion of neurotrophic factors such as S100b (Nishi et al., 2000). Thus we believe that the presence of 4.8% GFAP-positive cells in our cultures was sufficient to simulate a physiological environment and support neuronal survival and differentiation.

Previously, raphe cultures have not been extensively characterized for other neurotransmitter phenotypes. Our cultures displayed less than 1% TH positive monoaminergic cells, 8-fold less than the frequency of 5-HT cells. Since dopaminergic (TH positive) neurons are localized adjacent to 5-HT neurons in both the rostral and caudal embryonic rat rhombencephalon, while adrenergic neurons are entirely caudal (Dahlstrom and Fuxe, 1964; Hokfelt et al., 1984; Konig et al., 1988), the few TH positive cells detected in our cultures are probably dopaminergic. The predominant (over 50%) neuronal subtype present in our cultures was GABAergic. A small fraction of GAD-positive neurons are present in the adult rat DRN, suggesting that the neurons in our culture may arise from adjacent tissue (Abellan et al., 2000). Interestingly, 5-HT neurons express both GAD (Nanopoulos et al., 1982; Belin et al., 1983), GABA-R1 receptor subunit (Abellan et al., 2000) and vesicular glutamate transporter type 3 (vGLUT3) (Stornetta et al., 2005), suggesting that some of the 5-HT neurons in rostral raphe cultures may produce GABA. Consistent with this, amino acid neurotransmitters GABA, glycine, taurine and glutamate were reported in rostral raphe cultures from E15 rat embryos (12 DIV), and were found to regulate 5-HT metabolism (Becquet et al., 1993).

Primary cultures have the advantage of retaining many of the properties of differentiated neurons, in comparison to transformed cell lines that tend to de-differentiate with passage in culture. An important limitation of primary cultures of the rostral raphe region is the cellular heterogeneity due to the inherent complexity of this region. However, the presence of critical cell types also provides a more physiological environment. For example, the presence of glia results in greater neuronal survival, mimicking the physiological conditions found in the rostral raphe region of the brain. For this reason we have optimized the procedure to obtain the highest purity of 5-HT neurons, while preserving a physiological environment for their survival. The problem of heterogeneity is mitigated by examining cell-specific markers (e.g. serotonergic). Another important limitation of all primary cultures is that culture conditions (dispersed cells, media, target neurons, etc.) do not entirely replicate the developmental milieu of raphe neurons *in vivo*. For this reason we have measured several serotonergic markers to assess the differentiation of these embryonic neurons to the serotonergic phenotype.

3.5.2 Multiplex Q-PCR

One objective of establishing enriched raphe cultures was to obtain enough cells to perform quantitative measurement of RNA levels using Q-PCR. We examined three RNAs: the transcription factor Deaf-1, which was prevalent in these cultures and is widely expressed in the brain (Lemonde et al., 2003); the serotonin-specific marker TPH2; and the 5-HT1A receptor, a more weakly expressed protein that is predominantly expressed in 5-HT neurons (Hillion et al., 1994). Thus we predicted a

range of RNA abundances, from transcripts expressed in all cells (Deaf-1) to serotonin-specific RNA expression (TPH2, 5-HT1A), although 5-HT1A receptors have been detected in a small proportion of non-serotonergic raphe cells in adult DRN as well (Kirby et al., 2003; Beck et al., 2004; Marinelli et al., 2004). Although TPH2 is the predominant isoform expressed in the brainstem (Patel et al., 2004), there have been no specific studies done on this enzyme in raphe primary cultures. To quantify the RNA subtypes we performed a dual probe Q-PCR reaction to normalize the mRNA to GAPDH within each sample. Thus from each individual sample we could determine the relative abundance of the RNA transcript. Interestingly, the abundance of Deaf-1 and TPH2 were similar despite the more widespread cellular distribution of Deaf-1, suggesting that TPH2 RNA is 3.5-fold more abundant in 5-HT cells (assuming 8% proportion of 5-HT cells). On the other hand, 5-HT1A receptor RNA was 3-fold less abundant than TPH2, suggesting that in 5-HT neurons, 5-HT1A receptor and Deaf-1 RNA have similar expression (assuming 5-HT1A receptors are expressed in 5-HT neurons alone). These data are consistent with a greater synthesis of TPH2 enzyme compared to receptor (100 fmol/mg protein) or Deaf-1. However, we have assumed similar efficiencies of reverse transcription and PCR amplification to estimate the relative amounts of the target RNA (Figure 3.3C).

In summary we have established and characterized primary raphe cultures that provide several important advantages over previous cultures. Firstly, these cultures are enriched in 5-HT neurons, and have a low proportion of TH-positive cells. They also yield sufficient RNA to perform multiplex Q-PCR analysis on at least

three different transcripts with GAPDH as standard, over a 200-fold concentration range. Both ubiquitous and 5-HT-specific RNA's were quantified using this protocol.

3.6 REFERENCES

Abellan MT, Martin-Ruiz R, Artigas F. Local modulation of the 5-HT release in the dorsal striatum of the rat: an in vivo microdialysis study. Eur Neuropsychopharmacol, 2000; 10: 455-62.

Adell A, Celada P, Abellan MT, Artigas F. Origin and functional role of the extracellular serotonin in the midbrain raphe nuclei. Brain Res Brain Res Rev, 2002; 39: 154-80.

Aitken AR, Tork I. Early development of serotonin-containing neurons and pathways as seen in wholemount preparations of the fetal rat brain. J Comp Neurol, 1988; 274: 32-47.

Albert PR, Lemonde S. 5-HT1A receptors, gene repression, and depression: guilt by association. Neuroscientist, 2004; 10: 575-93.

Baker KG, Halliday GM, Tork I. Cytoarchitecture of the human dorsal raphe nucleus. J Comp Neurol, 1990; 301: 147-61.

Barnes NM, Sharp T. A review of central 5-HT receptors and their function. Neuropharmacology, 1999; 38: 1083-152.

Beck SG, Pan YZ, Akanwa AC, Kirby LG. Median and dorsal raphe neurons are not electrophysiologically identical. J Neurophysiol, 2004; 91: 994-1005.

Becquet D, Hery M, Francois-Bellan AM, Giraud P, Deprez P, Faudon M, Fache MP, Hery F. Glutamate, GABA, glycine and taurine modulate serotonin synthesis

and release in rostral and caudal rhombencephalic raphe cells in primary cultures. Neurochem Int, 1993; 23: 269-83.

Belin MF, Nanopoulos D, Didier M, Aguera M, Steinbusch H, Verhofstad A, Maitre M, Pujol JF. Immunohistochemical evidence for the presence of gamma-aminobutyric acid and serotonin in one nerve cell. A study on the raphe nuclei of the rat using antibodies to glutamate decarboxylase and serotonin. Brain Res, 1983; 275: 329-39.

Brewer GJ, Torricelli JR, Evege EK, Price PJ. Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. J Neurosci Res, 1993; 35: 567-76.

Czesak M, Lemonde S, Peterson EA, Rogaeva A, Albert PR. Cell-specific repressor or enhancer activities of Deaf-1 at a serotonin 1A receptor gene polymorphism. J Neurosci, 2006; 26: 1864-71.

Dahlstrom A, Fuxe K. Localization of monoamines in the lower brain stem. Experientia, 1964; 20: 398-9.

Galter D, Unsicker K. Regulation of the transmitter phenotype of rostral and caudal groups of cultured serotonergic raphe neurons. Neuroscience, 1999; 88: 549-59.

Galter D, Unsicker K. Brain-derived neurotrophic factor and trkB are essential for cAMP-mediated induction of the serotonergic neuronal phenotype. J Neurosci Res, 2000; 61: 295-301.

Hendricks TJ, Fyodorov DV, Wegman LJ, Lelutiu NB, Pehek EA, Yamamoto B, Silver J, Weeber EJ, Sweatt JD, Deneris ES. Pet-1 ETS gene plays a critical role in 5-HT neuron development and is required for normal anxiety-like and aggressive behavior. Neuron, 2003; 37: 233-47.

Hery M, Semont A, Fache MP, Faudon M, Hery F. The effects of serotonin on glucocorticoid receptor binding in rat raphe nuclei and hippocampal cells in culture. J Neurochem, 2000; 74: 406-13.

Hillion J, Catelon J, Raid M, Hamon M, De Vitry F. Neuronal localization of 5-HT1A receptor mRNA and protein in rat embryonic brain stem cultures. Brain Res Dev Brain Res, 1994; 79: 195-202.

Hokfelt T, Everitt BJ, Theodorsson-Norheim E, Goldstein M. Occurrence of neurotensinlike immunoreactivity in subpopulations of hypothalamic, mesencephalic, and medullary catecholamine neurons. J Comp Neurol, 1984; 222: 543-59.

Jacobs BL, Azmitia EC. Structure and function of the brain serotonin system. Physiol Rev, 1992; 72: 165-229.

Jacobsen KX, Staines WA. Vibration enhancement of slide-mounted immunofluorescence staining. J Neurosci Methods, 2004; 137: 71-7.

Kirby LG, Pernar L, Valentino RJ, Beck SG. Distinguishing characteristics of serotonin and non-serotonin-containing cells in the dorsal raphe nucleus: electrophysiological and immunohistochemical studies. Neuroscience, 2003; 116: 669-83.

Kohler C, Steinbusch H. Identification of serotonin and non-serotonin-containing neurons of the mid-brain raphe projecting to the entorhinal area and the hippocampal formation. A combined immunohistochemical and fluorescent retrograde tracing study in the rat brain. Neuroscience, 1982; 7: 951-75.

Konig N, Wilkie MB, Lauder JM. Tyrosine hydroxylase and serotonin containing cells in embryonic rat rhombencephalon: a whole-mount immunocytochemical study. J Neurosci Res, 1988; 20: 212-23.

Lautenschlager M, Holtje M, von Jagow B, Veh RW, Harms C, Bergk A, Dirnagl U, Ahnert-Hilger G, Hortnagl H. Serotonin uptake and release mechanisms in developing cultures of rat embryonic raphe neurons: age- and region-specific differences. Neuroscience, 2000; 99: 519-27.

Lemonde S, Turecki G, Bakish D, Du L, Hrdina PD, Bown CD, Sequeira A, Kushwaha N, Morris SJ, Basak A, Ou XM, Albert PR. Impaired repression at a 5-hydroxytryptamine 1A receptor gene polymorphism associated with major depression and suicide. J Neurosci, 2003; 23: 8788-99.

Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods, 2001; 25: 402-8.

Marinelli S, Schnell SA, Hack SP, Christie MJ, Wessendorf MW, Vaughan CW. Serotonergic and nonserotonergic dorsal raphe neurons are pharmacologically and electrophysiologically heterogeneous. J Neurophysiol, 2004; 92: 3532-7.

Nanopoulos D, Belin MF, Maitre M, Vincendon G, Pujol JF. Immunocytochemical evidence for the existence of GABAergic neurons in the nucleus raphe dorsalis. Possible existence of neurons containing serotonin and GABA. Brain Res, 1982; 232: 375-89.

Nishi M, Kawata M, Azmitia EC. Trophic interactions between brain-derived neurotrophic factor and s100beta on cultured serotonergic neurons. Brain Res, 2000; 868: 113-8.

O'Rahilly R, Muller F. Neurulation in the normal human embryo. Ciba Found Symp, 1994; 181: 70-82; discussion 82-9.

Patel PD, Pontrello C, Burke S. Robust and tissue-specific expression of TPH2 versus TPH1 in rat raphe and pineal gland. Biol Psychiatry, 2004; 55: 428-33.

Raymond JR, Mukhin YV, Gettys TW, Garnovskaya MN. The recombinant 5-HT1A receptor: G protein coupling and signalling pathways. Br J Pharmacol, 1999; 127: 1751-64.

Riad M, Garcia S, Watkins KC, Jodoin N, Doucet E, Langlois X, el Mestikawy S, Hamon M, Descarries L. Somatodendritic localization of 5-HT1A and preterminal axonal localization of 5-HT1B serotonin receptors in adult rat brain. J Comp Neurol, 2000; 417: 181-94.

Rumajogee P, Madeira A, Verge D, Hamon M, Miquel MC. Up-regulation of the neuronal serotoninergic phenotype in vitro: BDNF and cAMP share Trk B-dependent mechanisms. J Neurochem, 2002; 83: 1525-8.

Sotelo C, Cholley B, El Mestikawy S, Gozlan H, Hamon M. Direct Immunohistochemical Evidence of the Existence of 5-HT1A Autoreceptors on Serotoninergic Neurons in the Midbrain Raphe Nuclei. Eur J Neurosci, 1990; 2: 1144-54.

Steinbusch HW. Distribution of serotonin-immunoreactivity in the central nervous system of the rat-cell bodies and terminals. Neuroscience, 1981; 6: 557-618.

Stornetta RL, Rosin DL, Simmons JR, McQuiston TJ, Vujovic N, Weston MC, Guyenet PG. Coexpression of vesicular glutamate transporter-3 and gamma-aminobutyric acidergic markers in rat rostral medullary raphe and intermediolateral cell column. J Comp Neurol, 2005; 492: 477-94.

Tork I. Anatomy of the serotonergic system. Ann N Y Acad Sci, 1990; 600: 9-34; discussion 34-5.

Vertes RP. A PHA-L analysis of ascending projections of the dorsal raphe nucleus in the rat. J Comp Neurol, 1991; 313: 643-68.

Walther DJ, Peter JU, Bashammakh S, Hortnagl H, Voits M, Fink H, Bader M. Synthesis of serotonin by a second tryptophan hydroxylase isoform. Science, 2003; 299: 76.

CHAPTER 4

The following manuscript has been submitted:

Czesak M., Le François B., Deria M., Daigle M., Visvader J., and Albert P.R. Deaf-1 knockout mice: a model for 5-HT1A receptor dys-regulation by a functional promoter polymorphism

Journal of Neuroscience, December 2009

Revisions are currently being addressed

DEAF-1 KNOCKOUT MICE: A MODEL FOR 5-HT1A RECEPTOR DYS-REGULATION BY A FUNCTIONAL PROMOTER POLYMORPHISM

Margaret Czesak[1], Brice Le François[1], Mariam Deria[1], Mireille Daigle[1], Jane E. Visvader[2] and Paul R. Albert[1]

[1]Ottawa Health Research Institute (Neuroscience), University of Ottawa, Department of Cellular and Molecular Medicine, Ottawa, Canada, K1H 8M5

[2]The Walter and Eliza Hall Institute of Medical Research, Melbourne, Victoria 3050, Australia

Abbreviated Title: 5-HT1A receptor dys-regulation in Deaf-1 -/- mice

Keywords: Serotonin; Transcription; Promoter; Depression; Anxiety; 5-HT1A receptor

Acknowledgements: We thank Dr. Kelly McClellan for assistance with *in situ* hybridization and Matthew Mount for assistance with perfusions. These studies were supported by a grant from the Canadian Institutes of Health Research (CIHR, MOP-36437) to P.R.A.; M.C. was supported by a Canada Graduate Scholarship Doctoral Award from CIHR; M.D. was supported by Summer Studentships from National Science and Engineering Council of Canada. The Heart and Stroke Foundation Centre for Stroke Recovery supported some equipment used for these studies.

4.1 Abstract

Altered regulation of the serotonin-1A (5-HT1A) receptor gene is implicated in major depression and mood disorders. The functional 5-HT1A C(-1019)G promoter polymorphism (rs6295), which prevents the binding of Deaf-1/NUDR leading to dys-regulation of the receptor, has been associated with major depression. In cell models, Deaf-1 represses 5-HT1A autoreceptor expression in serotonergic raphe cells, but enhances post-synaptic 5-HT1A receptor expression in non-serotonergic neurons. Analogous to the human promoter, an imperfect palindrome Deaf-1 binding site was identified at -947 bp of the mouse 5-HT1A promoter. To address regulation by Deaf-1 *in vivo*, Deaf-1 knockout mice compared to wild-type siblings were examined for changes in 5-HT1A RNA and protein by quantitative RT-PCR, *in situ* hybridization and immunofluorescence. Deaf-1 knockouts were bred to a C57BL/6 background and loss of Deaf-1 coding RNA and protein were verified. In the dorsal raphe, Deaf-1 knockout mice displayed increased 5-HT1A mRNA and protein and reduced 5-HT levels, while other serotonergic markers such as 5-HT-positive cells and TPH2 RNA were unchanged. By contrast, 5-HT1A mRNA and 5-HT1A-positive cells were reduced in frontal cortex of Deaf-1-null mice, with no significant change in hippocampal 5-HT1A RNA or protein. These findings demonstrate region-specific regulation of brain 5-HT1A gene expression by Deaf-1 *in vivo*. The Deaf-1 deficient mice provide a model to study how the 5-HT1A G(-1019) allele may impair serotonergic neurotransmission in humans by de-repressing 5-HT1A autoreceptors, while reducing serotonin response through region-specific reduction in post-synaptic 5-HT1A receptor expression.

4.2 Introduction

Reduced activity of the brain serotonin system has been implicated in major depression and suicide (Charney et al., 1990, Mann, 1999, Jans et al., 2007) and antidepressant treatments including serotonin reuptake inhibitors (SSRI's) increase serotonergic neurotransmission, either directly or indirectly (Pineyro and Blier, 1999, Blier, 2003). The serotonin-1A (5-HT1A) somatodendritic autoreceptor inhibits the firing of raphe serotonin neurons to negatively regulate the serotonin system (Pineyro and Blier, 1999). Post-synaptic 5-HT1A receptors are strongly expressed in limbic and cortical brain regions (Chalmers and Watson, 1991; Pompeiano et al., 1992) and mediate serotonin action to reduce fear and anxiety. 5-HT1A-null mice display increased depression and anxiety behaviours (Heisler et al , 1998; Parks et al., 1998; Ramboz et al., 1998) and lack response to SSRI treatment (Santarelli et al., 2003), while transgenic over-expression of the 5-HT1A receptor decreases anxiety (Kusserow et al , 2004). Rescue of 5-HT1A receptor expression in early postnatal forebrain rescues the anxiety phenotype suggesting its role in development of the anxiety phenotype (Gross et al., 2002). Hence, the 5-HT1A receptor regulates the serotonin system and is implicated in mood disorders.

The 5-HT1A gene is regulated by a proximal promoter and an upstream repressor region that determines basal expression in neuronal cells (Ou et al., 2000, Lemonde et al., 2004). In the human 5-HT1A repressor region, we previously identified a functional C(-1019)G polymorphism associated with major depression and suicide, which has been replicated in independent studies (Le Francois et al., 2008, Kishi et al., 2009). The G-allele prevents the binding of the transcription factor

Deformed epidermal autoregulatory factor-1 (Deaf-1) (Lemonde et al., 2003). In serotonergic cells, Deaf-1 represses 5-HT1A autoreceptor expression, while in non-serotonergic neuronal cells, Deaf-1 enhances 5-HT1A promoter activity (Czesak et al., 2006). However, the opposite action of Deaf-1 on 5-HT1A expression *in vivo* has not been demonstrated. The G-allele should increase 5-HT1A autoreceptor levels to reduce 5-HT neuron firing, and decrease post-synaptic 5-HT1A receptors synergistically reducing serotonin neurotransmission. In depressed subjects, the homozygous 5-HT1A G/G(-1019) genotype was associated with increased 5-HT1A autoreceptor binding potential (Parsey et al., 2006b; Sullivan et al., 2009), consistent with increased 5-HT1A autoreceptors in post-mortem studies of depressed suicides (Stockmeier et al., 1998; Boldrini et al., 2008). Thus, the 5-HT1A G(-1019) allele may alter 5-HT1A receptor expression *in vivo* by blocking Deaf-1 function.

The role of Deaf-1 in regulating 5-HT1A receptor expression *in vivo* was addressed using the Deaf-1 -/- mouse (Hahm et al., 2004). In Deaf-1 null mice compared to littermate wild-type controls, 5-HT1A mRNA and protein levels were increased in the dorsal raphe region, while 5-HT levels were reduced and serotonin cell number and TPH2 mRNA were unchanged. By contrast, post-synaptic 5-HT1A receptors were down-regulated in the frontal cortex of Deaf-1 knockout mice. Thus, consistent with results in cell lines, we find a region-specific dys-regulation of 5-HT1A receptor expression in Deaf-1 -/- mice. The Deaf-1 knockout mice provide a new model for 5-HT1A receptor gene regulation that mimics the homozygous G(-1019) genotype in humans.

4.3 Materials and Methods

4.3.1 Animals

All animal studies were conducted in accordance to The University of Ottawa Animal Care Committee guidelines using protocol NSI-57. Animals were maintained on a 12 h light/dark cycle with lights on at 6:00 A.M., at 21°C with food and water *ad libitum*. Deaf-1 knockout mice were received from Dr. Jane Visvader (Hahm et al., 2004) and re-derived by embryo transfer (Taconic, Hudson, NY) on a mixed C57BL/6-BALB/C-129 background. Pilot experiments revealed no significant differences in 5-HT1A mRNA levels between mixed background Deaf-1 null (-/-) and wild-type (+/+) mice, but differences were observed compared to pure C57BL/6 mice (Supplementary Figure 4.1). Hence the mice were backcrossed for six generations onto the C57BL/6 background. Although litter size was decreased by 50% compared to wild-type C57BL/6 mice the allele distribution remained normal. All experiments were performed on adult (8-10 weeks) knockout mice with age-matched littermate wild-type controls from the C56BL/6J background, unless otherwise specified. Absence of Deaf-1 mRNA expression was verified using sequence-specific primers designed within the deleted mRNA sequence (Supplementary Figure 4.2A). Lack of functional Deaf-1 protein was verified by two-dimensional gel electrophoresis and Western blot analysis (Suppl. Figure 4.2B).

4.3.2 Genotyping

Tail DNA was isolated using DNA lysis buffer, pH 8.0 (5 mM EDTA, 0.2% SDS, 200 mM NaCl, 10 μg/ml proteinase K). Genotyping was done by PCR using the following

primers: forward primer, GTGCTCTCATCCTCATAGCCTC; reverse primer-1, 3'-GAAACAAGAAAACACATCGGGAGAATCA; reverse primer-2, AAGAATAGAACCACCACCCAGCT, with 267-bp wild-type and 421-bp knockout products. Each mouse used in this study was genotyped at weaning and verified at sacrifice.

4.3.3 Immunofluorescence

Mice were anesthetized by lethal intraperitoneal injection (0.01 ml/g body weight) of sodium pentobarbital (Somnitol, MTC Pharmaceuticals, Cambridge, Ontario) and perfused by cardiac infusion of 20 ml saline, then 20 ml 4% paraformaldehyde (PFA). Whole brains were isolated, post-fixed overnight in 4% PFA, washed with 10% sucrose changed twice daily for five consecutive days, and frozen. Coronal brain slices (12-μm) were prepared using the following coordinates (Paxinos and Franklin, 2001): rostral raphe (Bregma -4.2 to -4.96 mm), hippocampus (Bregma 1.46 to -2.30 mm), and prefrontal cortex (Bregma 2.96 to -2.80 mm). Sections were blocked for 1 h in blocking solution (Stockholm's PBS with 1% BSA, 10% goat serum, 0.1% Triton X-100), and incubated overnight with the following antibodies: 1:50 rabbit serum anti-5-HT1A (custom made primary antibody designed to the i2 loop of the 5-HT1A receptor sequence, Cedarlane, Hornby, Ontario) and 1:100 rat anti-5-HT antibody (Chemicon International, Temecula, CA). As control, preimmune rabbit serum was used at a dilution of 1:5000 (Cedarlane). Sections were washed 3x in PBS followed by 1-hr incubation at 22°C with secondary antibodies in blocking solution (1:1000) Alexa Fluor 488 donkey anti-rabbit IgG and Alexa Fluor 594 goat

anti-rat IgG (Molecular Probes, Eugene, OR). Images were acquired on an Axiovert S100 Zeiss microscope using Northern Eclipse imaging software (EMPIX, Toronto, ON, Canada). Blinded counts of positive-labeled cells were performed in triplicate using a constant area and minimum-threshold of 15% brightness (compared to background) in Adobe Photoshop CS3. Intensity of staining was quantified based on percent brightness of each cell and normalized to average of background brightness, for each section analyzed. A constant pixel area was selected in the brightest region of the cell body. Each staining was replicated three times per brain region, with at least three animals used per group.

4.3.4 In situ Hybridization

Plasmid containing a 417-bp sequence of the mouse 5-HT1A receptor cDNA for sense and antisense 5-HT1A riboprobes was a kind gift from Dr. Alexandre Bonnin (Bonnin et al., 2006). The plasmid was linearized and transcribed to incorporate digoxygenin-UTP (Roche Diagnostics, Laval, Quebec) to generate sense or antisense riboprobes using SP6 or T7 polymerases, respectively. *In situ* hybridization was done as described (Ferguson et al., 2005). Tissue sections were incubated overnight with riboprobes (1:1000) in hybridization buffer (1x SSC, deionized formamide, 10% dextran sulfate, 1 mg/ml rRNA, 1x Denhardt's solution). Slides were washed 3x for 30 min each, followed by two washes, 30 min each with 1x MABT (0.5M Maleic acid, 0.75M NaCl, 0.5% Tween). Slides were then blocked for 1 h at 22°C in blocking reagent (Roche Diagnostics) and followed by overnight incubation with 1:1000 anti-DIG antibody (Roche Diagnostics). Slides were washed 5x for 20 min with MABT,

equilibrated with pre-staining buffer (100 mM NaCl, 50 mM $MgCl_2$, 100 mM Tris pH 9.5, 0.1% Tween-20) 3x for 10 min at 22°C and developed using staining solution ((100 mM NaCl, 50 mM $MgCl_2$, 100 mM Tris pH 9.5, 0.1% Tween-20, 10% polyvinyl alcohol) containing 3.5µl/ml of BCIP (5-bromo-4-chloro-3-indolyl-phosphate, Roche Diagnostics) and 4.5 µl/ml of NBT (4-nitro blue tetrazolium chloride, Roche Diagnostics) for 2-24 h at 22°C and terminated by rinsing in PBS. Sections were examined using a Zeiss Axiophot II light microscope at 10x magnification. Images were acquired using Northern Eclipse software and a Axiovert S100 Zeiss microscope (EMPIX).

