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Characterization of the G-Protein Coupling Domains of the 5-HT1A Receptor

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CHARACTERIZATION OF THE G-PROTEIN COUPLING DOMAINS OF THE 5-HT1A RECEPTOR

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Dedication

To my parents,

Rani & Santosh Kushwaha,

My brother and sister,

Nirmal & Anita,

And my husband,

Michael,

I thank you for your endless encouragement, patience and love.

Acknowledgements

First and foremost, I would like to thank Dr. Paul Albert for providing me with guidance and an environment in which I was able to fulfill my potential as a graduate student. I have learned much from Dr. Albert. One lesson I will take with me is how to handle any situation with respect and dignity. As well as being an exceptional scientist, Dr. Albert is also an exceptional human being. He is kind, understanding, and encouraging. I will always look back on my years in his lab fondly.

I would like to thank the members of my advisory committee: Drs. Ian Lorimer, Johnny Ngsee, and Johne Liu, for providing valuable advice and support throughout my studies.

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I have been fortunate to have gained the friendship of many people outside the Albert lab as well. In particular I would like to thank Dr. David Park for the numerous times he has given me both professional and personal advice, but mostly I'd like to thank him for all the great 'chats'.

A last heart-felt thank you goes out to my family. To my parents, who have been my role-models for hard work, persistence and personal sacrifices, and who instilled in me the inspiration to set high goals and the confidence to achieve them. To my brother and sister, who have been my emotional anchors through not only my time in graduate school, but my entire life. Finally, to Michael, who has been proud and supportive of my work and who has shared the many uncertainties, challenges and sacrifices for completing this dissertation.

Abstract

The 5-HT1A receptor is expressed pre-synaptically as the primary somatodendritic autoreceptor on serotonergic raphe neurons, and post-synaptically in several brain regions. However, many of the 5-HT1A-mediated signaling pathways initially identified in non-neuronal cells transfected with the receptor have not been extensively studied in neuronal models. Our first study focused on signaling of the 5-HT1A autoreceptor in RN46A cells, a model of serotonergic raphe neurons that express endogenous 5-HT1A receptors. We found that in RN46A cells stably transfected with the wild-type 5-HT1A receptor, 5-HT1A receptor activation coupled to Gαi-mediated inhibition of forskolin-induced cAMP formation, Gβγ-mediated increase in $[Ca^{2+}]_I$, and induced a novel inhibition of phospho-p42/p44-mitogen activated protein kinase (MAPK) that was also Gβγ-dependent. The activity of 5-HT1A receptors containing mutations of PKC sites in the second (i2: T149A) or third intracellular loop (i3: T229A/S253G/T343A) was also tested. The signaling of the 5-HT1A i3 mutant was similar to the 5-HT1A wild-type receptor while the i2 and quadruple (i2/i3) mutants, which both comprise the T149A substitution, failed to couple to Gβγ-mediated increase in $[Ca^{2+}]_I$ or inhibition of MAPK, but did couple to Gαi-mediated inhibition of cAMP. Thus, the i2 domain of the 5-HT1A autoreceptor is crucial for coupling to Gβγ-mediated signaling.

Based on these findings and previous studies performed in our lab, we wanted to investigate the structure-function relationship of the T149 amino acid and adjacent residues in the coupling of G-proteins, in particular Gβγ subunits, to the 5-HT1A

receptor. Using a random mutagenesis approach, over 60 mutant 5-HT1A receptors were generated in the α-helical Ci2 loop sequence (^{143}DYVNKRTPRR^{152}) and tested for functionality. Mutant receptors were tested for Gβγ signaling to adenylyl cyclase II (ACII) or phospholipase C (PLC) and GαI coupling using sensitive assays to detect constitutive and agonist-induced coupling. Our data support an amphipathic α-helical model coiled at Pro150, with the positively charged face oriented cytoplasmically. The uncharged residues (T149, N146) in this face are required for Gβγ but not GαI coupling and may directly interact with Gβγ subunits. The hydrophobic face is oriented internally towards the C-terminal i3 (Ci3) and N-terminal i2 (Ni2) loops, and includes the critical Y144 that directs the specificity of coupling to both Gβγ and GαI pathways. Coordinate hydrogen and ionic bonding between functionally key residues Y144/K147 (Ci2), D133/R134 (Ni2 DRY motif), and E340 (Ci3) is predicted to stabilize the G-protein coupling domain. These results provide the first detailed evidence that the entire Ci2 domain is a critical determinant for receptor coupling to G-proteins, especially Gβγ subunits, to induce multiple responses.

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List of Abbreviations

-/-	knockout
5-CT	5-carboxytryptamine (a 5-HT receptor agonist)
5-HIAA	5-hydroxyindole acetic acid
5-HT	5-hydroxytryptamine, serotonin
5-HT1A	5-hydroxytryptamine 1A receptor, serotonin 1A receptor
5-HTP	5-hydroxytryptophan
5-HTT	serotonin transporter, SERT
5-HTTLPR	serotonin transporter long polymorphic repeat
8-OH-DPAT	8-hydroxy-2-(di-n-propylamino)tetralin
AC	adenylyl cyclase
Ala	alanine
Amp	ampicillin
AP-2	β2-adaptin
Arf6	ADP-ribosylation factor 6
ARNO	ADP-ribosylation factor nucleotide-binding site opener
Asn	asparagine
Asp	aspartate
ATP	adenosine-5'-triphosphate
β1AR	β1-adrenergic receptor
β2AR	β2-adrenergic receptor
βarr	β-arrestin
βARK	β-adrenergic receptor kinase
BSA	bovine serum albumin
bp	base pairs
Ca^{2+}	calcium
CAMKII	Ca^{2+}/calmodulin-dependent protein kinase II
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
CHO	Chinese Hamster Ovary
Ci2	carboxy-terminal of the second intracellular loop
Ci3	carboxy-terminal of the third intracellular loop
CNS	central nervous system
CO_2	carbon dioxide
cpm	counts per minute
DAG	diacylglycerol
DBD	DNA binding domain
dCTP	deoxycytosine triphosphate
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DOI	1-[2, 5-dimethoxy-4-iodophenyl]-2-aminopropane
DRN	dorsal raphe nucleus
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid

EGTA	ethylene glycol bis(2-aminoethyl ether)-N,N,N'N'-tetraacetic acid
ERK1/2	extracellular signal regulated kinases 1 and 2
FBS	fetal bovine serum
GABA	γ-aminobutyric acid
GDP	guanosine di-phosphate
GI	gastrointestinal
GIRK	G protein-coupled inwardly rectifying potassium channel
GnRH	gonadotropin releasing hormone
GPCR	G protein-coupled receptors
GRK	G protein-coupled receptor kinase
GRK-CT	carboxy-tail of GRK2
GTP	guanosine triphosphate
H-bond	hydrogen bond
HEK 293	human embryonic kidney 293 cells
HEPES	N-2-hydroxyethylpiperazine-N9-2-ethanesulfonic acid
i1	first intracellular
i2	second intracellular
i3	third intracellular
IBMX	3-isobutyl-1-methylxanthine
IP3	inositol triphosphate
JAK/STAT	Janus kinase/signal transducers and activators of transcription
kb	kilobases
KCl	potassium chloride
kDa	kilodalton
LSD	*d*-lysergic acid diethylamide
MAO A	monoamine oxidase A
MAO B	monoamine oxidase B
MAOI	monoamine oxidase inhibitors
MAPK	mitogen-activated protein kinase
MEK1/2	MAPK/ERK kinases 1 and 2
MDD	major depressive disorder
MDMA	3,4-methylenedioxymethamphetamine
$MgCl_2$	magnesium chloride
mGlu	metabotropic glutamate
MRN	median raphe nucleus
mRNA	messenger RNA
Ni2	amino-terminal of the second intracellular loop
Ni3	amino-terminal of the third intracellular loop
NP-40	nonidet P-40
NSF	*N*-ethylmaleimide-sensitive factor
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PET	positron emission tomography
Pet-1	PC12 ETS factor
PFC	prefrontal cortex
PGK-puro	phosphoglycerate kinase 1-puromycin resistance gene

PH	pleckstrin homology
PI	phosphoinositol
PIP_2	phosphatidylinositol-4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PLD	phospholipase D
PMA	phorbol 12-myristate 13-acetate
PM	plasma membrane
PTX	pertussis toxin
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
SERT	serotonin transporter, 5-HTT
SSRI	selective serotonin reuptake inhibitor
SV40	simian virus 40
TCA	tricyclic antidepressant
TBS	Tris-buffered saline
TH	tyrosine hydroxylase
TM	transmembrane domain
TPA	phorbol 12-myristate 13-acetate
TPH	tryptophan hydroxylase
TRH	thyrotropin releasing hormone
TSH	thyroid stimulating hormone
VIP	vasoactive intestinal peptide
VMAT	vesicular monoamine transporter

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Thesis Format

This thesis is written in manuscript format and is composed of two manuscripts listed below. The contribution of each author is also described below.

Chapter II: Coupling of 5-HT1A autoreceptors to inhibition of mitogen-activated protein kinase activation via Gβγ subunit signaling. N. Kushwaha and P. R. Albert. (2005) Eur J Neurosci. 21(3):721-32.

Authors' contribution: I was responsible for all the work in this chapter. The manuscript was written by myself and extensively revised by Dr. Albert.

Chapter III: Molecular determinants in the second intracellular loop of the 5-hydroxytryptamine1A (5-HT1A) receptor for G-protein coupling. N. Kushwaha, S. C. Harwood, A. Wilson, M. Berger, L.H. Tecott, B.L. Roth and P. R. Albert. (2006) Mol Pharmacol. 69(5):1518-1526.

Authors' contribution: I was responsible for most of the work in this chapter including the construction and sequencing of 5-HT1A receptor mutants, transfection, calcium and cAMP assays. Shannon Harwood assisted with the transfection and cAMP assays, and the sequencing of constructs to verify 5-HT1A receptor i2 loop mutations. Ariel Wilson assisted with several of the final cAMP assays. I generated all the models presented in the manuscript using the atomic coordinate file for the rat 5-HT1A receptor created by Bryan Roth. The manuscript was written by myself and extensively revised by Dr. Albert.

Chapter I. Introduction

Serotonin: An Historical Perspective

Serotonin (5-hydroxytryptamine, 5-HT) was originally isolated in the 1930's by Vittorio Erspamer who was investigating various amine substances capable of causing smooth muscle contraction. During his studies he came across an unknown secretory product in an extract of rabbit gastric mucosa. This substance was believed to originate from the enterochromaffin cells of the gastrointestinal (GI) tract and was named 'enteramine' (Erspamer and Vialli, 1937). In the late 1940's, Irvine Page, Maurice Rapport, and Arda Green isolated and characterized a powerful vasoconstrictor substance from bovine blood serum (Rapport et al., 1948a) and named this compound 'serotonin,' from 'sero' (Latin term for serum) and 'tonin' (Greek term for tonic). Page's group also succeeded in isolating a crystalline complex of serotonin (Rapport et al., 1948b), and Rapport went on to determine the chemical structure to be 5-hydroxytryptamine (Rapport, 1949). In 1952, it was reported that enteramine and serotonin were identical compounds (Erspamer and Asero, 1952). The following year Betty Twarog and Irvine Page discovered the presence of 5-HT in mammalian brain and suggested that it may function as a putative neurotransmitter in the central nervous system (CNS) (Twarog and Page, 1953). Serotonin had now entered the field of neuroscience.

Serotonin research exploded when synthetic 5-HT was made available for research by Upjohn Pharmaceutical and Abbott Laboratories in the early 1950's. As investigators began to focus on the function of 5-HT in the CNS, their findings were soon followed by the suggestion that serotonin played a role in mental illness (Wooley and Shaw, 1954). This hypothesis was primarily based on the finding that the vasoconstrictor actions of 5-HT on smooth muscle were antagonized by the structurally-similar

hallucinogen, lysergic acid diethylamide (LSD) (Woolley and Shaw, 1953). Additionally, depressive symptoms were found to be associated with the use of reserpine, an antihypertensive agent that depletes monoamine stores, including serotonin, in some individuals taking the drug for hypertension (Shore et al., 1955). Reserpine was also shown to modify the action of LSD in rodent brains by lowering their levels of 5-HT (Shore et al., 1955). Observations like these led to the hypothesis that affective disorders such as depression, were a result of deficiencies in serotonergic and/or catecholaminergic systems.

In the 1950's and 60's, the first effective drugs to treat depression were agents that enhanced serotonergic neurotransmission: the monoamine oxidase inhibitors (MAOIs) and tricyclic antidepressants (TCAs). The discovery of the mechanism of action and molecular targets of these first generation antidepressants led to the design of second and third generation serotonin-related drugs such as the selective serotonin reuptake inhibitors (SSRIs). Presently, SSRIs such as fluoxetine (Prozac®), paroxetine (Paxil®), and sertraline (Zoloft®), are prescribed for the treatment of depression and other mood disorders and rank among the most commonly prescribed medications worldwide.

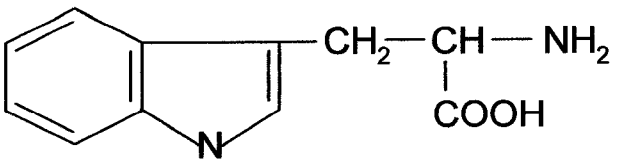
Biosynthesis and Metabolism of Serotonin

In the adult human serotonin is found in three main areas of the body: enterochromaffin cells of the gastrointestinal mucosa (90%); platelets (8-9%); and the CNS (1-2%) (Lambert et al., 1995). Peripherally, serotonin plays a major role in platelet homeostasis, motility of the GI tract, cardiovascular regulation, and carcinoid tumour secretion (tumours derived from the enterochromaffin cells). Centrally, the functions of

serotonin are numerous and involve the control of appetite, sleep, cognition, temperature regulation, pain perception, endocrine functions, mood and behavior (Buhot, 1997). This represents quite a broad spectrum of physiological and psychological effects, considering the fact that the average human adult possesses only about 10 mg of 5-HT (Lambert et al., 1995).

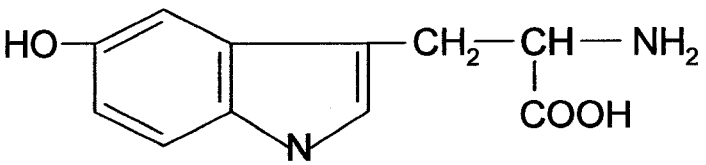
Serotonin is one of the main secretory products secreted by the pineal gland, where is serves as a precursor for melatonin. All other cells must synthesize 5-HT since it is unable to cross the blood brain barrier. The first step in this pathway is the active uptake of the essential amino acid L-tryptophan via a non-specific large neutral amino acid carrier. Plasma tryptophan arises primarily from the diet and depletion of dietary tryptophan can profoundly decrease the levels of brain 5-HT (Twarog, 1988). Serotonin is formed by the hydroxylation and decarboxylation of tryptophan (Figure 1). Serotonin and tryptophan belong to a group of aromatic compounds called indoles with a five-membered ring containing nitrogen joined to a benzene ring. Tryptophan is first hydroxylated by the rate limiting enzyme tryptophan-5-hydroxylase (TPH) to form 5-hydroxytryptophan (5-HTP). This step can be blocked by para-chlorophenylalanine which directly competes with tryptophan and binds to TPH in an irreversible manner. Recently, it has been realized that two isoforms of the TPH enzyme exist in the brain and are encoded by two different genes: *Tph1* and *Tph2* (Walther et al., 2003). TPH2 is found exclusively in the brain where it constitutes the most abundant isoform, while TPH1 is expressed both in the brain and peripheral tissues (Walther et al., 2003). Once synthesized, 5-HTP is almost immediately decarboxylated to 5-HT by the non-specific L-aromatic amino acid decarboxylase. Serotonin is then taken up into secretory vesicles by

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



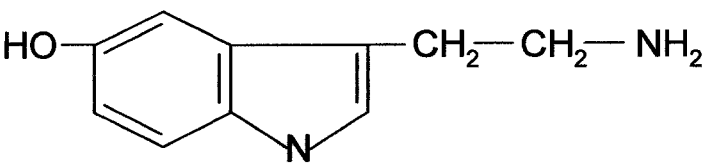
Tryptophan

Tryptophan Hydroxylase (TPH)



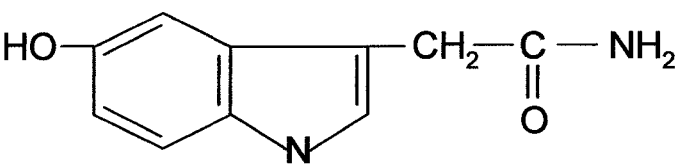
5-hydroxytryptophan (5-HTP)

L-aromatic amino acid decarboxylase



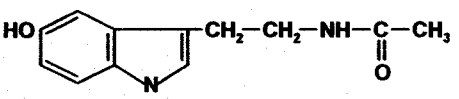
Serotonin (5-HT, 5-hydroxytryptamine)

Monoamine oxidase A (MAO-A) + Aldehyde dehydrogenase



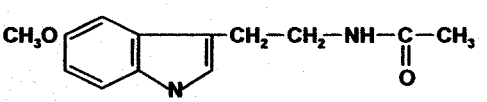
5-hydroxyindole acetic acid (5-HIAA)

5-HT-N-acetyl transferase (NAT) (Pineal)



N-acetyl serotonin

5-hydroxy indole O-methyl transferase



Melatonin

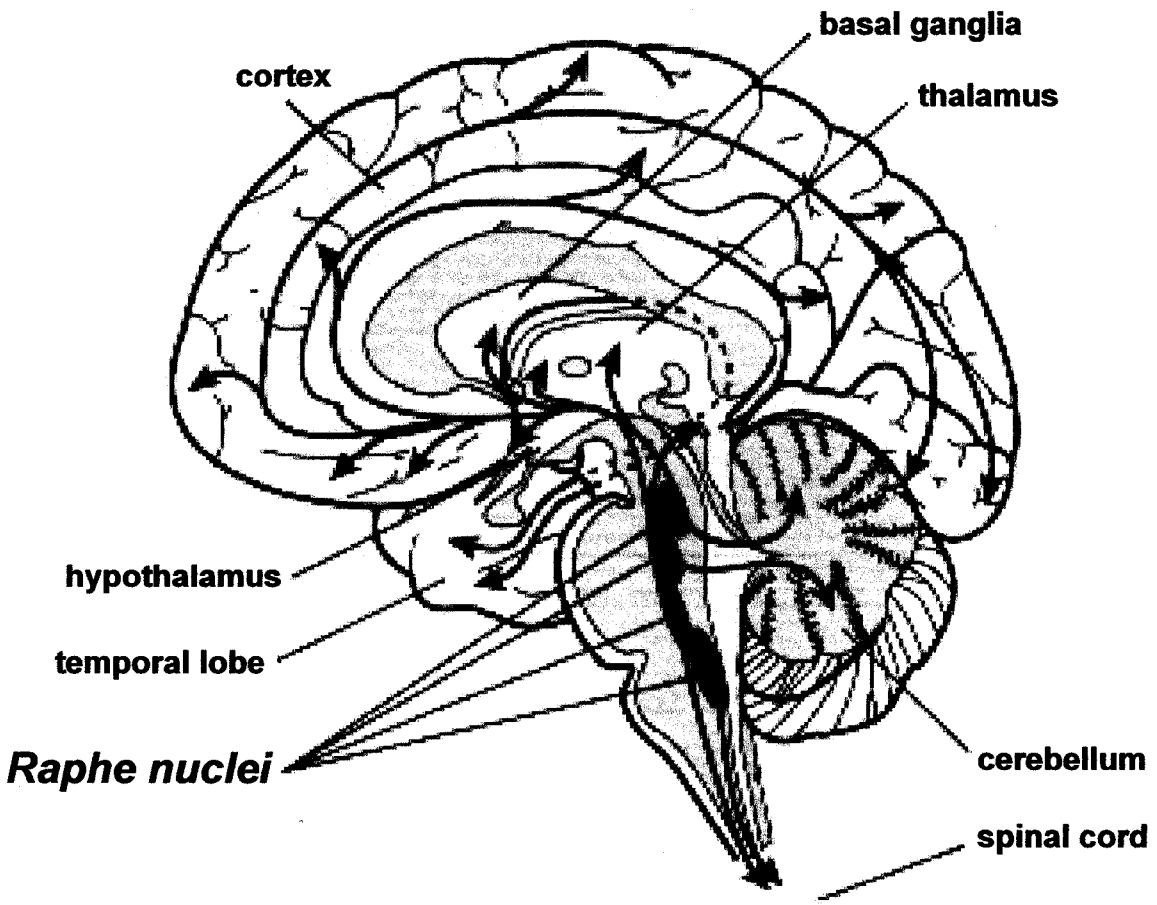
the vesicular monoamine transporter (VMAT) where it is stored for exocytotic release. If 5-HT is not sequestered into vesicles, it is rapidly inactivated by the mitochondrial enzyme monoamine oxidase A (MAO-A) and aldehyde dehydrogenase to the major metabolite, 5-hydroxyindole acetic acid (5-HIAA). 5-HIAA is excreted into the cerebral spinal fluid and eventually into the urine, and is used as a measurement to determine the synthesis of 5-HT in humans (Lambert et al., 1995).

The Central Serotonergic System

The serotonergic system consists of a diverse group of neurons with distinct morphologies, most of which are located in the brainstem raphe nuclei (Figure 2) and a smaller number in the reticular formation (Törk, 1990; Jacobs and Azmitia, 1992). These diffuse and complex axonal systems project to essentially all regions of the CNS, with higher densities in the cerebral cortex, limbic structures, basal ganglia, brainstem, and gray matter of the spinal cord (Törk, 1990; Jacobs and Azmitia, 1992). Serotonergic neurons appear early in development, on embryonic (E) days 10 to 12 in the mouse, E12 to E15 in the rat, and during the first month of gestation in primates (Törk, 1990; Gaspar et al., 2003). Although raphe neurons can synthesize 5-HT and begin to extend their diffuse axonal tracts one day after their generation, complete maturation of the serotonergic network occurs only after birth.

Using histofluorescence techniques, Dahlström and Fuxe (1964) revealed the preferential localization of serotonergic neurons in the brainstem and divided the 5-HT cell groups into nine clusters, B1-B9, the most caudal cluster being B1. These serotonergic cell groups can be further segregated into two subdivisions based on their

Figure 2. Schematic illustration of the CNS serotonergic system.



distribution and main projections: the caudal group, comprised of B1-B5, corresponding to the raphe pallidus, raphe magnus, raphe obscurus, and regions of the adjacent lateral reticular formation; and the rostral group, comprised of B6-B9, corresponding to the caudal linear, dorsal raphe and median raphe nuclei (Wallace and Lauder, 1983; Törk, 1990).

The rostral nuclei account for 85% of all serotonergic neurons in the brain (Hornung, 2003). Projections from these nuclei extensively innervate forebrain structures including those belonging to the limbic system (amygdala, thalamus, hypothalamus, olfactory bulb, basal ganglia, hippocampus, cingulate gyrus) (Törk, 1990; Jacobs and Azmitia, 1992). The caudal group of the raphe system account for a maximum of 15% of the total population of 5-HT neurons in the brain (Hornung, 2003). These neurons provide important descending projections to the spinal cord and the cerebellum.

The ascending serotonergic system (rostral nuclei) plays a crucial role in the control of complex brain functions including learning, memory, appetite, sleep/wake cycle, thermoregulation, stress response, and modulation of emotion (Buhot, 1997). The major ascending projections to the forebrain primarily originate from the dorsal raphe nucleus (DRN) and the median raphe nucleus (MRN). These nuclei provide two distinct classes of fibers, M- and D-type fibers respectively. M-type cell bodies (from the DRN) possess fine axons with small granular varicosities and are vulnerable to neurotoxic amphetamine derivatives such as para-chloroamphetamine (Mamounas et al., 1991). At the ultrastructural level the axons of DRN neurons do not form true chemical synapses, but rather contribute to the paracrine or volume transmission system (Hornung, 2003). D-type neurons (from the MRN) have beaded axons with large varicosities, are resistant

to neurotoxic agents (Mamounas et al., 1991), and have terminals that form true chemical synapses (Hornung, 2003). Both M and D fibers are found together in most brain regions especially cortical areas, whereas the striatum almost exclusively receives input from D fibers, and the dentate gyrus primarily M fibers. The functional significance of this neuronal fiber arrangement has yet to be determined.

In addition to being the two largest serotonergic nuclei, the DRN and MRN also possess non-serotonergic cell types, including γ-aminobutyric acid (GABA), peptide, and dopamine neurons (Hornung, 2003). The complexity of the serotonergic system is amplified by the colocalization of 5-HT with other neurotransmitters such as dopamine and GABA, or neuropeptides such as substance P, galanin, dynorphin, angiotensin, enkephalin, thyrotropin releasing hormone (TRH), and neurotensin (Pineyro and Blier, 1999). Neurotransmitters that 5-HT neurons contain and/or use may also play a role in models of diseases that implicate the serotonergic pathway, such as depression and anxiety.

Serotonin Receptors

Serotonin is implicated a wide variety of physiological and behavioural processes, and its multiple actions are mediated through a large array of highly conserved membrane-bound receptors. These receptors are found in the central and peripheral nervous systems, as well as in a number of non-neuronal tissues in the gastrointestinal, cardiovascular and blood systems. 5-HT receptors belong to the seven transmembrane-spanning G-protein coupled receptor (GPCR) superfamily, with the exception of the 5-HT3 receptor, which is a ligand-gated ion channel. The serotonin receptors are divided

into seven distinct classes (5-HT1 to 5-HT7) and more than fifteen different subtypes (5-HT1A/B/D/E/F, 5-HT2A/B/C, 5-HT3A/B/C, 5-HT4, 5-HT5A/B, 5-HT6, 5-HT7) based on operational (pharmacology, radioligand binding data), transduction (signaling pathways) and structural (gene sequence, amino acid homology, chromosomal location) characteristics (Hoyer et al., 1994; Hoyer and Martin, 1997; Hoyer et al., 2002).

The classification of 5-HT receptors began in 1957, when Gaddum and Picarelli (1957) demonstrated that serotonin-mediated smooth muscle contraction of guinea pig ileum was partially blocked by morphine (M), while the remainder of the response could be blocked by dibenzyline (D). These results led to the speculation that 5-HT activated two different receptors in the gut wall: a neurotropic "M" receptor located on parasympathetic ganglia (effect blocked by morphine and atropine) and a musculotropic "D" receptor located on smooth muscles (effect blocked by dibenzyline, lysergide, 2-bromolysergide and dihydroergotamine) (Gaddum and Picarelli, 1957). Subsequent experiments have shown that the "D" receptor and the 5-HT2 receptor are pharmacologically indistinguishable, and that the "M" receptor is identical to the 5-HT3 subtype (Hoyer et al., 2002).

This classification system held up well for three decades, but with the advances in radioligand binding techniques and synthesis of selective ligands for various receptor subtypes, as well as the advent of molecular cloning, modification of 5-HT receptor classification was inevitable. In 1974, Bennett and Aghajanian reported the first successful radioligand binding study of 5-HT receptors using [^{3}H]-lysergide. Further studies with tritiated-5-HT, -spiperone and -lysergide enabled Peroutka and Snyder (1979) to identify two receptors, named 5-HT1 (with nM affinity for 5-HT) and 5-HT2

(with nM affinity for spiperone, but μM affinity for 5-HT); lysergide had high (nM) affinities for both. Subsequently, 5-HT1 receptors were subdivided into 5-HT1A and 5-HT1B subtypes on the basis of spiperone exhibiting a high and low affinity, respectively (Pedigo et al., 1981), and 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT, DPAT) was designated as a selective 5-HT1A ligand (Gozlan et al., 1983; Middlemiss and Fozard, 1983).

In 1994, the NC-IUPHAR (Nomenclature Committee of the International Union of Pharmacology, for Serotonin Receptors) reclassified 5-HT receptors based on operational, transduction and structural information as mentioned above (Hoyer et al., 1994). Importantly, the current classification has been progressively adapted to accommodate new information obtained from both native and recombinant 5-HT receptors which are being studied in organisms ranging from *Caenorhabditis elegans* and *Drosophila melanogaster* to humans.

5-HT1 Receptor Class

The 5-HT1 receptor class is comprised of five subtypes: 5-HT1A, 5-HT1B, 5-HT1D, 5-HT1E, and 5-HT1F. These members are characterized by their ability to couple preferentially, although not exclusively, to pertussis toxin (PTX)-sensitive Gi/Go proteins to mediate a variety of actions including inhibition of adenylyl cyclase (AC) activity and subsequent cAMP formation. The genes of these receptors are intronless, and in humans, they share 40-63% sequence homology (Hoyer and Martin, 1997). The 5-HT1C receptor subtype was reclassified to 5-HT2C according to the operational, transduction and structural criteria set by NC-IUPHAR (Hoyer et al., 1994).

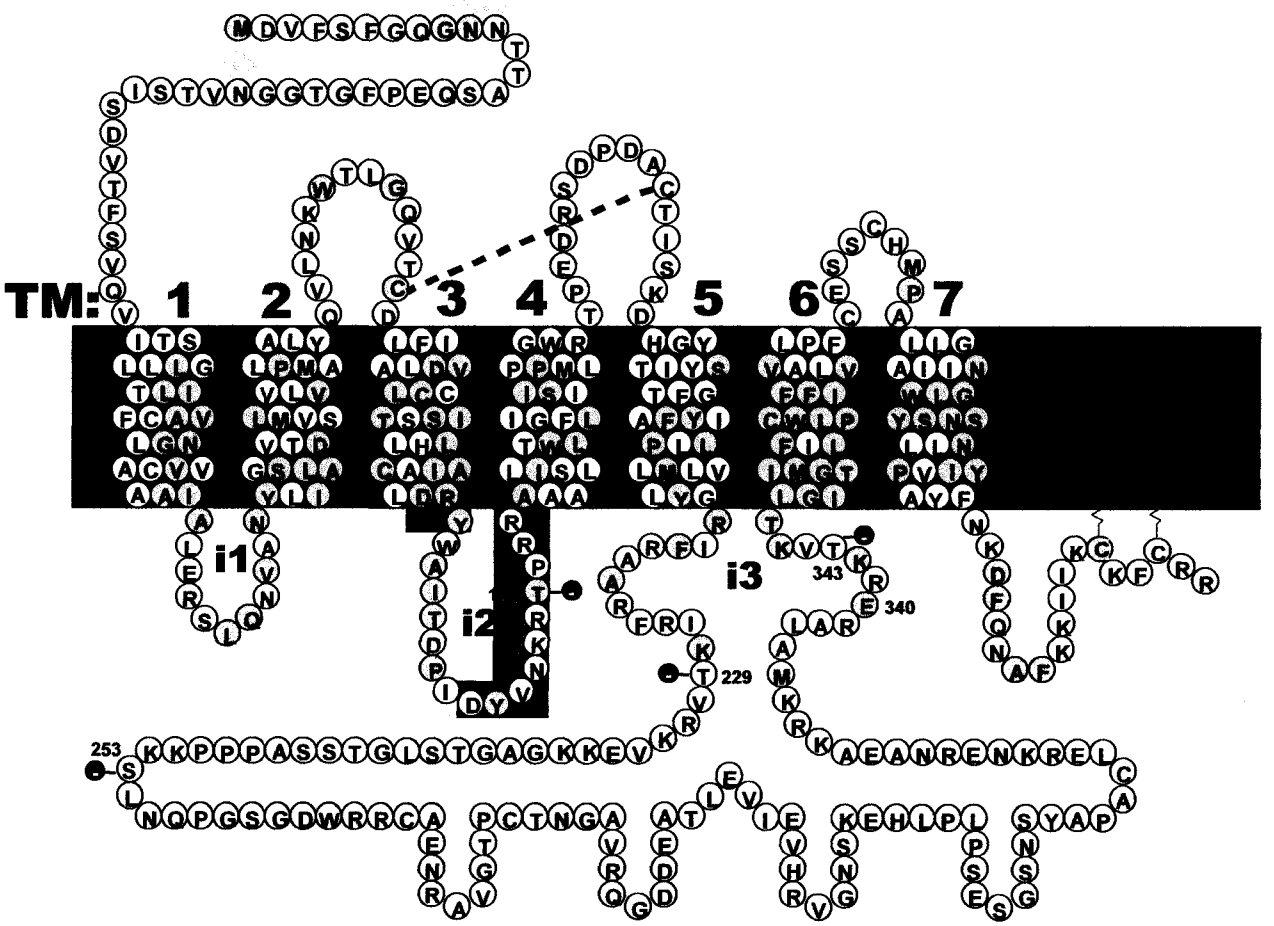
5-HT1A Receptors

The 5-HT1A receptor is one of the best-characterized GPCRs, primarily due to the early discovery of a selective ligand, 8-OH-DPAT, and the molecular cloning and sequencing of the human gene. The 5-HT1A receptor gene is located on chromosome 5q11.2-q13 and was originally isolated as an orphan receptor while screening a human genomic library using probes for the β2-adrenergic receptor with which it shares considerable amino acid sequence homology (Kobilka et al., 1987), and subsequently identified as the 5-HT1A receptor (Fargin et al., 1988). Since then, 5-HT1A receptor genes have been cloned from a number of organisms including rat (Albert et al., 1990), mouse (Charest et al., 1993), *Xenopus laevis* [Marracci, 1997 #472], *Fugu rubripes* (Yamaguchi and Brenner, 1997), and Drosophila (Nikoh et al., 2004). The human 5-HT1A receptor nucleic acid sequence is 88% and 80% homologous to that of rat and mouse sequences, respectively (Albert et al., 1990; Charest et al., 1993).

The 5-HT1A receptor protein is composed of 422 amino acids that has a core molecular weight of approximately 46 kilodaltons (kDa), and an isoelectric point of 8.8 (Raymond et al., 1999). As depicted in Figure 3, the 5-HT1A receptor contains an extracellular amino terminus, a cytoplasmic C-terminal domain and seven hydrophobic stretches of 20-24 amino acids predicted to form membrane-spanning α-helices (TM1-TM7). These transmembrane domains are connected by three intracellular (i1, i2, i3) and three extracellular (e1, e2, e3) loops. Recently, it was shown that unlike other receptors, the 5-HT1A receptor is constitutively palmitoylated at C-terminal cysteine residues (417 and 420), thereby forming a putative fourth intracellular loop (i4) and a short eighth alpha-helical domain (Papoucheva et al., 2004). The intracellular loops appear to be

Figure 3. Model of the rat 5-HT1A receptor.

The amino acid sequence of the rat 5-HT1A receptor is shown with the predicted transmembrane domains (TM) and intracellular loops (i1, i2, i3) as indicated. Shade residues are identical to those of the β2-adrenergic receptor, and the conserved DRY motif and the Ci2 α-helical region are boxed. Potential disulfide linkage (dashed line) and palmitoylation sites (squiggle) are indicated. PKC phosphorylation sites (T149, T229, S253, T343) and Ci3 residue E340 are numbered.



important in the coupling of the receptor to G-proteins. There are four specific protein kinase C (PKC) phosphorylation sites located within the second and third intracellular loops (Thr149 in the i2; Thr229, Ser253, Thr343 in the i3), that have been implicated in receptor desensitization (Lembo and Albert, 1995; Lembo et al., 1997; Wu et al., 2002). Two of these consensus sequences are also putative protein kinase A (PKA) phosphorylation sites (Thr229 and Thr343) (Liu and Albert, 1991; Raymond and Olsen, 1994). Three consensus sites for *N*-linked glycosylation are present on the amino-terminus of the receptor, and may account for the heavier forms of the receptor observed at 55 and 65 kDa by Western blot analysis. A disulfide bond may form between Cys109, located at the e1/TM3 junction, and Cys186, found in the e2, which could stabilize 5-HT1A receptor conformation (Figure 3). Within the transmembrane domains exist highly conserved residues that are important for agonist binding and determine receptor-ligand specificity (Figure 3) (Ostrowski et al., 1992; Raymond et al., 1999).

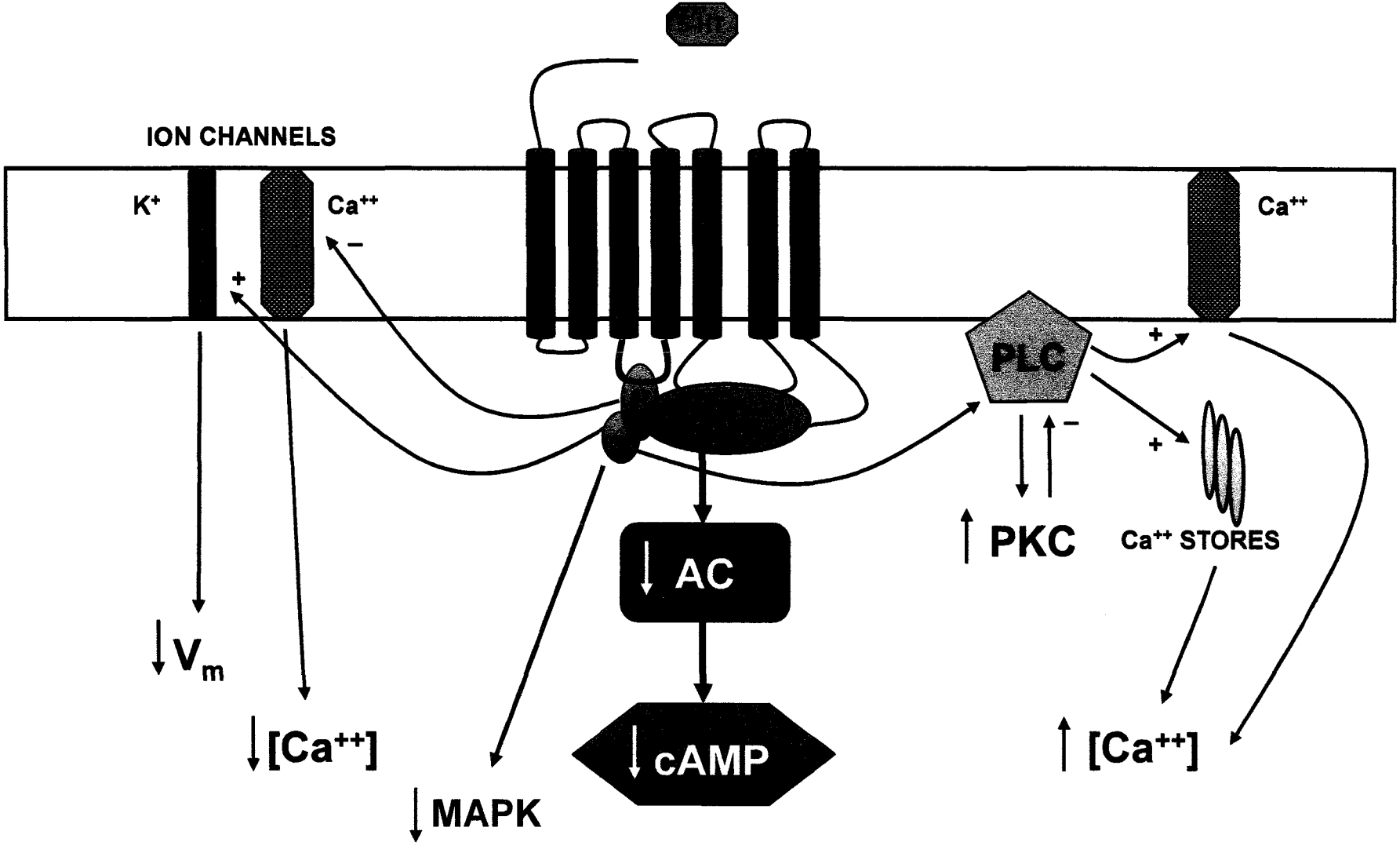
The 5-HT1A receptor is expressed mainly in the CNS, GI tract, spleen, and neonatal kidney (Kobilka et al., 1987; Fargin et al., 1989). In the brain, the 5-HT1A receptor is expressed presynaptically as the major autoreceptor localized to the soma and dendrites of serotonergic raphe neurons (Sotelo et al., 1990; Riad et al., 2000), and is also expressed postsynaptically throughout the central nervous system with highest densities in the hippocampus, entorhinal cortex, amygdala, and lateral septum (Chalmers and Watson, 1991; Pompeiano et al., 1992). As a classical autoreceptor, the 5-HT1A receptor is expressed on serotonergic raphe neurons and negatively regulates the release of 5-HT onto postsynaptic sites by modulating neurotransmitter synthesis, terminal release, and

rate of cell firing (Albert et al., 1996; Evrard et al., 1999). Hence, the 5-HT1A receptor represents a critical modulatory site for overall regulation of the serotonin system.

Signaling Pathways: The 5-HT1A receptor couples to PTX-sensitive Gi/Go proteins to mediate a variety of responses depending on the cell type (Figure 4) (Albert, 1994; Raymond et al., 1999). For example, in fibroblasts 5-HT1A receptors exhibit a 'stimulatory' phenotype in that they activate mitogen-activated protein kinase (MAPK)/extracellular signal regulated kinase (ERK) (Cowen et al., 1996; Garnovskaya et al., 1996; van Biesen et al., 1996a) and phospholipase C (PLC), leading to calcium mobilization and PKC activation (Liu and Albert, 1991; Lembo and Albert, 1995). By contrast, in neuronal cells including raphe neurons, hippocampal neurons, F11 (dorsal root ganglion X neuroblastoma) cells (Wu et al., 2002), and neuroendocrine pituitary GH4C1 cells, the 5-HT1A receptor inhibits adenylyl cyclase (AC) activity (De Vivo et al., 1986; De Vivo et al., 1990; Kushwaha et al., 2005), increases potassium conductance (Penington et al., 1993; Bayliss et al., 1997b), and inhibits calcium channels (Penington et al., 1991; Bayliss et al., 1997a) to inhibit neuronal activity. Additionally, the 5-HT1A receptor also decreases MAPK in neurons (Chen et al., 2002; Kushwaha et al., 2005), which is expected to inhibit gene expression, and this fits with the inhibitory signaling phenotype of neuronal 5-HT1A receptors. In serotonergic raphe neurons, these actions of the 5-HT1A autoreceptor combine to regulate the activity of the entire serotonergic system.

Figure 4. Signal transduction pathways of the 5-HT1A receptor.

The 5-HT1A receptor is a member of a family of receptors that couple to pertussis toxin-sensitive G proteins (G_i/G_o) to initiate inhibitory or stimulatory responses, depending on the cell type in which the receptor is expressed. When transfected into pituitary GH4C1 cells or endogenously expressed in neurons, the 5-HT1A receptor displays an inhibitory signaling phenotype, including reduced adenylyl cyclase activity, opening of K^+ channels, and inhibition of calcium influx induced by the dihydropyridine Ca^{2+} channel agonist BayK8644, leading to decreased $[Ca^{2+}]_i$ levels. However, when expressed in a variety of fibroblast-derived cells, such as Ltk-, HeLa, or Balb/c-3T3, the 5-HT1A receptor enhances PI turnover, releasing intracellular Ca^{2+} stores to increase $[Ca^{2+}]_i$. In raphe RN46A cells, the 5-HT1A receptor couples to the ubiquitous Gαi-mediated inhibition of AC, and to Gβγ-mediated calcium mobilization and a novel inhibition to MAPK activity.



Coupling to G-proteins: The 5-HT1A receptor mediates its actions almost exclusively through pertussis-toxin sensitive Gi/o-proteins. PTX comprises an ADP-ribosyltransferase that catalyzes ADP-ribosylation of a conserved C-terminal cysteine residue on Gαi/o subunits rendering them incapable of exchanging GDP for GTP, and thus uncoupling the G-protein from its receptor. Studies examining 5-HT1A receptor coupling to G-proteins in various expression systems has established a rank order of Gαi3 > Gαi2 ≥ Gαi1 ≥ Gαo > Gαz (Bertin et al., 1992; Raymond et al., 1993; Garnovskaya et al., 1997). Recent studies have further indicated that regional differences in the distribution of these G-proteins in the brain may also contribute to variations in 5-HT1A receptor signaling. Using immunoaffinity chromatography, 5-HT1A receptors from rat brain were found to interact with Go, Gi1, Gi3 and Gz in the hypothalamus, equally with Go and Gi3 in the cerebral cortex, predominantly with Go and weakly with Gi3 in the hippocampus, and exclusively with Gi3 in the anterior raphe (Mannoury la Cour et al., 2006).

Coupling to AC: The 5-HT1A receptor has been shown in numerous cell types to inhibit both basal and forskolin-stimulated AC activity, and in some cases can also stimulate certain AC subtypes. Inhibition of AC is most likely mediated by Gi2 or Gi3 in most cells, with minor roles for Gi1 and Go (Fargin et al., 1991; Raymond et al., 1993; Liu et al., 1994).

In contrast, it has also been shown that the 5-HT1A receptor can positively couple to AC in the hippocampus (Markstein et al., 1986; Shenker et al., 1987; Cadogan et al., 1994). Results form several studies point to the presence of ACII as a key factor for this

type of signaling. Using an antisense approach, Liu et al. (1999) demonstrated that ablation of Gi1 neuroendocrine GH4C1 cells results in accumulation of cAMP rather than depletion. Furthermore, this effect was PTX-sensitive suggesting the involvement of Gi/o proteins. More detailed studies performed in HEK 293 cells showed a requirement of ACII, Gi2 and Gβγ subunits for 5-HT1A receptor to increase cAMP in an agonist-independent manner (Albert et al., 1999). So it appears that the 5-HT1A receptor can stimulate cAMP accumulation in cells that express ACII via release of Gβγ subunits from PTX-sensitive Gαi2 proteins.

Coupling to PLC: The 5-HT1A receptor can activate the PLC pathway in a cell-specific manner. Activated PLC converts phosphatidylinositol-4, 5-bisphosphate (PIP2) to inositol triphosphate (IP3) and diacylglycerol (DAG). IP3 releases calcium from intracellular stores and DAG activates PKC. Transfected human 5-HT1A receptor can activate PLC in various cell lines including HeLa cells and Xenopus oocytes, as well as in cell lines that endogenously express the receptor such as T-cell-derived Jurkat cells (Fargin et al., 1989; Aune et al., 1993; Ni et al., 1997). In transfected Ltk- or BALB/c-3T3 fibroblasts and rat raphe RN46A cells, the rat 5-HT1A receptor can increase phosphoinositol (PI) hydrolysis and release calcium from intracellular stores, however, this effect is not observed in GH4C1 pituitary cells (Liu and Albert, 1991; Abdel-Baset et al., 1992; Kushwaha and Albert, 2005). The activation of PLC (especially subtypes β2 and β3) is mediated by Gβγ subunits (Wang et al., 2000).

Coupling to Ion Channels: The 5-HT1A receptor has been shown to couple to several types of ion channels including the activation of the inwardly rectifying potassium channels (GIRKs) (Zgombick et al., 1989; Penington et al., 1993; Bayliss et al., 1997b) and inhibition of voltage-dependent calcium channels (Penington et al., 1991; Bayliss et al., 1997a). Both these effects are Gβγ-mediated (Doupnik et al., 1996; Chen and Penington, 1997). Opening of GIRKs in neurons hyperpolarizes the cell leading to inhibition of cell firing. The 5-HT1A receptor has also been shown to inhibit both N- and P/Q-type calcium channels in raphe neurons (Penington et al., 1991; Bayliss et al., 1997a) which would result in a decrease in Ca^{2+}-dependent signaling in these cells. Using stable expression of full-length antisense Gαi/o cDNAs, Liu et al. (1994) demonstrated that the rat 5-HT1A receptor could inhibit dihydropyridine-mediated Ca^{2+} influx in GH4C1 cells and that this effect required Gαo. More recently, 5-HT1A receptors were shown to also inhibit AMPA receptor channels in cortical neurons, which would result in a suppression of glutamatergic signaling (Cai et al., 2002). This effect was found to occur through inhibition of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), which may be achieved by the inhibition of AC and PKA activity and subsequent stimulation of protein phosphatase 1 (PP1).

Coupling to MAPK: In fibroblast-derived cells and hippocampal HN2-5 cells, 5-HT1A receptor stimulates MAPK activation (Cowen et al., 1996; Garnovskaya et al., 1996; van Biesen et al., 1996b; Adayev et al., 2003). This signaling to the MAPK pathway is complex and appears to involve receptor internalization and potential cross talk with molecules of the receptor tyrosine kinase pathway (Luttrell, 2002). The 5-HT1A-

activation of ERK requires Gβγ subunits released from PTX-sensitive G-proteins (Garnovskaya et al., 1996). This results in a signaling cascade involving Src, Shc, phosphoinositide-3 kinase (PI3K), Sos, Grb2, and subsequent activation of Ras-Raf-MEK pathway. As well, a Ca^{2+}/calmodulin (CaM)-dependent step appears to be involved during endocytosis (Della Rocca et al., 1999).

In our studies we have characterized a novel 5-HT1A receptor signaling pathway in rat raphe RN46A cells to inhibit ERK1/2 phosphorylation; another signal initiated by Gβγ subunits (Kushwaha and Albert, 2005). Similarly, in hippocampal slices and extracts 5-HT1A mediated inhibition of ERK1/2 is also observed (Chen et al., 2002; Vanhoose et al., 2002).

5-HT1A Receptor Expression in Mental Illness: Major depressive disorder (MDD, major or unipolar depression) affects between 15-20% of the general population (Holden, 2000), with women twice as likely to develop the disease as men (Doris et al., 1999). Interestingly, in patients with depression, comorbidity with anxiety disorder occurs in 25 to 50% of individuals and is associated with increased severity of the disease (Kessler et al., 2002). Many drugs used to treat depression and anxiety target the serotonin system, a critical regulator of mood and emotion. Evidence suggests that hyperactivity of the serotonin system is involved in anxiety disorders (Gross and Hen, 2004), whereas depression may result from a decreased activity of the 5-HT system (Blier and de Montigny, 1999; Lesch and Heils, 2000).

Data from clinical studies implicates altered regulation of 5-HT1A receptors in depression and other mental illnesses. Examination of post-mortem brains from

depressed suicide victims revealed an elevated density of 5-HT1A autoreceptors in the raphe nuclei, with no change in postsynaptic 5-HT1A receptor sites compared to nonsuicide controls (Stockmeier et al., 1998). Additionally, PET imaging studies have confirmed a widespread reduction of postsynaptic 5-HT1A receptors in major depression (Sargent et al., 2000) and bipolar disorders (Drevets et al., 2000). These results suggest that an increase in 5-HT1A autoreceptor levels or decrease in postsynaptic receptors, leading to a reduction in serotonergic activity may predispose an individual to major depression. Recently, we have identified a C(-1019)G polymorphism in the promoter region of the 5-HT1A receptor gene that is associated with depressed patients and suicide victims (Lemonde et al., 2003). The polymorphism appears to derepress 5-HT1A autoreceptor expression to reduce serotonergic neurotransmission, possibly predisposing an individual to depression and suicide (Lemonde et al., 2003; Albert and Lemonde, 2004).

5-HT1A Autoreceptors in Antidepressant Action: Dys-regulation of both serotonin levels and serotonin receptor function, in particular the 5-HT1A receptor, has been implicated in neuropsychiatric disorders such as depression and anxiety. The drugs used to treat depression such as monoamine oxidase inhibitors (MAOIs), tricyclic antidepressants (TCAs) and selective serotonin reuptake inhibitors (SSRIs) in particular, target the serotonin system and act to boost synaptic serotonin levels (Stahl, 1998), suggesting that hypoactivity of the serotonergic system may predispose an individual to an episode of depression. SSRIs rapidly enter the brain and act by blocking the serotonin transporter (5-HTT) to reuptake 5-HT, which should immediately increase 5-HT

neurotransmission. Unfortunately, current antidepressant compounds require two to four weeks to demonstrate their effectiveness and are not always successful. This delay in antidepressant action may be due to negative feedback regulation of 5-HT neurotransmission by 5-HT1A autoreceptors. Acute treatment with antidepressant drugs (e.g. SSRI blockade of the 5-HTT) causes a marked enhancement of extracellular 5-HT both within the midbrain raphe and at postsynaptic sites (Adell and Artigas, 1991). Local dendro-dendritic release of 5-HT in the raphe nuclei from recurrent axonal collaterals activates the 5-HT1A autoreceptors which leads to inhibition of cell firing, accompanied by a reduction of terminal 5-HT release, thus attenuating the increase in extracellular 5-HT produced by reuptake blockade (Artigas et al., 1996). Long-term administration of SSRIs results in desensitization (or down-regulation) of raphe 5-HT1A autoreceptors, reducing the strength of this negative feedback which likely accounts for the therapeutic effect of the antidepressant treatment, i.e. by facilitating 5-HT neurotransmission (Blier and de Montigny, 1994). This chronic treatment with antidepressants selectively desensitizes 5-HT1A autoreceptors, whereas postsynaptic receptors are relatively resistant to agonist-induced desensitization (Blier and de Montigny, 1994; Pineyro and Blier, 1999).