4.3.5 RNA extraction/reverse transcription

Adult mice were euthanized by CO_2 asphyxiation followed by decapitation, removal and dissection of brains. For preparation of total RNA, brain samples were triturated and extracted in 600 µl TRIzol (Invitrogen, Burlington, Ontario, Canada), centrifuged in chloroform, and the supernatant precipitated and resuspended in 30 µL diethylpyrocarbonate-treated water to 0.5–2 µg/µL. Samples were treated with 1 µL of DNase/5 µg of total RNA for 30 min at 37°C using the Turbo DNA-free kit (Ambion, Foster City, CA), terminated in DNase Inactivation buffer, and quantified at OD_{260}. Reverse transcription was carried out using 0.05 µg/µl of DNase-treated RNA, with 1:10 random decamer oligonucleotides (Ambion) and 0.4 mM dNTP mix. Samples were incubated for 3 min at 70°C and annealed on ice. First-strand buffer (Ambion) was then added along with 25U of M-MLV reverse transcriptase (Ambion) and 10U of RNase inhibitor (Promega, Madison, WI, USA) and reactions were

incubated for 60 min at 42°C, and inactivated for 10 min at 95°C. Parallel reactions lacking M-MLV enzyme served as no reverse transcription controls. Newly synthesized cDNA was stored at -20°C.

4.3.6 Q RT-PCR

Quantitative Real-Time RT-PCR was performed as described (Czesak et al., 2007) using TaqMan Gene Expression Assay Kits (Applied Biosystems, Foster City, CA, USA). Product numbers are as follows: TPH2, Mm00557717_m1; Deaf-1, Mm00516805_m1; 5-HT1AR, Mm00434106_s1; and GAPDH, 4352339E. TPH2, Deaf-1 and 5-HT1A probes are FAM-labeled probes, while GAPDH is a VIC-labeled probe. GAPDH amplification was used as internal control and was included in every multiplex reaction. Each reaction contained 5 μL of TaqMan Universal PCR Mastermix (Applied Biosystems), 0.5 μL each of FAM- and VIC-labeled probes, 3 μL of nuclease-free water and 1 μL of sample. Standard curves ($r^2 \geq 0.996$) from serial dilution of the most concentrated cDNA sample spanned the range of test sample duplicates and at least one standard was included in duplicate in each experiment for quantification. Control samples without template DNA (no template controls) or reverse-transcriptase (no RT) controls were run for each gene analyzed. The cycling program used was: 95°C for 10 min; 40 cycles of 95°C for 15 s and 60°C for 60 s on the Rotor Gene 3000 cycler (Corbett Research, Sydney, Australia). Data were analyzed using Rotor Gene v6.0 software using the ΔΔCt method (Livak and Schmittgen, 2001). Briefly, relative amounts of each target gene to GAPDH were obtained as $10^{(CtT-CtG)}$ where CtT is the threshold cycle for the target gene and CtG is the threshold cycle for GAPDH in the same tube. In order to verify the deleted exon

sequence in Deaf-1 knockout mice (Suppl. Figure 2A), SYBR green technology was applied. The following probes were designed to the deleted sequence of the Deaf-1 gene: forward 5'-CTGTCGGAACATCAGTGGCA; reverse 5'-CACAGGCAACTCGCTGTCATAC. The amplification cycles were 92°C for 10min, 92°C for 30s, 58°C for 15s, 72°C for 20s (40 cycles), terminated at 72°C for 10 min. Ct values were quantified using SYBR green (Molecular Probes) incorporation following amplification with QtaqTM DNA polymerase mix (Clontech, Mountain View, CA). Averages were normalized to GAPDH.

4.3.7 Electrophoretic Mobility Shift Assay (EMSA)

EMSA protocol was conducted as previously described (Czesak et al., 2006). Briefly, sense and antisense oligonucleotides of the 26-bp Deaf-1 element with CC/GG 3'-overhangs were hybridized and labeled with [^{32}P]-dCTP using Klenow fragment DNA polymerase (Ou et al., 2000). Purified Deaf-1 protein (2.5 µg/reaction) was pre-incubated with or without competitor DNA in a 20 µl 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), 1 ug poly dI-dC, at 22°C for 20 min. and resolved on a non-denaturing 5% acrylamide/Tris-glycine gel at 4°C. Unlabeled doubled-stranded polyoma virus enhancer activator 3 (PEA3) (GGGATCCAGGAAGTGA) and E2F (ATTTAAGTTTCGCGCCTTTTC) sequences were used as nonspecific competitor.

4.3.8 Two dimensional SDS-PAGE

Prefrontal cortex was dissected, rinsed in ice–cold 10 mM BES pH 7.4, 0.3 M sucrose,

homogenized in isoelectric focusing (IEF) buffer (7M Urea, 2M Thiourea, 4% CHAPS, 1% DTT, 0.001% Bromophenol blue) and incubated for 90 min at 22°C. Samples were centrifuged at 10,000g for 5 min, supernatant precipitated in 90% acetone, centrifuged (2000g x 5 min.) and pellet resuspended in IEF buffer supplemented with biolytes. IPG (17-cm) strips (BioRad Laboratories Ltd. Mississauga, Ontario)(pH 3-10) were rehydrated overnight with 300 uL of IEF buffer containing 1 mg of total protein. Isoelectric focusing was done using the following conditions: desalting for 20 min at 500V; 2.5 hr ramp to 10,000 V; 40000 VH focusing at 10,000V. IPG strips were then treated for 15 min with a reducing solution (375 mM Tris-HCl pH 8.8, 20% glycerol, 2% SDS, 65 mM DTT) followed by 15-min incubation with an alkylation solution (375 mM Tris-HCl pH 8.8, 2% SDS, 20% glycerol, 215 mM iodoacetamide) and placed on top of a 12% SDS PAGE gel using a 0.5% agarose overlay. Gels were run for 5 hrs at 30 mA and transferred to PVDF membranes and exposed overnight at 4ºC with rabbit anti-Deaf-1 antibody (1:1000; custom raised against full-length Deaf-1 peptide, Cedarlane).

4.4 Results

4.4.1 Deaf-1 binding site of the mouse 5-HT1A promoter

Previous studies have identified the allele-specific recognition by Deaf-1 at the C-allele of a 26-bp palindrome sequence at -1019 bp of the human 5-HT1A promoter sequence (Lemonde et al., 2003; Czesak et al., 2006), but the corresponding site of the mouse 5-HT1A promoter sequence has not been identified. Based the location and palindrome structure of the human 5-HT1A Deaf-1 site, two imperfect

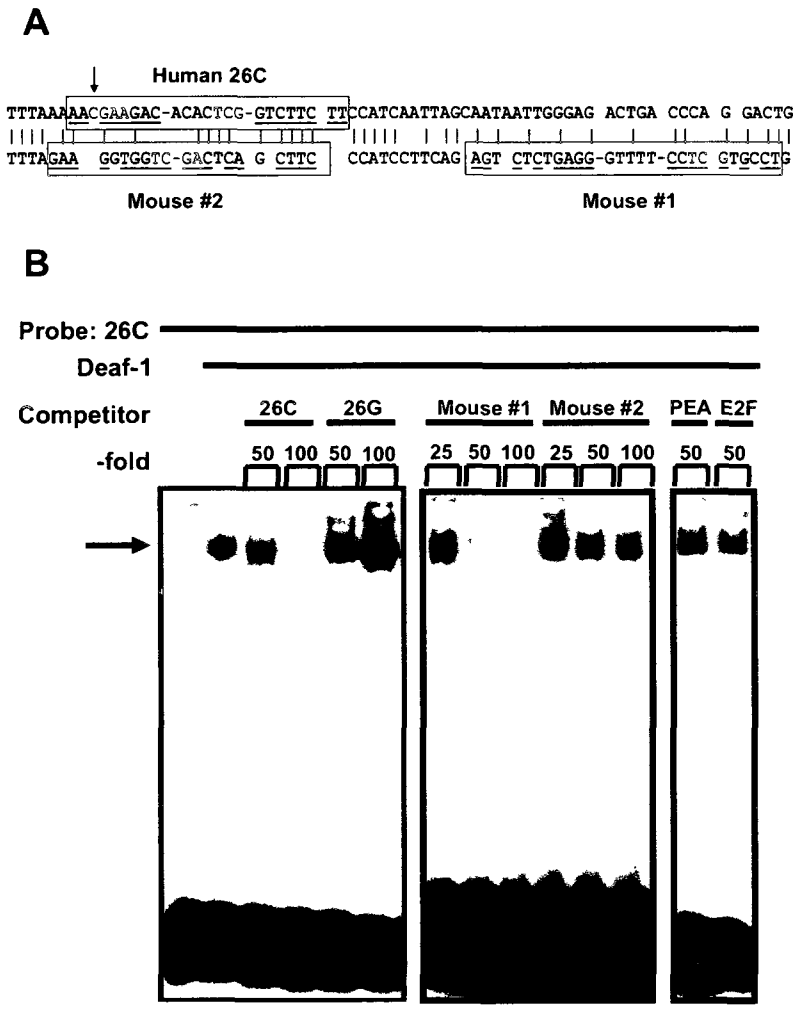


Figure.4.1: Deaf-1 binding site of the mouse 5-HT1A promoter.

A. 5-HT1A Promoter Alignment. Alignment (5'-3') of human (above, -1027/-959 bp)) and mouse (below, -971/-946 bp) 5-HT1A promoter regions showing human Deaf-1 site (26C, -1021/-998 bp), mouse palindrome #1 ((Mouse #1, -971/-947 bp) and mouse palindrome #2 (Mouse #2, -1003/-983 bp) in boxes. Consensus minimal Deaf-1 sites (TCG) are highlighted. Palindrome sequences are underlined, palindrome stems are separated by dashes, and palindrome gaps indicated by a space; an arrow indicates the location of C(-1019)G human 5-HT1A polymorphism. B. Electrophoretic Mobility Shift Assay. EMSA was done using purified recombinant Deaf-1 protein (5 μg/lane) incubated with labeled human 26-bp palindrome, in the absence or presence of the indicated fold excess of unlabelled competitor double-stranded primers. Deaf-1 protein binding is competed out by increasing concentrations of Mouse #1 but not Mouse #2 palindrome element. The C- or G-allele (26C or 26G) of human 5-HT1A 26-bp palindrome elements were used as positive or negative controls, respectively, and PEA3 and E2F elements were included as non-specific competitors.

palindrome mouse promoter sequences (Mouse#1 and #2) were identified containing the Deaf-1 TCG minimal consensus sequence (Huggenvik et al., 1998) (Figure 4.1A). Electrophoretic mobility shift assay (EMSA) was done using recombinant purified Deaf-1 protein bound to labelled 26-bp human 5-HT1A C-allele Deaf-1 site (26C) in the absence or presence of excess unlabelled double-stranded primers (Figure 4.1B). Deaf-1 binding was competed with equally potency by the human 26C and Mouse#1 primers, but not Mouse #2 primers. As a control, the human 26-bp G-allele palindrome (26G) that does not bind Deaf-1 or unrelated DNA elements for PEA3 or E2F, failed to compete Deaf-1 binding. Thus, the mouse 5-HT1A promoter contains a Deaf-1 binding element 947 bp upstream of translation start site.

4.4.2 Characterization of Deaf-1 deficient mice

The importance of Deaf-1 in regulation of the 5-HT1A receptor *in vivo* was addressed using Deaf-1 knockout mice (Hahm et al., 2004), which were backcrossed six generations from a mixed background onto the C57BL/6 background (see Methods). After genotyping, the presence of the knockout mutation in genomic DNA and mRNA was verified by DNA sequence analysis. In Deaf-1 -/- mice, the loss Deaf-1 coding RNA was shown by RT-PCR analysis (Supplementary Figure 4.2A) and the loss of Deaf-1 protein was resolved by 2D-gel electrophoresis/Western blot analysis (Supplementary Figure 4.2B). Morphological analysis of the brains of Deaf-1 -/- mice using Cresyl violet staining showed no gross alteration (Supplementary Figure 4.3).

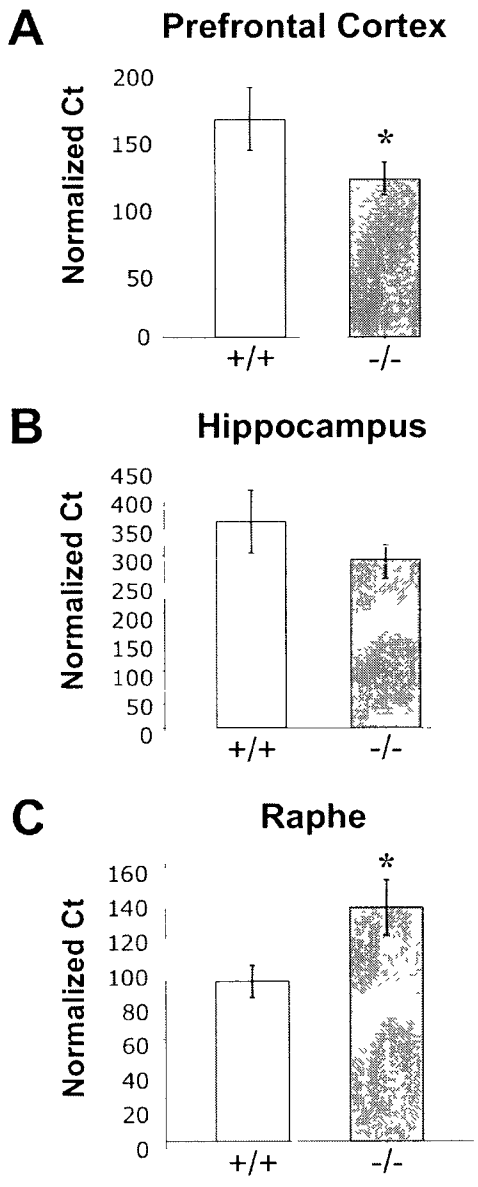


Figure 4.2: 5-HT1A mRNA expression alterations in brain areas of Deaf-1 knockout mice.

RNA isolated from prefrontal cortex (A), hippocampus (B), and rostral raphe region (C) was analyzed by Multiplex RT-PCR for 5-HT1A mRNA and GAPDH RNA and quantified as 5-HT1A RNA normalized to GAPDH RNA using the $10^{(CtT-CtG)}$ formula. Data are presented as mean ± S.E.M. n=4 mice/group, *$p<0.05$ by two-tailed unpaired t-test. In Deaf-1 knockout (-/-) compared to wild-type mice (+/+), 5-HT1A RNA was reduced by 38% in prefrontal cortex, 23% in hippocampus, and increased by 46% in raphe region.

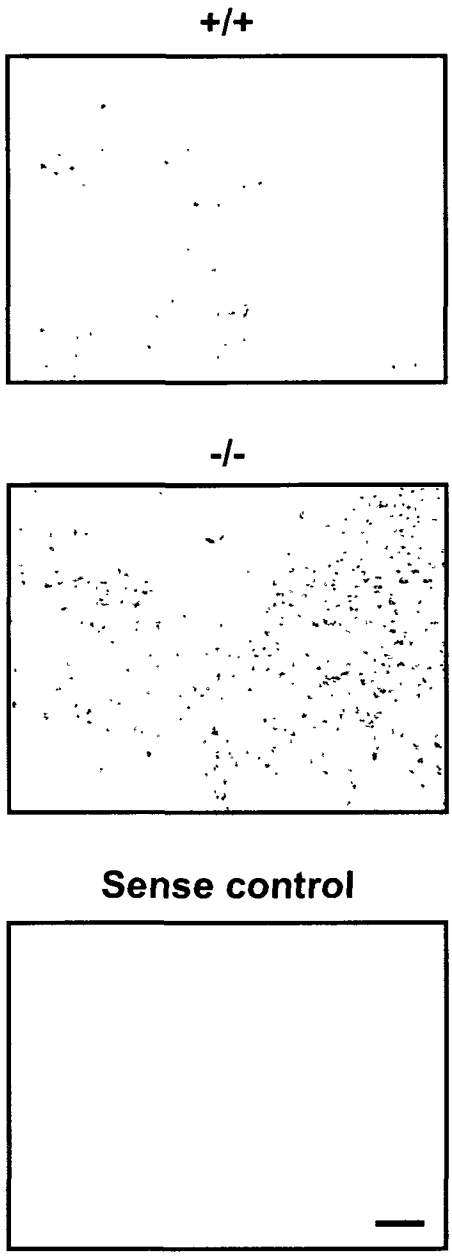


Figure 4.3: Increased 5-HT1A mRNA in Dorsal Raphe Nuclei of Deaf-1 -/- mice.

In situ hybridization showing representative sections of the dorsal raphe nuclei hybridized with 5-HT1A antisense RNA probe in wild-type (+/+) vs. Deaf-1 knockout (-/-) mice: increased 5-HT1A-positive cell counts were observed in Deaf-1 -/- mice. Sense probe was used as negative control. Representative images are shown at 20x magnification, size bar = 100 μm.

4.4.3 Altered 5-HT1A mRNA levels in Deaf-1 deficient mice

To address whether loss of Deaf-1 affects 5-HT1A receptor expression, brain tissues from Deaf-1-null (-/-) and wild-type (+/+) littermates were examined for levels of 5-HT1A receptor RNA using quantitative RT-PCR. Interestingly, we observed a significant 38% decrease in 5-HT1A mRNA levels in the frontal cortex when compared to wild-type littermate controls (Figure 4.2A) and a trend towards decreased 5-HT1A mRNA levels (23%) in the hippocampus (Figure 4.2B). Oppositely, there was a significant 46% increase in 5-HT1A mRNA in midbrain tissue from the rostral raphe region (Figure 4.2C). Although midbrain tissue dissection used was enriched for serotonin neurons (Czesak et al., 2007), *in situ* hybridization was performed to determine the precise localization of 5-HT1A RNA in the dorsal raphe nuclei (DRN) (Figure 4.3). Compared to wild-type littermates, 5-HT1A RNA was markedly up-regulated in the DRN of Deaf-1 null mice, consistent with the up-regulation of 5-HT1A autoreceptor expression in serotonin neurons of the DRN. By contrast, no change in 5-HT1A mRNA was observed in the median raphe nuclei of these mice (data not shown), implying that up-regulation of 5-HT1A receptor mRNA in Deaf-1 -/- mice is specific to the DRN.

4.4.4 5-HT1A protein in Deaf-1 knockout mice

To determine whether altered 5-HT1A mRNA corresponded with changes in 5-HT1A protein, immunofluorescence staining for 5-HT1A receptors was done in the dorsal raphe nuclei, prefrontal cortex, and dentate gyrus (DG) region of the hippocampus (Figure 4.4A). In Deaf-1 null mice, a significant increase in 5-HT1A

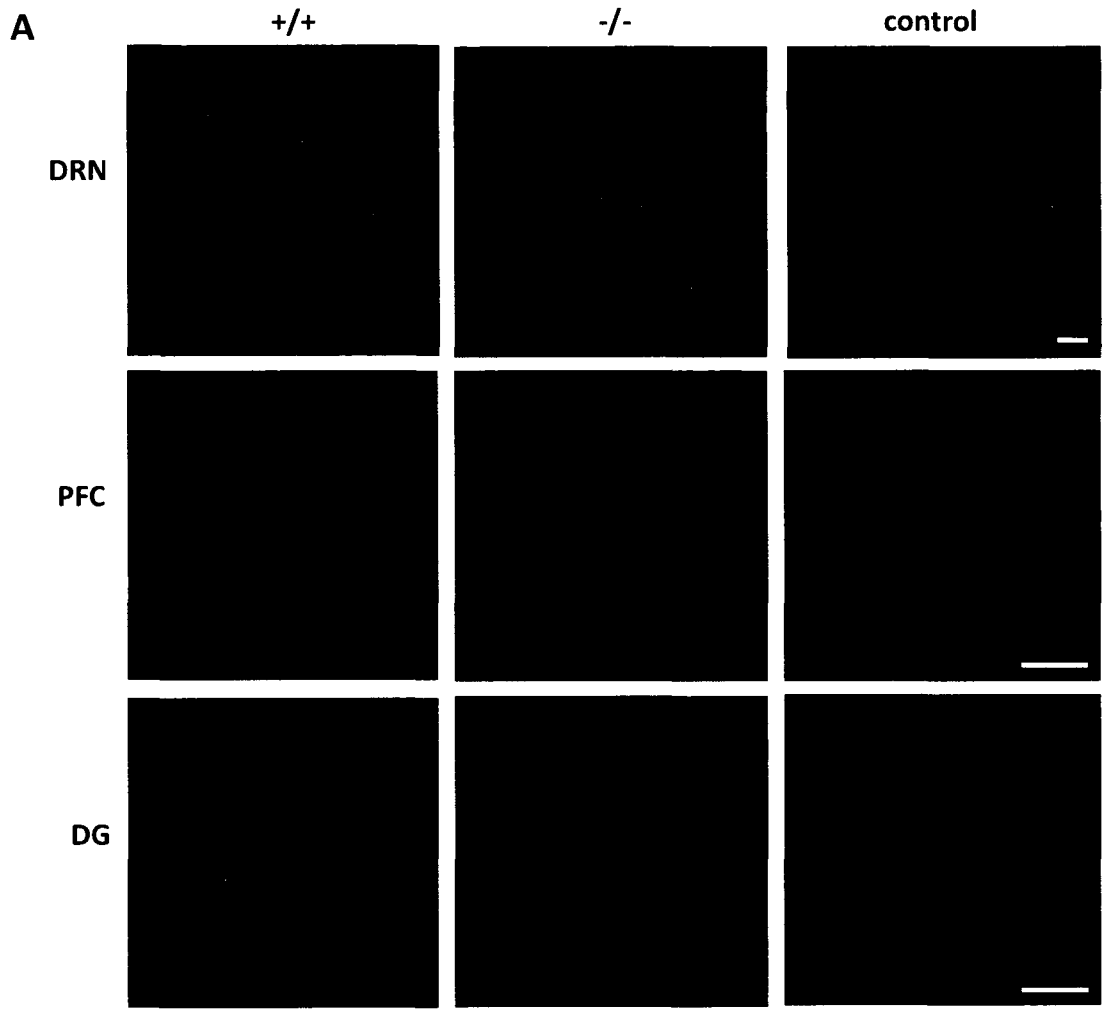


Figure 4.4-A: 5-HT1A protein expression in Deaf-1 knockout mice.

Immunofluorescence of 5-HT1A receptor immunoreactive labeling in wild-type (+/+) and Deaf-1 knockout (-/-) mice in Dorsal Raphe Nuclei (DRN), Prefrontal Cortex (PFC) and hippocampal Dentate Gyrus (DG), secondary antibody was used as a negative control Representative images are shown at 10x (DRN) and 20x magnification, size bar = 100 μm

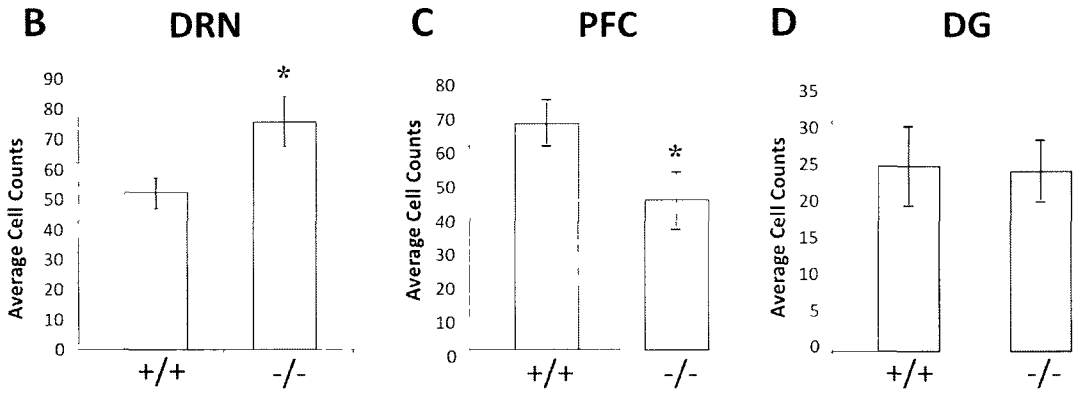


Figure 4.4-B,C,D: 5-HT1A protein expression in Deaf-1 knockout mice.

Quantitative analysis of 5-HT1A receptor-positive cells in B) dorsal raphe nuclei, DRN; C) prefrontal cortex, PFC; and D) hippocampus of wildtype (+/+) and Deaf-1 knockout (-/-) mice. Counts are presented as mean % of total cells ± S.E.M., n ≥ 3 mice, *$p<0.05$ by two-tailed unpaired t-test.

receptor-positive cells (by 36%) and 5-HT1A protein levels (by 16%) was observed in the DRN (Figure 4.4B), which correlated with the observed up-regulation of 5-HT1A mRNA (Figure 4.2C, Figure 4.3). The number of cells labelled with anti-GFAP antibody in the dorsal raphe region was comparable between knockout and wild-type mice (data not shown), implying no alteration in activated astroglia. Oppositely, in the prefrontal cortex a significant 34% decrease in 5-HT1A receptor labelling was observed (Figure 4.4B). However, no significant change in 5-HT1A-labelled cells was observed in the hippocampal dentate gyrus (Figure 4.4B). Thus, Deaf-1 deficiency led to brain region-specific changes in 5-HT1A receptor levels that mirrored changes in 5-HT1A mRNA.

4.4.5 Expression of 5-HT and TPH2 RNA in Deaf-1 knockout mice

As marker for serotonin neurons in the dorsal raphe nuclei, immunofluorescence was done using anti-5-HT antibody (Figure 4.5A). Although the intensity of the staining was reduced by 30% in serotonergic neurons of Deaf-1-null mice, there was no change in the number of 5-HT-positive cells (Figure 4.5B). In addition, 5-HT1A and 5HT were co-localized in the majority of DRN neurons, confirming the serotonergic identity of sections analyzed (Figure 4.6). As another marker of serotonin function, the level of tryptophan hydroxylase-2 (TPH2) mRNA in raphe tissue was quantified (Figure 4.7), since TPH2 is the rate-limiting enzyme for brain 5-HT synthesis. Levels of TPH2 mRNA were the same between Deaf-1 knockout and wild-type mice. Taken together these results indicate that the absence Deaf-1 did

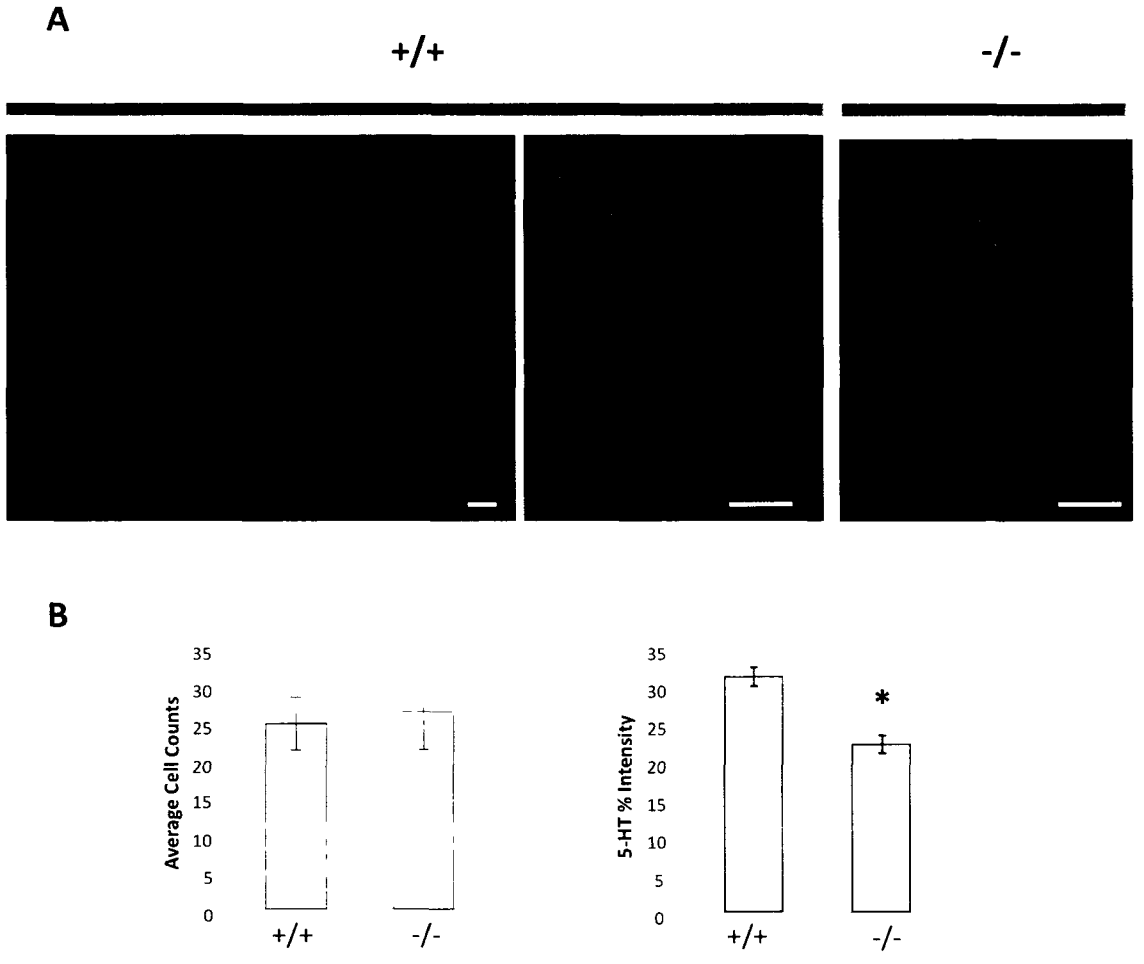


Figure 4.5: Serotonin (5-HT)-positive cells in the DRN.

A) Representative images of Immunofluorescent labeling of 5-HT-positive cells in wild-type (+/+) and Deaf-1 knockout (-/-) dorsal raphe nuclei at 20x magnification (scale bar = 100 μm). B) Quantification of 5-HT-positive cells and 5-HT intensity/cell in dorsal raphe sections. Cell counts are presented as mean % of total cells ± S.E.M. from combined rostral and caudal DRN; intensity is presented percent brightness above background. n ≥ 3 mice/group; *$p<0.05$ by two-tailed unpaired t-test.

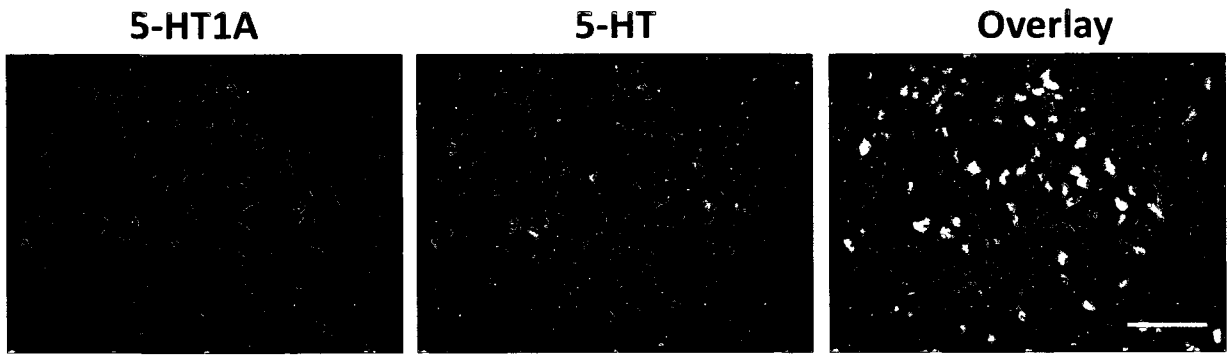


Figure 4.6: 5-HT1A expression in Serotonergic Cells.

Dorsal raphe nuclei of Deaf-1 control mice, showing co-localization of 5-HT1A with5-HT positive neurons Region expressing Almost 100% co-localization was observed in the region corresponding to the DRN (which was used in cell counts and analysis) 20X magnification was used, with n=3

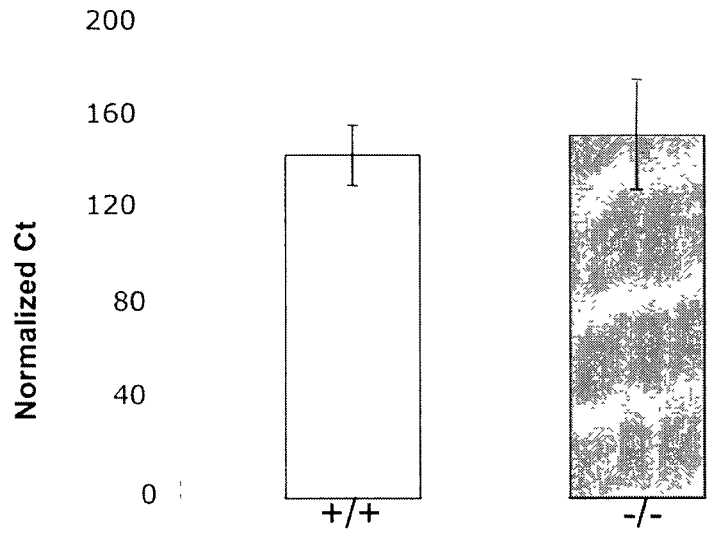


Figure 4.7: TPH2 mRNA expression in Dorsal Raphe region.

Multiplex Real-time PCR showing TPH2 RNA levels in the dorsal raphe region dissected from wild-type (+/+) and Deaf-1 knockout (-/-) mice. Data are normalized to GAPDH using the $10^{(Ct\Gamma-CtG)}$ method, and presented as mean ± S.E.M., n=3 mice/group.

not effect the number of serotonin neurons or the expression of TPH2, but reduced the level of 5-HT. This suggests that up-regulation of the 5-HT1A autoreceptor in Deaf-1 null mice did not affect serotonergic differentiation but may have reduced serotonin synthesis in the dorsal raphe nuclei.

4.5 Discussion

4.5.1 Deaf-1 knockout mice as a model 5-HT1A gene regulation

The identification of Deaf-1 as an allele-specific regulator of the human 5-HT1A receptor gene, and the association of the 5-HT1A G(-1019) allele with depression and suicide suggests that Deaf-1 may have an important role in the regulation of 5-HT1A receptor expression *in vivo*. Our previous results demonstrated that Deaf-1 has differential activity depending on the cell model, with repressor activity in serotonergic raphe RN46A cells, but enhancer activity in several non-serotonergic neuronal cell lines (Lemonde et al., 2003; Czesak et al., 2006), implying that Deaf-1 regulates the 5-HT1A autoreceptor differently than 5-HT1A receptors expressed on non-serotonergic neurons. To address the effect of Deaf-1 on 5-HT1A receptor gene regulation *in vivo*, we have examined the levels of 5-HT1A mRNA and protein in Deaf-1 knockout mice on the C57BL/6 genetic background. The C57BL/6 strain displays higher relative levels of forebrain 5-HT1A receptor RNA (Supplementary Figure 4.1) and responds to chronic SSRI treatment with increased hippocampal neurogenesis and reduced anxiety-like behaviour, responses that are abolished in 5-HT1A knockout mice (Santarelli et al., 2003), unlike the BALB/C strain (Holick et al., 2008). The C57BL/6 strain also has the higher activity Arg477 variant of TPH2

(Zhang et al., 2004) and the low-activity GK-5-HTT variant (Carneiro et al., 2009), suggesting a more active serotonin system. Furthermore, by analogy with the human 5-HT1A promoter, a palindrome site is located at -947 bp in the mouse 5-HT1A promoter that contains the minimal TCG consensus sequence (Huggenvik et al., 1998) and is specifically recognized by Deaf-1 protein. Although the level of nucleotide identity of human and mouse genes in this region is limited (Figure 4.1A), the presence of a Deaf-1 element in the same region suggests its importance in regulation of 5-HT1A gene transcription. Thus the Deaf-1 knockout mouse provides a useful model to study 5-HT1A receptor regulation *in vivo*.

In Deaf-1 null mice, 5-HT1A mRNA was increased in the dorsal raphe nuclei, consistent with the role of Deaf-1 as a repressor of 5-HT1A autoreceptor transcription in serotonergic neurons. Increased presynaptic 5-HT1A autoreceptor expression in Deaf-1 knockout mice would tend to reduce raphe firing rate and decrease serotonin release. Consistent with this, we observed reduced 5-HT levels in dorsal raphe neurons from Deaf-1 -/- mice, suggesting a restriction in serotonin synthesis despite the lack of change in TPH2 mRNA. Oppositely in 5-HT1A -/- mice, the lack of 5-HT1A autoreceptors results in increased raphe basal firing and fluoxetine-induced 5-HT release (He et al., 2001; Richer et al., 2002; Bortolozzi et al., 2004). Interestingly, the number of serotonin-positive cells was not altered in adult Deaf-1 knockout mice, suggesting that Deaf-1 deficiency does not significantly alter serotonergic differentiation. However, dysregulation of 5-HT1A autoreceptor levels and serotonin neurotransmission during development could play a role in the behavioural phenotype of adult mice (Gross et al., 2002). By contrast to the up-

regulation of 5-HT1A autoreceptors, 5-HT1A mRNA and 5-HT1A stained cells were reduced in the frontal cortex of Deaf-1 knockout mice, suggesting that Deaf-1 acts as an enhancer in the cortex, consistent with a dual regulation of pre- and post-synaptic 5-HT1A receptors by Deaf-1 *in vivo*. Although Deaf-1 enhanced 5-HT1A receptor transcription in non-serotonergic neurons, Hes1, which represses at the 26-bp 5-HT1A palindrome sequence, retained repressor activity in these cells (Czesak et al., 2006; Jacobsen et al., 2008). In contrast to Deaf-1 deficient mice, Hes1-/- mice display widespread up-regulation of 5-HT1A receptor RNA and protein in embryonic midbrain and hindbrain regions (Jacobsen et al., 2008). Thus, the loss of Deaf-1 regulation results in a dual action to decrease serotonergic neurotransmission: induction of 5-HT1A autoreceptors to decrease 5-HT neuronal activity; and decreased cortical 5-HT1A receptors to reduce serotonin responsiveness.

4.5.2 Deaf-1 knockout mice as a model for the human 5-HT1A C(-1019)G polymorphism

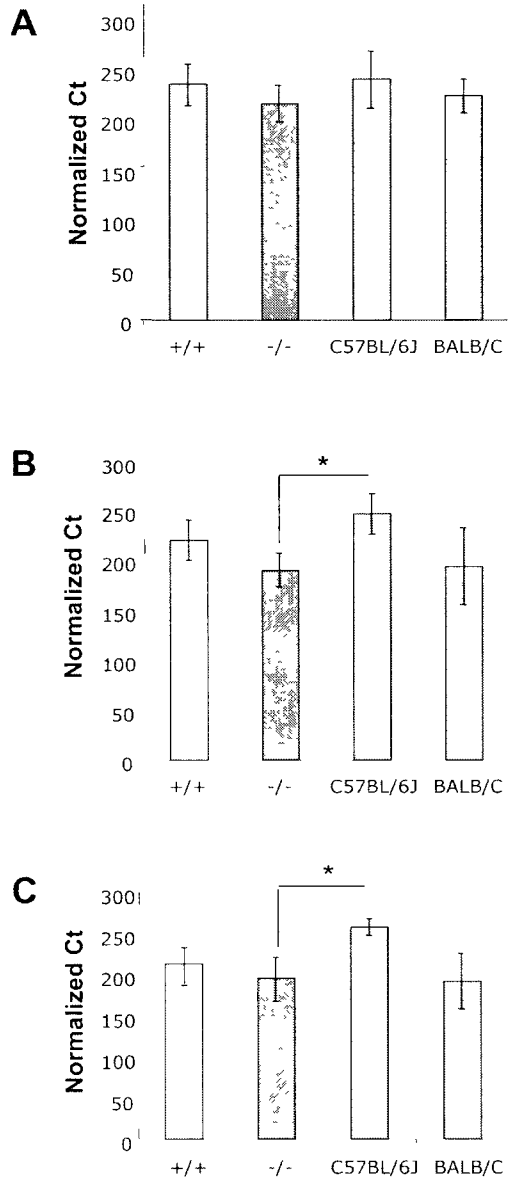
Reduced activity of the serotonin system is implicated in major depressive disorder (Jans et al., 2007), and antidepressant therapies act directly or indirectly to enhance serotonergic neurotransmission (Pineyro and Blier, 1999). Upon chronic treatment with SSRI, 5-HT1A autoreceptors become desensitized while post-synaptic 5-HT1A receptors remain responsive to enhance serotonergic neurotransmission, correlating with clinical improvement. The 5-HT1A C(-1019)G polymorphism is associated with depression and prevents Deaf-1 regulation of 5-HT1A transcription, which could reduce serotonergic neurotransmission (Le Francois et al., 2008).

Based on our results, the 5-HT1A G/G(-1019) genotype would be predicted confer differential changes in 5-HT1A receptor RNA and protein levels in pre- and post-synaptic brain regions. Recent studies support an up-regulation of 5-HT1A autoreceptors in antidepressant-free depressed patients with the G/G genotype (Parsey et al., 2006a; Sullivan et al., 2009), but not in healthy subjects (David et al., 2005). This suggests that reserve mechanisms are recruited to normalize 5-HT1A autoreceptor expression in healthy subjects, but are incompletely effective in depressed subjects. However, the effect of the G/G genotype on post-synaptic 5-HT1A receptors is less clear. The G/G genotype in depressed subjects was associated with no change or increased 5-HT1A receptors in some regions such as hippocampus (Parsey et al., 2006a; Sullivan et al., 2009), which could reflect a compensatory up-regulation due to reduced serotonergic neurotransmission in these subjects (Abbas et al., 2007). Several other studies report reduced 5-HT1A RNA or binding potential in the prefrontal cortex of subjects with major depression (Lopez et al., 1998; Stockmeier et al., 1998; Sargent et al., 2000; Drevets et al., 2007; Szewczyk et al., 2009), consistent with reduced transcription of the 5-HT1A receptor. Similarly, in female Cynomolgus monkeys, depressed status was associated with reduced 5-HT1A receptor levels in several forebrain regions (especially cingulate cortex, with lesser change in hippocampus and amygdala) with no change in the raphe region (Shively et al., 2006). In panic disorder, both pre- and post-synaptic 5-HT1A receptors are reduced (Neumeister et al., 2004), but recovery is associated with an increase in post-synaptic 5-HT1A receptors (Nash et al., 2008), suggesting their role in anxiety (Akimova et al., 2009). The G(-1019) allele is also

associated with reduced threat-induced reactivity of the amygdala and prefrontal cortex (Domschke et al., 2006; Fakra et al., 2009), suggesting a functional role for post-synaptic 5-HT1A receptor sites in fear response. In addition to the presence of the G(-1019) allele, reduction in Deaf-1 expression has also been associated with reduced 5-HT1A receptor expression in the prefrontal cortex of female depressed subjects (Szewczyk et al., 2009). Thus, multiple factors may influence the regulation of 5-HT1A receptor levels in the brains of healthy or depressed subjects.

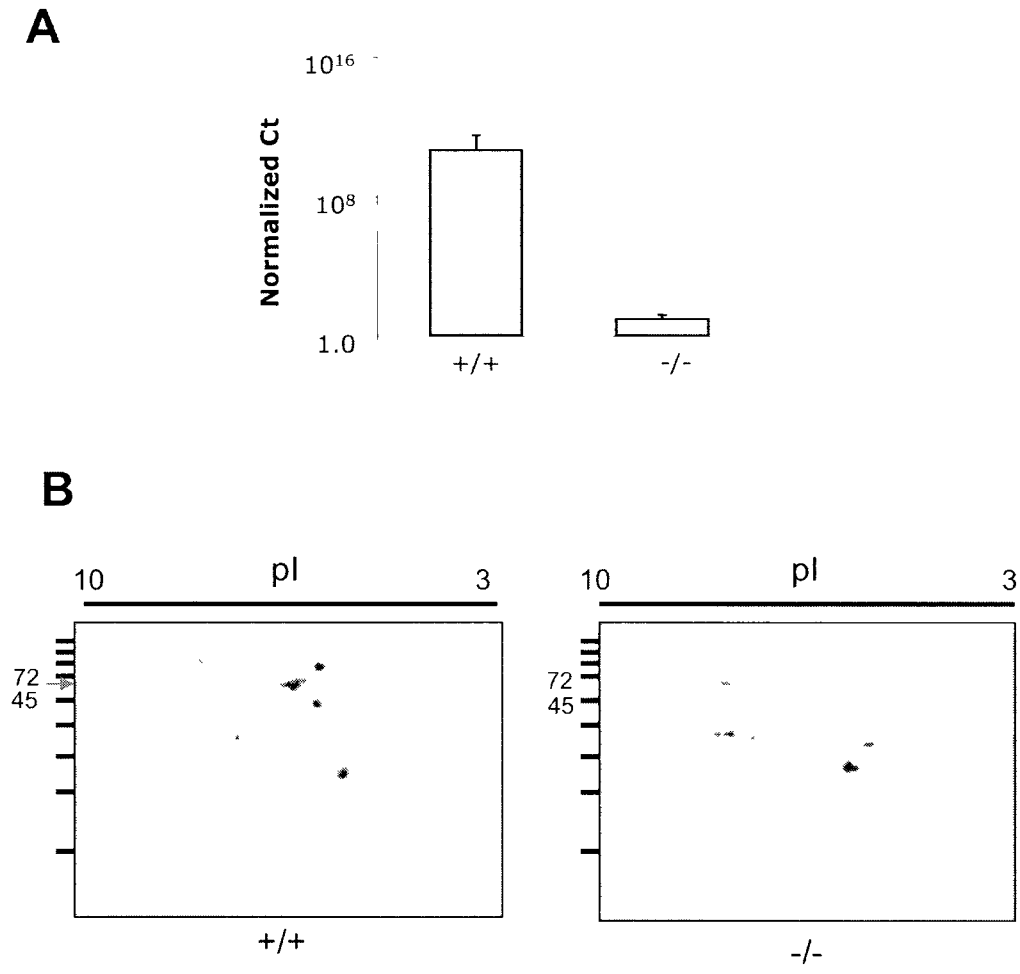
Recent evidence suggests that stressful life events in combination with the 5-HT1A G(-1019) genotype can increase the risk for depression and suicide (Zhang et al., 2008; Videtic et al., 2009). Since the C(-1019)G 5-HT1A polymorphism effects its regulation by Deaf-1 and Hes proteins throughout the lifetime, dys-regulation of the 5-HT1A receptor in embryonic or early post-natal brain may have long-term effects on adult behavioral phenotype (Gross et al., 2002), that could be modified by early life environmental events. However association studies are limited by frequency of the risk allele in the population size, heterogeneity of the genetic background as well as environmental influences, and are not always replicated. In addition, these studies do not shed light on the mechanisms of interaction between 5-HT1A genotype and environment. Hence better animal models are needed to address the etiology and treatment of depression. Deaf-1 deficient mice provide an animal model of perturbed 5-HT1A receptor regulation that corresponds to the expected effect of C(-1019)G genotype in humans. In addition to its association with depression, anxiety and suicide, the C(-1019)G polymorphism has been associated with reduced response to antidepressant treatment (Lemonde et al., 2004; Serretti

et al., 2004; Arias et al., 2005; Hong et al., 2006; Yu et al., 2006; Baune et al., 2008; Wang et al., 2008; Yevtushenko et al., 2009). In mice and rats, 5-HT1A receptors are implicated in the activity of chronic SSRI treatment to induce hippocampal neurogenesis and to reduce anxiety and depression-like behaviors (Santarelli et al., 2003; Greene et al., 2009). It is possible that the combination of reduced cortical 5-HT1A receptors and increased 5-HT1A autoreceptors in the Deaf-1-null mice may attenuate SSRI action. Thus, Deaf-1 deficient mice may serve as a useful model for investigating the actions of the C(-1019)G polymorphism on behaviour and antidepressant response (Le Francois et al., 2008).



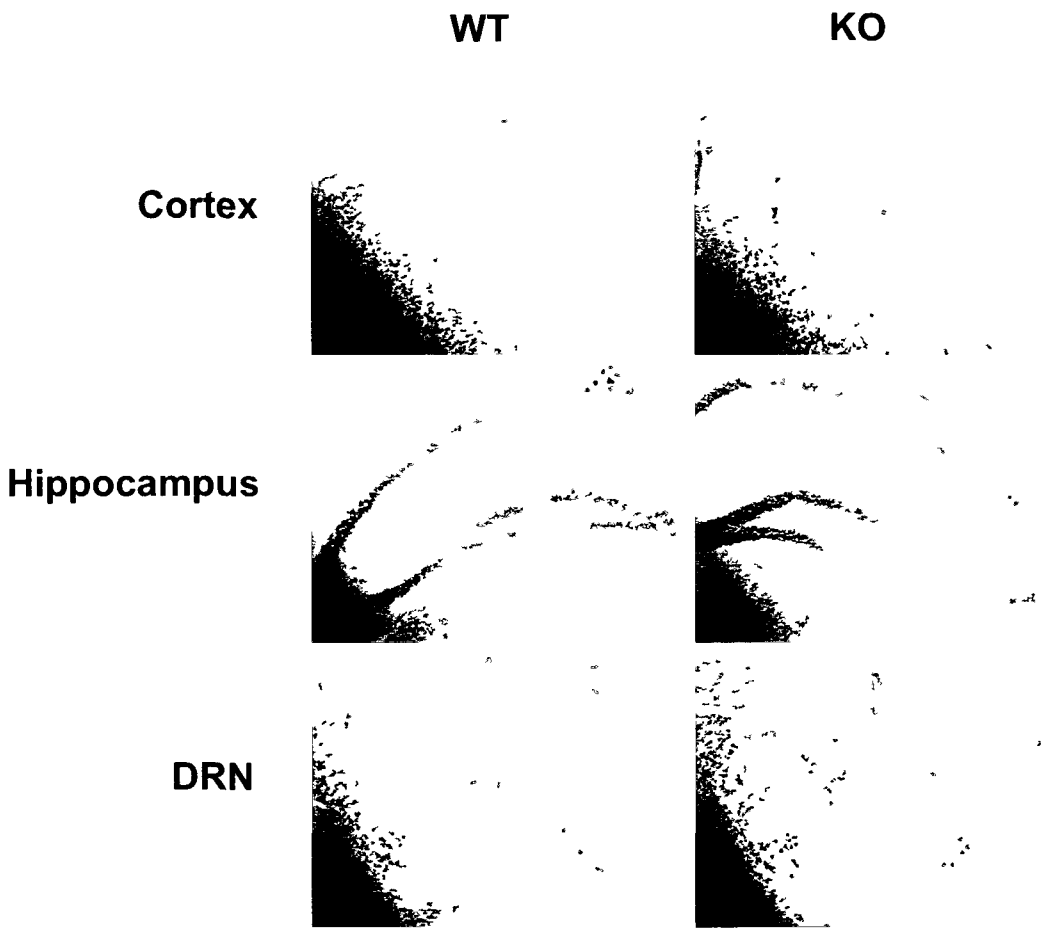
Supplemental Figure. 4.1: 5-HT1A mRNA expression in mixed and pure mouse strains.

Multiplex Real-time PCR analysis of 5-HT1A mRNA levels in mixed BALB/C-C57BL/6 Deaf-1 deficient mice (-/-) compared to wild-type littermate controls (+/+), pure BALB/C or pure C57BL/6 mice in A) Dorsal Raphe region (DRN), B) Frontal Cortex and C) Hippocampus. Data are normalized to GAPDH using the $10^{(CtT-CtG)}$ and presented as mean ± S.E.M. n=3 mice/group, *$p<0.05$ were considered statistically significant using the two-tailed unpaired t-test.