Furthermore, it has been proposed that 5-HT1A autoreceptor blockade (i.e. using pindolol, a 5-HT1A receptor antagonist) (Artigas et al., 2001) or desensitization (i.e. treatment with full or partial 5-HT1A receptor agonists) is a crucial step to accelerate and enhance the action of antidepressant compounds such as fluoxetine (Blier et al., 1997). Preventing the negative feedback by 5-HT1A receptor antagonism could enable a more rapid increase of synaptic 5-HT by mimicking autoreceptor desensitization produced by prolonged SSRI treatment (Artigas et al., 1996). Faster autoreceptor desensitization can

also be achieved with agonist treatment which directly stimulates the 5-HT1A receptors. These effects suggest that desensitization of raphe 5-HT1A autoreceptors is an essential component of the antidepressant effect (Blier and de Montigny, 1994).

Genetic manipulation of the 5-HT1A receptor: It was in late 1998 when three independent laboratories published descriptions of the generation of 5-HT1A receptor knockout mice (Heisler et al., 1998; Parks et al., 1998; Ramboz et al., 1998). Despite using mice with different genetic backgrounds (129/Sv, C57BL/6, Swiss-Webster) and different techniques, the 5-HT1A receptor knockouts displayed surprisingly similar behavioural phenotypes. Mice lacking the 5-HT1A receptor exhibited increased anxiety-related and antidepressant-like behaviours in several different paradigms (Heisler et al., 1998; Parks et al., 1998; Ramboz et al., 1998) associated with increased serotonergic neurotransmission (Parsons et al., 2001; Richer et al., 2002).

Behavioural characterization showed that the 5-HT1A knockout mice exhibited anxiety-like behaviour in conflict-based tests such as the open field (Heisler et al., 1998; Parks et al., 1998; Ramboz et al., 1998), elevated plus maze (Heisler et al., 1998; Ramboz et al., 1998), novelty-suppressed feeding (Zhuang et al., 1999), and novel object exploration tasks (Heisler et al., 1998). The 5-HT1A knockout mice also demonstrated antidepressant-like effects such as, increased mobility in the tail suspension (Heisler et al., 1998) and forced swim tests (Parks et al., 1998; Ramboz et al., 1998). In general, knockout mice were not different in their total activity levels from their wild-type counterparts (Heisler et al., 1998; Parks et al., 1998; Ramboz et al., 1998). Motor coordination, which

was tested by rotorod performance also revealed no significant differences (Heisler et al., 1998; Parks et al., 1998).

Since the knockout mice had the 5-HT1A receptor deleted in all brain regions it was difficult to assess which receptor population (pre-or postsynaptic) contributed to anxiety in the KO animals. This question was answered using an elegant conditional and tissue-specific receptor expression approach by Gross et al. (2002) who demonstrated that expression of the 5-HT1A receptor at postsynaptic sites (i.e. in the hippocampus, cortex, and amygdala) was sufficient to rescue the behavioural phenotype of the 5-HT1A knockout mice. In the rescue mice, the 5-HT1A receptor gene was driven by a doxycycline-inducible αCaMKII promoter which directed expression to postsynaptic areas. One weakness of using this approach is that 5-HT1A receptor binding in these regions did not precisely mimic the wild-type receptor binding pattern and so the CaMKII promoter may have driven 5-HT1A expression in inappropriate neuronal cell types in the forebrain. The findings also indicated that ablation of the 5-HT1A receptor in the forebrain structures of mice resulted in an anxiety-related phenotype. Moreover, it was shown that expression of the 5-HT1A receptor during a critical period of time of postnatal development (5-21 days) rescued the anxiety-like behaviour of the conditional knockout mice (Gross et al., 2002). Unfortunately, with this model the contribution of the raphe 5-HT1A autoreceptor to the anxiety and antidepressant-like behaviours could not directly be addressed.

5-HT1B/5-HT1Dβ Receptors

Initially, the 5-HT1B receptor was thought to exist only in rodent species (e.g. rat, mouse, hamster, opossum) (Hoyer et al., 1994; Sari, 2004), but molecular cloning and sequencing studies have shown that it is the homologue of the human 5-HT1Dβ receptor (human 5-HT1B) (Adham et al., 1992). There are slight structural differences between the two receptor subtypes with the rodent 5-HT1B receptor comprising of 386 amino acids, and the human 5-HT1B receptor containing 390 amino acids (Sari, 2004). The human 5-HT1B receptor is located on chromosome 6q13 (Hoyer et al., 2002). Despite a 97% overall sequence homology between rodent and non-rodent species homologues, these receptor subtypes display distinct pharmacological profiles. For example, some β-adrenergic antagonists derived of pindolol, bind 5-HT1B receptors with much higher affinity than 5-HT1Dβ receptors (Adham et al., 1992; Hamblin et al., 1992). These pharmacological differences between the two receptor subtypes were found to be due to a single amino acid change in the seventh transmembrane domain, asparagine (5-HT1B) versus threonine (5-HT1Dβ) (Asn355Thr) (Oksenberg et al., 1992).

Interestingly, the distribution pattern of 5-HT1B receptor protein and its encoding mRNA is quite different. In the brain, Northern blot analysis and *in situ* hybridization studies have identified 5-HT1B receptor mRNA in the raphe nuclei, striatum, Purkinje cell layer of the cerebellum, CA1 pyramidal neurons of the hippocampus, entorhinal and cingulate cortex, subthalamic nucleus, retinal ganglion cells, olfactory tubercle and nucleus accumbens (Maroteaux et al., 1992; Bruinvels et al., 1994). However, binding studies using the 5-HT1B-specific radioligand cyanopindolol or the 5-HT1D-specific radioligand SCM-G-TNH2, demonstrated receptor sites in the globus pallidus, substantia nigra, striatum, subiculum, and superior colliculi (Hoyer et al., 1985; Bruinvels et al.,

1994). Furthermore, studies using radioligand binding, *in situ* hybridization, viral transfection and immunocytochemistry at the ultrastructural level have demonstrated that 5-HT1B receptors are localized at nerve terminals, where they are thought to function as presynaptic receptors (Sari, 2004). 5-HT1B receptors act as autoreceptors on serotonergic axonal varicosities and terminals to inhibit 5-HT synthesis and release (Gothert, 1990). They are also expressed on non-serotonergic neurons as terminal heteroreceptors regulating the release of other neurotransmitters such as acetylcholine, glutamate, dopamine, and GABA (Gothert, 1990). Peripherally, 5-HT1B receptors are found on cerebral arteries and other vascular tissues where they mediate effects such as inhibition of noradrenaline release from sympathetic nerve terminals in rodent vena cava (Hoyer et al., 2002) and vasoconstriction of rat caudal arteries (Craig and Martin, 1993).

The signaling of the 5-HT1B receptor is very similar to that of the 5-HT1A receptor. The 5-HT1B receptor couples to PTX-sensitive Gi/Go proteins to initiate multiple effector pathways including inhibition of AC activity (Hoyer et al., 1994), the opening of GIRKs (Ghavami et al., 1997), activation of PLC (Albert, 1994; Lembo et al., 1999), and stimulation of DNA synthesis and cell growth (Seuwen et al., 1988; Pauwels et al., 1996).

5-HT1B receptors have been shown to be involved in several physiological functions and behaviors including locomotor activity, drug abuse reinforcement, migraine, anxiety and aggressive behaviors. Mice lacking 5-HT1B receptors (Saudou et al., 1994) display increased aggressiveness in the resident-intruder and maternal aggression tests (Saudou et al., 1994), increased exploratory activity in the open field (Ramboz et al., 1996) or when placed in a rich environment (Malleret et al., 1999), and

enhanced spatial memory performance in the Morris water maze (Malleret et al., 1999) than their wild-type counterparts. The 5-HT1B knockouts also exhibit increased susceptibility to cocaine addiction (Castanon et al., 2000) and elevated alcohol consumption (Crabbe et al., 1996). Constitutive inactivation of the 5-HT1B receptor, particularly during development, has led to compensatory changes in these KO mice such as region-specific decreases in 5-HTT levels (Ase et al., 2001), 5-HT hyperinnervation in the amygdalo-hippocampal nucleus and ventral hippocampus (Ase et al., 2001), and reduction in 5-HT2C receptor function (Clifton et al., 2003). Certainly, these adaptive changes could play a role in the observed behavioral phenotypes (e.g. aggressiveness), and might also explain better performance of the 5-HT1B$^{-/-}$ mice in some cognitive tests.

5-HT1Dα Receptors

The original pharmacology of the 5-HT1D receptor was based on a composite of two indistinguishable receptor subtypes. In 1992, two structurally distinct genes were cloned encoding human 5-HT1 receptors with pharmacological profiles closely resembling the 5-HT1D receptor (Weinshank et al., 1992). Since the operational profiles of these two new receptors were virtually identical, they were called 5-HT1Dα (renamed 5-HT1D) and 5-HT1Dβ (human 5-HT1B). The 5-HT1D receptor is located on chromosome 1p34.3-p36.3 and possesses an overall structural homology of 63% with the 5-HT1B receptor (Hoyer and Martin, 1997). Although 5-HT1D receptor levels are much lower than those of 5-HT1B receptors, the distribution pattern of the two subtypes is similar, with the exception of the cerebellum where 5-HT1D receptor expression is undetectable (Bonaventure et al., 1997). *In situ* hybridization experiments have shown

the presence of 5-HT1D mRNA in pyramidal cells of the olfactory tubercle (Bonaventure et al., 1998), and more recently, ligand binding studies suggest the presence of a 5-HT1D autoreceptor in the DRN (Stamford et al., 2000). Additionally, 5-HT1D receptors have been found in the human heart where they modulate 5-HT release (Hoyer and Martin, 1997).

The 5-HT1D receptor exhibits the conventional PTX-sensitive, negative coupling to AC when heterologously expressed in several cell lines (Gerhardt and van Heerikhuizen, 1997). Human 5-HT1D receptors have also been shown to increase calcium mobilization and inositol phosphate (IP) turnover (Zgombick et al., 1993).

5-HT1E Receptors

The human 5-HT1E receptor gene was independently cloned by several laboratories and encodes a protein of 365 amino acids (Levy et al., 1992; McAllister et al., 1992; Zgombick et al., 1992; Gudermann et al., 1993). Sequence comparison demonstrates that the 5-HT1E receptor has the highest homology with the 5-HT1F receptor, followed by the 5-HT1B and 5-HT1D receptors (McAllister et al., 1992; Zgombick et al., 1992). The 5-HT1E gene resides on chromosome 6q14-q15 (Hoyer et al., 2002).

In situ hybridization analysis of human and primate brains revealed the distribution of 5-HT1E mRNA transcripts in the striatum, hypothalamus, and cortex (Bruinvels et al., 1994). The protein distribution of the 5-HT1E receptor in the brain is inconclusive largely due to the lack of selective ligands and antibodies. Most distribution studies depict a combined localization of 5-HT1E/1F binding sites, and in human these

sites are detected in the cortex, striatum, hippocampus, and amygdala (Leonhardt et al., 1989; Fugelli et al., 1997). The physiological function of these receptors remains to be determined.

Agonist-stimulated 5-HT1E receptors, transfected into different mammalian cell lines, can inhibit forskolin-stimulated AC activity (Levy et al., 1992; McAllister et al., 1992; Zgombick et al., 1992; Gudermann et al., 1993). Yet, the percent inhibition mediated by the 5-HT1E (30-35%) is weak compared to other 5-HT1 receptors (50-60%). This observed difference may be the result of multiple receptor-G-protein interactions. In fact, it has been shown that the 5-HT1E receptor can couple to either Gi (to inhibit AC) or Gs (to stimulate AC) depending on receptor density (Adham et al., 1994). Coupling to other effector pathways such as PLC or intracellular calcium has not yet been reported.

5-HT1F Receptors

Little is known about the 5-HT1F receptor whose cDNA was first cloned from mouse and human (Hoyer et al., 1994). The 5-HT1F receptor gene has been mapped to chromosome 3p11 and its gene product consists of 366 amino acids. Sequence analysis reveals that the 5-HT1F receptor is most closely related to the 5-HT1E receptor (70% homology) when comparing the TM domains (Hoyer et al., 1994). In primate and rodent brains, the 5-HT1F mRNA has been detected in the DRN, hippocampus, and cortical regions (Adham et al., 1993b; Bruinvels et al., 1994). Recent binding studies on rodent, monkey, and human brain samples using a high-affinity, selective 5-HT1F receptor agonist LY334370, revealed specific sites in the cortex (layers 4-5), olfactory bulb and tubercle, nucleus accumbens, striatum, thalamus, hippocampus, subiculum, amygdala,

and cerebellum (granule layer) (Lucaites et al., 2005). Although the precise function of this receptor remains unclear, 5-HT1F localization may provide clues of its physiological role. For example, the presence of the 5-HT1F receptor in limbic regions such as the striatum and amygdala, may indicate a role in the regulation of mood and emotion as has been shown for other 5-HT receptors (e.g. 5-HT1A, 5-HT1B). Similarly, localization of the receptor in the cortex, subiculum, and hippocampus, may indicate a role for the receptor in learning and memory. Of course, more *in vivo* data is required to ascertain the importance and function of the 5-HTF receptor.

Like other members of its class, 5-HT1F receptors expressed in different cell lines (NIH-3T3, HeLa, or Ltk- cells) can mediate the inhibition of forskolin-induced AC to decrease cAMP accumulation (Adham et al., 1993b; Gerhardt and van Heerikhuizen, 1997). Additionally, the transfected 5-HT1F receptor can also activate PLC and calcium mobilization in Ltk- mouse fibroblasts (Adham et al., 1993a).

5-HT2 Receptor Class

This class of 5-HT receptors is comprised of three members, the 5-HT2A, 5-HT2B, and 5-HT2C receptors. These receptors exhibit 46-50% overall sequence homology and their genes lack introns at highly conserved positions (Gerhardt and van Heerikhuizen, 1997; Hoyer and Martin, 1997). The 5-HT2 receptors couple preferentially to Gq/11 proteins to activate PLC, which in turn converts PIP2 to IP3 and DAG; IP3 releases intracellular calcium stores and DAG activates PKC.

5-HT2A Receptors

The 5-HT2A receptor was originally described as the 'D' receptor by Gaddum and Picarelli (1957), and later defined as the 5-HT2 subtype (Peroutka and Snyder, 1979). The receptor has a widespread distribution pattern. The 5-HT2A receptor mediates several peripheral responses including smooth muscle contraction, platelet aggregation and increased vascular permeability (Hoyer et al., 1994). These receptors are also found in the frontal cortex, basal ganglia and spinal cord where they are reported to be involved in central functions such as regulation of hormone secretion, sexual activity, sleep, and locomotion (Hoyer et al., 1994; Gerhardt and van Heerikhuizen, 1997). Additionally, 5-HT2A receptors have been implicated in a number of neurological disorders including epilepsy, migraine, anxiety, depression, and schizophrenia (Saxena, 1995). Though 5-HT2A receptor knockout mice display no gross abnormalities, results suggest that these receptors may function in the development and/or maintenance of intestinal smooth muscle and epithelial cells (Fiorica-Howells et al., 2002). Recent behavioral studies indicate a reduced anxiety-like phenotype in 5-HT2A$^{-/-}$ mice, without affecting depression-related behaviours (Weisstaub et al., 2006). Further, rescue of 5-HT2A receptors in the cortex normalized anxiety responses suggesting, as with the 5-HT1A receptor, this brain region is important in the development and maintenance of proper anxiety behaviours (Gross et al., 2001; Weisstaub et al., 2006).

Although no selective 5-HT2A receptor agonists are yet available, ketanserin, spiperone and MDL100907 are quite potent and selective antagonists at the 5-HT2A receptor. DOI (1-[2, 5-dimethoxy-4-iodophenyl]-2-aminopropane) and α-methyl-5-HT are potent agonists, but cannot discriminate between the three 5-HT2 receptor subtypes.

Similarly, ritanserin, mianserin, metergoline, mesulergine and methiothepin are non-selective antagonists at this receptor.

Activation of the 5-HT2A receptor *in vivo* and in different cell lines leads to an increase in intracellular calcium via IP3-sensistive Ca^{2+} stores (Pritchett et al., 1988; Hoyer et al., 1994). In *Xenopus* oocytes, activation of exogenously expressed 5-HT2A receptors stimulates the opening of a Ca^{2+}-sensitive chloride (Cl^-) channel (Pritchett et al., 1988). Additionally, endogenously expressed 5-HT2A receptors have been shown to couple to several other signaling pathways including inhibition of AC (Garnovskaya et al., 1995), activation of AC (Berg et al., 1994), and activation of phospholipase A2 (Felder et al., 1990). The 5-HT2A receptor is also mitogenic in certain cell types (Julius et al., 1990), and recently, it has been shown to activate the JAK/STAT (Janus kinase/signal transducers and activators of transcription) pathway in rat vascular smooth muscle preparations (Banes et al., 2005).

5-HT2B Receptors

The 5-HT2B receptor (formerly known as 5-HT2F) was initially characterized by its ability to mediate fundic smooth muscle contraction in rat stomach (Vane, 1959). The 5-HT2B mRNA is present in the fundus (rat), heart, kidney, lung, small intestine, and brain (Hoyer and Martin, 1997). Centrally, 5-HT2B receptors have a pattern of expression restricted to the cerebellum, lateral septum, hypothalamus, and medial amygdala (Duxon et al., 1997b). Due to the lack of selective ligands, the function of these receptors remains ambiguous. However, it has been shown that 5-HT2B receptors possess anxiolytic properties when activated in rat amygdala (Duxon et al., 1997a). As

well, these receptors have been implicated in the initiation of migraine attacks (Hoyer and Martin, 1997).

Targeted inactivation of the 5-HT2B gene leads to embryonic and neonatal death caused by heart defects, suggesting that this receptor is an important regulator of cardiac myocyte proliferation and differentiation (Nebigil et al., 2000b). In cultured mouse myocytes, activating the 5-HT2B receptor enhances cell proliferation and reduces apoptosis. The 5-HT2B receptor is involved in 5-HT-mediated mitogenesis by inducing higher cyclin D1, via the MAPK pathway, and cyclin E expression levels (Nebigil et al., 2000a). It confers protection from apoptotic death by activating extracellular signal regulated kinases (ERKs) to inhibit Bax expression, and prevents cytochrome c release and caspase-9 and -3 activation by cross-talk to the phosphatidylinositol-3 kinase/Akt pathway (Nebigil et al., 2003). 5-HT2B receptors have also recently been shown to be required for proper eye development in *Xenopus* (De Lucchini et al., 2005).

Similar to the 5-HT2A receptor, activation of the 5-HT2B receptor results in stimulation of the PLC pathway and opening of Ca^{2+}-sensitive Cl^- channels when expressed in *Xenopus* oocytes (Foguet et al., 1992). Stimulation of the 5-HT2B receptor in mouse fibroblast Ltk- cells results in activation of p21ras and ERK1/2 which is thought to contribute to its mitogenic effects (Launay et al., 1996). The 5-HT2B receptor has also been shown to activate nitric oxide synthesis through a PDZ domain (Manivet et al., 2000).

5-HT2C Receptors

The 5-HT2C receptor was originally named 5-HT1C because its binding properties appeared to resemble 5-HT1A and 5-HT1B receptor sites. Subsequent cloning of the 5-HT2C receptor cDNA revealed that it was highly related to the 5-HT2A subtype, with which it shares substantial amino acid identity (Julius et al., 1990). Not surprisingly, the 5-HT2C and 5-HT2A receptors have very similar pharmacology making it difficult to distinguish their individual contributions to certain pharmacological actions. The 5-HT2C receptor was first identified in the choroid plexus, however, the functional significance of these receptors remains to be determined (Pazos et al., 1984). Unlike the 5-HT2A and 5-HT2B receptors, 5-HT2C receptors appear to be almost exclusively expressed in the CNS (Hoyer et al., 2002). Within brain tissue, 5-HT2C binding sites are widely distributed in neocortical areas, hippocampus, nucleus accumbens, amygdala, dorsal striatum and substantia nigra (Pompeiano et al., 1994; Pasqualetti et al., 1999). An overlapping distribution of 5-HT2C receptor mRNA and protein has been observed in monkey brain indicating a potential somatodendritic localization of these receptors (Lopez-Gimenez et al., 2001). Both 5-HT2C receptor mRNA and immunoreactivity have also been observed in the DRN, a critical region for providing forebrain serotonergic innervation (Wright et al., 1995; Clemett et al., 2000).

Interestingly, the 5-HT2C receptor is the only known GPCR whose mRNA undergoes post-transcriptional editing to generate different receptor isoforms (Burns et al., 1997). The editing sites of the 5-HT2C receptor are located in the i2 loop which contains important residues for G-protein interaction (Burns et al., 1997). In fact, receptor isoforms differ with respect to their G-protein coupling abilities and level of constitutive activation (Niswender et al., 1999), as well as their distribution. It has been

reported that 5-HT depletion increases expression of 5-HT2C mRNA isoforms encoding receptors with higher sensitivity to serotonin, indicating that mRNA editing may serve as a mechanism whereby 5-HT2C receptor activity is stabilized in the face of changing synaptic serotonergic input (Gurevich et al., 2002a). Abnormalities in 5-HT2C mRNA editing could also contribute to the pathology of certain psychiatric disorders, such as depression. Analysis of post-mortem brains from depressed suicide victims revealed altered patterns of 5-HT2C receptor mRNA isoforms in the prefrontal cortex (PFC), which were the opposite to those detected in mice chronically treated with the SSRI fluoxetine (Gurevich et al., 2002b). Thus, altering this mRNA editing system may be one way in which SSRIs mediate their therapeutic effects.

5-HT2C receptor KO mice have provided new insight into receptor function. These animals develop hyperphagia and obesity, experience spontaneous convulsions, suffer cognitive impairment, display compulsive-like behaviours, and are hyper-responsive to stress (Tecott et al., 1995; Nonogaki et al., 1998; Chou-Green et al., 2003a, b). Additional behavioral and physiological responses attributed to 5-HT2C receptor activation are hypoactivity, hypophagia, oral dyskinesia, and tonic inhibition of dopaminergic and adrenergic neurotransmission (Hoyer et al., 2002).

Like the other members of its class, 5-HT2C receptor stimulation leads to PLC activation, hydrolysis of PI and an increase in intracellular calcium levels. Increased $[Ca^{2+}]$ inside the cell results in the opening of Ca^{2+}-senstive Cl^- and K^+ channels, and increased diacylglycerol causes activation of PKCα (Saudou et al., 1994). Expression of recombinant 5-HT2C receptors in Chinese Hamster Ovary (CHO) cells activates the ERK1/2 cascade and may contribute to its growth-promoting abilities (Werry et al.,

2005). The 5-HT2C receptor also exhibits promiscuous coupling to other G-proteins. Stimulation of endogenously expressed 5-HT2C receptors in primary cultures of rat choroid plexus epithelial cells activates phospholipase D (PLD) in addition to phospholipase C. This activation of PLD is mediated by PTX-insensitive $\beta\gamma$ subunits and $G\alpha 13$, not $G\alpha q$ (McGrew et al., 2002).

5-HT3 Receptor Class: The 5-HT-Gated Ion Channel

The 5-HT3 receptor subtype, originally called the 'M' receptor by Gaddum and Picarelli (1957), was first recognized for its rapid depolarizing effect on enteric and peripheral sensory neurons. The 5-HT3 receptor is unique among 5-HT receptors in that it is a member of the ligand-gated ion channel superfamily, which includes the nicotinic acetylcholine receptor, the glycine receptor, and the GABA-A receptor (Hoyer et al., 2002). The receptors produce rapid depolarization by increasing membrane permeability to monovalent cations (Na^+, Ca^{2+} influx, K^+ efflux). As with other ligand-gated ion channels, the 5-HT3 receptor is a pentameric ion channel comprising five glycoprotein subunits and a large N-terminal extracellular domain. Thus far, two subunits of this receptor have been identified, 5-HT3A and 5-HT3B (Maricq et al., 1991; Davies et al., 1999).

The 5-HT3 receptors are found on both central and peripheral neurons. In the CNS, these receptors are located in the cortex, CA1 pyramidal cell layer of the hippocampus, the dorsal motor nucleus of the solitary tract, and the area postrema (Bloom and Morales, 1998). In the periphery, 5-HT3 receptors are present on pre- and postganglionic autonomic neurons and sensory neurons (Hoyer et al., 2002). The 5-HT3

receptors are involved in regulating the motility and secretion of the GI tract. In fact, 5-HT3 receptor antagonists are used to treat GI symptoms (nausea, vomiting) induced by chemotherapy and radiation treatments (Hoyer et al., 2002). Furthermore, 5-HT3 receptors have been implicated in the pathogenesis of the gastrointestinal disorder, irritable bowel syndrome. The precise function of 5-HT3 receptors in the CNS remains to be clarified. However, the use of 5-HT3 receptor antagonists in behavioral studies has suggested that this receptor possesses anxiolytic, antipsychotic and cognitive-enhancing actions and can facilitate the withdrawal from drugs of abuse (Bloom and Morales, 1998). In addition, activation of the 5-HT3 receptor has been demonstrated to modulate the release of various neurotransmitters in the brain including dopamine (Hoyer et al., 2002).

5-HT4 Receptor Class

The 5-HT4 receptor was initially characterized in rodent GI tract and brain (Bockaert et al., 1992). Since then multiple receptor isoforms as a result of alternative splicing events, have been identified. Peripherally, the receptors are also expressed in the bladder and heart. Consequently, a role for 5-HT4 receptors in disease states involving these organs has been postulated. Centrally, 5-HT4 receptor mRNA is largely present in the basal ganglia, the olfactory system and the hippocampal formation (Vilaro et al., 1996).

All 5-HT4 receptor variants couple positively to AC, with differing levels of constitutive activity (Hoyer et al., 2002). The resulting increase in cAMP production has been shown to activate PKA (Gerald et al., 1995). Additionally, these receptors mediate

the closure of K^+ channels resulting in prolonged depolarization, the opening of voltage-sensitive Ca2+channels and neurotransmitter release (Fagni et al., 1992).

5-HT5 Receptor Class

To date, two subtypes of the 5-HT5 receptor have been cloned, the 5-HT5A and 5-HT5B. The sequence similarity between the two subtypes is high (77%), while homology to other 5-HT receptors is relatively low. Both genes contain one intron at a highly conserved position located in the middle portion of the third intracellular loop.

The 5-HT5 receptor subtypes are differentially expressed. The 5-HT5A receptor is expressed throughout the CNS, whereas 5-HT5B receptors are restricted to the CA1 region of the hippocampus, the medial habenulae, and the raphe nuclei (Gerhardt and van Heerikhuizen, 1997). Results from 5-HT5A receptor KO mice suggest that these receptors modulate the activity of neural circuits involved specifically in exploratory behavior (Grailhe et al., 1999). The 5-HT5 receptors have a high affinity for LSD and data from 5-HT5A$^{-/-}$ studies imply that some of the psychotropic effects of LSD may be mediated by 5-HT5A receptors (Grailhe et al., 1999). Gene association studies have also implicated the 5-HT5A receptor gene in susceptibility to certain neuropsychiatric disorders such as schizophrenia and some affective disorders (Birkett et al., 2000; Dubertret et al., 2004).

These receptors have been transiently transfected into a number of cells lines displaying no effect on either PLC or AC signaling pathways (Matthes et al., 1993). However, recent studies using recombinant 5-HT5A receptors stably expressed in C6 glioma cells demonstrate coupling of the receptors to multiple pathways. The 5-HT5A

receptor was shown to inhibit cAMP accumulation and ADP-ribosyl cyclase activity, as well as regulate intracellular IP3-sensitive Ca^{2+} mobilization (Noda et al., 2003). It has been suggested that the 5-HT5 receptors could potentially interact with ion channels rather than with G-proteins. Recently, it was shown that 5-HT5A receptors expressed in *Xenopus* oocytes could couple to the inwardly rectifying K^+ channel, GIRK1 (Grailhe et al., 2001). These multiple signal transduction systems may induce complex changes in the serotonergic system in brain function.

5-HT6 Receptor Class

The 5-HT6 receptor gene was initially cloned from rat cDNA based on its homology to previously cloned GPCRs (Monsma et al., 1993). The rat and human receptors are 438 and 440 amino acids, respectively, and their protein sequences are 89% identical (Kohen et al., 1996). The rat and human gene sequences each contain two introns, one in the third intracellular loop and the other in the third extracellular loop (Kohen et al., 1996). The intron in the i3 loop appears at the same location as that described for the 5-HT5A and 5-HT5B receptors.

The distribution of 5-HT6 receptor mRNA has been determined by Northern blot analysis in the rat and human brain. Highest expression levels are detected in the striatum, amygdala, nucleus accumbens, hippocampus, cortex, and olfactory tubercle (Monsma et al., 1993; Kohen et al., 1996).

The 5-HT6 receptors exhibit high affinity for tricyclic antipsychotic drugs (which act as antagonists at these receptors) and can be detected with radiolabelled [^{125}I]-LSD. The primary signal transduction pathway of the 5-HT6 receptor is the stimulation of AC

via activation of Gαs (Ruat et al., 1993b). The 5-HT6 receptor expressed in HEK 293 cells interacts specifically with the Gs-sensitive ACVI, but not with ACI or ACVIII, which are calmodulin-sensitive and are not activated by Gs proteins *in vivo* (Baker et al., 1998).

Targeted gene disruption of the 5-HT6 receptor has served as a useful probe for its function. At present, the only detectable difference between 5-HT6 receptor KO and wild-type animals has been an increase in anxiety-like behavior in the elevated zero maze (Branchek and Blackburn, 2000). Additional studies are required to fully probe the changes in behavior and physiology in this knockout mouse. The high affinity of 5-HT6 receptors for several antipsychotics hints at a potential involvement in the etiology of psychiatric disorders.

5-HT7 Receptor Class

The 5-HT7 receptor was independently cloned by three separate laboratories in 1993 (Bard et al., 1993; Lovenberg et al., 1993; Ruat et al., 1993a). Using Northern blot analysis and *in situ* hybridization, 5-HT7 receptor mRNA has been detected in blood vessels, the GI tract, and the CNS (Bard et al., 1993; Lovenberg et al., 1993; Ruat et al., 1993a). In the brain, receptor mRNA is found chiefly in the hypothalamus, thalamus, hippocampus, and cortex. Immunolabelling and radioligand binding studies have confirmed receptor protein expression in these brain areas (Bonaventure et al., 2002; Bonaventure et al., 2004). Pharmacologically, the receptor shows high affinity for 5-carboxytryptamine (5-CT) and 5-HT, and relatively high affinity for the 5-HT1A receptor agonist, 8-OH-DPAT. Splice variants of the 5-HT7 receptor have been detected in rat

versus human, however, these variants do not seem to possess any functional differences (Heidmann et al., 1998; Krobert and Levy, 2002).

The 5-HT7 receptor couples positively to AC to stimulate cAMP production (Bard et al., 1993; Lovenberg et al., 1993; Ruat et al., 1993a). Recent studies have demonstrated that the receptor activates ERK1/2 through a Ras-dependent mechanism (Norum et al., 2003). Another group has shown that the 5-HT7 receptor can mediate ERK activation through a PKA-independent pathway that may involve using a cAMP-activated guanine nucleotide exchange factor, Epac1 (Lin et al., 2003). Activation of the 5-HT7 receptor directly stimulates ERKs in hippocampal neurons (Errico et al., 2001), an effect that may be important for hippocampal function and mood regulation.

The recent availability of selective 5-HT7 antagonists and knockout animals has elucidated important functional roles for this receptor. In the periphery, the 5-HT7 receptors mediate vasodilation and ileum peristalsis. The central 5-HT7 receptors are involved in thermoregulation, circadian rhythm, learning and memory, hippocampal signaling and sleep (Hedlund and Sutcliffe, 2004). Current research has focused on the hypothesis that this receptor may be involved in mood regulation, suggesting that the 5-HT7 receptor is another putative target in the treatment of depression.

The Serotonin Transporter: 5-HTT

In contrast to the diverse number of 5-HT receptors, there exists only one transporter for serotonin, the 5-HT transporter (5-HTT). The 5-HTT mediates reuptake of serotonin into the presynaptic neuron after 5-HT has been released into the synaptic cleft, thereby attenuating serotonergic neurotransmission. In adult brain, the 5-HTT is

located exclusively on serotonergic neurons with the highest densities found on the cell bodies of raphe neurons and in serotonergic projection areas such as neocortical regions, the entorhinal cortex, the CA3 region of the hippocampus, the amygdala, the striatum, the hypothalamus, and the substantia nigra (Hensler et al., 1994; Sur et al., 1996).

The 5-HTT is the target for certain drugs of abuse including the substituted amphetamine 3,4-methylenedioxymethamphetamine (MDMA, 'Ecstasy') (Bengel et al., 1998) and cocaine (Sora et al., 1998). It is also the therapeutic target of a wide variety of antidepressant drugs, especially the SSRIs such as fluoxetine, which prevent the reuptake of serotonin by blocking transporter function. Mice that lack the 5-HTT (Bengel et al., 1998) exhibit 8-14-fold higher levels of extracellular serotonin (Fabre et al., 2000) and display adaptive changes of several 5-HT receptors including the 5-HT1A, 5-HT1B, 5-HT2A and 5-HT2C (Fabre et al., 2000; Mannoury la Cour et al., 2001; Li et al., 2003). These 5-HTT knockout mice also exhibit increased depression- and anxiety-related behaviors (Holmes et al., 2003; Lira et al., 2003), which is paradoxical since in mature humans, chronic SSRI antidepressant treatment also produces a reduction of 5-HTT function, yet these agents act to improve anxiety- and depression-related symptoms. Additionally, there is increasing evidence that long and short variants of a repeat polymorphism in the 5'-untranlated region of the 5-HTT gene (5-HTTLPR) are prevalent in the general population and influence the level of 5-HTT gene transcription. The long 5-HTT allele is associated with negative behavioural traits such as anxiety, depression, and aggression (Lesch et al., 1996), whereas the short allele is associated with decreased 5-HTT expression and function (Lesch, 2004). The above data emphasizes that

interfering with or modulating the serotonin reuptake system can lead to behavioural consequences.

GPCR Structure and Function

The G-protein coupled receptor superfamily is one of the largest evolutionarily conserved family of proteins found in organisms ranging from humans to nematodes. In fact, GPCRs occupy about 3% of the human genome and are represented in over 5% of the *C. elegans* genome (Bargmann, 1998; Venter et al., 2001). These proteins function as signal transduction molecules converting a variety of external signals (light, odor, taste, neurotransmitters, hormones) into a cellular response. GPCRs mediate their actions by coupling to heterotrimeric G-proteins that are composed of Gα and Gβγ subunits. Since GPCRs are involved in a wide variety of physiological functions, modulation of receptor activity is one of the most successful areas of drug development. Drugs directed against GPCRs account for greater than 60% of currently marketed therapeutics, making about 25% of the Top 100-selling list. As new functions for GPCRs are discovered the number of drugs that target GPCRs can only be expected to increase.

General Structure: Up until recently most of our insight into the tertiary structure of GPCRs was derived from low resolution structures of the retinal light-sensing receptor rhodopsin (Schertler et al., 1993), or high resolution structures of the bacterial light-driven proton pump, bacteriorhodopsin (Henderson et al., 1990). In 2000, Palczewski and colleagues succeeded in generating three-dimensional crystals of inactive bovine rhodopsin for X-ray crystallography offering for the first time a true tertiary structural

model of a GPCR at atomic resolution (2.8Å) (Palczewski et al., 2000). Other structures representing similar inactive state conformations of bovine rhodopsin at 2.6-2.8Å resolution soon followed (Teller et al., 2001; Okada et al., 2002). In the highest resolution structure (Okada et al., 2002), the localization of individual water molecules in the inner cavity of the protein has been determined. As well as being one of the best characterized GPCRs, rhodopsin is also the only GPCR for which a crystal structure has been determined to date. Remarkably, the crystal model of rhodopsin confirmed much of our predictions and current knowledge of GPCR structure and function.

Despite the remarkable chemical diversity among their endogenous ligands, all GPCRs are predicted to share a common tertiary structure which includes an extracellular N-terminal domain, seven hydrophobic TMs connected by three extracellular (e1-e3) and three intracellular loops (i1-i3), and a C-terminal tail. This is shown schematically in Figure 3 which depicts the rat 5-HT1A receptor, a prototypical G-protein coupled receptor. A putative fourth intracellular loop is formed when the C-terminal segment is palmitoylated at Cys, as is observed with the 5-HT1A receptor (Papoucheva et al., 2004).

The TMs generally consist of 20-27 amino acids that are predicted to form α-helices. The crystal structure of bovine rhodopsin provides direct structural confirmation that the helices are arranged in a counterclockwise order (Palczewski et al., 2000). The rhodopsin structure also reveals that these α-helices are bent or kinked at Pro residues or Gly-Gly sequences (Palczewski et al., 2000). Generally, the presence of a proline residue disrupts the hydrogen (H)-bonding pattern within a helix, but in the case of the TMs this is not sufficient to cause a break in the helical structure. The bends and twists in the

helical TMs may be axis points for conformational changes (e.g. further bending and/or separation of TMs) associated with GPCR activation (Filipek et al., 2003).

In contrast to the TMs, the N-terminal domains (7-595 aa), loops (5-230 aa), and C-terminal tails (12-359 aa) vary in size, an indication of their diverse structures and functions (Ji et al., 1998). The N-terminus, extracellular loops, and TM domains, are known to be involved in ligand binding (Gether, 2000). Cytoplasmic regions of GPCRs, including the C-terminal domain and the intracellular loops (particularly i2 and i3), are known to be important for the G-protein recognition and activation (Wess, 1997).

GPCRs are glycoproteins, with all receptors cloned to date containing at least one putative consensus site for N-linked glycosylation (Asn-X-Ser/Thr) located in the extracellular domain (N-terminus, e2) (Strader et al., 1994). Three putative glycosylation sites are found on the N-terminal of the rat 5-HT1A receptor (Fig. 3). N-linked glycosylation has been shown for a number of receptors including the β2-adrenergic receptor (β2AR) and rhodopsin, to be critical for determining the level of receptor expression at the cell surface, but generally is not required for either ligand binding or functional activity (Strader et al., 1994).

Many GPCRs also contain two highly conserved Cys residues which appear to play a critical role in the overall structural integrity of the receptor. Conserved cysteines located in the second and third extracellular loops of the receptors (C106 and C184 in the β2AR; C109 and C187 in the 5-HT1A receptor) have been shown to stabilize the tertiary structure of both rhodopsin and β2AR (Strader et al., 1994). These Cys residues are predicted to form an intramolecular disulfide bridge which links e2 and e3, thereby constraining the conformation of the extracellular domain.

Although the GPCRs do not show high overall sequence homology, significant homology is found within the defined subfamilies (Gether, 2000). The three major subfamilies include the receptors related to the rhodopsin and the β2-adrenergic receptor (Family A), receptors related to the glucagon receptor (Family B), and receptors related to the metabotropic neurotransmitter receptors (Family C). The receptors for yeast pheromones form two minor unrelated subfamilies (Families D and E), and four cAMP receptors from *Dictyostelium discoideum* constitute another unique GPCR subfamily (Family F) (Gether, 2000).

Family A (rhodopsin/β2-adrenergic-like receptors) represents the largest and most studied GPCR family. This subfamily includes biogenic amine receptors (serotonin, adrenergic, dopamine, muscarinic, histamine), some peptide ligand receptors (endothelin, neurotensin, oxytocin, vasopressin, somatostatin, TRH), receptors for opsins, adenosine, and opioids, and the olfactory receptors, to name a few (Gether, 2000). Most Family A receptors possess a relatively short (about 40 aa) extracellular amino terminus and palmitoylated cysteine(s) in the carboxyl-terminal resulting in the formation of a putative fourth intracellular loop (Gether, 2000; Palczewski et al., 2000). Highly conserved key residues include a Asn-Pro-xx(x)-Tyr (NPxx(x)Y) motif found in TM7, and a Glu/Asp-Arg-Tyr (E/DRY) motif that are present at the boundary of TM3 and the i2 loop (Figure 3). The latter is very important for maintaining the receptor in an inactive conformational state, and will be further addressed below (see **i2 loop**).

Family B includes approximately 20 different receptors for peptide hormones and neuropeptides, such as vasoactive intestinal peptide (VIP), calcitonin, and glucagon. These receptors are characterized by their long (about 100 aa) extracellular N-terminus

containing several cysteines that presumably form a number of disulfide bridges (Gether, 2000). Notably, the important E/DRY motif and the palmitoylation site(s) are absent in Family B receptors.

Family C receptors include the metabotropic glutamate and GABA receptors, the calcium receptors, the pheromone and the taste receptors. These receptors possess an especially long (about 500-600 aa) N-terminus and a short, highly conserved third intracellular loop (Gether, 2000). The ligand binding site of Family C receptors is believed to lie in the long amino terminal domain. A KPxxY motif located in TM7, similar to the NPxxY sequence of Family A receptors, is conserved among many Family C receptors.

Ligand binding domains: GPCRs interact with an exceptionally diverse array of ligands which include biogenic amines, peptides, glycoproteins, lipids, nucleotides, ions, and proteases. There are several distinct ways in which high affinity ligands bind to their respective receptors; ligands may bind to the TM core exclusively, to both the TM core and extracellular loops, to extracellular loops and the N-terminal domain, or to the N-terminal region exclusively (Ji et al., 1998). The general mechanism of receptor activation may be divided into at least three steps: ligand binding, signal generation and transduction through the TM core, and transfer of the signal to intracellular effectors. It is believed that ligand-receptor interactions involve changes in hydrogen bonds, ion pairs and hydrophobic contacts (Ji et al., 1998).

Most of the information regarding ligand binding domains arises from chimeric receptor or site-directed mutagenesis studies. The 5-HT1A receptor is classified as a

GPCR that binds biogenic amines (e.g. serotonin, dopamine, epinephrine, acetylcholine, etc.). These receptors are characterized as having a common ligand binding site in the central TM core, where TM3 contains the primary site for ligand binding and TMs 5 and 6 are the signal generation sites (Ji et al., 1998).

Point mutations in the transmembrane domains of the human 5-HT1A receptor have identified specific residues required for the binding of its endogenous ligand 5-HT. Substitution of either Asp82 to Asn (located in TM2), Asp116 to Asn (TM3), or Ser199 to Ala (TM5), resulted in decreased affinity for 5-HT by 60-100-fold, without affecting the affinity for the β-receptor /5-HT1A receptor antagonist, pindolol (Ho et al., 1992). Interestingly, none of the mutations affected receptor-G-protein coupling indicating a key role for these residues in 5-HT binding and not receptor activation. All GPCRs cloned to date contain the highly conserved Asp residue located in the second transmembrane domain, while only those receptors that bind biogenic amines possess the Asp residue located in the third transmembrane region (Strader et al., 1994). Like the β2AR, the acidic carboxyl group of Asp116 and/or Asp82 is thought to act as a counter ion to the protonated amine group of 5-HT, thus stabilizing ligand-receptor interaction (Strader et al., 1994). Similar observations have been reported about equivalent Asp mutations in the β2-adrenergic, α2A-adrenergic, 5-HT2A, muscarinic M1 acetylcholine, and dopamine D2 receptors (Neve et al., 1991; Wang et al., 1991; Ostrowski et al., 1992; Wang et al., 1993; Wess, 1993).

Site-directed mutagenesis studies have also identified other transmembrane residues that are important for 5-HT1A receptor ligand binding. Substitution of the conserved Asn396 residue (in TM7) with Ala, Val, or Phe abrogated binding of the

specific 5-HT1A receptor agonist, 8-OH-DPAT (Chanda et al., 1993). Similarly, mutation of another conserved TM7 residue, Ser393 to Ala, also resulted in reduced 8-OH-DPAT binding (Chanda et al., 1993). Key recognition sites for pindolol, a β-adrenergic/5-HT1A receptor antagonist, also appear to be located in TM7. Mutation of Asn385, a highly conserved residue between the 5-HT1A and β-adrenergic receptors, resulted in a selective decrease in affinity of mutant 5-HT1A receptors for pindolol (about 100-fold), without any effect on the affinity for other agonists or antagonists (Guan et al., 1992).

The binding site of biogenic amine receptors appears to be contained within a hydrophilic crevice formed among its seven membrane-spanning segments. For the 5-HT1A receptor, critical residues from TMs 3, 5, 6, and 7 have been implicated to be important for ligand binding.

G-protein coupling domains

G-protein coupled receptors mediate their signals to effector molecules through the binding and activation of heterotrimeric G-proteins. Much of our knowledge of receptor domains involved in G-protein coupling has been obtained using chimeric receptor studies, mutational analysis and biochemical experiments. Taken together, these studies have demonstrated that multiple intracellular domains, particularly the i2 loop, the N- and C-terminal regions of the i3 loop (Ni3 and Ci3), and the C-tail, participate in the functional coupling and activation of G-proteins.

i1 Loop: There are relatively few studies indicating that the i1 loop is directly involved in G-protein coupling. Interestingly, the length of the i1 loop is highly conserved among GPCRs, suggesting that it may play more of a structural role (Wess, 1998). Consistent with this idea, β2AR receptors mutated at i1 loop residues displayed low or undetectable expression levels, presumably reflecting improper folding or insertion of the mutant proteins into the plasma membrane as a result of defective structural integrity of the i1 loop (O'Dowd et al., 1988).

Splice variants involving the i1 loop have been shown to couple to different signaling pathways. For example, using a chimeric receptor approach it was revealed that an inserted sequence of 16 aa in the i1 loop of human calcitonin receptor 1 (hCTR-1) abolished coupling of the receptor to Gq/11 and subsequent PLC activation, but still retained coupling to Gs and AC stimulation (Nussenzveig et al., 1994). Similarly, a corticotropin-releasing factor (CRF) receptor isoform (Type II CRF receptor) which contained a 29 aa i1 loop insertion, was shown to be greatly impaired in its coupling ability to Gs (Xiong et al., 1995). Similar strategies have been used to look at G-protein coupling properties of CCK-A and –B receptors, and C5a and fMLP receptors. These data suggest that the i1 loop can contribute to G-protein coupling and activation. However, whether i1 loop residues are direct contact sites for G-proteins or whether the i1 loop can induce conformation changes to reveal other receptor-G-protein interaction sites remains to be determined.

i2 Loop: There is considerable evidence demonstrating the importance of the i2 loop in G-protein coupling and activation. Much attention has focused on the functional role of a

highly conserved tripeptide sequence, 'DRY', that is found in most members of the rhodopsin family (Family A). If the motif is expanded to include E/DRY/W, the sequence conservation is greatly extended. The DRY motif usually occurs at the boundary of TM3 and the N-terminal of the i2 loop. One function of this sequence is to maintain the receptor in an inactive conformation via intermolecular interactions. The rhodopsin crystal model demonstrates that these residues participate in several hydrogen bonds with surrounding residues (Palczewski et al., 2000). The carboxylate of E134 forms a salt-bridge with the guanidium side chain of R135. This same R135 is also connected to residues E247 and T251 in TM6. Three Val residues (V137-V139) are close enough to partially mask the cytoplasmic side of E134 and R135. These are the critical constraints that have been suggested to keep rhodopsin in the inactive state.

The Arg residue within this structural motif is one of the most highly conserved residues among GPCRs and is found in 99% of all receptor sequences (Wess, 1998). Mutation of the conserved Arg in a number of GPCRs including rhodopsin, β2AR, V2 vasopressin receptor, M1 muscarinic receptor, and α1B-adrenergic receptor results in significantly reduced or abrogated G-protein coupling without affecting ligand binding affinities (Fraser et al., 1988; Franke et al., 1992; Rosenthal et al., 1993; Zhu et al., 1994; Scheer et al., 1996). The importance of this residue in proper signal transduction has been demonstrated by naturally occurring mutations of the conserved Arg. For instance, two mutations found in human rhodopsin, R135L and R135W, were identified in individuals with autosomal dominant retinitis pigmentosa (Sung et al., 1991). An Arg137His mutation of the V2 vasopressin receptor found in individuals that were affected with congenital nephrogenic diabetes insipidus, failed to couple to Gs and

stimulate AC activity (Rosenthal et al., 1993). These data suggest that the conserved Arg residue may be a key site for receptor-G-protein interaction. Studies with mutant M1 muscarinic receptors have demonstrated that a charge-conserving mutation (Arg to Lys) results in only a slight impairment of receptor function further suggesting that the conserved Arg residue interacts with an anionic (electron rich) site on the G-protein (Jones et al., 1995).

In contrast to the result presented above, mutation of the conserved Asp preceding the critical Arg in several GPCRs, including the M1 muscarinic and α1B-adrenergic receptors, did not result in a significant loss of G-protein coupling efficiency (Lu et al., 1997; Scheer et al., 1997). However, many of the Asp mutants were poorly expressed at the cell surface suggesting that this residue may play a role in proper receptor folding and trafficking (Lu et al., 1997; Scheer et al., 1997). Although the conserved aspartate may not directly interact with G-proteins, its neutralization seems to be necessary for the adjacent arginine side chain to achieve an orientation that is suitable for proper G-protein interactions (Wess, 1998). Consistently, analysis of rhodopsin and the α1B-adrenergic receptor mutants have shown that the substitution of the conserved Glu/Asp residue with other hydrophobic or neutral amino acids leads to constitutively active receptors (Scheer et al., 1996, 1997; Wess, 1998).

Mutational studies in the M1 receptor suggest that the Tyr residue of the DRY triad is not essential for efficient G-protein coupling (Lu et al., 1997). Furthermore, many of these Tyr mutant receptors were expressed at very low levels. It is hypothesized that the conserved Tyr forms intramolecular contacts that have a stabilizing function to render the receptor in an active conformation (Lu et al., 1997).

Another conserved amino acid appears to be a leucine/hydrophobic residue found within the middle portion of the i2 loop (corresponding to Ile141 in the rat 5-HT1A receptor, Figure 3). Substitution of this residue in several GPCRs, including the M1 and M3 muscarinic receptors (Moro et al., 1993), β2AR (Moro et al., 1993), gonadotropin releasing hormone (GnRH) (Arora et al., 1995), H2 histamine (Smit et al., 1996), and thyroid stimulating hormone (TSH) receptors (Kosugi et al., 1994; Neumann et al., 2005), results in defective G-protein coupling. It is possible that a bulky hydrophobic amino acid such as Leu, in the center of the i2 loop serves as a general site for G-protein binding.

Mutagenesis studies have implicated the second intracellular loop to be important for coupling selectivity of receptor/G-protein interactions. Using a random mutagenesis approach, Brann and co-workers studied in detail the role of the i2 domain in Gq-coupled muscarinic-m5 receptor signaling (Burstein et al., 1998). Interestingly, two sets of residues were identified in the i2 loop of the M5 muscarinic receptor; one set that played a role in constraining the receptor to an inactive state conformation, and one set that determined G-protein coupling and specificity (Burstein et al., 1998). The data suggests that the i2 loop is alpha-helical in nature, has two protein faces, and functions as a conformational "switch" that enables G-protein coupling (Burstein et al., 1998). Examination of the i2 domain of metabotropic glutamate (mGlu) receptors (family C GPCRs) revealed that the central portion of the i2 loop is responsible for the selective recognition of the C-terminal end of Gαq-subunits (Havlickova et al., 2003). For the TSH receptor, i2 loop residues 525-527 were shown to be critical for Gs coupling, whereas residues 528-532 were more important for Gq activation (Kosugi et al., 1994).

Chimeric receptor studies have also indicated a role for the i2 loop in G-protein selectivity. For example, muscarinic receptors (Gq-coupled M1 or Gi/o-coupled M2) substituted with the i2 and i3 loops of the Gs-coupled β1-adrenergic receptor (β1AR) have the G-protein coupling profile of β1AR with the ligand binding profile of the respective muscarinic receptor (Wong et al., 1990; Wong and Ross, 1994). Interestingly, hybrid receptors where only the i2 or i3 loop of the muscarinic receptor is replaced with either the i2 or i3 loop of the β1AR, couple to both Gq and Gs (M1/β1AR) or Gs and Gi (M2/β1AR) (Wong et al., 1990; Wong and Ross, 1994). It appears that the structural determinants required for efficient Gs coupling, at least for these receptors, resides in both the i2 and the i3 regions. Similar results have been obtained with mGluR1/mGluR3 glutamate hybrid receptors with regard to Gq-coupling (Gomeza et al., 1996).