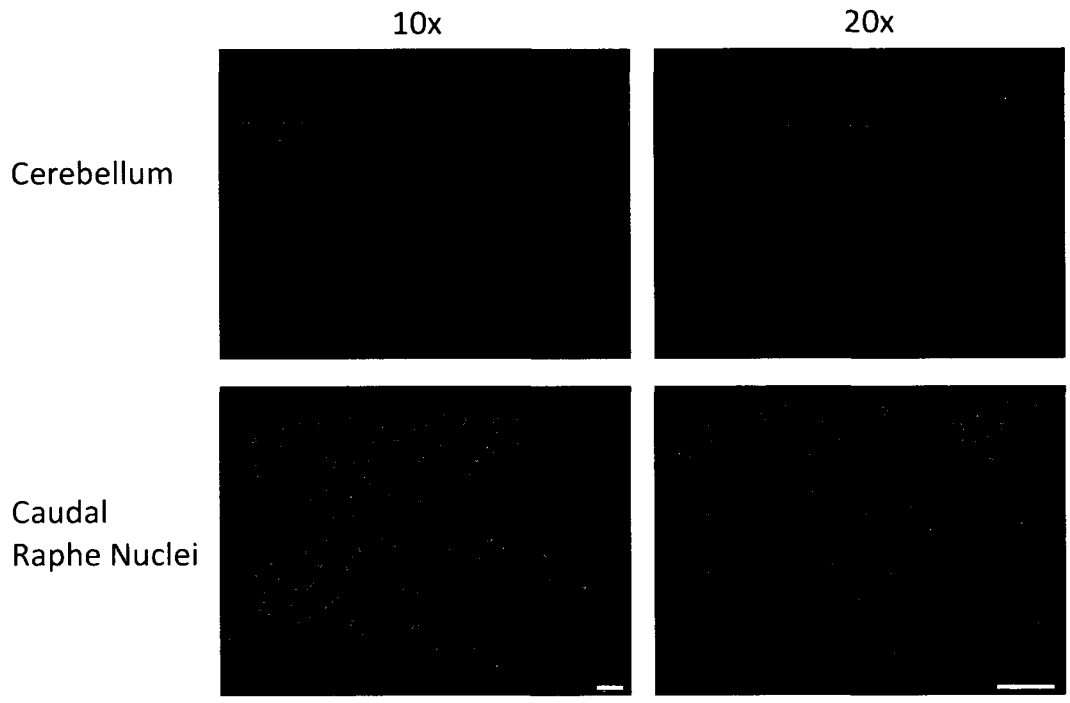
Supplemental Figure 4.2: Loss of Deaf-1 mRNA and protein expression in Deaf-1 -/- mice.

A) Deaf-1 RNA. Real-time PCR amplification of Deaf-1 mRNA from the frontal cortex of Deaf-1 wild-type (+/+) and knockout (-/-) mice using primers located within the deleted exon 4 cDNA sequence as indicated. Ct values were normalized to GAPDH. n=3 mice/group. B) Deaf-1 protein. Two-Dimensional SDS-PAGE showing Deaf-1 protein expression in frontal cortex tissue of wild-type (+/+) and knockout (-/-) mice. M.W. standards are shown on the ordinate, and pI range on the abscissa. Deaf-1 deficient mice lacked the 68-kDa Deaf-1 protein species.



Supplemental Figure 4.3: Brain morphology of Deaf-1 deficient mice.

Representative sections of frontal cortex, hippocampus and dorsal raphe nuclei from Deaf-1 knockout (-/-) mice compared to wild-type (+/+) mice were stained with Cresyl Violet Representative images are shown at 10x magnification, scale bar is 100 μm n=3 mice/group



Supplemental Figure 4.4: 5-HT1A receptor expression in the cerebellum.

Sections of cerebellum from wildtype Deaf-1 mice were stained with 5-HT1A antibody (1 100) and viewed at 10x and 20x magnification Lack of 5-HT1A receptor expression in the cerebellum corresponds to absence of 5-HT1A staining As positive antibody control, the caudal raphe nuclei (also from the same coronal section) was used for comparison

4.6 References

Abbas SY, Nogueira MI, Azmitia EC (2007) Antagonist-induced increase in 5-HT1A-receptor expression in adult rat hippocampus and cortex. Synapse 61:531-539.

Akimova E, Lanzenberger R, Kasper S (2009) The serotonin-1A receptor in anxiety disorders. Biol Psychiatry 66:627-635.

Arias B, Catalan R, Gasto C, Gutierrez B, Fananas L (2005) Evidence for a combined genetic effect of the 5-HT(1A) receptor and serotonin transporter genes in the clinical outcome of major depressive patients treated with citalopram. J Psychopharmacol 19:166-172.

Baune BT, Hohoff C, Roehrs T, Deckert J, Arolt V, Domschke K (2008) Serotonin receptor 1A-1019C/G variant: impact on antidepressant pharmacoresponse in melancholic depression? Neurosci Lett 436:111-115.

Blier P (2003) The pharmacology of putative early-onset antidepressant strategies. Eur Neuropsychopharmacol 13:57-66.

Boldrini M, Underwood MD, Mann JJ, Arango V (2008) Serotonin-1A autoreceptor binding in the dorsal raphe nucleus of depressed suicides. J Psychiatr Res 42:433-442.

Bonnin A, Peng W, Hewlett W, Levitt P (2006) Expression mapping of 5-HT1 serotonin receptor subtypes during fetal and early postnatal mouse forebrain development. Neuroscience 141:781-794.

Bortolozzi A, Amargos-Bosch M, Toth M, Artigas F, Adell A (2004) In vivo efflux of serotonin in the dorsal raphe nucleus of 5-HT1A receptor knockout mice. J Neurochem 88:1373-1379.

Carneiro AM, Airey DC, Thompson B, Zhu CB, Lu L, Chesler EJ, Erikson KM, Blakely RD (2009) Functional coding variation in recombinant inbred mouse lines reveals multiple serotonin transporter-associated phenotypes. Proc Natl Acad Sci U S A 106:2047-2052.

Chalmers DT, Watson SJ (1991) Comparative anatomical distribution of 5-HT1A receptor mRNA and 5-HT1A binding in rat brain--a combined in situ hybridisation/in vitro receptor autoradiographic study. Brain Research 561:51-60.

Charney DS, Krystal JH, Delgado PL, Heninger GR (1990) Serotonin-specific drugs for anxiety and depressive disorders. Annual Review of Medicine 41:437-446.

Czesak M, Burns AM, Lenicov FR, Albert PR (2007) Characterization of rat rostral raphe primary cultures: multiplex quantification of serotonergic markers. J Neurosci Methods 164:59-67.

Czesak M, Lemonde S, Peterson EA, Rogaeva A, Albert PR (2006) Cell-specific repressor or enhancer activities of Deaf-1 at a serotonin 1A receptor gene polymorphism. J Neurosci 26:1864-1871.

David SP, Murthy NV, Rabiner EA, Munafo MR, Johnstone EC, Jacob R, Walton RT, Grasby PM (2005) A functional genetic variation of the serotonin (5-HT)

transporter affects 5-HT1A receptor binding in humans. J Neurosci 25:2586-2590.

Domschke K, Braun M, Ohrmann P, Suslow T, Kugel H, Bauer J, Hohoff C, Kersting A, Engelien A, Arolt V, Heindel W, Deckert J (2006) Association of the functional -1019C/G 5-HT1A polymorphism with prefrontal cortex and amygdala activation measured with 3 T fMRI in panic disorder. Int J Neuropsychopharmacol 9:349-355.

Drevets WC, Thase ME, Moses-Kolko EL, Price J, Frank E, Kupfer DJ, Mathis C (2007) Serotonin-1A receptor imaging in recurrent depression: replication and literature review. Nucl Med Biol 34:865-877.

Fakra E, Hyde LW, Gorka A, Fisher PM, Munoz KE, Kimak M, Halder I, Ferrell RE, Manuck SB, Hariri AR (2009) Effects of HTR1A C(-1019)G on amygdala reactivity and trait anxiety. Arch Gen Psychiatry 66:33-40.

Ferguson KL, McClellan KA, Vanderluit JL, McIntosh WC, Schuurmans C, Polleux F, Slack RS (2005) A cell-autonomous requirement for the cell cycle regulatory protein, Rb, in neuronal migration. Embo J 24:4381-4391.

Greene J, Banasr M, Lee B, Warner-Schmidt J, Duman RS (2009) Vascular endothelial growth factor signaling is required for the behavioral actions of antidepressant treatment: pharmacological and cellular characterization. Neuropsychopharmacology 34:2459-2468.

Gross C, Zhuang X, Stark K, Ramboz S, Oosting R, Kirby L, Santarelli L, Beck S, Hen R (2002) Serotonin1A receptor acts during development to establish normal anxiety-like behaviour in the adult. Nature 416:396-400.

Hahm K, Sum EY, Fujiwara Y, Lindeman GJ, Visvader JE, Orkin SH (2004) Defective neural tube closure and anteroposterior patterning in mice lacking the LIM protein LMO4 or its interacting partner Deaf-1. Mol Cell Biol 24:2074-2082.

He M, Sibille E, Benjamin D, Toth M, Shippenberg T (2001) Differential effects of 5-HT1A receptor deletion upon basal and fluoxetine-evoked 5-HT concentrations as revealed by in vivo microdialysis. Brain Res 902:11-17.

Heisler LK, Chu HM, Brennan TJ, Danao JA, Bajwa P, Parsons LH, Tecott LH (1998) Elevated anxiety and antidepressant-like responses in serotonin 5-HT1A receptor mutant mice. Proc Natl Acad Sci U S A 95:15049-15054.

Holick KA, Lee DC, Hen R, Dulawa SC (2008) Behavioral Effects of Chronic Fluoxetine in BALB/cJ Mice Do Not Require Adult Hippocampal Neurogenesis or the Serotonin 1A Receptor. Neuropsychopharmacology 33:406-417.

Hong CJ, Chen TJ, Yu YW, Tsai SJ (2006) Response to fluoxetine and serotonin 1A receptor (C-1019G) polymorphism in Taiwan Chinese major depressive disorder. Pharmacogenomics J 6:27-33.

Huggenvik JI, Michelson RJ, Collard MW, Ziemba AJ, Gurley P, Mowen KA (1998) Characterization of a nuclear deformed epidermal autoregulatory factor-1 (DEAF-1)-related (NUDR) transcriptional regulator protein. Mol Endocrinol 12:1619-1639.

Jacobsen KX, Vanderluit J, Slack RS, Albert PR (2008) HES1 regulates 5-HT1A receptor gene transcription at a functional polymorphism: Essential role in developmental expression. Mol Cell Neurosci 38:349-358.

Jans LA, Riedel WJ, Markus CR, Blokland A (2007) Serotonergic vulnerability and depression: assumptions, experimental evidence and implications. Mol Psychiatry 12:522-543.

Kishi T, Tsunoka T, Ikeda M, Kawashima K, Okochi T, Kitajima T, Kinoshita Y, Okumura T, Yamanouchi Y, Inada T, Ozaki N, Iwata N (2009) Serotonin 1A receptor gene and major depressive disorder: an association study and meta-analysis. J Hum Genet.

Kusserow H, Davies B, Hortnagl H, Voigt I, Stroh T, Bert B, Deng DR, Fink H, Veh RW, Theuring F (2004) Reduced anxiety-related behaviour in transgenic mice overexpressing serotonin 1A receptors. Brain Res Mol Brain Res 129:104-116.

Le Francois B, Czesak M, Steubl D, Albert PR (2008) Transcriptional regulation at a HTR1A polymorphism associated with mental illness. Neuropharmacology 55:977-985.

Lemonde S, Rogaeva A, Albert PR (2004) Cell type-dependent recruitment of trichostatin A-sensitive repression of the human 5-HT1A receptor gene. J Neurochem 88:857-868.

Lemonde S, Turecki G, Bakish D, Du L, Hrdina PD, Bown CD, Sequeira A, Kushwaha N, Morris SJ, Basak A, Ou XM, Albert PR (2003) Impaired repression at a 5-hydroxytryptamine 1A receptor gene polymorphism associated with major depression and suicide. J Neurosci 23:8788-8799.

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402-408.

Lopez JF, Chalmers DT, Little KY, Watson SJ (1998) A.E. Bennett Research Award. Regulation of serotonin1A, glucocorticoid, and mineralocorticoid receptor in rat and human hippocampus: implications for the neurobiology of depression. Biological Psychiatry 43:547-573.

Mann JJ (1999) Role of the serotonergic system in the pathogenesis of major depression and suicidal behavior. Neuropsychopharmacology 21:99S-105S.

Nash JR, Sargent PA, Rabiner EA, Hood SD, Argyropoulos SV, Potokar JP, Grasby PM, Nutt DJ (2008) Serotonin 5-HT1A receptor binding in people with panic disorder: positron emission tomography study. Br J Psychiatry 193:229-234.

Neumeister A, Bain E, Nugent AC, Carson RE, Bonne O, Luckenbaugh DA, Eckelman W, Herscovitch P, Charney DS, Drevets WC (2004) Reduced serotonin type 1A receptor binding in panic disorder. J Neurosci 24:589-591.

Ou XM, Jafar-Nejad H, Storring JM, Meng JH, Lemonde S, Albert PR (2000) Novel dual repressor elements for neuronal cell-specific transcription of the rat 5-HT1A receptor gene. J Biol Chem 275:8161-8168.

Parks CL, Robinson PS, Sibille E, Shenk T, Toth M (1998) Increased anxiety of mice lacking the serotonin1A receptor. Proc Natl Acad Sci U S A 95:10734-10739.

Parsey RV, Olvet DM, Oquendo MA, Huang YY, Ogden RT, Mann JJ (2006a) Higher 5-HT1A receptor binding potential during a major depressive episode predicts poor treatment response: preliminary data from a naturalistic study. Neuropsychopharmacology 31:1745-1749.

Parsey RV, Oquendo MA, Ogden RT, Olvet DM, Simpson N, Huang YY, Van Heertum RL, Arango V, Mann JJ (2006b) Altered serotonin 1A binding in major depression: a [carbonyl-C-11]WAY100635 positron emission tomography study. Biol Psychiatry 59:106-113.

Paxinos G, Franklin K (2001) The Mouse Brain, 2nd Edition Edition. New York: Academic Press.

Pineyro G, Blier P (1999) Autoregulation of serotonin neurons: role in antidepressant drug action. Pharmacol Rev 51:533-591.

Pompeiano M, Palacios JM, Mengod G (1992) Distribution and cellular localization of mRNA coding for 5-HT1A receptor in the rat brain: correlation with receptor binding. Journal of Neuroscience 12:440-453.

Ramboz S, Oosting R, Amara DA, Kung HF, Blier P, Mendelsohn M, Mann JJ, Brunner D, Hen R (1998) Serotonin receptor 1A knockout: an animal model of anxiety-related disorder. Proc Natl Acad Sci U S A 95:14476-14481.

Richer M, Hen R, Blier P (2002) Modification of serotonin neuron properties in mice lacking 5-HT1A receptors. Eur J Pharmacol 435:195-203.

Santarelli L, Saxe M, Gross C, Surget A, Battaglia F, Dulawa S, Weisstaub N, Lee J, Duman R, Arancio O, Belzung C, Hen R (2003) Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants. Science 301:805-809.

Sargent PA, Kjaer KH, Bench CJ, Rabiner EA, Messa C, Meyer J, Gunn RN, Grasby PM, Cowen PJ (2000) Brain serotonin1A receptor binding measured by positron emission tomography with [11C]WAY-100635: effects of depression and antidepressant treatment. Arch Gen Psychiatry 57:174-180.

Serretti A, Artioli P, Lorenzi C, Pirovano A, Tubazio V, Zanardi R (2004) The C(-1019)G polymorphism of the 5-HT1A gene promoter and antidepressant response in mood disorders: preliminary findings. Int J Neuropsychopharmacol 7:453-460.

Shively CA, Friedman DP, Gage HD, Bounds MC, Brown-Proctor C, Blair JB, Henderson JA, Smith MA, Buchheimer N (2006) Behavioral depression and positron emission tomography-determined serotonin 1A receptor binding potential in cynomolgus monkeys. Arch Gen Psychiatry 63:396-403.

Stockmeier CA, Shapiro LA, Dilley GE, Kolli TN, Friedman L, Rajkowska G (1998) Increase in serotonin-1A autoreceptors in the midbrain of suicide victims with major depression-postmortem evidence for decreased serotonin activity. J Neurosci 18:7394-7401.

Sullivan GM, Ogden RT, Oquendo MA, Kumar JS, Simpson N, Huang YY, Mann JJ, Parsey RV (2009) Positron emission tomography quantification of serotonin-1A receptor binding in medication-free bipolar depression. Biol Psychiatry 66:223-230.

Szewczyk B, Albert PR, Burns AM, Czesak M, Overholser JC, Jurjus GJ, Meltzer HY, Konick LC, Dieter L, Herbst N, May W, Rajkowska G, Stockmeier CA, Austin MC (2009) Gender-specific decrease in NUDR and 5-HT1A receptor proteins in the prefrontal cortex of subjects with major depressive disorder. Int J Neuropsychopharmacol 12:155-168.

Videtic A, Zupanc T, Pregelj P, Balazic J, Tomori M, Komel R (2009) Suicide, stress and serotonin receptor 1A promoter polymorphism-1019C > G in Slovenian suicide victims. European Archives of Psychiatry and Clinical Neuroscience 259:234-238.

Wang L, Fang C, Zhang A, Du J, Yu L, Ma J, Feng G, Xing Q, He L (2008) The --1019 C/G polymorphism of the 5-HT(1)A receptor gene is associated with negative symptom response to risperidone treatment in schizophrenia patients. J Psychopharmacol 22:904-909.

Yevtushenko OO, Oros MM, Reynolds GP (2009) Early response to selective serotonin reuptake inhibitors in panic disorder is associated with a functional 5-HT1A receptor gene polymorphism. J Affect Disord.

Yu YW, Tsai SJ, Liou YJ, Hong CJ, Chen TJ (2006) Association study of two serotonin 1A receptor gene polymorphisms and fluoxetine treatment response in Chinese major depressive disorders. Eur Neuropsychopharmacol 16:498-503.

Zhang K, Xu Q, Xu Y, Yang H, Luo J, Sun Y, Sun N, Wang S, Shen Y (2008) The combined effects of the 5-HTTLPR and 5-HTR1A genes modulates the relationship between negative life events and major depressive disorder in a Chinese population. J Affect Disord.

Zhang X, Beaulieu JM, Sotnikova TD, Gainetdinov RR, Caron MG (2004) Tryptophan hydroxylase-2 controls brain serotonin synthesis. Science 305:217.

CHAPTER 5 — DISCUSSION

The 5-HT1A receptor is a key modulator of serotonergic neurotransmission, regulating both, pre-synaptic firing rates of serotonin, as well as its reuptake at post-synaptic sites (Pineyro and Blier, 1999). Furthermore, pharmacological evidence suggests that transcriptional regulation of the 5-HT1A receptor gene may contribute to the effectiveness of antidepressant treatments (Albert and Lemonde, 2004). As such, deciphering the transcriptional machinery involved in modulating basal expression of the 5-HT1A gene is crucial in strengthening our understanding of the etiology of major depression. For that reason, the main focus of my thesis was to elucidate specific mechanisms involved in transcriptional regulation of the 5-HT1A receptor gene, and how these mechanisms might be affected by depression, leading to altered levels of 5-HT1A receptors both *in vitro* and *in vivo* and resulting in reduced serotonergic activity.

My initial studies focused on characterizing the palindromic region at the C(-1019)G polymorphism of the human 5-HT1A promoter and its role in transcriptional regulation of the 5-HT1A receptor. The results in Chapter II focused on transfection studies characterizing the 26-bp regulatory region encompassing the C(-1019)G polymorphism in different neuronal cell lines. I confirmed the role of Deaf-1 as a transcriptional regulator of 5-HT1A gene expression in cell models of both pre- and post-synaptic 5-HT1A receptor expression and verified the importance of the normal C(-1019) allele in regulation of the 5-HT1A receptor gene by specific transcription factors. I have successfully characterized the dual properties of Deaf-1 in regulating the 5-HT1A promoter: in a serotonergic raphe cell model of presynaptic receptors, Deaf-1 functioned as a repressor of gene

transcription, while in post-synaptic 5-HT1A cell models, Deaf-1 exhibited enhancer activity. In the presence of the G(-1019) allele, however, Deaf-1 was not able to bind to the palindrome sequence, and did not regulate the 5-HT1A promoter. Thus, my data convincingly supports the role of Deaf-1 as a sequence-specific transcriptional regulator of the 5-HT1A receptor gene at this polymorphic site. Hes5 however remained a transcriptional repressor of the 5-HT1A promoter in both pre- and post-synaptic cell lines. In addition, the regulatory properties of Deaf-1 were unaffected by presence of Hes5, both pre- and post-synaptically, suggesting that Deaf-1 has the higher affinity for the palindrome element (Jacobsen et al., 2008b). Since Hes5 is only expressed in development (Akazawa et al., 1992; Lemonde et al., 2003) and is less affected by the presence of the C(-1019)G polymorphism, I focused my studies on examining the role of Deaf-1 in regulating 5-HT1A receptor expression. Deaf-1 exerted its regulatory properties through a mechanism that was TSA-sensitive, thus implicating HDAC as a potential partner in regulating 5-HT1A gene expression. The findings in Chapter II indicate that genetic alterations in the palindromic region of the 5-HT1A promoter lead to abnormal 5-HT1A receptor regulation both pre- and post-synaptically, which could result in reduced serotonergic activity *in vivo*. Cell-specific repressor and enhancer activities of Deaf-1 suggest distinct, cell-specific co-repressor or co-enhancer complexes are involved in mediating differential effects on chromatin remodeling.

Secondly, in Chapter III I developed a novel technique of culturing midbrain raphe primary cells, and to enrich for serotonin neurons. The rationale was to develop and characterize a new model for studying the pre-synaptic serotonergic

system *in vivo*, as an alternative to using the RN46A pre-synaptic cell line. While useful for biochemical studies, the RN46A cells have over time displayed reduced expression of serotonergic markers, and particularly 5-HT1A receptor RNA and protein. Thus, it was desirable to develop a cell culture model that more closely reflected regulation of the 5-HT1A receptor in normal raphe serotonin neurons. I quantified the types of neuronal markers present in my cell cultures, including various serotonergic markers, and established a much higher yield of 5-HT positive neurons then previously reported. More importantly, I was able to optimize a sensitive real-time RT-PCR technique for consistent and accurate detection of Deaf-1 and 5-HT1A mRNA within the rostral raphe region of the mouse brain, consistent with previous findings that implemented Deaf-1 in transcriptional regulation of the 5-HT1A receptor *in vitro*.

Finally in Chapter IV, I conducted experiments to establish the role of Deaf-1 as an important regulator of 5-HT1A receptor expression *in vivo*. I successfully identified a palindromic region within the mouse promoter sequence containing a minimal TCG Deaf-1 binding site and demonstrated its binding potential using EMSA. This evidence is the first to implicate Deaf-1 in regulating the mouse 5-HT1A receptor gene. Using Deaf-1 knockout mice, I discovered that the levels of 5-HT1A RNA and protein exhibited striking similarities in 5-HT1A gene expression patterns to those predicted using my cell culture models, and consistent with changes in 5-HT1A receptor levels reported in human depression. Specifically, mice lacking the functional Deaf-1 protein had altered 5-HT1A mRNA and protein levels that were increased pre-synaptically in the raphe nuclei and decreased in at least one post-

synaptic brain region, the prefrontal cortex. TPH2, a marker of a functional serotonergic system was not affected in the Deaf-1-deficient raphe, implying a direct effect of Deaf-1 on 5-HT1A receptor regulation, as opposed to global alteration in serotonergic differentiation. Further studies will be necessary to establish whether altered 5-HT1A receptor levels translate to a depressive-like behavioural phenotype.

It should be noted that the experimental design of the *in vitro* portion of my thesis was somewhat restricted by the availability of only one pre-synaptic RN46A cell line model, as well as a limited variety 5-HT1A-expressing cell lines available. As such, human gene segments were studied in rat or hybrid cell lines rather than real neurons and thus, some aspects of cell-specific expression may not have been detected in these assays. Hence in chapter III, I developed a more accurate pre-synaptic model system that provided a predominantly neuronal population of cells, derived from the rostral raphe rat region that can be utilized in the future to study 5-HT1A gene regulation or to establish a new pre-synaptic neuronal cell line (data not shown). Unfortunately, the yield of serotonin-positive cells in these cultures, while better than previous protocols (1%), was still quite low (8%) and not practical for most biochemical assays (Galter and Unsicker, 1999; Lautenschlager et al., 2000; Rumajogee et al., 2004). Finally, in chapter IV, several technical difficulties had to be addressed before proceeding with experiments, including re-derivation and backcrossing of the Deaf-1 knockout strain of mice. Once characterized, mice lacking Deaf-1 protein became an excellent model to study 5-HT1A regulation and provided

a model for examining the effects of the C(-1019)G polymorphism on 5-HT1A receptor expression, and ultimately behaviour.

The findings of this thesis provide new insight into the understanding of potential mechanisms involved in the etiology and treatment of major depression. Increased 5-HT1A autoreceptor expression is associated with depression, implying that a compromised regulatory mechanism is at play (Stockmeier et al., 1998). Moreover, down-regulation of this receptor is postulated to mediate the effects of antidepressants and result in enhanced serotonergic neurotransmission (Albert et al., 1996; Pineyro and Blier, 1999). In addition, a complete knockout of the 5-HT1A receptor gene consistently generates mice with an anxious phenotype (Heisler et al., 1998; Parks et al., 1998; Ramboz et al., 1998), while 5-HT1A gene over-expression at the pre-synaptic sites generates a depressive phenotype; implying differential mechanisms exist to regulate the two phenotypes (Richardson-Jones et al.). In light of these observations, both 5-HT1A enhancement pre-synaptically and its repression post-synaptically may be attributed to abnormal gene regulation by Deaf-1 which could partially explain the behavioural phenotypes observed with 5-HT1A knockout mouse models. Interestingly enough, the anxious phenotype observed using complete gene knockout models can be rescued by inducing early post-natal expression of the 5-HT1A receptor in hippocampal/prefrontal regions (Gross et al., 2002). As such, developmental regulation of the 5-HT1A receptor and its expression profile may contribute to the neurodevelopmental wiring of anxiety or depression pathways. As a result, expression of the 5-HT1A autoreceptor during early post-natal development could be a critical determinant of predisposition to

depression, regardless of the receptor levels in adulthood. Both Deaf-1 and Hes5 are expressed during development (Ohtsuka et al., 2001; Gross and McGinnis, 1996; Huggenvik et al., 1998), where they may function as key regulators of 5-HT1A receptor expression by interacting with either the LIM-domain protein family or the Notch signaling pathway, respectively (Bulchand et al., 2003); Sugihara et al., 1998). Since Hes5 is expressed in the dentate gyrus and subventricular zone of the hippocampus, and in the cortex in early post-natal development (Stump et al., 2002), it may function to inhibit dendritic elongation (Sestan et al., 1999; Redmond et al., 2000); Ohtsuka et al., 1999). As a result, Hes5-mediated repression of 5-HT1A expression may reduce inhibitory somatodendritic signaling of the 5-HT1A receptor during embryogenesis. In addition, Hes1 was shown to also repress 5-HT1A receptor expression (Jacobsen et al., 2008). In Hes1 -/- embryos, 5-HT1A receptor RNA is dramatically upregulated and its midbrain expression pattern is broadened, suggesting a widespread and adherent upregulation of 5-HT1A receptor expression and overlapping developmental functions of Hes1 and Hes5 in regulating 5-HT1A gene. Moreover, the presence of the C(-1019)G functional polymorphism throughout development may also affect the onset of 5-HT1A receptor expression during serotonergic differentiation, perhaps leading to long-term changes in serotonergic development and behavioural changes in the adult. Hence, the Deaf-1 -/- mice may not model the full extent of changes in 5-HT1A receptor expression that occur due to the C(-1019)G polymorphism. Future approaches will focus on deciphering the molecular role of both Hes5 and Deaf-1 in early developmental regulation of the 5-HT1A receptor and the serotonergic system.