Several chimeric studies have shown that substitution of the i2 loop alone can confer new G-protein coupling profiles to recipient receptors. Hybrid vasopressin receptors comprising the Gs-coupled V2 receptor with the i2 loop of the Gq/11-coupled V1a receptor, resulted in mutant receptors that coupled to both Gs and Gq/11 proteins (Liu and Wess, 1996). Similar data has been generated for α2A/α2B-adrenergic receptors (Nasman et al., 1997), α2A-adrenergic/β2AR hybrids (Eason and Liggett, 1996), and β2AR/thrombin and D2/thrombin chimeric receptors (Wess, 1998). Finally, studies with hybrid M2/M3 muscarinic receptors have identified four hydrophilic M3 receptor residues, S168, R171, R176, and R183, that are essential for selective recognition of Gq/11 proteins (Blin et al., 1995).

Additionally, short peptides derived from the i2 loop of several GPCRs have been shown to mimic or inhibit receptor activation. Synthetic peptides from the i2 loop of

rhodopsin are able to inhibit the interaction between the receptor and its cognate G-protein, transducin (Konig et al., 1989). Similarly, in the Gs-coupled β1AR, a synthetic peptide corresponding to the entire i2 loop abrogated hormone-dependent AC activation (Palm et al., 1989). In contrast, a nine-amino acid peptide corresponding to the C-terminal of the i2 loop (Ci2) of the Gi-coupled M2 receptor inhibited forskolin-induced AC activity and stimulated GTPase activity in membrane preparations (McClue et al., 1994). Studies performed with the 5-HT1A receptor demonstrated that a synthetic peptide representing the entire i2 loop could directly inhibit forskolin-stimulated AC activity in a PTX-independent manner (Varrault et al., 1994; Thiagaraj et al., 2002).

i3 Loop: There is a considerable amount of evidence indicating that the i3 loop, like the i2 loop, is a key determinant of receptor/G-protein coupling specificity. In some of the early studies using chimeric approaches with adrenergic, muscarinic and dopaminergic receptors, exchange of the i3 loop between functionally similar receptor subtypes resulted in hybrid receptors that exhibited G-protein specificity of the "donor" receptor without retaining the G-protein coupling of the "acceptor" receptor (Wess, 1998; Wong, 2003). For example, replacement of the Gi-coupled M2 receptor i3 loop with that of the Gq-coupled M3 receptor resulted in a chimeric receptor that exclusively coupled to Gq and not to Gi (Wess et al., 1990). Similarly, for αAR-βAR and D2-D3 hybrid receptors, there appeared to be a switch in the specificity of G-protein coupling (Kobilka et al., 1988; Van Leeuwen et al., 1995). Conversely, for many other chimeric receptors, exchange of the i3 loop does not appear to be sufficient to replace the specificity of G-protein coupling. In these cases, the hybrid receptors acquired the G-protein specificity

of the donor GPCR, but still retained the ability to couple to the G-protein of the acceptor GPCR (Wong, 2003). In addition, many of these chimeric receptors were less active than their wild-type counterparts and coupled to G-proteins with reduced efficacy (Wong et al., 1990; Wong and Ross, 1994; Wess, 1998). These findings indicate that although the i3 loop is very important for both G-protein selectivity and coupling, it is not the sole contributor to these properties. This is consistent with the prediction that the i3 loop acts cooperatively with other receptor domains, like the i2 loop, to properly recognize and couple to G-proteins.

Several lines of experimental evidence have established that the most important regions of the i3 loop for G-protein coupling are located at the N- and C-terminal ends (Ni3 and Ci3, respectively), just adjacent to transmembrane domains TM5 and TM6. For example, synthetic peptides derived from the Ni3 and Ci3 regions of the dopamine D2 receptor attenuated the dopaminergic inhibition of adenylyl cyclase (Malek et al., 1993; Voss et al., 1993). Similar peptide studies have been performed for other GPCRs including rhodopsin, adrenergic and muscarinic receptors (Cheung et al., 1991; Franke et al., 1992; Ikezu et al., 1992; Okamoto and Nishimoto, 1992).

The Ni3 appears to be crucial for coupling to various G-proteins. Mutation of the Ni3 region of the β2AR (residues 222-229) was shown to abolish coupling to Gs (Ostrowski et al., 1992; Strader et al., 1994). Similarly, mutation of the Ni3 region of the α2AR (which preferentially couples to Gi/o, but can also interact with Gs) to the corresponding 5-HT1A receptor sequence prevented coupling to Gs, while Gi coupling remained intact (Eason and Liggett, 1995). Similar substitution with the β2AR Ni3 sequence had no effect on Gi coupling and Gs coupling was still preserved, albeit slightly

reduced. Hybrid studies with angiotensin II receptors, the Gq/11-coupled AT1 and the Gi/o-coupled AT2, showed that the Ni3 region was important for coupling to Gq/11 proteins (Wang et al., 1995). Random mutagenesis experiments of the m5 muscarinic receptor Ni3 domain (Hill-Eubanks et al., 1996) showed that a limited number of primary hydrophobic or non-charged residues in the Ni3 play a key role in determining G-protein coupling. These residues are predicted to form the hydrophobic face of an amphipathic α-helix (Strader et al., 1994).

The Ci3 region, like the Ni3, is also important in G-protein recognition. Peptides derived from the C-terminal region of aminergic receptors can mimic G-protein activation (Palm et al., 1989; Okamoto et al., 1991; Okamoto and Nishimoto, 1992). The Ci3 region also appears central in regulating the activation state of certain GPCRs, including Gs-coupled receptors like the β2AR or dopamine D1 receptors (Strader et al., 1994; Jensen et al., 1996; Bourne, 1997). For example, mutation of Ci3 residue Phe264 to Ile confers on the D1 receptor constitutive activity that is typical of the D5 receptor (Charpentier et al., 1996). In contrast, the opposite mutation in the D5 receptor silences constitutive activity (Charpentier et al., 1996). Similarly, mutation of a conserved Thr residue in Ci3 of the α2AR also increases the agonist-independent coupling to Gi (Ren et al., 1993). Conversely, mutation of residues V344E and T343A in the Ci3 domain of the 5-HT1A receptor did not generate constitutively active mutant receptors but rather allowed coupling to Gs in addition to Gi/o proteins (Malmberg and Strange, 2000).

To identify potential receptor/G-protein contact sites, Liu et al. showed that the Gi/o-coupled m2 muscarinic receptor can couple to mutant Gαq proteins that have had their last 5 amino acids changed to the corresponding Gαi or Gαo residues (Liu et al.,

1995). Mutational analysis of a number of mutant m2 receptors revealed that the ability of the m2 receptor to interact with the mutant Gq protein was dependent on the presence of a 4aa motif located in the Ci3 region, which corresponds to Val385, Thy386, Ile387 and Leu389 (or "VTIL"). Similarly, these amino acids correspond to an "AALS" motif in the Gq-coupled m1, m3 and m5 receptors which, together with the Ni3 and i2 loop, plays an important role in the recognition of Gq/11 proteins (Blin et al., 1995). It is thought that upon receptor activation and subsequent reorientation of the TM, the "VTIL" motif becomes accessible to the G-protein (Wess, 1998).

C-Tail: Deletion studies involving the C-terminal tail have suggested that this domain may be involved in the basal activity or coupling in some GPCR subfamilies (Wess, 1998). Recently, the C-tail region of the 5-HT1A receptor was shown to have 2 cysteine palmitoylation sites (417 and 420) that were identified as being critical residues for Gi protein coupling. Mutation of one or both residues resulted in significant reduction or abrogation of 5-HT-induced inhibition of forskolin-stimulated cAMP formation, as well as Gβγ-dependent activation of ERK signaling (Papoucheva et al., 2004). The C-terminus is also known as an important interaction domain for GPCR-associated proteins (reviewed in (Bockaert et al., 2004). Those interacting with the GPCR C-terminus are the most numerous and the best studied.

It is generally thought that receptor activation involves disruption of stabilizing intramolecular interactions that induces the TMs to separate from one another which may lead to the creation a G-protein binding "pocket" (Bourne, 1997). At least one of the key

events in the activation process involves the protonation of the highly conserved aspartic acid or glutamic acid in the D/ERY motif at the TM3/i2 loop junction (Gether et al., 2002). It is believed that this event relieves the constraints that normally hold the receptor in an inactive conformation. Consistently, spin label studies demonstrate that upon activation, TM3 and TM6 move apart from one another (Bourne, 1997; Gether et al., 2002). Furthermore, many point mutations in these regions, especially in the E/DRY/i2 and TM6/i3 domains, result in constitutive activity suggesting that these intracellular regions which are predicted to form amphipathic alpha-helices may function to prevent receptor activation by holding the intracellular loops apart until agonist binding occurs.

Although the data presented above has provided valuable insights, what is needed to best determine the sites involved in G-protein coupling and activation is a crystal structure of an activated GPCR bound to its cognate G-protein.

Desensitization

Despite their vast diversity, most GPCRs are regulated by a common desensitization mechanism. Desensitization can be defined as the attenuation in responsiveness of receptors to repeated or sustained stimulation. This adaptive cellular response can limit the potentially harmful effects that may result from persistent receptor activation. Desensitization can be divided into three main phases: uncoupling (which takes seconds to minutes), internalization (minutes to hours) and downregulation or degradation (hours to days). The major regulatory molecules involved in the

desensitizing mechanism are second messenger kinases (e.g. PKA and PKC), G protein receptor kinases (GRKs) and the arrestins (Lefkowitz, 1998; Perry and Lefkowitz, 2002).

Two principal paradigms have been established as mechanisms for GPCR desensitization: heterologous and homologous desensitization. In heterologous desensitization, second messenger kinases such as PKA and PKC, phosphorylate the GPCR on specific serine/threonine residues that are usually found on the receptor's intracellular loops and/or C-tail (Lefkowitz, 1998). The minimal phosphorylation consensus sequence for PKA is RRX(S/T), and for PKC is (S/T)X(K/R) or (K/R)X(S/T) (Kennelly and Krebs, 1991). Phosphorylation of the receptor results in conformational changes that impair receptor/G-protein interaction (Lefkowitz, 1998). Heterologous desensitization is viewed as generalized and non-specific, since any stimulus, including GPCRs themselves, that increases cAMP, which in turn activates PKA, or DAG, which activates PKC, has the potential to result in the phosphorylation and subsequent desensitization of a receptor containing the appropriate PKA and/or PKC sites.

Homologous or "agonist-specific" desensitization is mediated by the GRK-arrestin system. First, an agonist-occupied or activated receptor is phosphorylated by GRKs, which in turn, promotes the binding of an arrestin that can sterically inhibit receptor/G-protein interaction (Pierce et al., 2002). The foremost reason GRKs can discriminate between inactive and agonist-occupied receptors is because the latter can catalytically activate the enzymes themselves (Pierce et al., 2002; Gainetdinov et al., 2004). There are seven GRKs known to date, and they are classified into three subfamilies: GRK1 and -7; GRK2 and -3; and GRK4, -5 and -6 (Pitcher et al., 1998). GRK1 (rhodopsin kinase) and GRK7 are primarily visual, GRK4 is mainly expressed in

the testes and the remaining GRKs, GRK2/βARK1 (β-adrenergic receptor kinase 1), GRK3/βARK2 (β-adrenergic receptor kinase 2), GRK5 and GRK6 have a wide distribution (Gainetdinov et al., 2004).

In order to phosphorylate their membrane-bound receptor substrates, GRKs must also be targeted to the plasma membrane (PM). GRK1, -4, -5, -6 and -7 are predominantly associated with the plasma membrane, some of these via covalent modifications of their carboxy termini: GRK1 is farnesylated; GRK4 and GRK6 are palmitoylated; the C-terminal domain of GRK7 contains a CAAX sequence that is geranylgeranylated; and GRK5 contains lipids comprising PIP2 that allow interaction with the PM. In contrast, GRK2 and GRK3 are primarily located in the cytosol. A pleckstrin homology (PH) domain found in the C-terminal regions of GRK2 and -3 binds both Gβγ subunits and membrane PIP2. Upon agonist binding, free Gβγ subunits, released from receptor-activated G-proteins, and PIP2 coordinately bind the PH domain and target the GRKs to the stimulated receptor (Lefkowitz, 1998; Gainetdinov et al., 2004). Furthermore, it has been shown that both PKA and PKC can activate GRK2 through phosphorylation, thereby adding yet another layer of complexity to the mechanisms of desensitization (Winstel et al., 1996; Cong et al., 2001).

Arrestin binding follows phosphorylation of an agonist-occupied receptor by a GRK. Like GRKs, arrestins also recognize, and are activated by, stimulated receptors. Four arrestins have been identified thus far: Visual and cone arrestins are expressed exclusively in the retina, while βarrestin-1 (βarr1/arrestin-2) and βarrestin-2 (βarr2/arrestin-3) are ubiquitously expressed (Pierce et al., 2002). In addition to the

receptor desensitization mechanism described above, arrestins are also active participants in agonist-promoted endocytosis.

Endocytosis (also known as internalization or sequestration) can occur using clathrin-coated pits, caveolae or non-coated vesicles. βarrestin-dependent receptor endocytosis is the best characterized mechanism for GPCR internalization. βarrestins target agonist-occupied receptors to clathrin-coated vesicles by interacting with the clathrin adaptor protein β2-adaptin (AP-2) and clathrin itself (Goodman et al., 1996; Laporte et al., 1999). The vesicles are then pinched off the plasma membrane by the GTPase, dynamin. Once internalized, receptors can be recycled rapidly, targeted to endosomes and recycled slowly, or targeted to lysosomes for degradation. Although endocytosis is not required for rapid uncoupling and desensitization, it has been shown to promote receptor resensitization and signaling (Daaka et al., 1998; Ferguson, 2001).

Recent studies have shown that arrestins can also positively regulate GPCR signaling by acting as adaptor or scaffolding proteins to propagate new signals from the very receptors they are attempting to desensitize (Perry and Lefkowitz, 2002). Arrestins have been shown to interact with and recruit numerous cell signaling proteins to activated receptors including c-Src, members of ERK/MAPK kinase cascades, Mdm2, ADP-ribosylation factor 6 (Arf6), ADP-ribosylation factor nucleotide-binding site opener (ARNO), and *N*-ethylmaleimide-sensitive factor (NSF) (Perry and Lefkowitz, 2002; Gainetdinov et al., 2004). The consequence of this type of signaling is an area of active investigation.

Finally, the physiological relevance of the GRK-arrestin system in the desensitization process is accentuated by the observation that all GRK or arrestin

knockout mice display supersensitivity to various GPCR-mediated signaling pathways (Pierce et al., 2002).

Desensitization of the 5-HT1A receptor has been shown to be mediated by PKC and PKA, with some evidence indicating that GRKs may also play a role. There are four PKC phosphorylation sites in the 5-HT1A receptor; one in the i2 loop (T149A) and three in the i3 loop (Thr229, Ser253, Thr343) that have been implicated in receptor desensitization (Lembo and Albert, 1995; Lembo et al., 1997; Wu et al., 2002). Two of these consensus sequences are also putative PKA phosphorylation sites (Thr229 and Thr343) (Liu and Albert, 1991; Raymond and Olsen, 1994). PKC is a direct target of phorbol esters such as phorbol 12-myristate 13-acetate (PMA)/ phorbol 12-myristate 13-acetate (TPA). Stimulation of PKC by phorbol esters in cells expressing the 5-HT1A receptor results in rapid receptor phosphorylation on at least 2 of the 4 PKC sites, and desensitization of several 5-HT1A-mediated signaling pathways (Raymond, 1991). 5-HT1A receptors expressed in HeLa cells were shown to be rapidly uncoupled from G-proteins upon pretreatment with phorbol esters, and that this uncoupling was reversed by PKC inhibitors (Harrington et al., 1994). In CHO and P11 rat pituitary cells transfected with the 5-HT1A receptor, PKC activation decreased the receptor's ability to inhibit AC (Raymond, 1991; Hensler et al., 1996). In contrast, treatment of stably transfected Ltk- fibroblasts and GH4C1 cells with TPA had no effect on 5-HT1A-mediated AC inhibition (Liu and Albert, 1991; Lembo and Albert, 1995; Lembo et al., 1997). In Ltk- cells, acute preactivation of PKC by TPA resulted in abrogation of 5-HT1A mediated calcium mobilization and PI hydrolysis (Lembo and Albert, 1995). This desensitization was virtually eliminated in cells expressing a 5-HT1A receptor mutant that had all three PKC

sites in the i3 loop removed, indicating the importance of these phosphorylation sites in receptor desensitization. The Thr149 site located in the i2 loop of the 5-HT1A receptor has also been implicated in coupling to N-type calcium channels. Mutation of Thr149Ala confers resistance to the receptor to low concentrations of PMA, suggesting that this single residue is important for receptor uncoupling in situations where PKC is weakly activated (Wu et al., 2002). Conversely, at high PMA concentrations which maximally activate PKC, it appears that downstream PKC sites play a larger role in uncoupling the receptor from G-proteins and the calcium channel.

There is also evidence that PKA plays a role in 5-HT1A receptor phosphorylation and desensitization in multiple cell types. Liu and Albert (1991) were the first to show that PKA activation could potentiate PKC-mediated desensitization of the 5-HT1A receptor in fibroblast Ltk- cells. This is consistent with the observation that stimulation of PKA in CHO cells also increased PKC actions on 5-HT1A-mediated AC inhibition (Raymond and Olsen, 1994). In HeLa cells, forskolin-induced activation of PKA resulted in a loss of high-affinity 5-HT1A receptor binding sites and a desensitization of receptor-mediated AC inhibition (Harrington et al., 1994).

Agonist treatment of the 5-HT1A receptor can also lead to its desensitization. Exposure of the 5-HT1A receptor to agonist such as 5-HT, 8-OH-DPAT, or 5-CT results in rapid uncoupling of the receptor from G-proteins and downstream signaling pathways (van Huizen et al., 1993; Harrington et al., 1994; Hensler et al., 1996). Interestingly, agonist-induced desensitization of the 5-HT1A receptor in Sf9 insect cells was largely unaffected by PKC or PKA inhibitors, but was blocked by heparin, an inhibitor of GRK

activity (Nebigil et al., 1995). In this case, agonist-induced phosphorylation and desensitization of the 5-HT1A receptor might be mediated by a GRK.

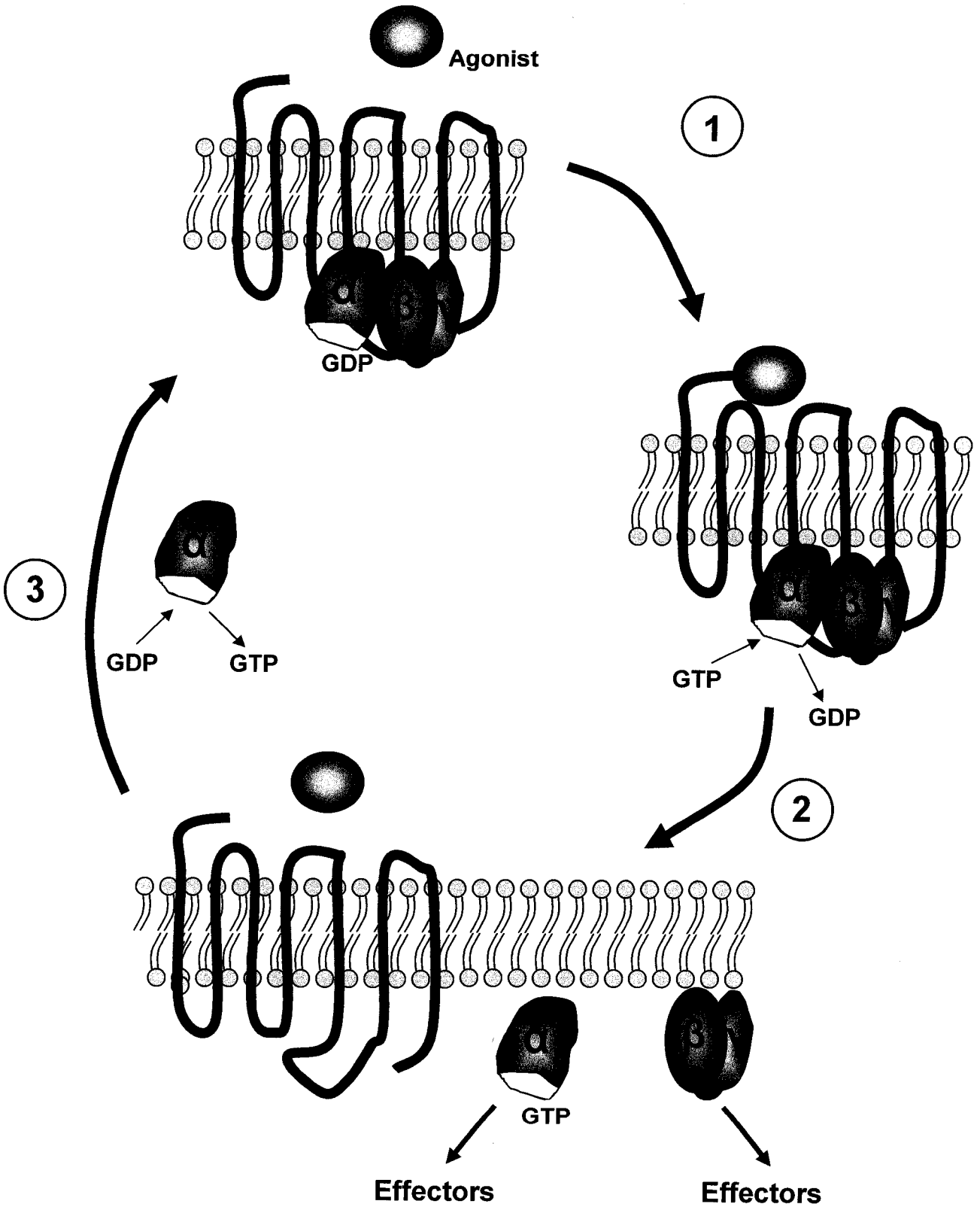
G-Proteins

The G Protein Cycle

Intracellular signaling from GPCRs begins with heterotrimeric guanine-nucleotide-binding proteins (G-proteins) consisting of α, β, and γ subunits (Gilman, 1987). These subunits are associated with the intracellular GPCR domains. The α-subunit cycles between binding of GDP and GTP, and this cycling is controlled in large part by the receptor (Figure 5). When the receptor is inactive (no ligand bound) the α-subunit is bound to GDP and complexed with the βγ dimer. Receptor activation leads to a conformational change in the α subunit such that GDP is released and quickly replaced by GTP, which is highly concentrated in the cytoplasm. The GTP bound α subunit assumes an active conformation, it dissociates from both the receptor and βγ subunits and then goes on to bind and activate effectors. Older models of G protein function assumed that the α-subunit was the predominant player in activation of downstream events and that the main role of βγ dimers was in docking of the α-subunit. However, many studies have since uncovered multiple roles for the βγ complex in mediating GPCR signaling, and in fact, it appears that βγ may regulate even more functions than the α-subunit (see below) (Clapham and Neer, 1997).

All α-subunits contain an intrinsic GTPase activity. Hydrolysis of GTP to GDP results in dissociation from effector molecules and reassociation with βγ subunits. The

Figure 5. The G-Protein Cycle. When GPCRs are in the inactive state they bind heterotrimeric G-proteins, maintaining them in the inactive state through GDP binding to the alpha subunit. 1. Agonist binding activates the receptor and induces conformational changes that promote the dissociation of GDP from the G-protein alpha subunit. GDP is rapidly replaced by GTP. 2. GTP binding to the Gα subunit promotes dissociation from the Gβγ subunit and the receptor. These dissociated forms of the G protein subunits are considered the active forms and they are capable of activating various downstream effectors that translate agonist binding into intracellular signals. These effectors translate the binding of agonist to receptor into cellular responses such as the opening or closing of ion channels or changes in gene transcription. 3. Hydrolysis of GTP to GDP by intrinsic GTPase activity of the α subunit results in reassociation with the βγ dimer and with the receptor.



rate of GTP hydrolysis by the α-subunit therefore controls the timing and extent of activation of α and βγ effectors. The intrinsic rate of GTP hydrolysis varies between different α-subunits, but is also controlled by two separate groups of accessory proteins: regulators of G protein signaling (RGSs), which inhibit G protein signaling by promoting GTPase activity, and guanine nucleotide dissociation inhibitors (GDIs) which have the opposing effect by slowing exchange of GDP for GTP and preventing association with Gβγ subunits (Hepler, 1999; Ross and Wilkie, 2000).

G Protein Families

To date at least 20 different α, 6 β, and 14 γ genes have been identified, and numerous different combinations have been found to be associated with each other *in vivo*. However, all possible combinations do not appear to exist naturally. Based on the primary sequence of the α subunit, G proteins have been divided into four main families: Gs, Gi/o, Gq, and $G_{12/13}$ (Simon et al., 1991). Because G proteins can combine with multiple different GPCRs, and also because multiple combinations of α, β, and γ subunits exist, it is normal for one class, or even one subtype, to be involved in multiple different physiological processes.

Gs Family

The Gs pathway was among the very first intracellular signaling pathway to be described. Most typically, Gs proteins stimulate the activity of adenylyl cyclase (AC), leading to an increase in the production of cAMP. A hallmark of Gs members is that cholera toxin (CTX) increases their GTPase activity by ADP-ribosylating Arg201,

effectively rendering them constitutively active. Most Gs proteins are expressed ubiquitously, however, some display a more selective expression profile, such as α_{olf}, which is strongly expressed within sensory neurons of the olfactory epithelium (Jones and Reed, 1989). More recent work has identified functions for $G\alpha_s$ beyond simply activating AC. Some Gαs members can also elicit the activation of Ca^{+2} channels, while others have been implicated in activation of the small GTPase Rap and MAPK pathways (Post and Brown, 1996; Wan and Huang, 1998). An extra large version of αs (Gαxl, 92KDa) associates with the trans-Golgi network where it stimulates secretory vesicle formation (Kehlenbach et al., 1994).