Although Hes1 and Hes5 are primarily developmental transcription factors, Deaf-1 is expressed widely throughout the brain at all stages of life and may play an important role in regulating 5-HT1A receptor gene expression in the adult (Lemonde et al., 2003). In my proposed model, the G(-1019) allele prevents the binding and repression of the 5-HT1A gene by Deaf-1, leading to decreased serotonergic neurotransmission pre-synaptically, and thus an increased risk of depression. Although the C(-1019)G polymorphism de-represses the 5-HT1A receptor presynaptically, it appears to decrease 5-HT1A receptor expression postsynaptically in at least some brain regions (prefrontal cortex) to further decrease 5-HT neurotransmission in subjects with the homozygous G-allele. In addition, my data suggest that Deaf-1 has the ability to function as an enhancer of 5-HT1A receptor promoter activity in post-synaptic (non-serotonergic) 5-HT1A expressing cell lines such as mouse septal SN-48, neuroblastoma hybrid NG108-15, rat hippocampal H19-7 as well as human neuroblastoma SKN-SH cells. As such, the cellular context appears to determine the regulatory properties of Deaf-1. Although reasons for this cell specificity are still not fully understood, both Deaf-1-mediated repression (Michelson et al., 1999) and activation (Huggenvik et al., 1998) of genes has been previously reported. Interestingly, the localization of Deaf-1 appears to vary depending on the cellular context: in pre-synaptic RN46A cells and primary raphe cultures Deaf-1 has a nuclear and perinuclear localization, while in post-synaptic cells Deaf-1 localization is mainly cytosolic (Lemonde et al., 2003, data not shown). It is not surprising that Deaf-1 has the capability to "shuttle" within the cellular compartments since it contains both a nuclear import and export signal

(Huggenvik et al., 1998; Jensik et al., 2004). Thus, the subcellular localization of Deaf-1 in a given cell type may effect its rate of translocation into the nucleus and hence, the rate of gene transcription (Michelson et al., 1999).

While the exact mechanism that determines Deaf-1 activity in cells remains to be elucidated, both repressor and enhancer activities of Deaf-1 are abolished at the G-allele resulting in the upregulation or downregulation of 5-HT1A promoter activity, respectively. Consistent with a role in human depression, our recent collaborative study showed that both Deaf-1 and 5-HT1A receptor proteins are co-localized in neurons of the prefrontal cortex of female depressed suicides (Appendix C) (Szewczyk et al., 2008). In addition, levels of Deaf-1 and 5-HT1A are decreased in prefrontal cortex from depressed females, suggesting that not only does Deaf-1 function as a transcriptional enhancer of 5-HT1A gene expression in this post-synaptic region of the brain, but also that dys-regulation of Deaf-1 expression potentially contributes to a depressive phenotype (Appendix D) (Szewczyk et al., 2008). This study further suggests that loss of Deaf-1 activity leads to reduced 5-HT1A gene transcription post-synaptically, consistent with an enhancer function for Deaf-1 in these cells. In addition, this study also implicates gender-specific changes in Deaf-1 and 5-HT1A protein expression in depression. Interestingly, pre-menstrual dysphoric disorder (PMDD), which effects 3-8% of women during reproductive age can be successfully treated with SSRIs, thus supporting the hypothesis that PMDD is associated with a dysregulation of the serotonergic system (Steiner and Born, 2000; Eriksson et al., 2001; Carr and Ensom, 2002). Additional

experiments using the Deaf-1 knockout mice will be needed to determine if Deaf-1 affects 5-HT1A receptor expression in a gender-specific manner.

Post-translational modification of histones has a fundamental role in regulating gene expression. Histone deacetylases (HDACs) regulate the pattern of histone acetylation and may be involved in deacetylation of non-histone proteins as well as recruitment of proteins to form complexes involved in regulation of gene expression. Deaf-1 provides a unique example of a transcription factor that has the intrinsic ability to repress or enhance gene expression by direct binding to its DNA regulatory element and recruitment of HDAC for both activities as evidenced by TSA-mediated inhibition of Deaf-1 activity at the 5-HT1A promoter. Since HDAC is often a co-repressor of transcription, its role in regulating Deaf-1 activity remains unclear. It is possible that the dual role of Deaf-1 in regulating the 5-HT1A receptor may depend on ligand-induced conformational change, and recruitment of histone deacetylase (for repression) or acetylase (for enhancement). Other transcriptional regulators can also act as either repressors or activators, depending on the developmental lineage of a particular cell (Bessis et al., 1997; Kallunki et al., 1998; Seth and Majzoub, 2001). For instance, the RE-1 silencing transcription factor (REST) is expressed ubiquitously in non-neuronal cells and neuronal precursors and functions to silence neurally expressed genes containing the neural restrictive silencer element (NRSE; binding element for REST). However, during post-natal development (postmitotic), REST can behave as either a repressor and an enhancer of gene expression, depending on the cellular context (Kallunki et al., 1998). Evidence also suggests that the location of the binding element of certain

transcription factors may play a part in establishing their regulatory properties. For instance, REST, when placed no further than 50 bp away from the transcriptional start site of the nicotinic acetylcholine receptor beta2-subunit gene, behaves as an enhancer in neuronal cells, but always functions as a silencer in non-neuronal cells regardless of position (Bessis et al., 1997). This suggests that activity of this regulator may be dependent on its concentration levels in a given cell type. For instance, 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). Deaf-1 may function is a similar concentration-dependent manner. In addition, multiple splice variants of REST have been identified in neurons (Palm et al., 1998), some with activating properties (Shimojo and Hersh, 2006), giving it the ability to repress and enhance neuronal gene expression in the brain, depending on the gene and its cellular context. As such, the unique dual properties of Deaf-1 may also be the result of potential cell-type specific splice-variant isoforms of Deaf-1. One study reports that a homoeodomain protein called Barx2 was found to exhibit region-specific DNA-binding properties in that the N-terminal region was essential to mediate repression of cell adhesion molecules, while the C-terminal domain was required for their activation (Edelman et al., 2000), thus making this regulator both a repressor and an activator. Similarly, Deaf-1 has previously been characterized as having tissue-specific variations in molecular weight (Huggenvik et al., 1998) and preliminary findings from our laboratory suggest alternative splice variants of this protein may be present depending on the cellular context (data not shown).

Post-translational modifications of Deaf-1, such as phosphorylation, sumoylation, methylation or ubiquitination may also contribute to the opposing regulatory mechanisms of Deaf-1, especially since several sites responsible for these modifications have already been identified in the Deaf-1 genetic sequence (Jensik et al., 2004). Oppositely, post-translational modifications of the 5-HT1A receptor such as methylation, may also alter its gene expression pattern by effecting the binding of various regulators including Deaf-1. Interestingly enough, the C(-1019)G polymorphism is located within a CpG sequence, which is often the target site of methylation. Unfortunately, preliminary data suggests that methylating the sequence does not prevent Deaf-1 from binding to the promoter (Appendix B). However, other CpG-rich regions within the palindromic sequence may become methylated and potentially alter the binding of Deaf-1 in an indirect manner (data not shown).

Cell-specific regulation of the 5-HT1A receptor by Deaf-1 implies that this transcription factor also recruits cell-specific regulatory factors to repress or enhance transcription. Interestingly enough, Deaf-1 contains a helix-loop-helix motif that is responsible for dimerization (Bottomley et al., 2001), which may direct heterodimerization between Deaf-1 and itself or other SAND-containing proteins that have differential pre- and post-synaptic expression patterns. In order to further our understanding of the mechanism involved in Deaf-1 mediated regulation of the 5-HT1A receptor gene, we preformed a yeast-2-hybrid experiment by screening a human cDNA brain library using the full length Deaf-1 sequence (data not shown). Surprisingly, over 400 positive interactions resulted from this experiment, and

although we subcloned and sequenced 30 positive clones, no relevant interacting partners were identified, prompting us to seek alternative approaches in addressing this issue.

In an alternative attempt to identify Deaf-1-interacting proteins, we performed immunprecipitation (IP) experiments in order to successfully isolate potential interacting partners of Deaf-1. By employing a technique that utilized cobalt beads, we obtained a relatively clean pull down of His-tagged Deaf-1 proteins transiently transfected into Hek-293 cells and identified several potential co-factors (Appendix E) by using MALDI-TOF Mass Spectrometry analysis of molecular bands. Amongst those isolated, several potential factors were identified, which included: bromodomain 4 (BRD4), nuclear fragile x mental retardation interacting partner protein 2 (NUFP2), transducin-like enhancer of split-3 (TLE); P66B (a transcriptional repressor that is part of the MeCP2 complex); histone deacetylase-2 (HDAC-2) and the REST (Appendix F). Although interesting, these results need to be further verified, due to the 5-HT1A-negative cell line that was used, the potential "stickiness" of beads used as well as the use of a transient transfection approach that may not mimic *endogenous Deaf-1* accurately. One technique that will be applied in the future to help in confirming the specificity of each target identified, is known as the SILAC (Stable isotope labeling with amino acids in cell culture) which will allow for either endogenous or stably-transfected Deaf-1 to be incorporated into an appropriate cell type and treated with light or heavy amino acid medium to detect the relative abundance of newly synthesized proteins (Ong et al., 2002; Trinkle-Mulcahy et al., 2008). However, other co-repressors that do not directly

interact with Deaf-1 may be more difficult to identify. For instance, the interaction with HDAC may include the recruitment of its corepressor Sin3A/B, and would require protein-complex immunoprecipitation (Co-IP) in order to be identified.

To avoid problems associated with the use of cell lines as models, Deaf-1 knockout mice provided an alternative *in vivo* model of studying regulation of the 5-HT1A receptor by Deaf-1. However, initial characterization of Deaf-1 knockout mice quickly revealed the influence of background mouse "strain" on the outcome of results. More specifically, Deaf-1 knockout mice were backcrossed from a C57BL/6 strain (Hahm et al., 2004) onto a BALB/C background by the original group for breeding purposes. Our initial findings established that these Deaf-1 knockout mice displayed a loss of the previously reported developmental phenotype such as excencephaly and in addition, exhibited comparable levels of 5-HT1A receptor gene expression with only a "trend" for decrease at the post-synaptic sites. Despite the fact that some knockout models such as the 5-HT1A knockout mice, produce similar phenotypes when generated on a variety of homogeneous background strains (Heisler et al., 1998; Parks et al., 1998), other mouse models report strain-related variability of data, particularly on mixed backgrounds (Gordon and Hen, 2004; Beaulieu et al., 2008; Carola et al., 2008). It appears that in the case of Deaf-1 knockout mice strain was also a factor, which prompted us to backcross the mice onto the original C57BL/6 mouse strain (Hahm et al., 2004). Upon further characterization, it became evident that lack of Deaf-1 protein alters 5-HT1A gene expression in a brain region-specific manner, as predicted by my studies in cell lines. More specifically, 5-HT1A mRNA and protein were both upregulated in the

raphe and downregulated in post-synaptic tissues including the cortex and dentate gyrus of the hippocampus, confirming the dual regulatory properties of Deaf-1. Interestingly enough, level of TPH2 RNA was not altered in these mice, suggesting Deaf-1 has a direct effect on fine-tuning the regulation of 5-HT1A but not altering other aspects of the serotonergic system. On the other hand, the intensity of 5-HT staining in pre-synaptic neurons was slightly decreased in Deaf-1 -/- mice, suggesting that although the number of serotonergic neurons has not changed, production of 5-HT may be reduced. Further experiments including HPLC analysis will provide a more quantitative measure of the level of 5-HT in the dorsal raphe tissue.

The Deaf-1 -/- mice provide a new model for studying the effects of Deaf-1 deficiency on the serotonergic system *in vivo,* that appears to generate a 5-HT1A phenotype mimicking that of humans with the C(-1019)G polymorphism and diagnosed with depression. A recent study has developed a mouse model which mimics the presynaptic effects of the human 5-HT1A C(-1019)G polymorphism (Richardson-Jones et al.). The authors of this study generate mice that express "high" or "low" levels of 5-HT1A gene expression by manipulating only the levels of 5-HT1A autoreceptors without affecting the heteroreceptors located at post-synaptic sites. Although the increase in presynaptic 5-HT1A receptor levels inhibited basal raphe firing, these mice lacked a detectable anxious phenotype, as previously reported for the full 5-HT1A knockout mice, indirectly implicating the post-synaptic 5-HT1A receptor as an important regulator of anxiety (Heisler et al., 1998; Parks et al., 1998; Ramboz et al., 1998; Gross et al., 2002). Interestingly,

elevated pre-synaptic 5-HT1A receptor levels observed in the 'high mice" resulted in decreased response to acute stress, increased behavioural despair (a hallmark phenotype of depression) and lack of antidepressant response, thus modeling patients with the 5-HT1A risk allele (Le François et al., 2008). It is likely that the Deaf-1 knockout mice, which display a similar molecular pattern of 5-HT1A autoreceptor over-expression, will exhibit depressive-like behaviours similar to the "high" mouse model. Additional experiments are required to establish a correlation between the lack of Deaf-1 protein expression and behavioural despair.

Human genetic studies are limited due to the restricted number of human samples available for testing. In addition, environmental, ethnic and regional differences make it difficult to obtain a large sample size with entirely matched controls. These considerations are particularly important in the case of depression, a heterogeneous disorder that is most likely affected by multiple loci in combination with potential environmental influences (Fava and Kendler, 2000). As an alternative, the mouse has served as an excellent tool for conducting genetic and environmental manipulations in order to study the effects on behaviour or drug treatment responses (Heisler et al., 1998; Palm et al., 1998; Olivier et al., 2001; Parsons et al., 2001; Groenink et al., 2003). A variety of behavioural tests have been designed to monitor anxiety-like and depressive behaviours in mice, and several of those can be utilized in future studies to correlate Deaf-1 protein deficiency with "depressive" or "anxiety"-like behaviour. The "tail-suspension test" relies on a hallmark behavioural phenotype of a depressed mouse that results in the loss of struggle observed when suspended by its tail (Steru et al., 1985; Cryan et al., 2005).

The "forced swim test" forces mice to swim in an inescapable cylinder of water, and a "depressive state" can be quantified by measuring the time spent in an immobile posture (Porsolt et al., 1979). This test is often accompanied by a pre-test to further induce a state of behavioural despair (i.e. loud noise, shaking), which will cause quicker immobility times during actual testing. The "elevated-plus maize" test (Ramboz et al., 1998) and the "novelty suppressed feeding" test (Overstreet et al., 2003) are additional behavioural paradigms which can be used to study levels of "anxiety" behaviour in Deaf-1 knockout mice. Moreover, each of the above mentioned tests can be combined with acute or chronic antidepressant treatment to determine the effects of gene mutations on drug response rates (Schramm et al., 2001; Santarelli et al., 2003). In this respect, the novelty suppressed feeding test is the most clinically relevant, since it responds to chronic antidepressant treatment only.

Strong pharmacological evidence suggests that selective desensitization of raphe 5-HT1A autoreceptors is a key adaptive change that contributes to the effectiveness of chronic antidepressant treatment. However, in humans, response to treatment occurs only in 67% of cases (Hirschfeld et al., 1997), while remission is even less frequent (30-40%) and this could be attributed in part to heritable factors (Lerer and Macciardi, 2002; Zanardi et al., 2001; Lemonde et al., 2004). In fact, several studies have already correlated genetic mutations such as the C(-1019)G polymorphism with a decreased response to anti-depressant treatments (Lemonde et al., 2004; Parsey et al., 2006a; Anttila et al., 2007; Kraus et al., 2007). In addition, mice overexpressing the pre-synaptic 5-HT1A receptor, and those lacking the gene

all together both fail to respond to antidepressant (SSRI) treatment (Richardson-Jones et al.; Santarelli et al., 2003). By establishing a depressive and/or anxious phenotype of the Deaf-1 knockout mouse, and then being able to address whether there is a decreased response to antidepressant treatment, we will be able to address whether Deaf-1 is a key factor in the MDD phenotype, and validate whether it is also required for effective antidepressant response. If the Deaf-1 -/- mice are antidepressant resistant, this may provide a useful model to test novel treatment approaches for treatment-resistant depression.

A possibility exists, that the signaling cascades involved in 5-HT1A signaling pathways may also be altered in Deaf-1 knockout mice, thus affecting 5-HT1A gene expression. Recent studies have demonstrated that chronic antidepressant therapy upregulates the cAMP cascade in hippocampus and cerebral cortex, increasing the levels of PKA and upregulating the function and expression of CREB (Popoli et al., 2001). As such, inhibition of cAMP and PKA signaling following activation of 5-HT1A autorereceptors in Deaf-1 knockout mice may lead to cellular changes in additional proteins that modulate 5-HT1A gene transcription. Further investigation using PKA or PKC activators and inhibitors will be necessary to address this possibility.

The role of antidepressant treatment in adult neurogenesis has been an important but controversial topic in recent years. It is now established that behavioural effects of chronic antidepressants require hippocampal neurogenesis and are mediated by an increased synaptic plasticity in the dentate gyrus (Santarelli et al., 2003; Sahay and Hen, 2007). Oppositely, antidepressant treatments have the potential to reverse loss of neurons in the prefrontal cortex and the hippocampus of

depressed and anxious individuals (Sheline et al., 1996) establishing a level of co-dependency between neurogenesis and depression treatment. However, genetic predisposition can play a key role in decreasing neurogenesis as in the case of the BDNF Val(66)Met mutant mouse model (Chen et al., 2006). As such, it would be very interesting to assess the effect of Deaf-1 protein deficiency on the development of new neurons in the hippocampus in the Deaf-1 knockout model, something that has never been addressed in humans harboring the 5-HT1A C(-1019)G polymorphism.

In an alternative approach, it would be interesting to re-visit the behavioural studies and build on previous findings that suggest expression of the 5-HT1A receptor at an early post-natal period is crucial for a normal anxiety phenotype in the adult (Gross et al., 2002; Lo Iacono and Gross, 2008). As an example, the lack of Deaf-1 protein may have an effect on the response to early post-natal stress, especially since the 5-HT1A receptor is a major regulator of the HPA axis (Hanley and Van de Kar, 2003). Mice can be subjected to maternal deprivation and the effect of the resulting glucocorticoid up-regulation can be studied at an adult stage (McEwen, 1999; Liu et al., 2000; Meaney, 2001). In fact, pre-disposition to depression has already been associated with early life stress and the 5-HTT polymorphism (Caspi et al., 2003), and there is some evidence for 5-HT1A C(-1019)G genotype/environment interaction in suicide completion (Videtic et al., 2006; Videtic et al., 2009). Thus, it is possible that loss of Deaf-1 protein may influence the developmental regulation of 5-HT1A gene expression leading to increased pre-disposition to depression especially when combined with elevated stress at an early stage in post-natal development.

One major drawback of studies correlating depression with a single genetic variation, such as the C(-1019)G polymorphism, is the heterogeneity of the disorder that is most likely to involve small contributions from multiple genetic variations. 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. For instance, the T(-102)C polymorphism in the 5-HT2A receptor has been associated suicidal thoughts in depressed subjects (Du et al., 2000) and 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). As such, other available mouse models may become useful tools for studying the additive effects of several genetic factors on regulation of the serotonerigic system in the Deaf-1 knockout mice. In addition to full knockout mouse models, including Pet-1, SERT, TPH2, Lmx1b, and various 5-HT-receptor subtype knockouts, mice that harbor genetic variations including Val(66)Met mutation in the BDNF gene (Chen et al., 2006), C(1473)G mutation in the TPH2 gene (Cervo et al., 2005; Osipova et al., 2009) or the R(441)H mutation in the TPH2 gene (Beaulieu et al., 2008) can also serve as excellent models for crossing. As a result, the added effects of multiple genetic abnormalities on the regulation of the serotonergic system can be studied.

In conclusion, the cell specific properties of Deaf-1 mediated transcriptional regulation of the 5-HT1A receptor observed in cell lines and in the Deaf-1-null mice provides a new model that could account for brain region specific alterations in 5-

HT1A receptor expression and serotonergic neurotransmission implicated in depression and anxiety disorders. 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 both *in vitro* and *in vivo* analysis of the 5-HT1A receptor regulation, and the implication of the C(-1019)G functional polymorphism as an important regulator responsible for "fine-tuning" the regulation of the 5-HT1A receptor both pre- and post-synaptically. Additionally, the *in vivo* work presented in this thesis correlates the loss of Deaf-1 protein with abnormal 5-HT1A gene expression. This molecular phenotype is similar to that observed with human depressed subjects harboring the C(1019)G polymorphism and may provide a potential new mouse model for studying the effects of genetic predisposition. This is also the first study to identify a potential Deaf-1 binding site in the mouse and implicate Deaf-1 as a direct transcriptional regulator of the mouse 5-HT1A gene. Finally, my pioneering studies on the characterization of Deaf-1 and its associated binding proteins may lead to new insights into the transcriptional regulatory mechanisms involved in the predisposition to mental illness which may stimulate research into analysis of these mechanisms in other candidate genes relevant to mental illness.

APPENDICES

APPENDIX A — COPYRIGHT PERMISSION

ELSEVIER LICENSE
TERMS AND CONDITIONS

Mar 31, 2010

This is a License Agreement between Margaret Czesak ("You") and Elsevier ("Elsevier") provided by Copyright Clearance Center ("CCC") The license consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

Supplier	Elsevier Limited The Boulevard,Langford Lane Kidlington,Oxford,OX5 1GB,UK
Registered Company Number	1982084
Customer name	Margaret Czesak
Customer address	421 Vendevale Avenue Ottawa, ON K2S0M6
License Number	2399691502800
License date	Mar 31, 2010
Licensed content publisher	Elsevier
Licensed content publication	Journal of Neuroscience Methods
Licensed content title	Characterization of rat rostral raphe primary cultures Multiplex quantification of serotonergic markers
Licensed content author	Margaret Czesak, Ariel M Burns, Federico Remes Lenicov, Paul R Albert
Licensed content date	15 August 2007
Volume number	
Issue number	
Pages	0
Type of Use	Thesis / Dissertation
Portion	Full article
Format	Both print and electronic
You are an author of the Elsevier article	Yes
Are you translating?	No
Order Reference Number	

PERMISSION INVOICE

Inv. # P03J 18112

April 9, 2010

Margaret Czesak

CAMBRIDGE UNIVERSITY PRESS

REFERENCE

Journal:	International Journal of Neuropsychopharmacoly, 2009 Mar;12(2):155-68
Author:	Margaret Czesak et al.
Title:	Gender-Specific Decrease in NUDR and 5-HT1A Receptor Proteins in the Prefrontal Cortex of Subjects with
Selection/pp.:	figure

Additional

USE

Reprint Title:	Transcriptional Regulation of the 5-HT1A Receptor
Publisher:	University of Ottawa, Ontario, Canada
Format:	dissertation / thesis
Quantity (Limit*):	n.a.
Avail. Date:	2010

RIGHTS/ACKNOWLEDGEMENT

Permission is granted for nonexclusive rights throughout the World in the English language for interior text editorial use in the format described above only. Please fully acknowledge our material and indicate the copyright notice as it appears in our publication, followed by the phrase "Reprinted with the permission of Cambridge University Press."

All requests from third parties to reproduce this material must be forwarded to Cambridge University Press.

FEES/RESTRICTIONS

$0.00

*You must re-apply for permission if this print run is exceeded. This permission is restricted to the indicated format and excludes reproduction in any other medium; for additional use, you must apply for permission separately. This permission does not allow reprinting of any material copyrighted by or credited in our publication to another source; Cambridge disclaims all liability in connection with the use of such material without proper consent. A COPY OF THIS INVOICE MUST ACCOMPANY PAYMENT. Payment is due upon publication or within 12 months, whichever is sooner. Make check payable to Cambridge University Press, Attn: Rights and Permissions. (CUP Fed. I.D. #: 13 -1599108.)

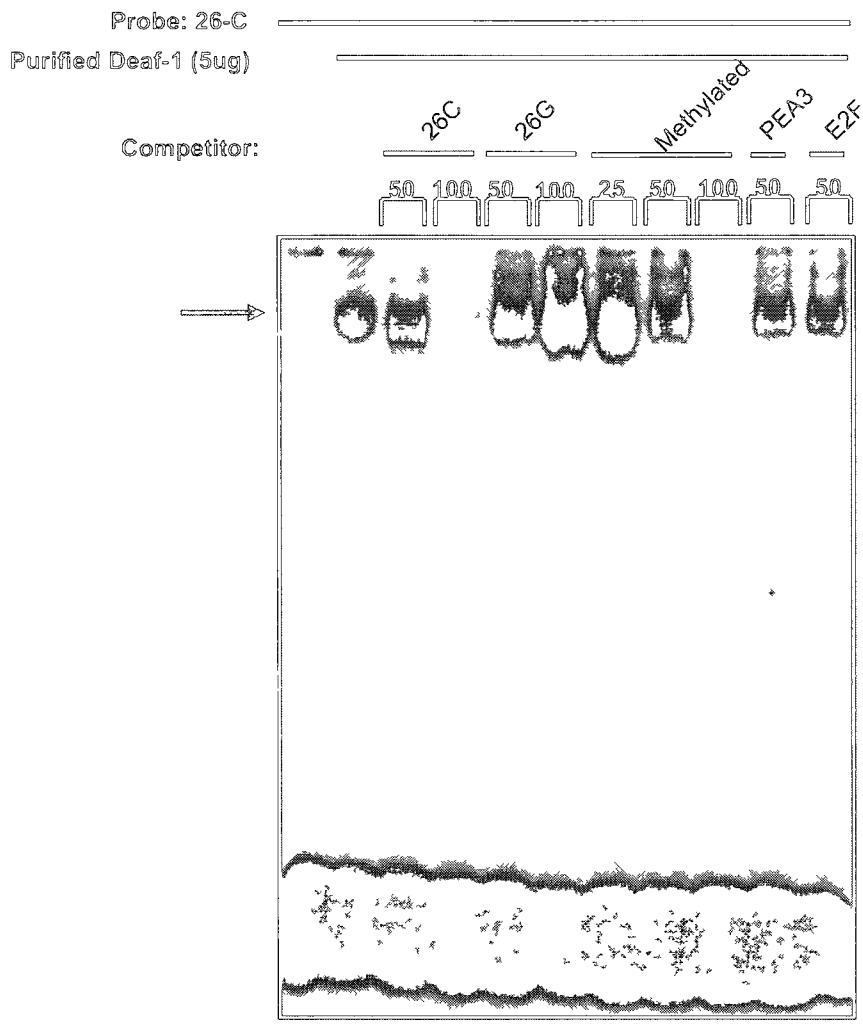
This permission does not supersede permission that may be required from the original source indicated in our publication.

This permission requires that you send zero (0) copies of your publication directly to our author and zero (0) copy of your publication to this office upon availability.

Authorization: _____

Adam Hirschberg
Rights and Permissions Associate

APPENDIX B

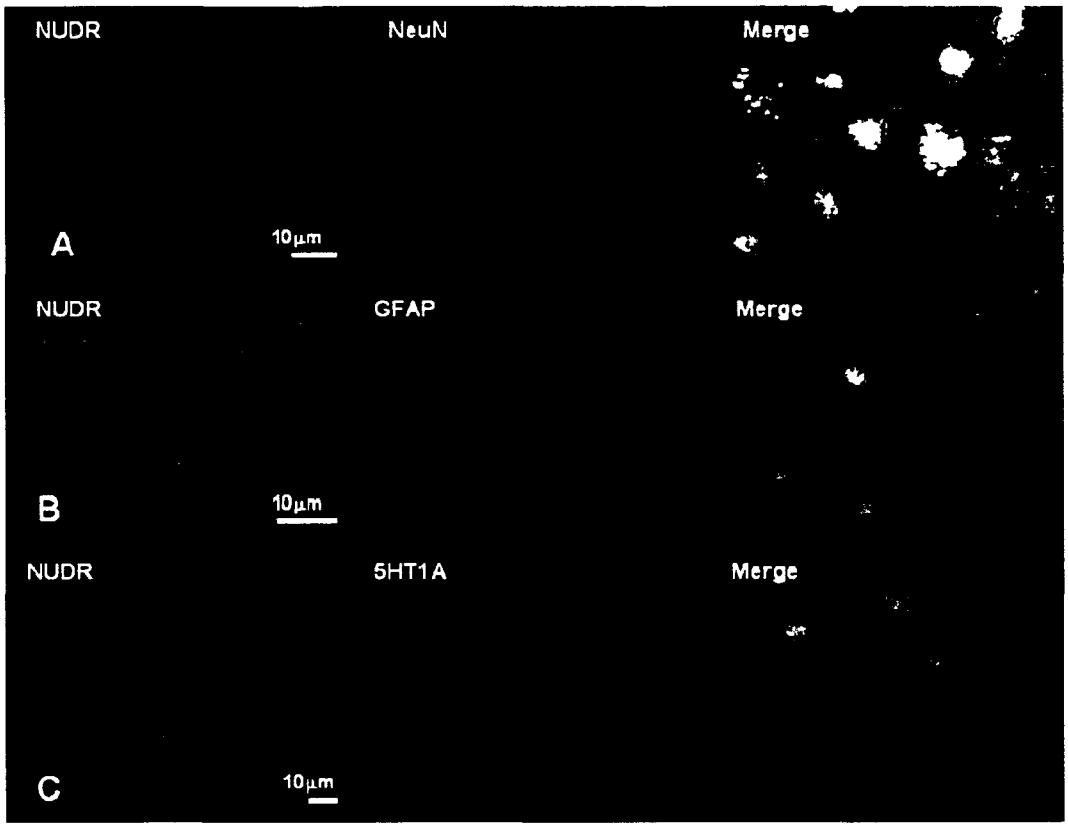


B-Figure 6.1: Effect of 5-HT1A promoter methylation on Deaf-1 binding.

Electronic Mobility Shift Assay shows no difference in binding affinity of purified Deaf-1 protein to the methylated 26-bp human promoter sequence versus the 26-bp promoter sequence that is not methylated (26C) Each sequence competes out the labeled probe with equal affinity as compared to the 26-bp sequence containing the polymorphism (26G), which does not compete out Deaf-1 PEA3 and E2F are used as non-specific competitors

Methods EMSA protocol was performed as described in the materials and methods section of Chapter 4

APPENDIX C

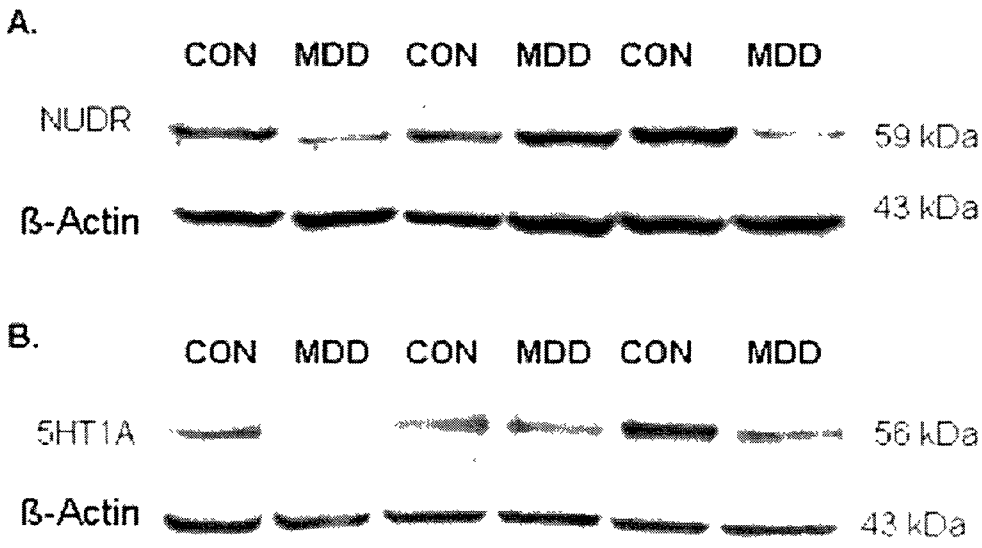


C – Figure 6.2: Colocalization of NUDR immunoreactivity in neurons and glia

A) Co-labeling of NUDR immunoreactivity (green) with the neuronal marker NeuN (red) NUDR protein (yellow) is localized to neuronal nuclei and perinuclear areas B) Co-labeling of NUDR immunoreactivity (green) with the astrocytic marker GFAP (red) NUDR (yellow) is localized in glial nuclei C) Colocalization of NUDR immunoreactivity (green) and immunoreactivity for HT1A receptor (red) NUDR immunoreactivity (yellow) is present in nuclei of majority of cells expressing 5-HT1A receptor immunoreactivity NUDR=Deaf-1 Copyright Szewczyk et al , 2009, Cambridge Press

Methods: Immunohistochemistry was used to examine the expression and laminar distribution of Deaf-1 in the human PFC of normal control subjects Immunostaining was performed as described in Szewczyk et al , 2009, using primary antibody characterized in Lemonde et al , 2003 and Czesak et al , 2006

APPENDIX D

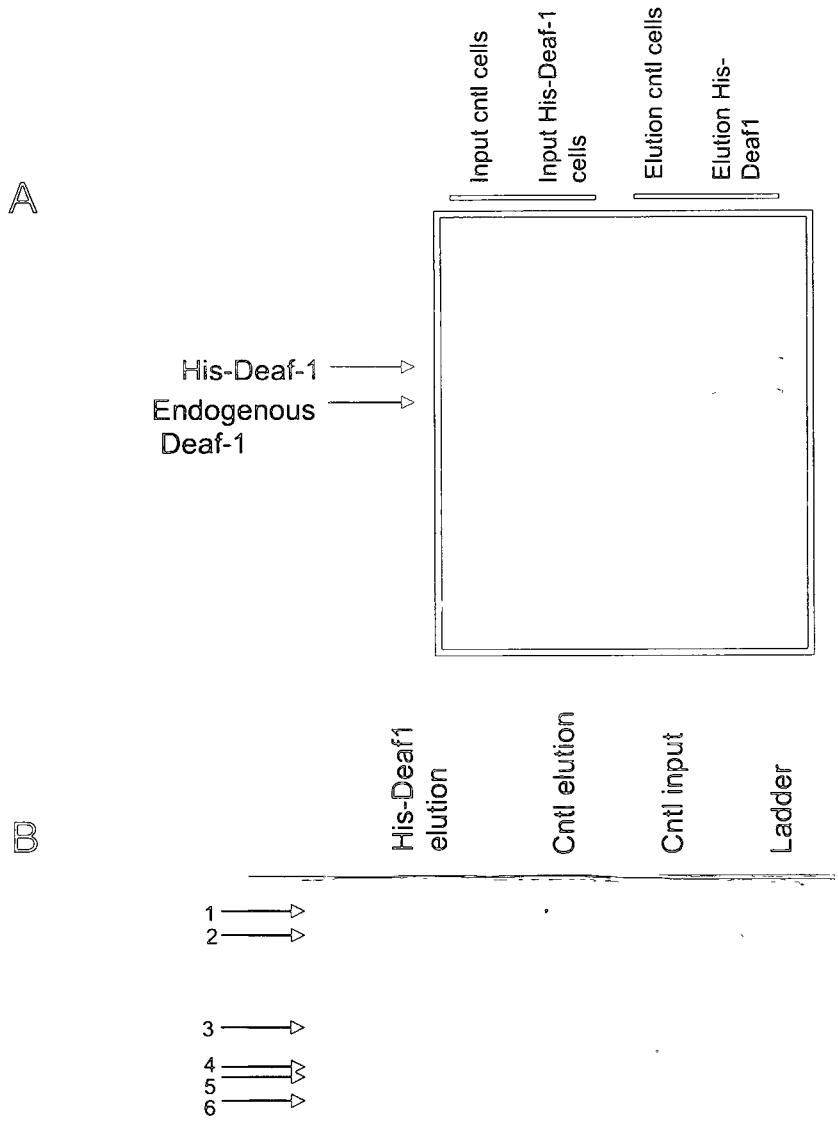


D – Figure 6.3 NUDR and 5-HT1A protein expression in human prefrontal cortex.

Representative Western blots showing the immunolabeling of Deaf-1/NUDR (A) and 5-HT1A receptor (B) and β -actin in prefrontal cortex (PFC) of 3 pairs of control and matched MDD subjects. Copyright Szewczyk et al., 2009, Cambridge Press.

Methods: Western blot was used to examine the protein expression of Deaf-1 in the human PFC of normal control and matched MDD subjects. Western blot was performed as described in Szewczyk et al., 2009, using primary antibody characterized in Lemonde et al., 2003 and Czesak et al., 2006.

APPENDIX E


A
Input cntl cells
Input His-Deaf-1 cells
Elution cntl cells
Elution His-Deaf1
His-Deaf-1
Endogenous Deaf-1
B
His-Deaf1 elution
Cntl elution
Cntl input
Ladder
1
2
3
4
5
6

E – Figure 6.4: Immunoprecipitation of His-Tagged Deaf-1 from Hek-293 cells.

A) Western blot showing His-tagged Deaf-1 successfully Immunoprecipitated from Hek-293 using His-Pur Cobalt bead technology. Custom-made rabbit anti-Deaf-1 antibody was used to detect protein signal. Input lanes show Hek-293 cell lysate with or without His-Deaf-1 transfected. Elution lanes show transiently transfected empty pcDNA3 construct lacking Deaf-1 or pcDNA construct with His-tagged Deaf-1 sequence, post immunoprecipitation with cobalt beads. B) Coomassie staining of SDS-PAGE shows "His-Deaf-1 elution" lane with all protein bands immunoprecipitated onto gel. "Cntl input" lane shows Hek-293 cell lysate and "cntl elution" shows transiently transfected empty pcDNA3 vector. Bands indicated by numbers were cut from gel and analyzed via MALDI-TOF spectroscopy.

Methods: Custom made Deaf-1 primary antibody was used at a dilution of 1·2000 as characterized in the materials and methods section of Chapter 4. His-Pur Cobalt beads were used according to company protocol (Pierce Inc). Briefly, Hek-293 cells were transiently transfected with His-tagged Deaf-1 in pcDNA3 vector for 48 hours. Empty pcDNA3 vector was transiently transfected as control. Whole cell lysates where collected in RIPPA buffer with protease inhibitors. Lysate was diluted 10x and pre-cleared with bead slurry for 30 minutes. Lysate was incubated with 50µl of HisPur beads at room temperature for 2 hours. Beads were washed and immunocomplexes dissociated by adding elution buffer and boiling for 5 min. 25ul of supernatant was run on SDS-PAGE and stained using Cooomassie or transferred onto nitrocellose membrane for Western blot analysis.

APPENDIX F

Table 6.1: MALDI-TOF Mass Spectrometry.

Highlighted (red) proteins are potential Deaf-1 interacting partners. All target proteins shown in this table are unique targets, never seen in control experiments. Proteins that are omitted from chart are those that have been identified in control IP's, chromatin associated or found in Sepharose Bead Proteome.

Type of Stain Band Type Band Size	Protein Identified	Molecular Weight of Protein	# of peptides
Coomassie Stain Band #1 200 kDa	• Deaf-1 • **BRD4 bromodomain 4** • CD2L7 cell division cycle 2-related protein kinase 7 • TBR1 T brain protein	60 kDa 152 kDa 164 kDa 74 kDa	13 5 3 1
Coomassie Stain Band #2 180-200 kDa	• DOCK7 dedicator of cytokinesis protein 7 • **BRD4 human bromodomain 4** • CD2L7 cell division cycle 2-related protein kinase 7	242 kDa 152 kDa 164 kDa	14 6 3
Coomassie Stain Band #3 80 kDa	• Deaf-1 (transfected) • **NUFP2 nuclear fragile x mental retardation interacting partner protein 2** • **TLE transducin-like enhancer of split 3**	80 kDa 76 kDa 83 kDa	39 10 2
Coomassie Stain Band #4 60 kDa (upper band)	• Deaf-1 • Q5PU80 protein phosphatase 2A • ZKSC1 zincfinger protein	60 kDa 61 kDa 63 kDa	8 3 2
Coomassie Stain Band #5 60 kDa (lower band)	• Deaf-1 • **P66B transcriptional repressor (MeCP2 complex)** • NRBP nuclear receptor binding protein • TF65 transcription factor p65 • SMAD4 tumor repressor • ADA4ZD insulin like growth factor 2 mRNA binding protein 2	60 kDa 65 kDa 59 kDa 60 kDa 60 kDa 65 kDa	10 3 2 1 1 1
Coomassie Stain Band #6 50 kDa	• **HDAC2 histone deacetylase 2** • SMAD4 tumor repressor • **REST transcription factor** • IF2B1 insulin growth factor 2 mRNA binding protein 1	55 kDa 60 kDa 53 kDa 63 kDa	2 1 1 1

REFERENCES

Akazawa C, Sasai Y, Nakanishi S, Kageyama R (1992) Molecular characterization of a rat negative regulator with a basic helix-loop-helix structure predominantly expressed in the developing nervous system. J Biol Chem 267:21879-21885.

Albert PR, Lemonde S (2004) 5-HT1A Receptors, Gene Repression, and Depression: Guilt by Association. Neuroscientist 10:575-593.

Albert PR, Zhou QY, Van Tol HH, Bunzow JR, Civelli O (1990) Cloning, functional expression, and mRNA tissue distribution of the rat 5-hydroxytryptamine1A receptor gene. J Biol Chem 265:5825-5832.

Albert PR, Lembo P, Storring JM, Charest A, Saucier C (1996) The 5-HT1A receptor: signaling, desensitization, and gene transcription. Neuropsychopharmacology 14:19-25.

Albert PR, Morris SJ, Ghahremani MH, Storring JM, Lembo PM (1998) A putative alpha-helical G beta gamma-coupling domain in the second intracellular loop of the 5-HT1A receptor. Ann N Y Acad Sci 861:146-161.

Alexandre C, Popa D, Fabre V, Bouali S, Venault P, Lesch KP, Hamon M, Adrien J (2006) Early life blockade of 5-hydroxytryptamine 1A receptors normalizes sleep and depression-like behavior in adult knock-out mice lacking the serotonin transporter. J Neurosci 26:5554-5564.

Amin AH, Crawford TB, Gaddum JH (1954) The distribution of substance P and 5-hydroxytryptamine in the central nervous system of the dog. J Physiol 126:596-618.

Anttila S, Huuhka K, Huuhka M, Rontu R, Hurme M, Leinonen E, Lehtimaki T (2007) Interaction between 5-HT1A and BDNF genotypes increases the risk of treatment-resistant depression. J Neural Transm 114:1065-1068.

Arango V, Underwood MD, Boldrini M, Tamir H, Kassir SA, Hsiung S, Chen JJ, Mann JJ (2001) Serotonin 1A receptors, serotonin transporter binding and serotonin transporter mRNA expression in the brainstem of depressed suicide victims. Neuropsychopharmacology 25:892-903.

Arias B, Catalan R, Gasto C, Gutierrez B, Fananas L (2005a) Evidence for a combined genetic effect of the 5-HT(1A) receptor and serotonin transporter genes in the clinical outcome of major depressive patients treated with citalopram. J Psychopharmacol 19:166-172.

Arias B, Catalan R, Gasto C, Gutierrez B, Fananas L (2005b) Evidence for a combined genetic effect of the 5-HT1A receptor and serotonin transporter genes in the clinical outcome of major depressive patients treated with citalopram. J Psychopharmacol 19:166-172.

Arias B, Arranz MJ, Gasto C, Catalan R, Pintor L, Gutierrez B, Kerwin RW, Fananas L (2002) Analysis of structural polymorphisms and C-1018G promoter variant of the 5-HT(1A) receptor gene as putative risk factors in major depression. Mol Psychiatry 7:930-932.

Artigas F, Perez V, Alvarez E (1994) Pindolol induces a rapid improvement of depressed patients treated with serotonin reuptake inhibitors. Arch Gen Psychiatry 51:248-251.

Artigas F, Romero L, de Montigny C, Blier P (1996) Acceleration of the effect of selected antidepressant drugs in major depression by 5-HT1A antagonists. Trends Neurosci 19:378-383.

Austin MC, Whitehead RE, Edgar CL, Janosky JE, Lewis DA (2002) Localized decrease in serotonin transporter-immunoreactive axons in the prefrontal cortex of depressed subjects committing suicide. Neuroscience 114:807-815.

Azizkhan JC, Jensen DE, Pierce AJ, Wade M (1993) Transcription from TATA-less promoters: dihydrofolate reductase as a model. Crit Rev Eukaryot Gene Expr 3:229-254.

Bae S, Bessho Y, Hojo M, Kageyama R (2000) The bHLH gene Hes6, an inhibitor of Hes1, promotes neuronal differentiation. Development 127:2933-2943.

Baker KG, Halliday GM, Tork I (1990) Cytoarchitecture of the human dorsal raphe nucleus. J Comp Neurol 301:147-161.

Barker HE, Smyth GK, Wettenhall J, Ward TA, Bath ML, Lindeman GJ, Visvader JE (2008) Deaf-1 regulates epithelial cell proliferation and side-branching in the mammary gland. BMC Dev Biol 8:94.

Barnes NM, Sharp T (1999) A review of central 5-HT receptors and their function. Neuropharmacology 38:1083-1152.

Basel-Vanagaite L, Attia R, Yahav M, Ferland RJ, Anteki L, Walsh CA, Olender T, Straussberg R, Magal N, Taub E, Drasinover V, Alkelai A, Bercovich D, Rechavi G, Simon AJ, Shohat M (2006) The CC2D1A, a member of a new gene family

with C2 domains, is involved in autosomal recessive non-syndromic mental retardation. J Med Genet 43:203-210.

Beaulieu JM, Zhang X, Rodriguiz RM, Sotnikova TD, Cools MJ, Wetsel WC, Gainetdinov RR, Caron MG (2008) Role of GSK3 beta in behavioral abnormalities induced by serotonin deficiency. Proc Natl Acad Sci U S A 105:1333-1338.

Bengel D, Murphy DL, Andrews AM, Wichems CH, Feltner D, Heils A, Mossner R, Westphal H, Lesch KP (1998) Altered brain serotonin homeostasis and locomotor insensitivity to 3, 4-methylenedioxymethamphetamine ("Ecstasy") in serotonin transporter-deficient mice. Mol Pharmacol 53:649-655.

Bennett JL, Aghajanian GK (1974) d-LSD binding to brain homogenates: possible relationship to serotonin receptors. Life Sci 15:1935-1944.

Benninghoff J, Gritti A, Rizzi M, Lamorte G, Schloesser RJ, Schmitt A, Robel S, Genius J, Moessner R, Riederer P, Manji HK, Grunze H, Rujescu D, Moeller HJ, Lesch KP, Vescovi AL Serotonin depletion hampers survival and proliferation in neurospheres derived from adult neural stem cells. Neuropsychopharmacology 35:893-903.

Benovic JL, Strasser RH, Caron MG, Lefkowitz RJ (1986) Beta-adrenergic receptor kinase: identification of a novel protein kinase that phosphorylates the agonist-occupied form of the receptor. Proc Natl Acad Sci U S A 83:2797-2801.

Bessis A, Champtiaux N, Chatelin L, Changeux JP (1997) The neuron-restrictive silencer element: a dual enhancer/silencer crucial for patterned expression of a nicotinic receptor gene in the brain. Proc Natl Acad Sci U S A 94:5906-5911.

Birmingham DJ, Nagaraja HN, Rovin BH, Spetie L, Zhao Y, Li X, Hackshaw KV, Yu CY, Malarkey WB, Hebert LA (2006) Fluctuation in self-perceived stress and increased risk of flare in patients with lupus nephritis carrying the serotonin receptor 1A -1019 G allele. Arthritis Rheum 54:3291-3299.

Blier P, de Montigny C (1985) Serotoninergic but not noradrenergic neurons in rat central nervous system adapt to long-term treatment with monoamine oxidase inhibitors. Neuroscience 16:949-955.

Blier P, de Montigny C (1990) Differential effect of gepirone on presynaptic and postsynaptic serotonin receptors: single-cell recording studies. J Clin Psychopharmacol 10:13S-20S.

Blier P, de Montigny C (1994) Current advances and trends in the treatment of depression. Trends Pharmacol Sci 15:220-226.

Blier P, Bergeron R (1995) Effectiveness of pindolol with selected antidepressant drugs in the treatment of major depression. J Clin Psychopharmacol 15:217-222.

Blier P, Ward NM (2003) Is there a role for 5-HT(1A) agonists in the treatment of depression? Biol Psychiatry 53:193-203.

Blier P, de Montigny C, Chaput Y (1990) A role for the serotonin system in the mechanism of action of antidepressant treatments: preclinical evidence. J Clin Psychiatry 51 Suppl:14-20; discussion 21.

Boldrini M, Underwood MD, Mann JJ, Arango V (2008) Serotonin-1A autoreceptor binding in the dorsal raphe nucleus of depressed suicides. J Psychiatr Res 42:433-442.

Bottomley MJ, Collard MW, Huggenvik JI, Liu Z, Gibson TJ, Sattler M (2001) The SAND domain structure defines a novel DNA-binding fold in transcriptional regulation. Nat Struct Biol 8:626-633.

Bourne HR, Sanders DA, McCormick F (1991) The GTPase superfamily: conserved structure and molecular mechanism. Nature 349:117-127.

Bouwman P, Philipsen S (2002) Regulation of the activity of Sp1-related transcription factors. Mol Cell Endocrinol 195:27-38.

Bradley PB, Engel G, Feniuk W, Fozard JR, Humphrey PP, Middlemiss DN, Mylecharane EJ, Richardson BP, Saxena PR (1986) Proposals for the classification and nomenclature of functional receptors for 5-hydroxytryptamine. Neuropharmacology 25:563-576.

Briscoe J, Sussel L, Serup P, Hartigan-O'Connor D, Jessell TM, Rubenstein JL, Ericson J (1999) Homeobox gene Nkx2.2 and specification of neuronal identity by graded Sonic hedgehog signalling. Nature 398:622-627.

Brown P, Molliver ME (2000) Dual serotonin (5-HT) projections to the nucleus accumbens core and shell: relation of the 5-HT transporter to amphetamine-induced neurotoxicity. J Neurosci 20:1952-1963.

Bruss M, Kostanian A, Bonisch H, Gothert M (2005) The naturally occurring Arg219Leu variant of the human 5-HT1A receptor: impairment of signal transduction. Pharmacogenet Genomics 15:257-264.

Bruss M, Buhlen M, Erdmann J, Gothert M, Bonisch H (1995) Binding properties of the naturally occurring human 5-HT1A receptor variant with the Ile28Val substitution in the extracellular domain. Naunyn Schmiedebergs Arch Pharmacol 352:455-458.

Buhot MC (1997) Serotonin receptors in cognitive behaviors. Curr Opin Neurobiol 7:243-254.

Bulchand S, Subramanian L, Tole S (2003) Dynamic spatiotemporal expression of LIM genes and cofactors in the embryonic and postnatal cerebral cortex. Dev Dyn 226:460-469.

Burnet PW, Eastwood SL, Lacey K, Harrison PJ (1995) The distribution of 5-HT1A and 5-HT2A receptor mRNA in human brain. Brain Res 676:157-168.

Carola V, Frazzetto G, Pascucci T, Audero E, Puglisi-Allegra S, Cabib S, Lesch KP, Gross C (2008) Identifying molecular substrates in a mouse model of the serotonin transporter x environment risk factor for anxiety and depression. Biol Psychiatry 63:840-846.

Carr RR, Ensom MH (2002) Fluoxetine in the treatment of premenstrual dysphoric disorder. Ann Pharmacother 36:713-717.

Cases O, Seif I, Grimsby J, Gaspar P, Chen K, Pournin S, Muller U, Aguet M, Babinet C, Shih JC, et al. (1995) Aggressive behavior and altered amounts of brain serotonin and norepinephrine in mice lacking MAOA. Science 268:1763-1766.

Caspi A, Sugden K, Moffitt TE, Taylor A, Craig IW, Harrington H, McClay J, Mill J, Martin J, Braithwaite A, Poulton R (2003) Influence of life stress on depression: moderation by a polymorphism in the 5-HTT gene. Science 301:386-389.

Ceci A, Baschirotto A, Borsini F (1994) The inhibitory effect of 8-OH-DPAT on the firing activity of dorsal raphe serotoninergic neurons in rats is attenuated by lesion of the frontal cortex. Neuropharmacology 33:709-713.