Gi/o Family

This large family of PTX-sensitive α subunits are characterized by their ability to inhibit AC, leading to a decrease in cAMP-mediated signaling. PTX induces ADP-ribosylation of a C-terminal cysteine residue which prevents interaction with the receptor, effectively inactivating the $\alpha_{i/o}$ proteins. Rods and cones contain unique members of the Gi/o family called transducins, which couple the capture of light energy by rhodopsin to activation of cGMP-phosphodiesterase (Arshavsky et al., 2002). Many taste receptor cells also contain a transducin like α protein termed gustducin. Gustducin appears to specifically mediate bitter taste reception by activating a taste-specific phosphodiesterase (Margolskee, 2002). In addition to their well characterized ability to inhibit AC, αi/o subunits have also been found to couple to K^+ channels, STAT3 via c-Src activation and to Rap pathways (Neves et al., 2002).

Gq Family

Members of the Gq family, which are insensitive to PTX, are best known for their activation of β-isoforms of PLC, followed by the hydrolysis of the membrane PIP2 to release IP3 and DAG. IP3 and DAG go on to promote calcium mobilization from intracellular stores, and to stimulate PKC, respectively. Five members of this family have been identified: αq, α11, α14, α15, and α16. Traditional models predict that members of this family are functionally redundant and that their downstream effects are limited to activation of PLCβ and calcium/PKC. However, emerging evidence suggests that Gαq proteins possess distinct functions and many of their effects are due to actions independent of these effectors (Hubbard and Hepler, 2006). For example, the expression profile for αq and α11 are so widespread that every tissue examined to date has been found to express either one or both (Nakamura et al., 1991; Wilkie et al., 1991). Whereas the expression of α14 is limited to liver, testis, kidney, lung, spleen, and bone marrow (Nakamura et al., 1991; Wilkie et al., 1991; Zigman et al., 1994). The expression of α15 and α16 are even more restricted. They are found only in cells of hematopoietic origin (Amatruda et al., 1991; Wilkie et al., 1991). These unique tissue distributions are suggestive of independent and tissue-specific functions. Gαq has also been implicated in the activation of phospholipase D (Xie et al., 2002) and can activate the protein kinase PYK2 to go on and induce NF-κB transcription (Shi and Kehrl, 2001).

$G_{12/13}$ Family

Perhaps the least well understood family, these PTX-insensitive G proteins share 45% sequence similarity to other α subunits and 67% identity with each other. In many

cases the receptors which couple to G_{12} and G_{13} are still not clear, but it is known that both the lysophosphatidic acid receptor (LPA) and the thromboxane A2 receptor can be activators (Offermanns et al., 1994; Gohla et al., 1998). Moreover, the physiological outcome of $G_{12/13}$ activation is often not known either. Recent work has indicated however, that $G_{12/13}$ may be involved in angiotensin II (Ang-II)-mediated cardiac hypertrophy. Inhibition of $G_{12/13}$ prevents Ang-II from inducing the production of reactive oxygen species (ROS). This data has linked $G_{12/13}$ to the ROS-dependent activation of c-Jun N-Terminal kinase (JNK), p38 MAPK, and NFAT (Fujii et al., 2005; Nishida et al., 2005). Interestingly, G_{13} has also been shown to have a very similar role to that of Gαq in the activation of phospholipase D and NF-κB (Fujii et al., 2005; Nishida et al., 2005).

Gα Structure

Gα proteins contain a conserved GTPase domain and a highly divergent helical domain. The GTPase region, which is highly similar to that of the small G proteins such as $p21^{Ras}$, is buried deep within the protein by the helical domain. Slight differences in this region may underlie the observed variations in rates of spontaneous GDP release, the rate limiting step in G protein activation. The tetrapeptide motif TCAT, present in the GTPase domain, is critical for guanine nucleotide binding. Mutations to this region enhance the rate of spontaneous GDP release (Thomas et al., 1993; Posner et al., 1998) . Such a mutation has been detected in Gs from male patients with pseudohypoparathyroidism and precocious puberty (Iiri et al., 1994). GDP-bound Gα subunits contain a hydrophobic pocket which binds the βγ dimmers. Displacement of

GDP for GTP eliminates this pocket and reduces the affinity between α and βγ (Lambright et al., 1994).

Crystal structures of active and inactive α subunits as well as the heterotrimeric complex have been determined and have provided tremendous insight into the mechanics of G protein activation, interaction with receptors, interaction with βγ subunits, and interaction/activation of effectors. Comparison of inactive and active α structure revealed the presence of three regions of flexibility, termed switch regions I, II, and III. Each of these regions becomes more tightly ordered under the GTP-bound active state (Noel et al., 1993; Lambright et al., 1994).

Interaction with receptors is determined in large part by the five most C-terminal residues of the α subunit. As mentioned, when Gi/o proteins are ADP-ribosylated in this region by PTX they are no longer able to bind their receptors and are rendered effectively inactive. Numerous mutations in this region have been described that affect interaction with receptors (Conklin et al., 1996; Kostenis et al., 1997; Blahos et al., 1998).. Antibodies directed against the α-subunit C-terminal region are capable of disrupting interactions between receptors and G proteins (McFadzean et al., 1989). Moreover, synthetic peptides corresponding to the C-terminus competitively inhibit receptor-G protein binding (Gilchrist et al., 1998). Because α subunits with identical C-termini can exhibit distinct receptor coupling profiles, it is clear that other regions also affect interaction with receptors. Residues influencing receptor coupling have also been found in the N-terminus and in various sections of the helical region (McFadzean et al., 1989; Taylor et al., 1994; Lee et al., 1995; Onrust et al., 1997; Blahos et al., 1998). Importantly, it has been shown that β and γ subunits can contribute to receptor

interactions as well (Kisselev et al., 1995a; Kisselev et al., 1995b; Yasuda et al., 1996; McIntire et al., 2001).

Gβγ Proteins

The first effectors discovered for G proteins were AC and retinal cGMP phosphodiesterase, both of which are activated by the dissociated α subunit. Because no clear role for βγ subunits could be established in terms of activation of effectors, it was assumed for quite some time that the primary function of these proteins was simply to turn off the activated α protein and to aid in membrane localization. The importance of the βγ dimer in transmitting signals downstream from GPCRs began to be appreciated when in 1987 it was shown that they could activate muscarinic K^+ receptors in cardiac cells (Logothetis et al., 1987). Since then numerous βγ effectors have been identified and indeed it appears that the number of these may even be greater than that for α subunits (for review see Clapham and Neer, 1997).

Potassium channels known to be regulated by βγ interaction belong to the GIRK family. These channels are commonly found on cardiac cells, endocrine tissues, and are widespread throughout the brain. Opening of GIRK channels inhibits the firing of excitable cells by hyperpolarizing the cell membrane. In cardiac pacemaker cells binding of acetylcholine to m2 muscarinic receptors slows heart rate by βγ activation of a GIRK1 (Sakmann et al., 1983; Yatani et al., 1987). PTX-sensitive Gβγ proteins are responsible for gating of GIRKs (Logothetis et al., 1987). Channels are formed by dimers of different GIRK subunits. GIRK1-GIRK2 and GIRK1-GIRK4 channels are distributed ubiquitously in the brain but are particularly concentrated in the cerebellum, locus

coereleus, substantia nigra, and hippocampus. A strain of mice termed *weaver* mice suffer from a profound ataxia due to a loss of cerebellar granule neurons (Patil et al., 1995). This cell loss is caused by a mutation in GIRK2.

Voltage-gated Ca^{2+} channels are essential for action potential-induced neurotransmitter release. Activation of various GPCRs can inhibit the opening of N, T and P/Q type Ca^{2+} channels (Hille, 1994). Direct intracellular perfusion of Gβγ in sensory neurons delays the kinetics of activation of calcium currents (Herlitze et al., 1996; Ikeda, 1996). Whether or not the effects of βγ dimmers on Ca^{2+} channels is a direct or indirect effect is not yet well understood.

A number of effectors are shared between Gα and Gβγ subunits. For example, both Gα and Gβγ can stimulate PLCβ activity. PTX can prevent activation of PLCβ in some instances but not in others, suggesting that both Gi/o and Gq heterodimers participate. No αi/o subunits have been found to be capable of activating PLCβ. However βγ subunits could stimulate multiple isoforms of PLCβ (Rhee and Choi, 1992; Smrcka and Sternweis, 1993; Wu et al., 1993; Jiang et al., 1994), suggesting that PTX-sensitive PLCβ activation is largely mediated by βγ subunits. PTX-insensitive activation of PLCβ is mediated by $G\alpha_{q/11}$. The dynamics of activation by Gβγ and the relative sensitivity to either Gβγ or $G\alpha_{q/11}$ varies with different members of the PLCβ family (PLCβ 1-4). Distinct regions of PLCβ mediate binding to Gβγ and Gα subunits. Truncation of a large portion of the C-terminus eliminates activation by $G\alpha_{q/11}$ but not Gβγ (Wu et al., 1993).

Adenylyl cyclase is another effector that is common to both Gα and Gβγ. Multiple isoforms of AC exist and are differentially regulated by Gβγ. AC-1 is inhibited

by Gβγ while AC-II and AC-IV are activated (Tang and Gilman, 1991). Inhibition of AC-1 by Gβγ is not simply a consequence of sequestering $G\alpha_S$. There is a direct binding between AC-1 and Gβγ and this binding appeared to be required for inhibition (Taussig et al., 1994). The precise binding site for Gβγ has not been identified, but it appears to lie somewhere within the C1a domain of AC (Dessauer and Gilman, 1996). Similarly, the site on Gβγ required for AC binding is not known. Some AC isoforms are stimulated by increases in intracellular Ca^{2+} (AC-I, AC-III, and AC-VII), while others are inhibited by this increase (AC-V and AC-VI) (Tang and Gilman, 1992). Since βγ subunits can influence Ca^{2+} levels indirectly through PLCβ and IP3, they can also have more wide-ranging effects on multiple AC isoforms. Moreover, PKC, the other major downstream messenger from PLCβ, can also activate AC-I, AC-III, and AC-VII, giving yet another means by which G proteins can influence AC activities (Morimoto and Koshland, 1994).

Most GPCRs are regulated by phosphorylation by members of the GRK family (GRK1-7) (Haga et al., 1994). Phosphorylation on serine/threonine residues in the carboxy-terminal tail region or the third intracellular loop of ligand bound receptors by GRKs causes desensitization by uncoupling the receptor from G proteins. Also, GRK phosphorylated sites on receptors provide binding sites for β-arrestins, which begin the process of receptor internalization and degradation (or resensitization) (Reiter and Lefkowitz, 2006). GRKs 2 and 3 are activated by Gβγ binding (Pitcher et al., 1992). It appears that at least in the case of GRK2 and GRK3, direct binding of Gβγ is required for recruitment to the membrane (Daaka et al., 1997).

A number of GPCRs have been found to influence mitogenic signals through the cell cycle. In yeast, the homologues of β and γ subunits, STE4 and STE18, are involved

in the mating pheromone response which involves an arrest in the cell cycle (Whiteway et al., 1989). In mammalian cells Gβγ proteins influence mitogenic responses through the MAP kinase pathway. There are both PTX-sensitive and PTX-insensitive G protein-mediated activation of the MAP kinase cascade. The PTX-insensitive activation appears to involve $G\alpha_q$ in a Ras-independent pathway (Pace et al., 1995). The PTX-sensitive MAP kinase activation involves the βγ components of Gi/o proteins working in a Ras-dependent manner (Crespo et al., 1994; Koch et al., 1994). The precise mechanism by which Gβγ activates this Ras pathway is not yet clear but it may involve recruitment of c-Src followed by increased Shc/Grb2/Sos complex formation. Yet another means by which Gβγ proteins may influence MAP kinase activity is via the activation of Phosphoinositide-3 kinase (PI3K). PI3K is a major signaling molecule involved in numerous cellular events including cell survival, cell proliferation, and cytoskeletal reorganization. Activation of MAP kinase by Gβγ can be repressed by inhibitors of PI3K, or by expressing a dominant negative PI3K (Hawes et al., 1996). Whether this dependency on PI3K represents a direct or indirect link is not precisely known. Activation of PI3K also allows Gβγ subunits to influence antiapoptotic signaling. One of the major targets of PI3K is Akt/PKB, which activates survival pathways and inhibits pro-apoptotic pathways (Brunet et al., 2001). Pro-survival signaling from the β2-adrenergic receptor is known to involve this Gβγ-PI3K-Akt pathway and is relevant to the survival of cardiac myocytes (Zhu et al., 2001).

Gβγ Structure

Dimers of Gβ and Gγ can be considered to act effectively as monomers because they cannot be dissociated except under strong non-physiological denaturing conditions. Gβ contains two discrete structural domains, a small N-terminal α-helical domain, and the remainder of the protein which contains seven repeating β strand sequences termed WD repeats. Crystal structures have revealed that these β strands of the WD repeat region organize into a propeller like structure with seven blades (Wall et al., 1995; Lambright et al., 1996; Sondek et al., 1996; Lodowski et al., 2003; Tesmer et al., 2005). Each blade of the propeller is composed of four β strands. The propeller is closed at the seventh blade by association between the extreme C-terminus and regions of the α-helical N-terminal domain. The propeller region is oriented towards the switch II region of Gα, a region that is conformationally altered by GTP binding. The amino terminus of the smaller γ subunit forms a coiled-coil with the N-terminal α-helical region of the β subunit. The remainder of the γ subunit makes multiple contacts with residues of blades 5, 6, and 7. These multiple points of contact explain the tight association between β and γ.

Rationale for Proposed Studies

The 5-HT1A receptor is a crucial modulator of the brain serotonergic system and has been implicated in mood disorders such as depression and anxiety. The 5-HT1A receptor couples to Gαi/o and Gβγ proteins to mediate intracellular signals. It appears that Gαi subunits are mainly involved in inhibition of adenylyl cyclase, whereas multiple effectors are regulated by Gβγ subunits, including PLC, calcium mobilization and ACII.

Studies with 5-HT1A receptors mutated at different PKC sites revealed a Thr149Ala mutation in the i2 loop that generated a receptor that was selectively impaired. The T149A mutant receptor coupled to Gαi-induced inhibition of cAMP, but failed to couple to any of the Gβγ-induced pathways including PLC-induced calcium mobilization and inhibition of Ca^{2+} channel activation (Lembo et al., 1995; Lembo et al. 1997).

We hypothesized that the Thr149 residue is located in a structural domain that couples the 5-HT1A receptor to Gβγ subunits.

We propose to examine:

1) the signaling of PKC site mutants in a raphe neuronal cell line, RN46A, which endogenously expresses the 5-HT1A receptor and is a model of presynaptic receptor function; and
2) the structure-activity relationship of the Ci2 domain to 5-HT1A receptor Gβγ-mediated signaling using a random mutagenesis approach

Our ultimate goal is the development of receptor-and effector-selective inhibitors of Gβγ signaling which should have increased specificity and fewer side-effects than current antidepressant therapies involving 5-HT1A receptor blockers.

Chapter II.

Coupling of 5-HT1A autoreceptors to inhibition of mitogen-activated protein kinase activation via Gβγ subunit signaling.

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Abstract

The 5-HT1A receptor is expressed pre-synaptically as the primary somatodendritic autoreceptor on serotonergic raphe neurons, and post-synaptically in several brain regions. Signaling of the 5-HT1A autoreceptor was studied in RN46A cells, a model of serotonergic raphe neurons that express endogenous 5-HT1A receptors. In undifferentiated RN46A cells stably transfected with the wild-type 5-HT1A receptor, 5-HT1A receptor activation inhibited forskolin-induced cAMP formation (by 50%), increased $[Ca^{2+}]_i$, and induced a novel inhibition (up to 60%) of phospho-p42/p44-mitogen activated protein kinase (MAPK). Upon differentiation of non-transfected or 5-HT1A transfected RN46A cells, agonist-mediated inhibition of MAPK was enhanced. These actions were blocked by pretreatment with pertussis toxin (PTX) indicating mediation via Gi/Go proteins and the calcium response was blocked by pre-activation of protein kinase C (PKC). In cells over-expressing the $G\beta\gamma$ scavenger carboxyl-terminal domain of G protein receptor kinase 2 (GRK-CT), 5-HT1A receptor activation inhibited cAMP formation, but coupling to calcium mobilization and inhibition of MAPK was abolished. The activity of 5-HT1A receptors containing mutations of PKC sites in the second (i2: T149A) or third intracellular loop (i3: T229A/S253G/T343A) was tested. At comparable levels of receptor expression, the signaling of the 5-HT1A i3 mutant was similar to the 5-HT1A wild-type receptor while the i2 and quadruple (i2/i3) mutants failed to couple to $G\beta\gamma$-mediated increase in $[Ca^{2+}]_i$ or inhibition of MAPK, but did couple to $G\alpha i$ mediated inhibition of cAMP. Thus, the i2-domain of the 5-HT1A autoreceptor is crucial for coupling to $G\beta\gamma$ subunits and their subsequent responses (e.g. calcium mobilization and inhibition of MAPK activity).

Introduction

The serotonin (5-HT) system originates from neurons of the raphe nuclei that project widely to regulate a number of physiological processes including mood, emotion, sleep, stress, etc. (Törk, 1990; Jacobs & Azmitia, 1992). The 5-HT1A receptor is expressed presynaptically as the major autoreceptor localized to the soma and dendrites of serotonergic raphe neurons (Sotelo *et al.*, 1990; Riad *et al.*, 2000), and is also expressed postsynaptically throughout the central nervous system. Altered regulation of the 5-HT1A autoreceptor is implicated in depression and suicide (Stockmeier *et al.*, 1998; Mann, 1999; Lemonde *et al.*, 2003), and chronic, but not acute treatment with antidepressants selectively desensitizes 5-HT1A autoreceptors, whereas postsynaptic receptors are relatively resistant to agonist-induced desensitization (Blier & de Montigny, 1994; Pineyro & Blier, 1999). In addition, pre- and post-synaptic 5-HT1A receptors display differences in pharmacology (Castro *et al.*, 2000) and coupling (Blier *et al.*, 1993), although only a single intronless 5-HT1A gene is present. Thus, understanding the signaling mechanisms of 5-HT1A autoreceptors in raphe cells could elucidate mechanisms of antidepressant action (Albert *et al.*, 1996).

The 5-HT1A receptor couples to PTX-sensitive Gi/Go proteins to mediate a variety of responses depending on the cell type (Albert, 1994; Raymond *et al.*, 1999). In fibroblasts, 5-HT1A receptors activate phospholipase C (PLC), leading to calcium mobilization and PKC activation. Activation of PKC phosphorylates the 5-HT1A receptor (Raymond, 1991) and abrogates Gβγ-mediated signaling to increase PLC activity, but does not alter Gαi-mediated inhibition of cAMP (Liu & Albert, 1991). Three PKC sites in the 5-HT1A receptor i3 loop (T229/ S253/T343) are required for PKC-

induced receptor uncoupling (Lembo & Albert, 1995). The i2 PKC site (T149) is necessary for 5-HT1A receptor coupling to $G\beta\gamma$ responses like Ca^{2+} mobilization, but not $G\alpha i$-induced inhibition of cAMP production (Lembo *et al.*, 1997). Additionally, the 5-HT1A receptor also activates MAPK in fibroblast cells (Cowen *et al.*, 1996; Garnovskaya *et al.*, 1996; van Biesen *et al.*, 1996). By contrast, in neuronal cells including raphe neurons, hippocampal neurons, F11 cells (Wu *et al.*, 2002), and neuroendocrine pituitary GH4C1 cells, the 5-HT1A receptor inhibits adenylyl cyclase (AC) activity (De Vivo & Maayani, 1986; 1990), increases potassium conductance (Penington *et al.*, 1993; Bayliss *et al.*, 1997a), and inhibits N-type calcium channels (Penington *et al.*, 1991; Bayliss *et al.*, 1997b) to inhibit neuronal activity. However, the coupling of 5-HT1A autoreceptors to several biochemical pathways in raphe neurons has not been elucidated due to their low abundance and the cellular heterogeneity of raphe nuclei.

We have investigated actions of 5-HT1A receptors in RN46A cells, a presynaptic model derived from rat E13 raphe 5-HT neurons (White *et al.*, 1994; Eaton *et al.*, 1995). We report for the first time the signaling of the 5-HT1A autoreceptor in raphe cells to inhibition of cAMP, calcium mobilization, and have identified a novel mechanism for inhibition of MAPK. The latter pathway is of particular interest because of the important role MAPK plays in signaling of neurotrophins to mediate neuronal differentiation.

Materials and Methods

Materials. Restriction enzymes, total p42/44 MAPK anti-serum, anti-phospho-p42/44 MAPK (T202/Y204) antibody and anti-phospho-MEK 1/2 (Ser217/221) antibody were purchased from New England Biolabs (Mississauga, ON). Anti-RGS-His_6 was acquired from Qiagen (Santa Clarita, CA). Forskolin, 12α-O-tetradecanoyl phorbol 13β-acetate (TPA), 3-isobutyl-1-methylxanthine (IBMX), 5-HT, PTX, EGTA, puromycin, and ±8-hydroxy-2-(di-n-propylamino)tetralin (DPAT) were purchased from Sigma. Enhanced chemiluminescence (ECL) detection kits, [^{3}H]-DPAT (135 Ci/mmol) and [α-^{32}P]-dCTP (2200 Ci/mmol) were obtained from Amersham (Arlington Heights, IL). Fura-2-AM was obtained from Molecular Probes (Eugene, OR). Hygromycin B, U73122, and U73343 were from Calbiochem (San Diego, CA). [^{125}I]-succinyl cAMP (2200 Ci/mmol) and polyvinylidene difluoride (PVDF) membrane were purchased from NEN Life Science Products (Boston, MA). Anti-3', 5'-cAMP antibody was obtained from ICN Biomedicals, Inc. (Aurora, Ohio). The Quikchange™ XL Site-Directed Mutagenesis was purchased from Stratagene (La Jolla, CA).

Plasmids. To generate the 5-HT1A expression plasmids a 1.9 kb BamHI/XbaI fragment of the rat 5-HT1A receptor gene (wild-type receptor clone designated 1A-1) or receptor mutants (i2 and i3 clones) were subcloned into BamHI/XbaI-digested pcDNA3 (Invitrogen). The wild-type receptor clone termed 1A-2 expressed a zinc-inducible vector, pZEM-3 (carrying the mouse metallothionein promoter), that contained a BamHI/PstI fragment of the 5-HT1A receptor gene ligated to its BglII site (Albert *et al.*,

1990). The quadruple 5-HT1A receptor mutant (QM) containing mutations at all four putative PKC phosphorylation sites, was generated using the triple mutant (i3) plasmid as a template. To incorporate the T149A mutation into the i3 mutant vector site-directed mutagenesis (Quikchange™ XL Site-Directed Mutagenesis, Stratagene, La Jolla, CA) was accomplished using the oligonucleotides, Sense: 5'-GTGAACAAAAGGGCTC CCCGGCG-3' and Anti-sense: 5'-CGCCGGGGAGCCCTTTTGTTCAC-3'. The QM plasmid was verified by DNA sequencing. Generation of the His_6-tagged GRK-CT construct has been described previously (Ghahremani *et al.*, 1999).

Cell Culture and Transfection. Rat raphe RN46A cells were maintained in Neurobasal medium (Invitrogen) supplemented with 0.5 mM L-glutamine (Wisent Inc.) and 8% fetal calf serum (FCS) at 33°C in 5% CO_2. Unless otherwise indicated, data are from studies done in non-differentiated cells. For differentiation, RN46A cells were cultured in F12/DMEM (Invitrogen) supplemented with 0.5 mM L-glutamine, N-2 Supplement (Invitrogen), 750 mg/L pyruvic acid (Sigma), 55 mg/L ovalbumin (Sigma), and 1% FCS for 4-5 days at 39°C in 5% CO_2. RN46A cells plated at 50% confluence were co-transfected with 10 μg of the indicated 5-HT1A receptor plasmids (1A: wild-type; i2 mut: i2 mutant; i3 mut: i3 mutant; QM: quadruple mutant) and 1μg of PGK-puro (gift of Dr. Michael McBurney, OHRI) using either Pfx-7 or Lipofectamine 2000 lipid mixtures according to the manufacturer's protocol (Invitrogen). Cells were selected for puromycin resistance (5 μg/ml) for 2-3 weeks and antibiotic-resistant clones were picked, expanded, and tested for the expression of corresponding 5-HT1A receptors using Northern Blot analysis. Clone 1A-GRK-CT was generated from 1A-2-expressing cells that were co-

transfected with 10 μg of His_6-tagged GRK-CT plasmid and 1 μg of pY3 (ATCC) using Lipofectamine 2000 (Invitrogen). Cells were selected for hygromycin B resistance (200 μg/ml) for 2-3 weeks and antibiotic-resistant clones were picked, expanded, and tested for the expression of recombinant GRK-CT protein using Western Blot analysis.

Ligand Binding. Cell membranes were prepared from 15-cm dishes by replacing the growth medium with ice-cold hypotonic buffer (15 mM Tris-HCl, pH 7.4, 2.5 mM $MgCl_2$, 0.2 mM EDTA). After swelling for 10-15 min at 4°C, the cells were scraped from the plates, sonicated on ice, centrifuged (14,000 rpm for 20 min) and resuspended in ice-cold TME buffer (75 mM Tris-HCl, pH 7.4, 12.5 mM $MgCl_2$, 1 mM EDTA). Aliquots of thawed and sonicated membrane preparation (100 μg/tube) were added to triplicate tubes containing 200 μl TME and 10 nM [^{3}H]-DPAT (Amersham) without or with 5-HT (10 μM) to determine total vs. non-specific binding at room temperature (30 min). Reactions were terminated by filtration through GF/C (Whatman) filters, washing with 3 x 4 ml of ice-cold buffer (50 mM Tris-HCl, pH 7.4) and 3 ml of scintillation fluid added to filters to quantify radioactivity by liquid scintillation counting. Protein was assayed with the Bio-Rad protein assay kit with bovine serum albumin as a standard.

cAMP Assay. Measurement of cAMP was performed as described previously (Albert *et al.*, 1990). Briefly, cells plated into 6-well 35-mm dishes were washed once with 1 ml serum-free DMEM. The cells were then incubated in 1 ml/well of serum-free DMEM containing 20mM HEPES, pH 7.2, 100 μM IBMX and various test compounds for 15

min at 33°C. The buffer was collected and stored at -20°C until assayed for cAMP using a specific radioimmunoassay (ICN) as described (Albert *et al.*, 1990).