Celada P, Puig MV, Casanovas JM, Guillazo G, Artigas F (2001) Control of dorsal raphe serotonergic neurons by the medial prefrontal cortex: Involvement of serotonin-1A, GABA(A), and glutamate receptors. J Neurosci 21:9917-9929.

Cervo L, Canetta A, Calcagno E, Burbassi S, Sacchetti G, Caccia S, Fracasso C, Albani D, Forloni G, Invernizzi RW (2005) Genotype-dependent activity of tryptophan hydroxylase-2 determines the response to citalopram in a mouse model of depression. J Neurosci 25:8165-8172.

Chanda PK, Minchin MC, Davis AR, Greenberg L, Reilly Y, McGregor WH, Bhat R, Lubeck MD, Mizutani S, Hung PP (1993) Identification of residues important

for ligand binding to the human 5-hydroxytryptamine1A serotonin receptor. Mol Pharmacol 43:516-520.

Chaput Y, de Montigny C, Blier P (1991) Presynaptic and postsynaptic modifications of the serotonin system by long-term administration of antidepressant treatments. An in vivo electrophysiologic study in the rat. Neuropsychopharmacology 5:219-229.

Charest A, Wainer BH, Albert PR (1993) Cloning and differentiation-induced expression of a murine serotonin1A receptor in a septal cell line. J Neurosci 13:5164-5171.

Chen H, Ovchinnikov D, Pressman CL, Aulehla A, Lun Y, Johnson RL (1998) Multiple calvarial defects in lmx1b mutant mice. Dev Genet 22:314-320.

Chen Y, Penington NJ (1997) QEHA27, a peptide that binds to G-protein beta gamma-subunits, reduces the inhibitory effect of 5-HT on the Ca2+ current of rat dorsal raphe neurons. Neurosci Lett 224:87-90.

Chen ZY, Jing D, Bath KG, Ieraci A, Khan T, Siao CJ, Herrera DG, Toth M, Yang C, McEwen BS, Hempstead BL, Lee FS (2006) Genetic variant BDNF (Val66Met) polymorphism alters anxiety-related behavior. Science 314:140-143.

Cheng L, Chen CL, Luo P, Tan M, Qiu M, Johnson R, Ma Q (2003) Lmx1b, Pet-1, and Nkx2.2 coordinately specify serotonergic neurotransmitter phenotype. J Neurosci 23:9961-9967.

Christensen J, Cotmore SF, Tattersall P (1999) Two new members of the emerging KDWK family of combinatorial transcription modulators bind as a

heterodimer to flexibly spaced PuCGPy half-sites. Mol Cell Biol 19:7741-7750.

Cote F, Fligny C, Bayard E, Launay JM, Gershon MD, Mallet J, Vodjdani G (2007) Maternal serotonin is crucial for murine embryonic development. Proc Natl Acad Sci U S A 104:329-334.

Cote F, Thevenot E, Fligny C, Fromes Y, Darmon M, Ripoche MA, Bayard E, Hanoun N, Saurini F, Lechat P, Dandolo L, Hamon M, Mallet J, Vodjdani G (2003) Disruption of the nonneuronal tph1 gene demonstrates the importance of peripheral serotonin in cardiac function. Proc Natl Acad Sci U S A 100:13525-13530.

Cowen DS, Molinoff PB, Manning DR (1997) 5-hydroxytryptamine1A receptor-mediated increases in receptor expression and activation of nuclear factor-kappaB in transfected Chinese hamster ovary cells. Mol Pharmacol 52:221-226.

Craven SE, Lim KC, Ye W, Engel JD, de Sauvage F, Rosenthal A (2004) Gata2 specifies serotonergic neurons downstream of sonic hedgehog. Development 131:1165-1173.

Cryan JF, Mombereau C, Vassout A (2005) The tail suspension test as a model for assessing antidepressant activity: review of pharmacological and genetic studies in mice. Neurosci Biobehav Rev 29:571-625.

Culpepper L, Kovalick LJ (2008) A review of the literature on the selegiline transdermal system: an effective and well-tolerated monoamine oxidase

inhibitor for the treatment of depression. Prim Care Companion J Clin Psychiatry 10:25-30.

Czesak M, Lemonde S, Peterson EA, Rogaeva A, Albert PR (2006) Cell-specific repressor or enhancer activities of Deaf-1 at a serotonin 1A receptor gene polymorphism. J Neurosci 26:1864-1871.

Dahlstrom A, Fuxe K (1964) Localization of monoamines in the lower brain stem. Experientia 20:398-399.

Dannlowski U, Ohrmann P, Bauer J, Kugel H, Baune BT, Hohoff C, Kersting A, Arolt V, Heindel W, Deckert J, Suslow T (2007) Serotonergic genes modulate amygdala activity in major depression. Genes Brain Behav 6:672-676.

David SP, Murthy NV, Rabiner EA, Munafo MR, Johnstone EC, Jacob R, Walton RT, Grasby PM (2005) A functional genetic variation of the serotonin (5-HT) transporter affects 5-HT1A receptor binding in humans. J Neurosci 25:2586-2590.

de Montigny C, Aghajanian GK (1978) Tricyclic antidepressants: long-term treatment increases responsivity of rat forebrain neurons to serotonin. Science 202:1303-1306.

Deecher DC, Beyer CE, Johnston G, Bray J, Shah S, Abou-Gharbia M, Andree TH (2006) Desvenlafaxine succinate: A new serotonin and norepinephrine reuptake inhibitor. J Pharmacol Exp Ther 318:657-665.

Ding YQ, Marklund U, Yuan W, Yin J, Wegman L, Ericson J, Deneris E, Johnson RL, Chen ZF (2003) Lmx1b is essential for the development of serotonergic neurons. Nat Neurosci 6:933-938.

Dohlman HG, Thorner J, Caron MG, Lefkowitz RJ (1991) Model systems for the study of seven-transmembrane-segment receptors. Annu Rev Biochem 60:653-688.

Domschke K, Braun M, Ohrmann P, Suslow T, Kugel H, Bauer J, Hohoff C, Kersting A, Engelien A, Arolt V, Heindel W, Deckert J (2006) Association of the functional -1019C/G 5-HT1A polymorphism with prefrontal cortex and amygdala activation measured with 3 T fMRI in panic disorder. Int J Neuropsychopharmacol 9:349-355.

Doris A, Ebmeier K, Shajahan P (1999) Depressive illness. Lancet 354:1369-1375.

Drago A, Ronchi DD, Serretti A (2007) 5-HT1A gene variants and psychiatric disorders: a review of current literature and selection of SNPs for future studies. Int J Neuropsychopharmacol:1-21.

Drevets WC, Frank E, Price JC, Kupfer DJ, Greer PJ, Mathis C (2000) Serotonin type-1A receptor imaging in depression. Nucl Med Biol 27:499-507.

Drevets WC, Thase ME, Moses-Kolko EL, Price J, Frank E, Kupfer DJ, Mathis C (2007) Serotonin-1A receptor imaging in recurrent depression: replication and literature review. Nucl Med Biol 34:865-877.

Du L, Bakish D, Lapierre YD, Ravindran AV, Hrdina PD (2000) Association of polymorphism of serotonin 2A receptor gene with suicidal ideation in major depressive disorder. Am J Med Genet 96:56-60.

Dumas S, Darmon MC, Delort J, Mallet J (1989) Differential control of tryptophan hydroxylase expression in raphe and in pineal gland: evidence for a role of translation efficiency. J Neurosci Res 24:537-547.

Edelman DB, Meech R, Jones FS (2000) The homeodomain protein Barx2 contains activator and repressor domains and interacts with members of the CREB family. J Biol Chem 275:21737-21745.

Elgin SC, Grewal SI (2003) Heterochromatin: silence is golden. Curr Biol 13:R895-898.

Encinas JM, Vaahtokari A, Enikolopov G (2006) Fluoxetine targets early progenitor cells in the adult brain. Proc Natl Acad Sci U S A 103:8233-8238.

Eriksson E, Andersch B, Ho HP, Landen M, Sundblad C (2001) [Serotonin uptake inhibitors provide rapid relief from premenstrual dysphoria. New findings shed light on how serotonin modulates sex hormone-related behavior]. Lakartidningen 98:3524-3530.

Exton JH (1996) Regulation of phosphoinositide phospholipases by hormones, neurotransmitters, and other agonists linked to G proteins. Annu Rev Pharmacol Toxicol 36:481-509.

Fabre V, Beaufour C, Evrard A, Rioux A, Hanoun N, Lesch KP, Murphy DL, Lanfumey L, Hamon M, Martres MP (2000) Altered expression and functions of serotonin 5-HT1A and 5-HT1B receptors in knock-out mice lacking the 5-HT transporter. Eur J Neurosci 12:2299-2310.

Fakra E, Hyde LW, Gorka A, Fisher PM, Munoz KE, Kimak M, Halder I, Ferrell RE, Manuck SB, Hariri AR (2009) Effects of HTR1A C(-1019)G on amygdala reactivity and trait anxiety. Arch Gen Psychiatry 66:33-40.

Fanelli RJ, Schuurman T, Glaser T, Traber J (1990) Ipsapirone: a novel anxiolytic and selective 5-HT1A receptor ligand. Prog Clin Biol Res 361:461-467.

Fargin A, Raymond JR, Lohse MJ, Kobilka BK, Caron MG, Lefkowitz RJ (1988) The genomic clone G-21 which resembles a beta-adrenergic receptor sequence encodes the 5-HT1A receptor. Nature 335:358-360.

Fava M, Kendler KS (2000) Major depressive disorder. Neuron 28:335-341.

Ferguson SS (2001) Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling. Pharmacol Rev 53:1-24.

Ferguson SS, Downey WE, 3rd, Colapietro AM, Barak LS, Menard L, Caron MG (1996) Role of beta-arrestin in mediating agonist-promoted G protein-coupled receptor internalization. Science 271:363-366.

Filip M, Bader M (2009) Overview on 5-HT receptors and their role in physiology and pathology of the central nervous system. Pharmacol Rep 61:761-777.

Fink KB, Gothert M (2007) 5-HT receptor regulation of neurotransmitter release. Pharmacol Rev 59:360-417.

Fior R, Henrique D (2005) A novel hes5/hes6 circuitry of negative regulation controls Notch activity during neurogenesis. Dev Biol 281:318-333.

Fisher PM, Meltzer CC, Ziolko SK, Price JC, Moses-Kolko EL, Berga SL, Hariri AR (2006) Capacity for 5-HT1A-mediated autoregulation predicts amygdala reactivity. Nat Neurosci 9:1362-1363.

Freitag CM, Domschke K, Rothe C, Lee YJ, Hohoff C, Gutknecht L, Sand P, Fimmers R, Lesch KP, Deckert J (2006) Interaction of serotonergic and noradrenergic gene variants in panic disorder. Psychiatr Genet 16:59-65.

Fuller RW (1990) Serotonin receptors and neuroendocrine responses. Neuropsychopharmacology 3:495-502.

Gaddum JH, Picarelli ZP (1957) Two kinds of tryptamine receptor. Br J Pharmacol Chemother 12:323-328.

Galter D, Unsicker K (1999) Regulation of the transmitter phenotype of rostral and caudal groups of cultured serotonergic raphe neurons. Neuroscience 88:549-559.

Gaspar P, Cases O, Maroteaux L (2003) The developmental role of serotonin: news from mouse molecular genetics. Nat Rev Neurosci 4:1002-1012.

Gilman AG (1987) G proteins: transducers of receptor-generated signals. Annu Rev Biochem 56:615-649.

Gobbi G, Murphy DL, Lesch K, Blier P (2001) Modifications of the serotonergic system in mice lacking serotonin transporters: an in vivo electrophysiological study. J Pharmacol Exp Ther 296:987-995.

Goodbourn S (1990) Negative regulation of transcriptional initiation in eukaryotes. Biochim Biophys Acta 1032:53-77.

Gordon JA, Hen R (2004) The serotonergic system and anxiety. Neuromolecular Med 5:27-40.

Goridis C, Rohrer H (2002) Specification of catecholaminergic and serotonergic neurons. Nat Rev Neurosci 3:531-541.

Gothert M (1990) Presynaptic serotonin receptors in the central nervous system. Ann N Y Acad Sci 604:102-112.

Groenink L, van Bogaert MJ, van der Gugten J, Oosting RS, Olivier B (2003) 5-HT1A receptor and 5-HT1B receptor knockout mice in stress and anxiety paradigms. Behav Pharmacol 14:369-383.

Gross C, Zhuang X, Stark K, Ramboz S, Oosting R, Kirby L, Santarelli L, Beck S, Hen R (2002) Serotonin1A receptor acts during development to establish normal anxiety-like behaviour in the adult. Nature 416:396-400.

Gross CT, McGinnis W (1996) DEAF-1, a novel protein that binds an essential region in a Deformed response element. Embo J 15:1961-1970.

Gutknecht L, Waider J, Kraft S, Kriegebaum C, Holtmann B, Reif A, Schmitt A, Lesch KP (2008) Deficiency of brain 5-HT synthesis but serotonergic neuron formation in Tph2 knockout mice. J Neural Transm 115:1127-1132.

Hadjighassem MR, Austin MC, Szewczyk B, Daigle M, Stockmeier CA, Albert PR (2009) Human Freud-2/CC2D1B: a novel repressor of postsynaptic serotonin-1A receptor expression. Biol Psychiatry 66:214-222.

Hahm K, Sum EY, Fujiwara Y, Lindeman GJ, Visvader JE, Orkin SH (2004) Defective neural tube closure and anteroposterior patterning in mice lacking the LIM protein LMO4 or its interacting partner Deaf-1. Mol Cell Biol 24:2074-2082.

Hajos M, Hajos-Korcsok E, Sharp T (1999) Role of the medial prefrontal cortex in 5-HT1A receptor-induced inhibition of 5-HT neuronal activity in the rat. Br J Pharmacol 126:1741-1750.

Hanley NR, Van de Kar LD (2003) Serotonin and the neuroendocrine regulation of the hypothalamic--pituitary-adrenal axis in health and disease. Vitam Horm 66:189-255.

Hariri AR, Mattay VS, Tessitore A, Kolachana B, Fera F, Goldman D, Egan MF, Weinberger DR (2002) Serotonin transporter genetic variation and the response of the human amygdala. Science 297:400-403.

Hasler G, Bonwetsch R, Giovacchini G, Toczek MT, Bagic A, Luckenbaugh DA, Drevets WC, Theodore WH (2007) 5-HT1A receptor binding in temporal lobe epilepsy patients with and without major depression. Biol Psychiatry 62:1258-1264.

Hatakeyama J, Sakamoto S, Kageyama R (2006) Hes1 and Hes5 regulate the development of the cranial and spinal nerve systems. Dev Neurosci 28:92-101.

Hausdorff WP, Lohse MJ, Bouvier M, Liggett SB, Caron MG, Lefkowitz RJ (1990) Two kinases mediate agonist-dependent phosphorylation and desensitization of the beta 2-adrenergic receptor. Symp Soc Exp Biol 44:225-240.

Heisler LK, Chu HM, Brennan TJ, Danao JA, Bajwa P, Parsons LH, Tecott LH (1998) Elevated anxiety and antidepressant-like responses in serotonin 5-HT1A receptor mutant mice. Proc Natl Acad Sci U S A 95:15049-15054.

Hendricks T, Francis N, Fyodorov D, Deneris ES (1999) The ETS domain factor Pet-1 is an early and precise marker of central serotonin neurons and interacts with a conserved element in serotonergic genes. J Neurosci 19:10348-10356.

Hendricks TJ, Fyodorov DV, Wegman LJ, Lelutiu NB, Pehek EA, Yamamoto B, Silver J, Weeber EJ, Sweatt JD, Deneris ES (2003) Pet-1 ETS gene plays a critical role in 5-HT neuron development and is required for normal anxiety-like and aggressive behavior. Neuron 37:233-247.

Hensler JG (2003) Regulation of 5-HT1A receptor function in brain following agonist or antidepressant administration. Life Sci 72:1665-1682.

Hirata H, Tomita K, Bessho Y, Kageyama R (2001) Hes1 and Hes3 regulate maintenance of the isthmic organizer and development of the mid/hindbrain. EMBO J 20:4454-4466.

Hirschfeld RM, Keller MB, Panico S, Arons BS, Barlow D, Davidoff F, Endicott J, Froom J, Goldstein M, Gorman JM, Marek RG, Maurer TA, Meyer R, Phillips K, Ross J, Schwenk TL, Sharfstein SS, Thase ME, Wyatt RJ (1997) The National

Depressive and Manic-Depressive Association consensus statement on the undertreatment of depression. JAMA 277:333-340.

Hjorth S, Suchowski CS, Galloway MP (1995) Evidence for 5-HT autoreceptor-mediated, nerve impulse-independent, control of 5-HT synthesis in the rat brain. Synapse 19:170-176.

Hjorth S, Bengtsson HJ, Milano S (1996) Raphe 5-HT1A autoreceptors, but not postsynaptic 5-HT1A receptors or beta-adrenoceptors, restrain the citalopram-induced increase in extracellular 5-hydroxytryptamine in vivo. Eur J Pharmacol 316:43-47.

Hornung JP (2003) The human raphe nuclei and the serotonergic system. J Chem Neuroanat 26:331-343.

Hoyer D, Clarke DE, Fozard JR, Hartig PR, Martin GR, Mylecharane EJ, Saxena PR, Humphrey PP (1994) International Union of Pharmacology classification of receptors for 5-hydroxytryptamine (Serotonin). Pharmacol Rev 46:157-203.

Hsiung SC, Adlersberg M, Arango V, Mann JJ, Tamir H, Liu KP (2003) Attenuated 5-HT1A receptor signaling in brains of suicide victims: involvement of adenylyl cyclase, phosphatidylinositol 3-kinase, Akt and mitogen-activated protein kinase. J Neurochem 87:182-194.

Huang YY, Battistuzzi C, Oquendo MA, Harkavy-Friedman J, Greenhill L, Zalsman G, Brodsky B, Arango V, Brent DA, Mann JJ (2004) Human 5-HT1A receptor C(-1019)G polymorphism and psychopathology. Int J Neuropsychopharmacol 7:441-451.

Huggenvik JI, Michelson RJ, Collard MW, Ziemba AJ, Gurley P, Mowen KA (1998) Characterization of a nuclear deformed epidermal autoregulatory factor-1 (DEAF-1)-related (NUDR) transcriptional regulator protein. Mol Endocrinol 12:1619-1639.

Hyman S (2000) Mental illness: genetically complex disorders of neural circuitry and neural communication. Neuron 28:321-323.

Ishibashi M, Ang SL, Shiota K, Nakanishi S, Kageyama R, Guillemot F (1995) Targeted disruption of mammalian hairy and Enhancer of split homolog-1 (HES-1) leads to up-regulation of neural helix-loop-helix factors, premature neurogenesis, and severe neural tube defects. Genes Dev 9:3136-3148.

Ito T (2007) Role of histone modification in chromatin dynamics. J Biochem 141:609-614.

Jacobs BL, Azmitia EC (1992) Structure and function of the brain serotonin system. Physiol Rev 72:165-229.

Jacobsen KX, Vanderluit JL, Slack RS, Albert PR (2008) HES1 regulates 5-HT1A receptor gene transcription at a functional polymorphism: essential role in developmental expression. Mol Cell Neurosci 38:349-358.

Jarriault S, Le Bail O, Hirsinger E, Pourquie O, Logeat F, Strong CF, Brou C, Seidah NG, Isra l A (1998) Delta-1 activation of notch-1 signaling results in HES-1 transactivation. Mol Cell Biol 18:7423-7431.

Jensik PJ, Huggenvik JI, Collard MW (2004) Identification of a nuclear export signal and protein interaction domains in deformed epidermal autoregulatory factor-1 (DEAF-1). J Biol Chem 279:32692-32699.

Jorgensen HS (2007) Studies on the neuroendocrine role of serotonin. Dan Med Bull 54:266-288.

Kageyama R, Ohtsuka T (1999) The Notch-Hes pathway in mammalian neural development. Cell Res 9:179-188.

Kageyama R, Ohtsuka T, Hatakeyama J, Ohsawa R (2005) Roles of bHLH genes in neural stem cell differentiation. Exp Cell Res 306:343-348.

Kallunki P, Edelman GM, Jones FS (1998) The neural restrictive silencer element can act as both a repressor and enhancer of L1 cell adhesion molecule gene expression during postnatal development. Proc Natl Acad Sci U S A 95:3233-3238.

Kashani AH, Qiu Z, Jurata L, Lee SK, Pfaff S, Goebbels S, Nave KA, Ghosh A (2006) Calcium activation of the LMO4 transcription complex and its role in the patterning of thalamocortical connections. J Neurosci 26:8398-8408.

Keller MB (2002) Rationale and options for the long-term treatment of depression. Hum Psychopharmacol 17 Suppl 1:S43-46.

Keller MB, Boland RJ (1998) Implications of failing to achieve successful long-term maintenance treatment of recurrent unipolar major depression. Biol Psychiatry 44:348-360.

Kessler RC (1997) The effects of stressful life events on depression. Annu Rev Psychol 48:191-214.

Kinney GG, Taber MT, Gribkoff VK (2000) The augmentation hypothesis for improvement of antidepressant therapy: is pindolol a suitable candidate for testing the ability of 5HT1A receptor antagonists to enhance SSRI efficacy and onset latency? Mol Neurobiol 21:137-152.

Kobilka BK, Frielle T, Collins S, Yang-Feng T, Kobilka TS, Francke U, Lefkowitz RJ, Caron MG (1987) An intronless gene encoding a potential member of the family of receptors coupled to guanine nucleotide regulatory proteins. Nature 329:75-79.

Koller G, Bondy B, Preuss UW, Zill P, Soyka M (2006) The C(-1019)G 5-HT1A promoter polymorphism and personality traits: no evidence for significant association in alcoholic patients. Behav Brain Funct 2:7.

Kraus MR, Al-Taie O, Schafer A, Pfersdorff M, Lesch KP, Scheurlen M (2007) Serotonin-1A receptor gene HTR1A variation predicts interferon-induced depression in chronic hepatitis C. Gastroenterology 132:1279-1286.

Krishnan KR (2007) Revisiting monoamine oxidase inhibitors. J Clin Psychiatry 68 Suppl 8:35-41.

Kuo MH, Allis CD (1998) Roles of histone acetyltransferases and deacetylases in gene regulation. Bioessays 20:615-626.

Kusserow H, Davies B, Hortnagl H, Voigt I, Stroh T, Bert B, Deng DR, Fink H, Veh RW, Theuring F (2004) Reduced anxiety-related behaviour in transgenic mice

overexpressing serotonin 1A receptors. Brain Res Mol Brain Res 129:104-116.

Lanfumey L, Hamon M (2004) 5-HT1 receptors. Curr Drug Targets CNS Neurol Disord 3:1-10.

Latham JA, Dent SY (2007) Cross-regulation of histone modifications. Nat Struct Mol Biol 14:1017-1024.

Lautenschlager M, Holtje M, von Jagow B, Veh RW, Harms C, Bergk A, Dirnagl U, Ahnert-Hilger G, Hortnagl H (2000) Serotonin uptake and release mechanisms in developing cultures of rat embryonic raphe neurons: age- and region-specific differences. Neuroscience 99:519-527.

Le Francois B, Czesak M, Steubl D, Albert PR (2008) Transcriptional regulation at a HTR1A polymorphism associated with mental illness. Neuropharmacology 55:977-985.

Lee SK, Jurata LW, Nowak R, Lettieri K, Kenny DA, Pfaff SL, Gill GN (2005) The LIM domain-only protein LMO4 is required for neural tube closure. Mol Cell Neurosci 28:205-214.

Lehtinen V, Joukamaa M (1994) Epidemiology of depression: prevalence, risk factors and treatment situation. Acta Psychiatr Scand Suppl 377:7-10.

Lembo PM, Albert PR (1995) Multiple phosphorylation sites are required for pathway-selective uncoupling of the 5-hydroxytryptamine1A receptor by protein kinase C. Mol Pharmacol 48:1024-1029.

Lemonde S, Du L, Bakish D, Hrdina P, Albert PR (2004) Association of the C(-1019)G 5-HT1A functional promoter polymorphism with antidepressant response. Int J Neuropsychopharmacol 7:501-506.

Lemonde S, Turecki G, Bakish D, Du L, Hrdina PD, Bown CD, Sequeira A, Kushwaha N, Morris SJ, Basak A, Ou XM, Albert PR (2003) Impaired repression at a 5-hydroxytryptamine 1A receptor gene polymorphism associated with major depression and suicide. J Neuroscience 23:8788-8799.

Lerer B, Macciardi F (2002) Pharmacogenetics of antidepressant and mood-stabilizing drugs: a review of candidate-gene studies and future research directions. Int J Neuropsychopharmacol 5:255-275.

Lesch KP, Bengel D, Heils A, Sabol SZ, Greenberg BD, Petri S, Benjamin J, Muller CR, Hamer DH, Murphy DL (1996) Association of anxiety-related traits with a polymorphism in the serotonin transporter gene regulatory region. Science 274:1527-1531.

Levin GM, Bowles TM, Ehret MJ, Langaee T, Tan JY, Johnson JA, Millard WJ (2007) Assessment of Human Serotonin 1A Receptor Polymorphisms and SSRI Responsiveness. Mol Diagn Ther 11:155-160.

Liu D, Diorio J, Day JC, Francis DD, Meaney MJ (2000) Maternal care, hippocampal synaptogenesis and cognitive development in rats. Nat Neurosci 3:799-806.

Liu Y, Edwards RH (1997) The role of vesicular transport proteins in synaptic transmission and neural degeneration. Annu Rev Neurosci 20:125-156.

Lo Iacono L, Gross C (2008) Alpha-Ca2+/calmodulin-dependent protein kinase II contributes to the developmental programming of anxiety in serotonin receptor 1A knock-out mice. J Neurosci 28:6250-6257.

Lopez-Figueroa AL, Norton CS, Lopez-Figueroa MO, Armellini-Dodel D, Burke S, Akil H, Lopez JF, Watson SJ (2004) Serotonin 5-HT1A, 5-HT1B, and 5-HT2A receptor mRNA expression in subjects with major depression, bipolar disorder, and schizophrenia. Biol Psychiatry 55:225-233.

Lopez-Munoz F, Alamo C (2009) Monoaminergic neurotransmission: the history of the discovery of antidepressants from 1950s until today. Curr Pharm Des 15:1563-1586.