Measurement of Intracellular Calcium. As described previously (Liu & Albert, 1991), cells were grown to 80% confluence, harvested with trypsin/EDTA, resuspended in 3ml of serum-free DMEM with the calcium indicator Fura-2 AM, 3μM) and incubated for 30 min at 37°C with shaking (100 rpm). The cells were washed once with HBBS-Ca^{2+}, resuspended in 2 ml of buffer, and subjected to fluorometric measurement. Changes in fluorescence ratio were recorded on a Perkin-Elmer Cetus LS-50 spectrofluorometer (Buckinghamshire, UK) and analyzed by computer, based on a K_d value of 227nM for the Fura-2/Ca^{2+}complex. Calibration of R_{max} was performed by the addition of 0.1% Triton X-100 and 20mM Tris base and of R_{min} by the addition of 10mM EGTA. Experimental compounds were added directly to cuvette at the specified concentrations at the indicated times.

Western Blot Analysis. Cells (3 x 10^5 cells/well) were harvested on ice in 200μl of RIPA lysis buffer (10mM Tris-HCl pH 8.0, 1.5mM $MgCl_2$, 5mM KCl, 0.5mM DTT, 0.5mM phenylmethylsulfonyl fluoride, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 5μg of Leupeptin/ml). Lysates were equally loaded and separated on 10-12% polyacrylamide gels and transferred onto PVDF membranes for 1 h at 250 mA at 4°C. Blots were incubated overnight at 4°C in a blocking solution containing 5% nonfat dry milk dissolved in TBS-T (10mM Tris-HCl, 150mM NaCl, pH 8.0, 0.05% Tween 20). Membranes were then incubated overnight at 4°C with primary antibody, followed by 40

min incubation at room temperature with horseradish peroxidase-conjugated secondary antibody. The immunoreactive products were visualized using enhanced chemiluminescence (ECL, Amersham). Immunoreactive bands were digitally quantified using UN-SCAN-IT software (Silk Scientific Inc., Orem, UT).

Statistical Analysis. The data are presented as mean ± SEM of at least three independent experiments. The data were analyzed by either Student's t-test or ANOVA with a *post hoc* Bonferroni test as indicated using the GraphPad Prism program (San Diego, CA).

Results

Transfection of wild-type and mutant 5-HT1A receptors in RN46A cells

Rat raphe RN46A cells express low levels of endogenous 5-HT1A receptors (2.4 ± 0.6 fmol/mg protein, Table 1) and were stably transfected with rat 5-HT1A receptor expression plasmids (1A: wild-type; i2-mut, i2 mutant; i3-mut, i3 mutant; QM, quadruple mutant). The i2 and i3 5-HT1A receptor mutants contain mutations in putative PKC consensus phosphorylation sites (to non-phosphate acceptor residues) located in the second (i2: T149A) and third (i3: T229A/S253G/T343A) intracellular loops of the receptor, respectively. The quadruple mutant (T149A/T229A/S253G/T343A) carries mutations at all four of these PKC sites. Receptor expression levels were determined by saturation binding analysis of membrane preparations from positive RN46A clones (Table 1). RN46A clones transfected with the wild-type 5-HT1A-pcDNA3 construct (i.e., 1A-1) displayed low (8.7 ± 0.9 fmol/mg protein, Table 1) receptor number compared to clones obtained using the inducible promoter vector with basal (non-induced) receptor expression of 53.0 ± 8.6 fmol/mg protein (clone 1A-2). Receptor expression in RN46A clones transfected with mutant 5-HT1A receptor was the same or greater than in clones expressing wild-type receptor (Table 1). This level of 5-HT1A receptor density is somewhat below the physiological range, estimated at 207 ± 17 fmol/mg protein in membranes from rat brainstem/raphe nuclei using radio-labeled DPAT (Hall *et al.*, 1985).

Table 1. Summary of 5-HT1A receptor signaling properties in RN46A cell lines.

For receptor density, membranes were prepared from untransfected RN46A cells and RN46A cells stably transfected with wild-type (1A) or mutant (i2, i3, and QM mutants) 5-HT1A receptors and subjected to binding analysis using [^{3}H]-DPAT. Only the wild-type receptor clone named 1A-2 carried the zinc-inducible mouse metallothionein promoter (see Material & Methods for details). Unless otherwise indicated, data are from studies done in non-differentiated cells. For cAMP inhibition, the percent inhibition of (10 μM) forskolin-stimulated cAMP accumulation by DPAT (1 μM) was calculated as [(F-FD)/F] x 100, where the level of cAMP after forskolin (F) or forskolin and DPAT (FD) was measured. Agonist-induced changes in $[Ca^{2+}]_i$, were assayed in the absence or presence of BAY K8644, are indicated as % over basal or BAY K8644-induced $[Ca^{2+}]_i$; NR, no response to treatment(s). 5-HT1A-induced MAPK inhibition was calculated as [(pMAPK$_{CON}$/Total MAPK$_{CON}$) – (pMAPK/Total MAPK)]/(pMAPK$_{CON}$/Total MAPK$_{CON}$) x 100, where the levels of phosphorylated MAPK before (pMAPK$_{CON}$) or after (pMAPK) DPAT (1 μM) treatment were measured using densitometric analysis of phospho-p42 and phospho-p44 MAPK bands. The phospho-MAPK levels were normalized to their respective total p42 and p44 MAPK bands (e.g. pMAPK/Total MAPK). In all cases, data are expressed as mean ±SEM of at least three independent experiments. For cAMP inhibition, asterisks denote a significant difference compared with RN46A control cells (*P <0.01, **P<0.001; by ANOVA +Bonferroni *post-hoc* test).

Cell line	Receptor Density (fmol/mg)	% cAMP Inhibition	% DPAT-Induced $[Ca^{2+}]_I$ (>basal)	% DPAT-Induced $[Ca^{2+}]_I$ (>BAYK8644)	% Phospho-MAPK Inhibition[a]
RN46A	2.4 ± 0.6	15 ± 3	NR	NR	33 ± 10[b]
1A-1	8.7 ± 0.9	54 ± 9*	27 ± 2	22 ± 7	61 ± 7
1A-2	53.0 ± 8.6	50 ± 3*	127 ± 9	132 ± 6	55 ± 8
i2 mutant-1	71.5 ± 3.8	83 ± 10**	NR	NR	-20 ± 11
i2 mutant-2	167.5 ± 9.8	54 ± 4**	NR	NR	10 ± 8
i3 mutant-1	88.1 ± 8.1	75 ± 8**	144 ± 11	120 ± 8	44 ± 5
i3 mutant-2	60.1 ± 5.4	50 ± 4*	64 ± 9	86 ± 4	30 ± 7
QM-1	79.5 ± 7.2	79 ± 5**	NR	NR	10 ± 6
QM-2	50.0 ± 3.6	48 ± 6*	NR	NR	7 ± 4
GRK-CT	5.5 ± 2.3	17 ± 2	NR	NR	10± 3
1A-GRK-CT	47.0 ± 7.2	56 ± 6*	NR	NR	1 ± 12

[a]: MAPK inhibition after *10 min* DPAT treatment in *differentiated* RN46A clones

[b]: MAPK inhibition after *5 min* DPAT treatment in *differentiated* RN46A cells

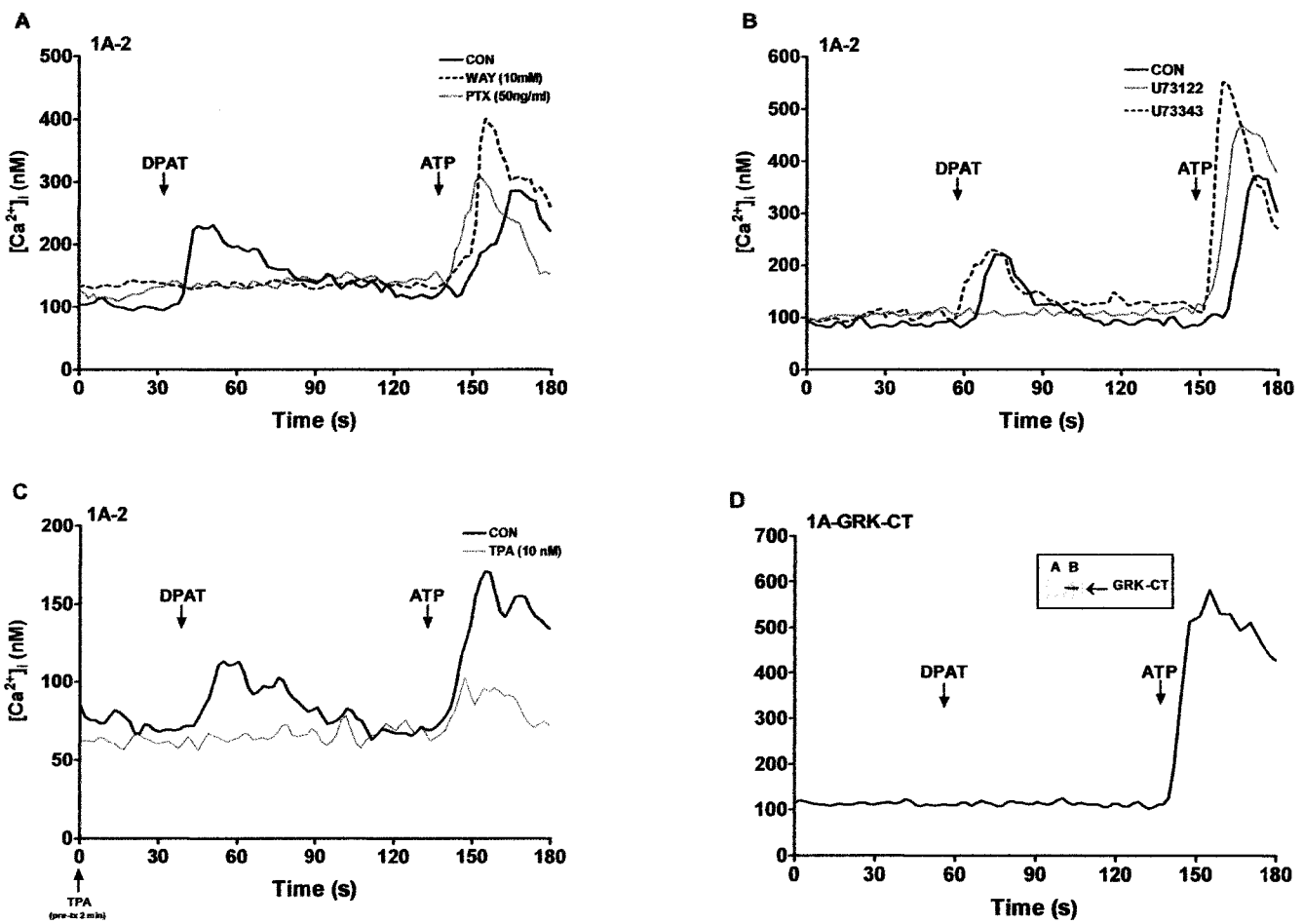
Inhibitory coupling to cAMP formation

The RN46A cells and clones were subjected to functional analysis of 5-HT1A receptor signaling. In non-transfected RN46A cells, 5-HT1A agonist DPAT (1 μM) induced a modest (15%) but reproducible inhibition of forskolin-induced cAMP formation, consistent with the low receptor density in non-differentiated RN46A cells (Table 1). In transfected cells, mutant 5-HT1A receptors each mediated 50-83% inhibition of forskolin-stimulated cAMP levels compared to 50% inhibition in clones expressing either low (clone 1A-1) or physiological (clone 1A-2) levels of wild-type 5-HT1A receptors (Table 1). Thus, the mutant receptors coupled at least as efficaciously as wild-type 5-HT1A receptors to inhibit AC.

5-HT1A receptor-induced calcium mobilization

Coupling of wild-type and mutant 5-HT1A receptors to $[Ca^{2+}]_i$ was examined in cells loaded with the calcium indicator Fura-2. In non-transfected raphe RN46A cells, DPAT had no detectable effect on $[Ca^{2+}]_i$, while in cells with intermediate level of wild-type 5-HT1A receptors (clone 1A-1), DPAT induced a small 25% increase in intracellular Ca^{2+} levels (Table 1). Interestingly, in RN46A cells expressing elevated levels of 5-HT1A receptors (clone 1A-2), DPAT-induced Ca^{2+} mobilization was enhanced approximately 2-fold (Fig. 1A, Table 1). The DPAT-induced calcium response was abrogated by the selective 5-HT1A antagonist WAY 100,635 and by pretreatment with PTX, consistent with 5-HT1A receptor coupling to Gi/Go proteins (Fig. 1A). ATP, which activates endogenous Gq-coupled P2-purinergic receptors, served as a positive

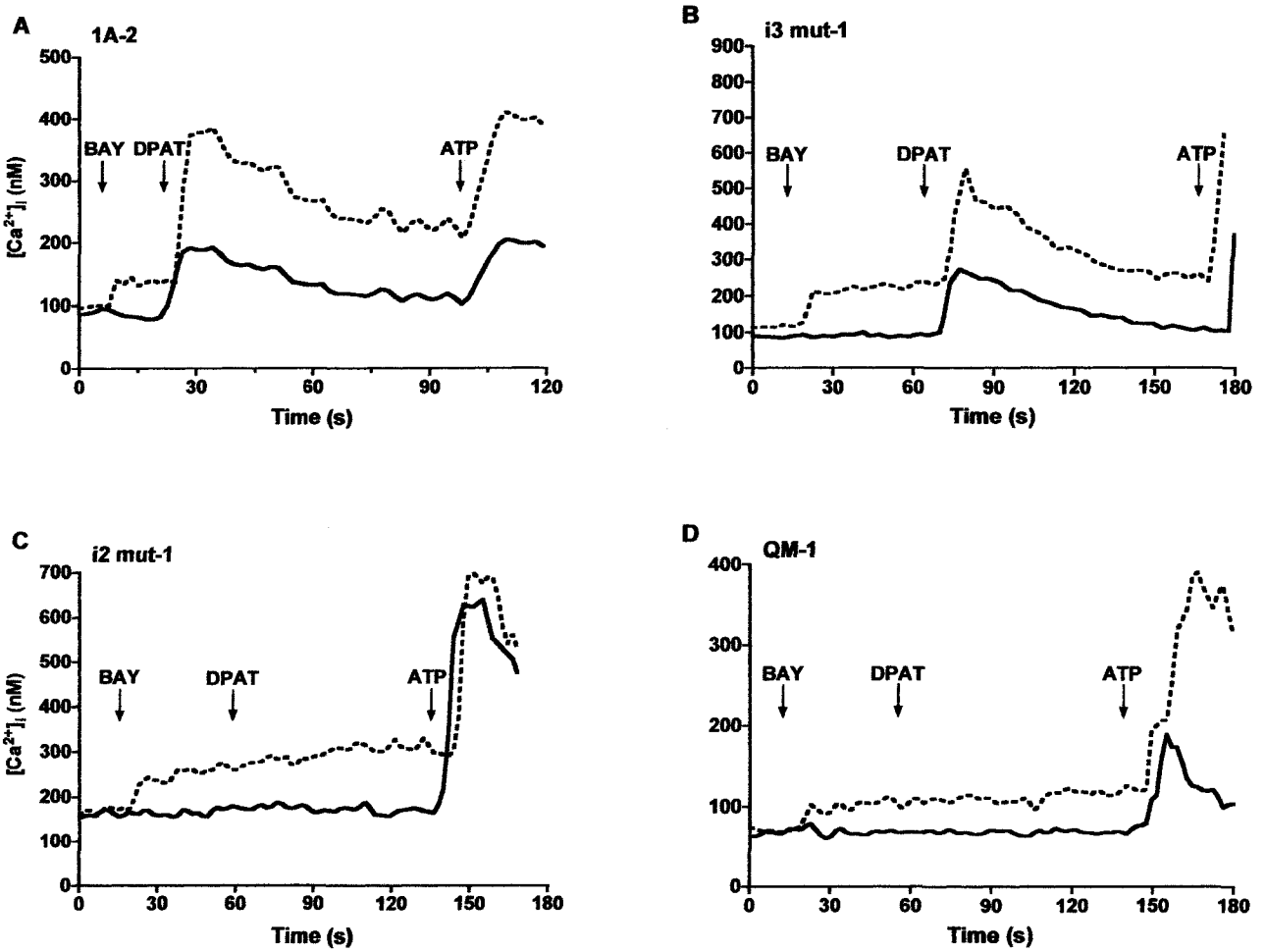
Figure 1. DPAT-induced calcium mobilization in RN46A cells is mediated via coupling of Gi/Go protein βγ-subunits to PLCβ and is TPA-sensitive. **A)** Changes in intracellular calcium in response to DPAT (1 μM) or ATP (10 μM) were measured (see Materials and Methods) in RN46A cells expressing the wild-type 5-HT1A receptor (1A-2, CON). These DPAT-induced changes in $[Ca^{2+}]_i$ were blocked by the 5-HT1A-specific antagonist WAY 100,635 (10 μM; thin line), and were sensitive to PTX treatment (50 ng/ml, 16 h; dashed line). **B)** DPAT-induced changes in $[Ca^{2+}]_i$ were blocked by a two minute pretreatment with an inhibitor of PLCβ, U73122 (10 μM; gray line), but not the inactive analogue U73343 (10 μM; dashed line). **C)** 1A-2 cells were pre-treated with TPA for two minutes (10 nM) and changes in intracellular calcium in response to DPAT (1 μM) or ATP (10 μM) were measured. **D)** DPAT-induced calcium mobilization is blocked by inhibition of Gβγ by GRK-CT. Clone 1A-2 was stably transfected with His_6-tagged carboxyl-terminal of GRK2 (1A-GRK-CT). **Inset)** Western blot analysis of total cell lysates (50 μg/lane) of 1A-2 (Lane A) and 1A-GRK-CT (Lane B) using an antibody against the His_6-tag of His-GRK-CT. The arrow indicates the 24-kDa recombinant His-GRK-CT protein. The graphs shown are representatives, but similar results were obtained from at least three independent experiments for each clone (see Table 1).



control for cell responsiveness. The PLCβ inhibitor U73122 (10 μM) completely blocked DPAT-induced calcium mobilization whereas the inactive analogue U73343 had no effect, indicating that it is mediated via PLC activation (Fig. 1B). Interestingly the Gq-mediated ATP response was relatively resistant to U73122, suggesting different mechanisms of PLC activation for 5-HT1A and P2 receptors. As observed in fibroblast cells expressing 5-HT1A receptors (Liu & Albert, 1991), low concentrations of PKC activator TPA completely blocked DPAT-induced calcium mobilization and partially blocked the ATP-induced change (Fig. 1C). Stable transfection of 1A-2 cells with GRK-CT (Ghahremani *et al.*, 1999), which binds and inactivates free Gβγ subunits (Koch *et al.*, 1994) selectively blocked DPAT-induced calcium mobilization, but not ATP action (Fig. 1D). Positive clones expressing His-GRK-CT protein were verified by Western blot (Fig. 1D, *Inset*). Importantly, GRK-CT expression did not significantly affect the binding characteristics of endogenous 5-HT1A receptors in these clones (Table 1) or their ability to inhibit cAMP accumulation, a Gαi-mediated pathway (Table 1). This indicates that 5-HT1A receptors coupled to PLCβ via Gβγ subunits, whereas P2-purinergic coupling via Gq was Gβγ-independent. Since Gαq activates all PLC-β isoforms, while 5-HT1A-mediated Gβγ mobilization is selective for PLC-β2 and -β3 (Exton, 1996), this may confer greater sensitivity to the inhibitor U73122. Taken together, the results indicate that the 5-HT1A receptor couples to PLCβ via Gi/Go protein Gβγ subunits to induce calcium mobilization in RN46A cells.

In order to address 5-HT1A receptor domains involved in DPAT-mediated calcium mobilization, RN46A clones expressing i2 and i3 mutants were examined. Similar to the wild-type 5-HT1A receptor clone 1A-2 (Fig. 2A), a DPAT-induced

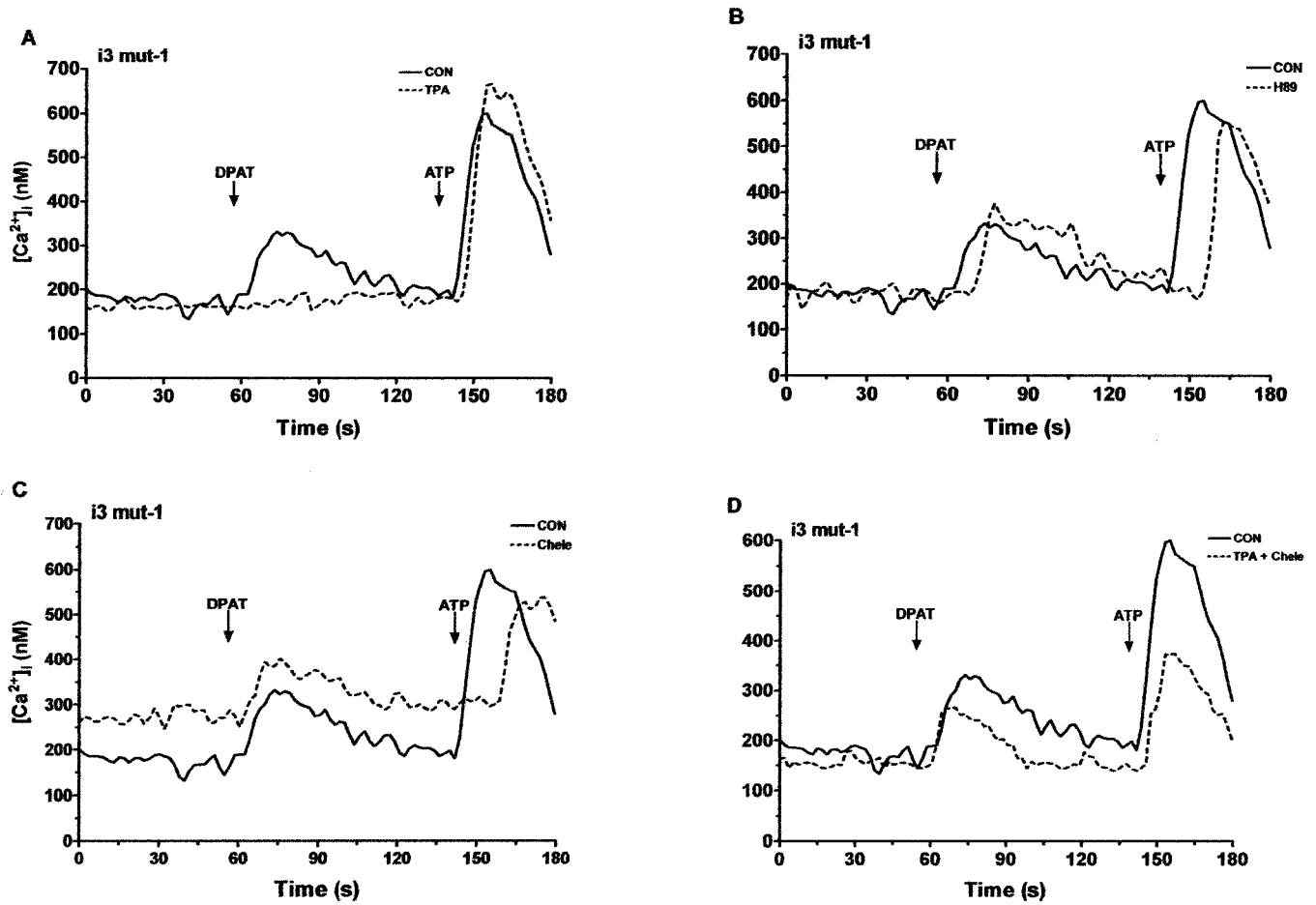
Figure 2. Agonist-mediated calcium mobilization in RN46A cells expressing 5-HT1A wild-type and mutant receptors. RN46A cells expressing the wild-type 5-HT1A (1A-2, ***A***), mutant 5-HT1A receptors (i3, ***B***; i2, ***C***; QM, ***D***) were treated with vehicle (0.1% ethanol, CON; *solid line*) or with L-type calcium channel agonist BAY K8644 (1 μM, +BAY; *dashed line*), and changes in intracellular calcium in response to DPAT (1 μM) or ATP (10 μM) were measured (*see Materials and Methods*). The graphs shown are representatives, but similar results were obtained from at least three independent experiments for each clone (see Table 1).



calcium response was observed in the 5-HT1A receptor i3-mutant clones (Fig. 2B, Table 1) and was blocked by 5-HT1A antagonist, PTX, and PLCβ inhibitor (data not shown). By contrast, the Ca^{2+} response was abolished in RN46A cells expressing high levels of either the i2 (Fig. 2C) or quadruple mutant 5-HT1A receptors (Fig. 2D). In all clones, L-type Ca^{2+} channel agonist BayK8466 (BAY) increased $[Ca^{2+}]_i$ by 2-fold and this response was not blocked by 5-HT1A receptor activation as observed in GH4C1 pituitary cells (Liu & Albert, 1991). These results suggest that, as observed in fibroblasts, the T149 residue of the i2 loop of the 5-HT1A receptor is a critical site for Gβγ-mediated coupling to calcium mobilization in RN46A cells. Similar results were obtained for two independent clones expressing each type of 5-HT1A receptor (i.e., wild-type, i2, i3, or QM receptors), but only one set of data is shown.

Finally, we compared the sensitivity of the 5-HT1A-induced calcium response to acute pre-activation of PKC using TPA in RN46A cells expressing either wild-type or i3 mutant 5-HT1A receptor. In both cell types, 10 nM TPA completely blocked DPAT-induced calcium mobilization in cells (Fig. 1C, 3A). Thus, the mutation of i3 PKC phosphorylation sites did not confer measurable resistance to TPA treatment as observed in Ltk- fibroblasts (Lembo & Albert, 1995), suggesting that additional sites mediate PKC action in RN46A cells. To further address whether PKC indeed mediated TPA-induced uncoupling, inhibitors of PKC (10 μM chelerythrine) or PKA (10 μM H89) were used. Alone, chelerythrine or H89 had no effect on DPAT-induced calcium responses (Fig. 3B, C, data shown for i3 mut). However, chelerythrine (10 μM) reversed TPA-induced uncoupling of wild-type (not shown) and i3 mutant 5-HT1A receptors (Fig. 3D). Thus

Figure 3. TPA-induced uncoupling of 5-HT1A receptor mutant lacking i3 PKC sites: blockade by PKC inhibitor. RN46A cells expressing the 5-HT1A i3 receptor mutant (i3 mut-1) were pretreated for 2 min with the indicated compounds and changes in intracellular calcium in response to DPAT (1 μM) or ATP (10 μM) were measured. ***A***) TPA (10 nM, 2 minutes) induced uncoupling of the 5-HT1A i3 triple mutant. ***B***) Lack of effect of H89, a PKA inhibitor (10 μM; dotted line). ***C***) Lack of effect of chelerythrine, a PKC inhibitor (10 μM; dotted line). ***D***) Combination of chelerythrine (10 μM) and TPA (10 nM) blocked TPA-mediated uncoupling of DPAT-induced response.