Lucas G, Rymar VV, Du J, Mnie-Filali O, Bisgaard C, Manta S, Lambas-Senas L, Wiborg O, Haddjeri N, Pineyro G, Sadikot AF, Debonnel G (2007) Serotonin(4) (5-HT(4)) receptor agonists are putative antidepressants with a rapid onset of action. Neuron 55:712-725.

Luscher C, Jan LY, Stoffel M, Malenka RC, Nicoll RA (1997) G protein-coupled inwardly rectifying K+ channels (GIRKs) mediate postsynaptic but not presynaptic transmitter actions in hippocampal neurons. Neuron 19:687-695.

Malberg JE, Eisch AJ, Nestler EJ, Duman RS (2000) Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus. J Neurosci 20:9104-9110.

Mann JJ (2003) Neurobiology of suicidal behaviour. Nat Rev Neurosci 4:819-828.

Marinelli S, Schnell SA, Hack SP, Christie MJ, Wessendorf MW, Vaughan CW (2004) Serotonergic and nonserotonergic dorsal raphe neurons are pharmacologically and electrophysiologically heterogeneous. J Neurophysiol 92:3532-3537.

Martin GR, Eglen RM, Hamblin MW, Hoyer D, Yocca F (1998) The structure and signalling properties of 5-HT receptors: an endless diversity? Trends Pharmacol Sci 19:2-4.

Marziniak M, Mossner R, Kienzler C, Riederer P, Lesch KP, Sommer C (2007) Functional polymorphisms of the 5-HT1A and 5-HT1B receptor are associated with clinical symptoms in migraineurs. J Neural Transm 114:1227-1232.

McEwen BS (1999) Stress and hippocampal plasticity. Annu Rev Neurosci 22:105-122.

McLarren KW, Theriault FM, Stifani S (2001) Association with the nuclear matrix and interaction with Groucho and RUNX proteins regulate the transcription repression activity of the basic helix loop helix factor Hes1. J Biol Chem 276:1578-1584.

Meaney MJ (2001) Maternal care, gene expression, and the transmission of individual differences in stress reactivity across generations. Annu Rev Neurosci 24:1161-1192.

Mehta KM, Yaffe K, Brenes GA, Newman AB, Shorr RI, Simonsick EM, Ayonayon HN, Rubin SM, Covinsky KE (2007a) Anxiety symptoms and decline in physical

function over 5 years in the health, aging and body composition study. J Am Geriatr Soc 55:265-270.

Mehta M, Ahmed Z, Fernando SS, Cano-Sanchez P, Adayev T, Ziemnicka D, Wieraszko A, Banerjee P (2007b) Plasticity of 5-HT 1A receptor-mediated signaling during early postnatal brain development. J Neurochem 101:918-928.

Meltzer CC, Price JC, Mathis CA, Butters MA, Ziolko SK, Moses-Kolko E, Mazumdar S, Mulsant BH, Houck PR, Lopresti BJ, Weissfeld LA, Reynolds CF (2004) Serotonin 1A receptor binding and treatment response in late-life depression. Neuropsychopharmacology 29:2258-2265.

Meyer-Lindenberg A, Weinberger DR (2006) Intermediate phenotypes and genetic mechanisms of psychiatric disorders. Nat Rev Neurosci 7:818-827.

Michelson RJ, Collard MW, Ziemba AJ, Persinger J, Bartholomew B, Huggenvik JI (1999) Nuclear DEAF-1-related (NUDR) protein contains a novel DNA binding domain and represses transcription of the heterogeneous nuclear ribonucleoprotein A2/B1 promoter. J Biol Chem 274:30510-30519.

Mizutani T, Taniguchi Y, Aoki T, Hashimoto N, Honjo T (2001) Conservation of the biochemical mechanisms of signal transduction among mammalian Notch family members. Proc Natl Acad Sci U S A 98:9026-9031.

Montgomery SA (1997) Fast-onset antidepressants. Int Clin Psychopharmacol 12 Suppl 3:S1-5.

Moses-Kolko EL, Wisner KL, Price JC, Berga SL, Drevets WC, Hanusa BH, Loucks TL, Meltzer CC (2008) Serotonin 1A receptor reductions in postpartum depression: a positron emission tomography study. Fertil Steril 89:685-692.

Murphy DL (1990) Neuropsychiatric disorders and the multiple human brain serotonin receptor subtypes and subsystems. Neuropsychopharmacology 3:457-471.

Neff CD, Abkevich V, Packer JC, Chen Y, Potter J, Riley R, Davenport C, DeGrado Warren J, Jammulapati S, Bhathena A, Choi WS, Kroeger PE, Metzger RE, Gutin A, Skolnick MH, Shattuck D, Katz DA (2009) Evidence for HTR1A and LHPP as interacting genetic risk factors in major depression. Mol Psychiatry 14:621-630.

Neumeister A, Bain E, Nugent AC, Carson RE, Bonne O, Luckenbaugh DA, Eckelman W, Herscovitch P, Charney DS, Drevets WC (2004) Reduced serotonin type 1A receptor binding in panic disorder. J Neurosci 24:589-591.

Newberry NR (1992) 5-HT1A receptors activate a potassium conductance in rat ventromedial hypothalamic neurones. Eur J Pharmacol 210:209-212.

Noor A, Windpassinger C, Patel M, Stachowiak B, Mikhailov A, Azam M, Irfan M, Siddiqui ZK, Naeem F, Paterson AD, Lutfullah M, Vincent JB, Ayub M (2008) CC2D2A, Encoding A Coiled-Coil and C2 Domain Protein, Causes Autosomal-Recessive Mental Retardation with Retinitis Pigmentosa. Am J Hum Genet 82:1011-1018.

Ogbourne S, Antalis TM (1998) Transcriptional control and the role of silencers in transcriptional regulation in eukaryotes. Biochem J 331 (Pt 1):1-14.

Ohtsuka T, Sakamoto M, Guillemot F, Kageyama R (2001) Roles of the basic helix-loop-helix genes Hes1 and Hes5 in expansion of neural stem cells of the developing brain. J Biol Chem 276:30467-30474.

Ohtsuka T, Ishibashi M, Gradwohl G, Nakanishi S, Guillemot F, Kageyama R (1999) Hes1 and Hes5 as notch effectors in mammalian neuronal differentiation. EMBO J 18:2196-2207.

Oki M, Aihara H, Ito T (2007) Role of histone phosphorylation in chromatin dynamics and its implications in diseases. Subcell Biochem 41:319-336.

Olivier B, Pattij T, Wood SJ, Oosting R, Sarnyai Z, Toth M (2001) The 5-HT(1A) receptor knockout mouse and anxiety. Behav Pharmacol 12:439-450.

Ong SE, Blagoev B, Kratchmarova I, Kristensen DB, Steen H, Pandey A, Mann M (2002) Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. Mol Cell Proteomics 1:376-386.

Osipova DV, Kulikov AV, Popova NK (2009) C1473G polymorphism in mouse tph2 gene is linked to tryptophan hydroxylase-2 activity in the brain, intermale aggression, and depressive-like behavior in the forced swim test. J Neurosci Res 87:1168-1174.

Ostrowski J, Kjelsberg MA, Caron MG, Lefkowitz RJ (1992) Mutagenesis of the beta 2-adrenergic receptor: how structure elucidates function. Annu Rev Pharmacol Toxicol 32:167-183.

Ou XM, Storring JM, Kushwaha N, Albert PR (2001) Heterodimerization of mineralocorticoid and glucocorticoid receptors at a novel negative response element of the 5-HT1A receptor gene. J Biol Chem 276:14299-14307.

Ou XM, Jafar-Nejad H, Storring JM, Meng JH, Lemonde S, Albert PR (2000) Novel dual repressor elements for neuronal cell-specific transcription of the rat 5-HT1A receptor gene. J Biol Chem 275:8161-8168.

Overstreet DH, Commissaris RC, De La Garza R, 2nd, File SE, Knapp DJ, Seiden LS (2003) Involvement of 5-HT1A receptors in animal tests of anxiety and depression: evidence from genetic models. Stress 6:101-110.

Palm K, Belluardo N, Metsis M, Timmusk T (1998) Neuronal expression of zinc finger transcription factor REST/NRSF/XBR gene. J Neurosci 18:1280-1296.

Parks CL, Shenk T (1996) The serotonin 1a receptor gene contains a TATA-less promoter that responds to MAZ and Sp1. Journal of Biological Chemistry 271:4417-4430.

Parks CL, Robinson PS, Sibille E, Shenk T, Toth M (1998) Increased anxiety of mice lacking the serotonin1A receptor. Proc Natl Acad Sci U S A 95:10734-10739.

Parsey RV, Olvet DM, Oquendo MA, Huang YY, Ogden RT, Mann JJ (2006a) Higher 5-HT1A receptor binding potential during a major depressive episode predicts

poor treatment response: preliminary data from a naturalistic study. Neuropsychopharmacology 31:1745-1749.

Parsey RV, Oquendo MA, Ogden RT, Olvet DM, Simpson N, Huang YY, Van Heertum RL, Arango V, Mann JJ (2006b) Altered serotonin 1A binding in major depression: a [carbonyl-C-11]WAY100635 positron emission tomography study. Biol Psychiatry 59:106-113.

Parsons LH, Kerr TM, Tecott LH (2001) 5-HT(1A) receptor mutant mice exhibit enhanced tonic, stress-induced and fluoxetine-induced serotonergic neurotransmission. J Neurochem 77:607-617.

Patel TD, Zhou FC (2005) Ontogeny of 5-HT1A receptor expression in the developing hippocampus. Brain Res Dev Brain Res 157:42-57.

Pattyn A, Simplicio N, van Doorninck JH, Goridis C, Guillemot F, Brunet JF (2004) Ascl1/Mash1 is required for the development of central serotonergic neurons. Nat Neurosci 7:589-595.

Pedersen AG, Baldi P, Chauvin Y, Brunak S (1999) The biology of eukaryotic promoter prediction--a review. Comput Chem 23:191-207.

Pedigo NW, Yamamura HI, Nelson DL (1981) Discrimination of multiple [3H]5-hydroxytryptamine binding sites by the neuroleptic spiperone in rat brain. J Neurochem 36:220-226.

Penington NJ, Kelly JS (1990) Serotonin receptor activation reduces calcium current in an acutely dissociated adult central neuron. Neuron 4:751-758.

Penington NJ, Kelly JS, Fox AP (1991) A study of the mechanism of Ca2+ current inhibition produced by serotonin in rat dorsal raphe neurons. J Neurosci 11:3594-3609.

Peroutka SJ (1993) 5-Hydroxytryptamine receptors. J Neurochem 60:408-416.

Peroutka SJ, Snyder SH (1979) Multiple serotonin receptors: differential binding of [3H]5-hydroxytryptamine, [3H]lysergic acid diethylamide and [3H]spiroperidol. Mol Pharmacol 16:687-699.

Pineyro G, Blier P (1999) Autoregulation of serotonin neurons: role in antidepressant drug action. Pharmacol Rev 51:533-591.

Popoli M, Mori S, Brunello N, Perez J, Gennarelli M, Racagni G (2001) Serine/threonine kinases as molecular targets of antidepressants: implications for pharmacological treatment and pathophysiology of affective disorders. Pharmacol Ther 89:149-170.

Porsolt RD, Bertin A, Blavet N, Deniel M, Jalfre M (1979) Immobility induced by forced swimming in rats: effects of agents which modify central catecholamine and serotonin activity. Eur J Pharmacol 57:201-210.

Raap DK, Van de Kar LD (1999) Selective serotonin reuptake inhibitors and neuroendocrine function. Life Sci 65:1217-1235.

Ramboz S, Oosting R, Amara DA, Kung HF, Blier P, Mendelsohn M, Mann JJ, Brunner D, Hen R (1998) Serotonin receptor 1A knockout: an animal model of anxiety-related disorder. Proc Natl Acad Sci U S A 95:14476-14481.

Rapport MM (1949) Serum vasoconstrictor (serotonin) the presence of creatinine in the complex; a proposed structure of the vasoconstrictor principle. J Biol Chem 180:961-969.

Rapport MM, Green AA, Page IH (1948) Serum vasoconstrictor, serotonin; chemical inactivation. J Biol Chem 176:1237-1241.

Redmond L, Oh SR, Hicks C, Weinmaster G, Ghosh A (2000) Nuclear Notch1 signaling and the regulation of dendritic development. Nat Neurosci 3:30-40.

Richardson-Jones JW, Craige CP, Guiard BP, Stephen A, Metzger KL, Kung HF, Gardier AM, Dranovsky A, David DJ, Beck SG, Hen R, Leonardo ED 5-HT1A autoreceptor levels determine vulnerability to stress and response to antidepressants. Neuron 65:40-52.

Richelson E (1991) Biological basis of depression and therapeutic relevance. J Clin Psychiatry 52 Suppl:4-10.

Rogaeva A, Galaraga K, Albert PR (2007) The Freud-1/CC2D1A family: Transcriptional regulators implicated in mental retardation. J Neurosci Res 85:2833-2888.

Rotondo A, Nielsen DA, Nakhai B, Hulihan-Giblin B, Bolos A, Goldman D (1997) Agonist-promoted down-regulation and functional desensitization in two naturally occurring variants of the human serotonin1A receptor. Neuropsychopharmacology 17:18-26.

Rumajogee P, Verge D, Hanoun N, Brisorgueil MJ, Hen R, Lesch KP, Hamon M, Miquel MC (2004) Adaption of the serotoninergic neuronal phenotype in the

absence of 5-HT autoreceptors or the 5-HT transporter: involvement of BDNF and cAMP. Eur J Neurosci 19:937-944.

Sahay A, Hen R (2007) Adult hippocampal neurogenesis in depression. Nat Neurosci 10:1110-1115.

Santarelli L, Saxe M, Gross C, Surget A, Battaglia F, Dulawa S, Weisstaub N, Lee J, Duman R, Arancio O, Belzung C, Hen R (2003) Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants. Science 301:805-809.

Sargent PA, Kjaer KH, Bench CJ, Rabiner EA, Messa C, Meyer J, Gunn RN, Grasby PM, Cowen PJ (2000) Brain serotonin1A receptor binding measured by positron emission tomography with [11C]WAY-100635: effects of depression and antidepressant treatment. Arch Gen Psychiatry 57:174-180.

Sawiniec J, Borkowski K, Ginalska G, Lewandowska-Stanek H (2007) Association between 5-hydroxytryptamine 1A receptor gene polymorphism and suicidal behavior. Przegl Lek 64:208-211.

Saxena PR (1972) The effects of antimigraine drugs on the vascular responses by-5-hydroxytryptamine and related biogenic substances on the external carotid bed of dogs: possible pharmacological implications to their antimigraine action. Headache 12:44-54.

Saxena PR, van Houwelingen P, Bonta IL (1971) The effects of mianserin hydrochloride on the vascular responses evoked by 5-hydroxytryptamine and related vasoactive substances. Eur J Pharmacol 13:295-305.

Schramm NL, McDonald MP, Limbird LE (2001) The alpha(2a)-adrenergic receptor plays a protective role in mouse behavioral models of depression and anxiety. J Neurosci 21:4875-4882.

Serretti A, Mandelli L, Giegling I, Schneider B, Hartmann AM, Schnabel A, Maurer K, Moller HJ, Rujescu D (2007) HTR2C and HTR1A gene variants in German and Italian suicide attempters and completers. Am J Med Genet B Neuropsychiatr Genet 144:291-299.

Sestan N, Artavanis-Tsakonas S, Rakic P (1999) Contact-dependent inhibition of cortical neurite growth mediated by notch signaling. Science 286:741-746.

Seth KA, Majzoub JA (2001) Repressor element silencing transcription factor/neuron-restrictive silencing factor (REST/NRSF) can act as an enhancer as well as a repressor of corticotropin-releasing hormone gene transcription. J Biol Chem 276:13917-13923.

Sharp T, Umbers V, Gartside SE (1997) Effect of a selective 5-HT reuptake inhibitor in combination with 5-HT1A and 5-HT1B receptor antagonists on extracellular 5-HT in rat frontal cortex in vivo. Br J Pharmacol 121:941-946.

Sheline YI, Wang PW, Gado MH, Csernansky JG, Vannier MW (1996) Hippocampal atrophy in recurrent major depression. Proc Natl Acad Sci U S A 93:3908-3913.

Shenoy SK, Lefkowitz RJ (2003) Trafficking patterns of beta-arrestin and G protein-coupled receptors determined by the kinetics of beta-arrestin deubiquitination. J Biol Chem 278:14498-14506.

Shih JC, Chen K (1999) MAO-A and -B gene knock-out mice exhibit distinctly different behavior. Neurobiology (Bp) 7:235-246.

Shimojo M, Hersh LB (2006) Characterization of the REST/NRSF-interacting LIM domain protein (RILP): localization and interaction with REST/NRSF. J Neurochem 96:1130-1138.

Smale ST (1997) Transcription initiation from TATA-less promoters within eukaryotic protein-coding genes. Biochim Biophys Acta 1351:73-88.

Smidt MP, Asbreuk CH, Cox JJ, Chen H, Johnson RL, Burbach JP (2000) A second independent pathway for development of mesencephalic dopaminergic neurons requires Lmx1b. Nat Neurosci 3:337-341.

Sotelo C, Cholley B, S. EM, Gozlan H, Hamon M (1990) Direct immunohistochemical evidence of the existence of 5-HT1A autoreceptors on serotoninergic neurons in the midbrain raphe nuclei. European Journal of Neuroscience 2:1144-1154.

Spadaccini R, Perrin H, Bottomley MJ, Ansieau S, Sattler M (2006) Structure and functional analysis of the MYND domain. J Mol Biol 358:498-508.

Stahl SM (1998) Basic psychopharmacology of antidepressants, part 1: Antidepressants have seven distinct mechanisms of action. J Clin Psychiatry 59 Suppl 4:5-14.

Steiner M, Born L (2000) Diagnosis and treatment of premenstrual dysphoric disorder: an update. Int Clin Psychopharmacol 15 Suppl 3:S5-17.

Steru L, Chermat R, Thierry B, Simon P (1985) The tail suspension test: a new method for screening antidepressants in mice. Psychopharmacology (Berl) 85:367-370.

Stockmeier CA, Shapiro LA, Dilley GE, Kolli TN, Friedman L, Rajkowska G (1998) Increase in serotonin-1A autoreceptors in the midbrain of suicide victims with major depression-postmortem evidence for decreased serotonin activity. J Neurosci 18:7394-7401.

Storring JM, Charest A, Cheng P, Albert PR (1999) TATA-driven transcriptional initiation and regulation of the rat serotonin 5-HT1A receptor gene. J Neurochem 72:2238-2247.

Strobel A, Gutknecht L, Rothe C, Reif A, Mossner R, Zeng Y, Brocke B, Lesch KP (2003) Allelic variation in 5-HT(1A) receptor expression is associated with anxiety- and depression-related personality traits. J Neural Transm 110:1445-1453.

Stump G, Durrer A, Klein AL, Lutolf S, Suter U, Taylor V (2002) Notch1 and its ligands Delta-like and Jagged are expressed and active in distinct cell populations in the postnatal mouse brain. Mech Dev 114:153-159.

Sugihara TM, Bach I, Kioussi C, Rosenfeld MG, Andersen B (1998) Mouse deformed epidermal autoregulatory factor 1 recruits a LIM domain factor, LMO-4, and CLIM coregulators. Proc Natl Acad Sci U S A 95:15418-15423.

Sunahara RK, Dessauer CW, Gilman AG (1996) Complexity and diversity of mammalian adenylyl cyclases. Annu Rev Pharmacol Toxicol 36:461-480.

Suzuki Y, Tsunoda T, Sese J, Taira H, Mizushima-Sugano J, Hata H, Ota T, Isogai T, Tanaka T, Nakamura Y, Suyama A, Sakaki Y, Morishita S, Okubo K, Sugano S (2001) Identification and characterization of the potential promoter regions of 1031 kinds of human genes. Genome Res 11:677-684.

Swaminathan V, Reddy BA, Ruthrotha Selvi B, Sukanya MS, Kundu TK (2007) Small molecule modulators in epigenetics: implications in gene expression and therapeutics. Subcell Biochem 41:397-428.

Szewczyk B, Albert PR, Burns AM, Czesak M, Overholser JC, Jurjus GJ, Meltzer HY, Konick LC, Dieter L, Herbst N, May W, Rajkowska G, Stockmeier CA, Austin MC (2008) Gender-specific decrease in NUDR and 5-HT1A receptor proteins in the prefrontal cortex of subjects with major depressive disorder. Int J Neuropsychopharmacol in press.

Tomita K, Ishibashi M, Nakahara K, Ang SL, Nakanishi S, Guillemot F, Kageyama R (1996) Mammalian hairy and Enhancer of split homolog 1 regulates differentiation of retinal neurons and is essential for eye morphogenesis. Neuron 16:723-734.

Tork I (1990) Anatomy of the serotonergic system. Ann N Y Acad Sci 600:9-34; discussion 34-35.

Trillat AC, Malagie I, Mathe-Allainmat M, Anmella MC, Jacquot C, Langlois M, Gardier AM (1998) Synergistic neurochemical and behavioral effects of fluoxetine and 5-HT1A receptor antagonists. Eur J Pharmacol 357:179-184.

Trinkle-Mulcahy L, Boulon S, Lam YW, Urcia R, Boisvert FM, Vandermoere F, Morrice NA, Swift S, Rothbauer U, Leonhardt H, Lamond A (2008) Identifying specific protein interaction partners using quantitative mass spectrometry and bead proteomes. J Cell Biol 183:223-239.

Tse E, Smith AJ, Hunt S, Lavenir I, Forster A, Warren AJ, Grutz G, Foroni L, Carlton MB, Colledge WH, Boehm T, Rabbitts TH (2004) Null mutation of the Lmo4 gene or a combined null mutation of the Lmo1/Lmo3 genes causes perinatal lethality, and Lmo4 controls neural tube development in mice. Mol Cell Biol 24:2063-2073.

Twarog BM, Page IH (1953) Serotonin content of some mammalian tissues and urine and a method for its determination. Am J Physiol 175:157-161.

Utley RT, Ikeda K, Grant PA, Cote J, Steger DJ, Eberharter A, John S, Workman JL (1998) Transcriptional activators direct histone acetyltransferase complexes to nucleosomes. Nature 394:498-502.

van Biesen T, Luttrell LM, Hawes BE, Lefkowitz RJ (1996) Mitogenic signaling via G protein-coupled receptors. Endocr Rev 17:698-714.

van Doorninck JH, van Der Wees J, Karis A, Goedknegt E, Engel JD, Coesmans M, Rutteman M, Grosveld F, De Zeeuw CI (1999) GATA-3 is involved in the development of serotonergic neurons in the caudal raphe nuclei. J Neurosci 19:RC12.

Veraksa A, Kennison J, McGinnis W (2002) DEAF-1 function is essential for the early embryonic development of Drosophila. Genesis 33:67-76.

Verge D, Daval G, Patey A, Gozlan H, el Mestikawy S, Hamon M (1985) Presynaptic 5-HT autoreceptors on serotonergic cell bodies and/or dendrites but not terminals are of the 5-HT1A subtype. Eur J Pharmacol 113 463-464.

Videtic A, Zupanc T, Pregelj P, Balazic J, Tomori M, Komel R (2009) Suicide, stress and serotonin receptor 1A promoter polymorphism-1019C > G in Slovenian suicide victims European Archives of Psychiatry and Clinical Neuroscience 259 234-238.

Videtic A, Pungercic G, Pajnic IZ, Zupanc T, Balazic J, Tomori M, Komel R (2006) Association study of seven polymorphisms in four serotonin receptor genes on suicide victims Am J Med Genet B Neuropsychiatr Genet 141 669-672

Walther DJ, Peter JU, Bashammakh S, Hortnagl H, Voits M, Fink H, Bader M (2003) Synthesis of serotonin by a second tryptophan hydroxylase isoform. Science 299 76

Wayne Dyer (2004) The Power of Intention Learning to Co-Create Your World Your Way Hay House Inc

Weiger WA (1997) Serotonergic modulation of behaviour a phylogenetic overview Biol Rev Camb Philos Soc 72 61-95

Weissman MM, Bland R, Joyce PR, Newman S, Wells JE, Wittchen HU (1993) Sex differences in rates of depression cross-national perspectives. J Affect Disord 29 77-84

Whitaker-Azmitia PM (2001) Serotonin and brain development role in human developmental diseases Brain Res Bull 56 479-485

Williams JB (2001) Standardizing the Hamilton Depression Rating Scale: past, present, and future. Eur Arch Psychiatry Clin Neurosci 251 Suppl 2:II6-12.

Wu S, Comings DE (1999) A common C-1018G polymorphism in the human 5-HT1A receptor gene. Psychiatr Genet 9:105-106.

Wu X, Kushwaha N, Albert PR, Penington NJ (2002) A critical protein kinase C phosphorylation site on the 5-HT(1A) receptor controlling coupling to N-type calcium channels. J Physiol 538:41-51.

Youdim MB, Finberg JP (1991) New directions in monoamine oxidase A and B selective inhibitors and substrates. Biochem Pharmacol 41:155-162.

Zanardi R, Serretti A, Rossini D, Franchini L, Cusin C, Lattuada E, Dotoli D, Smeraldi E (2001) Factors affecting fluvoxamine antidepressant activity: influence of pindolol and 5-HTTLPR in delusional and nondelusional depression. Biol Psychiatry 50:323-330.

Zawel L, Lu H, Cisek LJ, Corden JL, Reinberg D (1993) The cycling of RNA polymerase II during transcription. Cold Spring Harb Symp Quant Biol 58:187-198.

Zetzsche T, Preuss UW, Bondy B, Frodl T, Zill P, Schmitt G, Koutsouleris N, Rujescu D, Born C, Reiser M, Moller HJ, Meisenzahl EM (2008) 5-HT1A receptor gene C -1019 G polymorphism and amygdala volume in borderline personality disorder. Genes Brain Behav 7:306-313.

Zhao J (2007) Sumoylation regulates diverse biological processes. Cell Mol Life Sci 64:3017-3033.

Zhao ZQ, Scott M, Chiechio S, Wang JS, Renner KJ, Gereau RWt, Johnson RL, Deneris ES, Chen ZF (2006) Lmx1b is required for maintenance of central serotonergic neurons and mice lacking central serotonergic system exhibit normal locomotor activity. J Neurosci 26:12781-12788.