PKC-mediated uncoupling of the 5-HT1A receptor in RN46A cells was independent of PKC sites located in the i3 loop.

5-HT1A-induced inhibition of p42/44 MAPK phosphorylation

Activation of MAPK was assayed by Western blot analysis using an antibody specific for the phosphorylated forms of p42/44 MAPK (ERK1/2). In serum-starved, undifferentiated, non-transfected RN46A cells, phospho-MAPK levels were unchanged upon acute DPAT treatment (Fig. 4A, lanes 1-2). In contrast, undifferentiated cells either overexpressing the wild-type 1A or the i3 mutant receptor exhibited decreased p42 and p44 activation with acute DPAT treatment (e.g. 59 ± 9% and 52 ± 10%, respectively after 5 min treatment) (Fig. 4A, C), and this inhibition was more pronounced following differentiation (Fig. 4B, D, Table 1). In general, the time to achieve maximal inhibition by agonist treatment also differed between undifferentiated and differentiated cells (5' vs. 10', respectively). In addition, the inhibition of MAPK persisted for longer periods (>60') of time in the differentiated cells. DPAT-induced MAPK inhibition was absent in undifferentiated and differentiated clones expressing either the i2 mutant 5-HT1A receptor (Fig. 4E, F, Table 1) or the quadruple mutant receptor (Fig. 4G, H). The 5-HT1A-mediated inhibition of phospho-ERK1/2 levels was blocked by the selective 5-HT1A antagonist WAY 100,635, and largely inhibited by PTX pretreatment, implicating a role for Gi/Go proteins (Fig. 5). In differentiated 1A-1 cells, WAY100,635 and G-protein uncoupler PTX tended to increase basal MAPK levels and the combination of racemic DPAT and WAY100,635 produced a significant increase (Fig. 5). Increase in basal MAPK by these treatments may reflect an inhibition of constitutive activity of the

Figure 4. 5-HT1A-mediated inhibition of MAPK activity in RN46A cells. Undifferentiated (left column) or differentiated (right column) RN46A cells expressing the wild-type 5-HT1A (RN46A, ***A***; 1A-1, ***A, B***), and mutant 5-HT1A receptors (i3-1, ***C, D***; i2-1, ***E, F***; QM-1, ***G, H***) were treated with vehicle (C, sterile H_2O) or DPAT (1 μM, ***bolded line***) for the indicated times. Western blot analysis was performed on cell lysates with a specific anti-phospho-p42/44 MAPK antibody (T202/Y204). Immunoreactivity to total MAPK was used as a loading control.

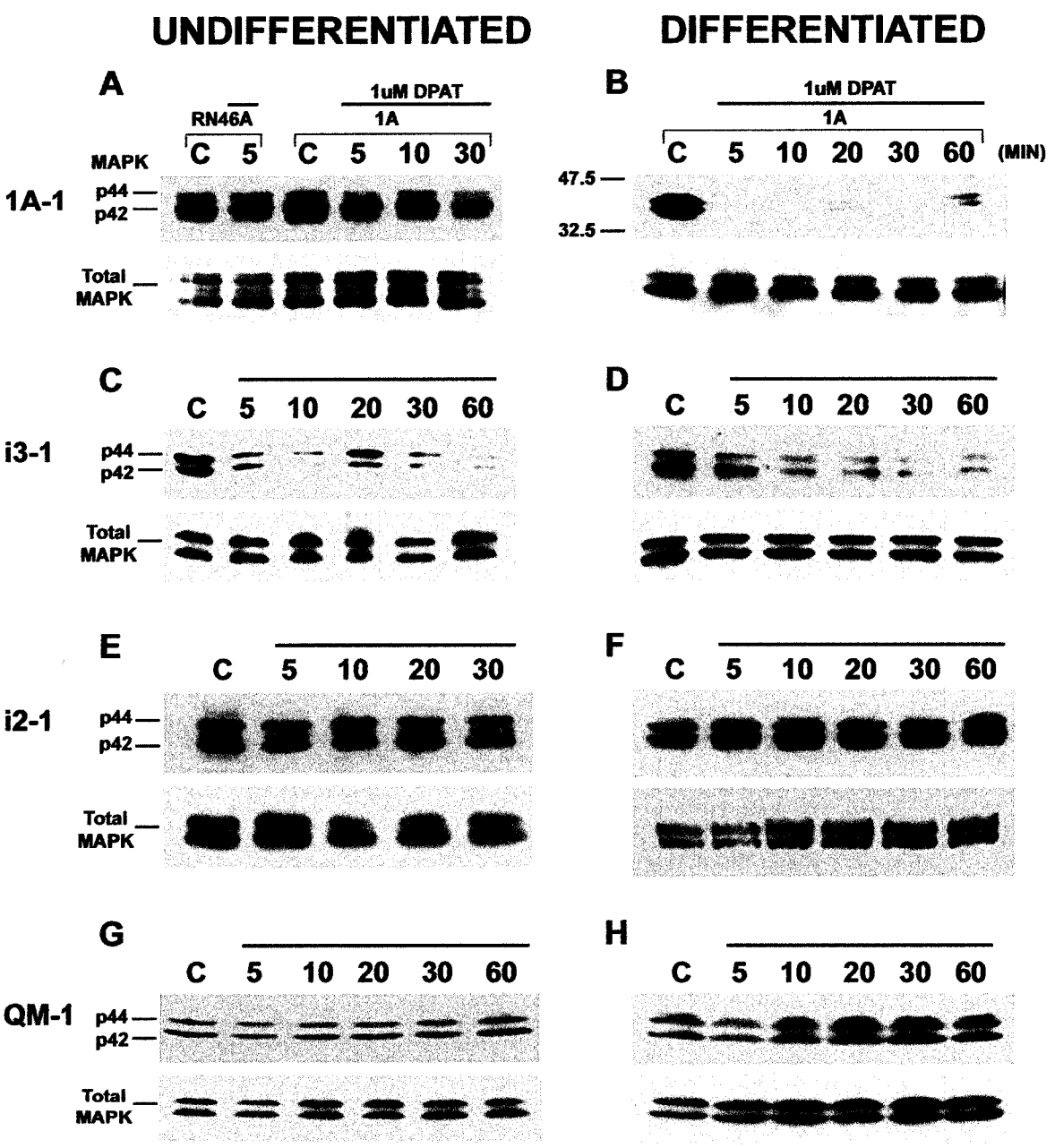
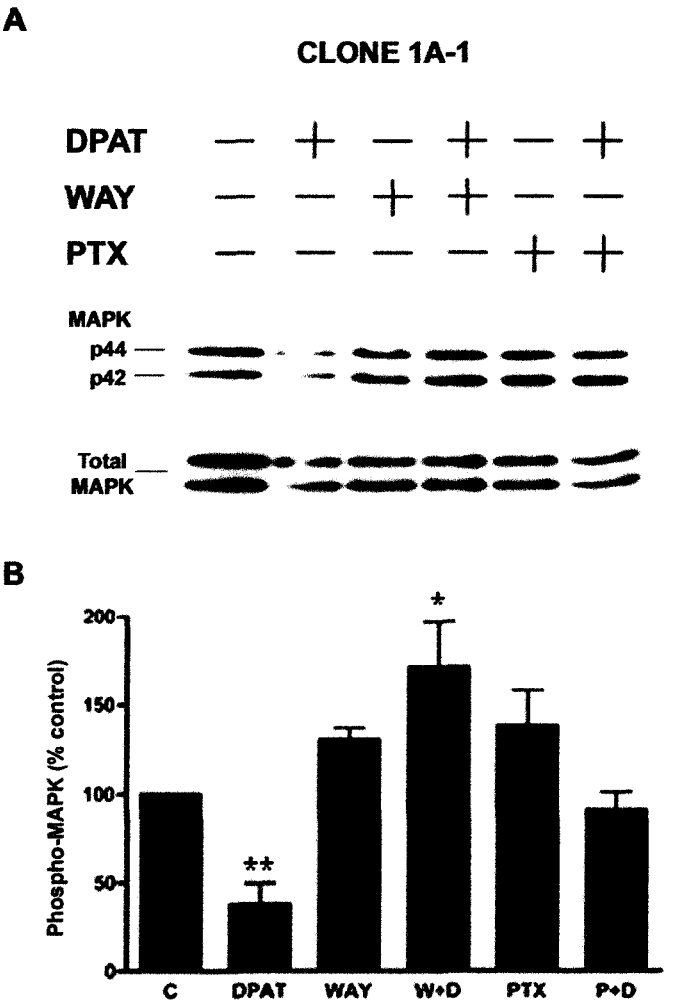


Figure 5. 5-HT1A-induced inhibition of MAPK activity in RN46A cells is mediated via Gi/Go proteins. ***A***) RN46A cells expressing wild-type 5-HT1A receptor (clone 1A-1) were differentiated for 4-5 days at 39°C and then treated with WAY 100,635 (10 μM) or PTX (50 ng/ml, 16h) in the presence or absence of DPAT (1μM, 10 min). Cells were treated with: vehicle (C, *Lane 1*); DPAT for 10 min (*Lane 2*); WAY alone (*Lane 3*); WAY +DPAT (*Lane 4*); PTX alone (*Lane 5*); PTX +DPAT (*Lane 6*). Western blot analysis was performed on cell lysates with a specific anti-phospho-p42/44 MAPK antibody (T202/Y204). Total MAPK immunoreactivity was used as a loading control. ***B***) 5-HT1A-induced MAPK inhibition was calculated as $[(pMAPK_{CON}/Total\ MAPK_{CON}) - (pMAPK/Total\ MAPK)]/(pMAPK_{CON}/Total\ MAPK_{CON}) \times 100$, where the levels of phosphorylated MAPK before ($pMAPK_{CON}$) or after (pMAPK) DPAT (1 μM) treatment were measured using densitometric analysis of phospho-p42 and phospho-p44 MAPK bands. The phospho-MAPK levels were normalized to their respective total p42 and p44 MAPK bands (e.g. pMAPK/Total MAPK). Asterisks denote a significant difference compared to control cells ($^{*}P < 0.05$, $^{**}P < 0.001$; by ANOVA +Bonferroni *post-hoc* test). Data are expressed as mean ± SEM of at least three independent experiments.



5-HT1A receptor (Newman-Tancredi *et al.*, 1997; Albert *et al.*, 1999) or of its activation by 5-HT secreted from differentiated cells (White *et al.*, 1994).

Mechanism of inhibitory coupling to MAPK

To further investigate upstream protein kinases involved in 5-HT1A inhibition of MAPK phosphorylation, MAPK/ERK kinases 1 and 2 (MEK1/2) phosphorylation was examined. In contrast to non-differentiated RN46A cells (Fig. 4A), endogenous 5-HT1A receptors found on differentiated non-transfected RN46A cells were able to inhibit both phosphorylated p42/44 MAPK and MEK levels (73 ± 10% and 60 ± 7% of control, respectively) after a 5 min treatment with DPAT (Fig. 6). Similarly, DPAT-induced inhibition of MAPK and MEK (45 ± 8% and 39 ± 4% of control, respectively) was also observed in cells overexpressing the wild-type 5-HT1A receptor (Fig 7). This 5-HT1A-mediated inhibition of MAPK and MEK was lost in cells overexpressing both GRK-CT and wild-type receptor (Fig. 8) indicating that this response is Gβγ-sensitive.

Figure 6. Agonist stimulation of 5-HT1A inhibits phosphorylation of MEK1/2 in differentiated RN46A cells. *A*) RN46A cells endogenously expressing wild-type 5-HT1A receptor were differentiated for 4-5 days at 39°C and then treated with vehicle (C) or DPAT (1μM) for the indicated times. Western blot analysis was performed on cell lysates with anti-phospho-p42/44 MAPK antibody (T202/Y204). Blots were then stripped and reincubated with a specific anti-phospho-MEK1/2 antibody (Ser 217/221). Total MAPK staining was used as a loading control. ***B***) Data from densitometric analysis are expressed as percent inhibition of basal phospho-MAPK and phospho-MEK levels following DPAT treatment. Asterisks denote a significant difference compared to control cells (*P <0.05; by ANOVA +Bonferroni *post-hoc* test).

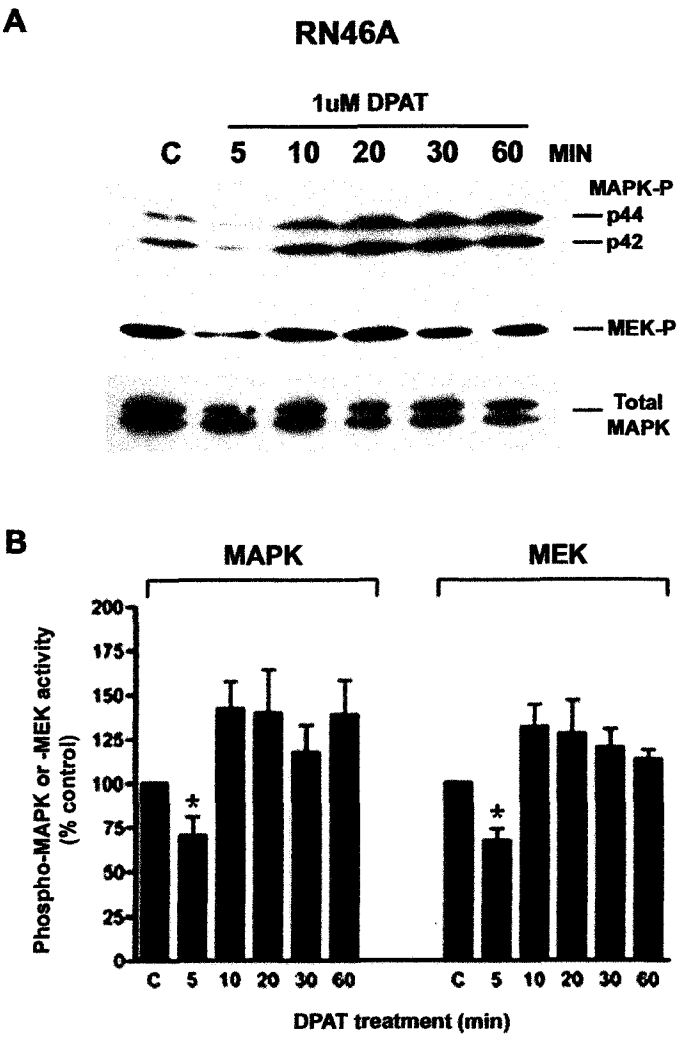
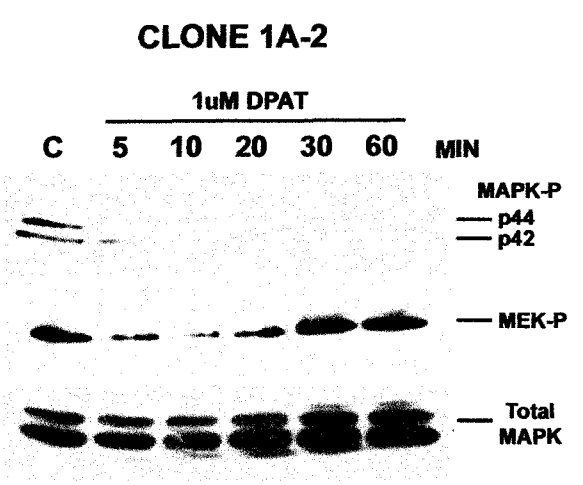


Figure 7. 5-HT1A-mediated inhibition of phospho-MEK1/2 in differentiated RN46A cells. ***A***) RN46A cells expressing wild-type 5-HT1A receptor (clone 1A-2) were differentiated for 4-5 days at 39°C and then treated with vehicle (C) or DPAT (1μM) for the indicated times. Western blot analysis was performed on cell lysates with anti-phospho-p42/44 MAPK antibody (T202/Y204). Blots were then stripped and incubated with a specific anti-phospho-MEK1/2 antibody (Ser 217/221). Total MAPK immunoreactivity was used as a loading control. ***B***) Densitometric analysis data are expressed as percent inhibition of basal phosphorylated p42/p44 MAPK and MEK levels by DPAT. Asterisks denote a significant difference compared to control cells (*P <0.05, **P <0.001; by ANOVA +Bonferroni *post-hoc* test).

A



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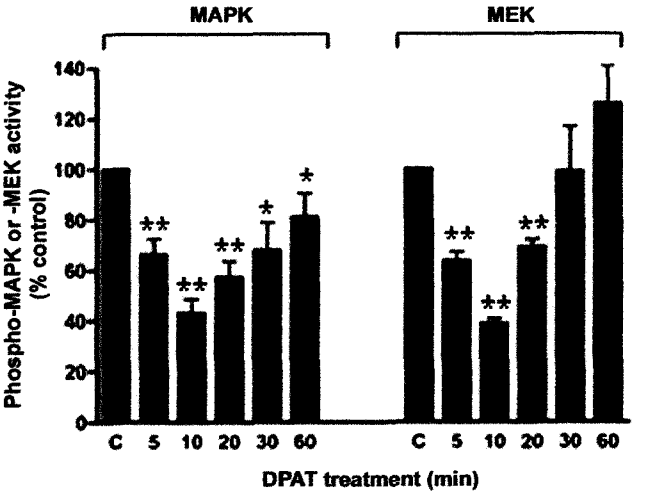
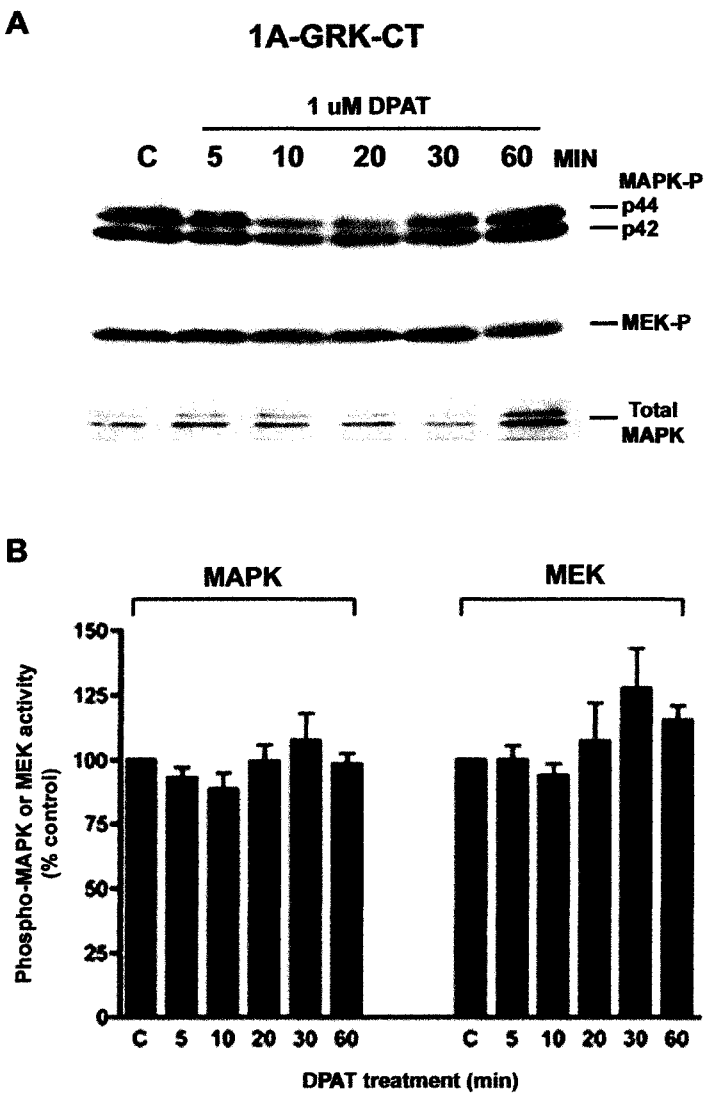


Figure 8. 5-HT1A-mediated inhibition of active ERK1/2 and MEK1/2 is blocked in differentiated RN46A cells expressing GRK-CT. ***A***) RN46A cells stably transfected with both the 5-HT1A receptor and His_6-tagged carboxyl-terminal of GRK2 (1A-GRK-CT) which is known to bind and sequester free Gβγ subunits, were differentiated for 4-5 days at 39°C and then treated with vehicle (C) or DPAT (1μM) for the indicated times. Western blot analysis was performed on cell lysates with specific antibodies against phospho-ERK1/2 (T202/Y204) and phospho-MEK1/2 (Ser 217/221) antibodies. Total MAPK immunoreactivity was used as a loading control. ***B***) Densitometric analysis data are expressed as percent inhibition of basal phosphorylated p42/p44 MAPK and MEK levels by DPAT (*P <0.05; by ANOVA +Bonferroni *post-hoc* test).



Discussion

Differences in the signaling and regulation of pre- and post-synaptic 5-HT1A receptors could play important roles in serotonergic regulation. The enhanced sensitivity of 5-HT1A autoreceptors to homologous desensitization could be mediated by differences in receptor reserve (Castro *et al.*, 2000), G protein coupling specificity (Blier *et al.*, 1993), or differences in signaling pathways to regulate long-term adaptative changes in receptor expression (Lemonde *et al.*, 2003). Hence we have investigated 5-HT1A autoreceptor signaling in raphe RN46A cells, as a model of presynaptic 5-HT neurons.

Inhibition of AC by 5-HT1A Receptor in Raphe Neurons

Inhibition of AC activity is a ubiquitous 5-HT1A receptor signaling pathway. The 5-HT1A receptors couple to inhibition of forskolin- or prostaglandin E1-stimulated AC activity in crude rodent hippocampal membrane preparations (De Vivo & Maayani, 1986; 1990) and in transfected fibroblast and neuronal cell lines (Raymond *et al.*, 1999). In man, 5-HT1A receptors decreased forskolin-induced AC activity in raphe and hippocampal membranes and weakly in cortical membranes (Palego *et al.*, 1999; Marazziti *et al.*, 2002). In 1A-1 RN46A cells, a 50% inhibition of forskolin-stimulated cAMP levels was mediated at 5-HT1A receptor density as low as 8.7 fmol/mg protein, compared with receptor densities of greater than 200 fmol/mg in raphe nuclei (Hall *et al.*, 1985; Li *et al.*, 2000). This is consistent with receptor reserve reported for pre-synaptic 5-HT1A inhibition of raphe action potential firing (Cox *et al.*, 1993). By contrast, post-synaptic 5-HT1A receptors in hippocampal membranes appear to lack detectable receptor

reserve for inhibition of forskolin-stimulated AC (Yocca *et al.*, 1992). Our results in RN46A cells suggest that receptor reserve for coupling to inhibition of forskolin-stimulated AC is a property of the 5-HT1A autoreceptor.

Activation of Calcium Mobilization in RN46A Cells

In neurons, both 5-HT1A autoreceptors and post-synaptic receptors inhibit activation of N-type and P/Q-type calcium channels and activate GIRK channels (Penington *et al.*, 1991; 1993; Bayliss *et al.*, 1997b; a) to reduce calcium influx. Similarly the 5-HT1A receptor inhibits calcium influx in several neuroendocrine cell lines (Liu & Albert, 1991; Singh *et al.*, 1996; Wu *et al.*, 2002), but in RN46A cells neither basal nor BayK8644-stimulated calcium entry via L-type calcium channels was inhibited. Instead, in RN46A clones with high levels of 5-HT1A receptors, the receptor coupled to $G\beta\gamma$-dependent activation of PLCβ resulting in calcium mobilization. The 5-HT1A receptor couples to PLC to enhance phosphatidyl inositol turnover and calcium mobilization fibroblast-derived cell lines, Xenopus oocytes and immune cells (Albert, 1994; Khan *et al.*, 1995; Ni *et al.*, 1997), but in hippocampus and raphe nuclei the receptor appears to inhibit phosphatidyl inositol turnover (Claustre *et al.*, 1991; Johnson *et al.*, 1997). Hence 5-HT1A-mediated calcium mobilization in RN46A cells is unexpected, but appears to be a minor pathway *in vivo*.

The receptor domains involved in 5-HT1A coupling were characterized using PKC site mutants (Lembo & Albert, 1995; Lembo *et al.*, 1997). As shown previously in Ltk- and GH4C1 clones, the i2-, i3-, and QM-5-HT1A mutants displayed equal or greater 5-HT1A receptor density and increased cAMP inhibition, indicating an intact or

enhanced receptor-Gαi coupling compared to wild-type 5-HT1A receptors. In Ltk- cells we found that 5-HT1A-induced calcium mobilization was present in the i3 mutant but resistant to PKC-induced uncoupling (Lembo & Albert, 1995). However in RN46A cells, the i3 mutant 5-HT1A receptors were as sensitive as wild-type receptors to TPA. The sensitivity of the i3 mutant receptor to TPA may reflect elevated PKC activity or different isoforms of PKC present in RN46A neuronal versus fibroblast cells. Another site of PKC action could be the i2 site (T149), mutation of which conferred resistance to TPA-induced uncoupling to inhibition of N-type calcium channels in neuronal F11 cells (Wu *et al.*, 2002). The T149A 5-HT1A mutant displays attenuated coupling selectively to Gβγ-mediated signaling pathways, including calcium channel inhibition and activation of PLCβ and AC type II (Lembo *et al.*, 1997; Albert *et al.*, 1999). Consistent with this, the i2 5-HT1A mutants failed to induce calcium mobilization. Hence the role of T149 in PKC action to uncouple 5-HT1A signaling could not be addressed.

Novel inhibition of MAPK activation induced by 5-HT1A

In fibroblast cell lines (e.g. HEK-293, CHO), the 5-HT1A receptor rapidly activates ERK via a PTX-sensitive pathway that involves Gβγ subunits, phosphatidyl inositol 3'-kinase and Src kinase, the Shc and Grb2 adapter proteins, mSos, Ras, and Raf (Cowen *et al.*, 1996; Garnovskaya *et al.*, 1996). By contrast, in RN46A cells 5-HT1A receptor activation inhibited MAPK phosphorylation via inhibition of MEK1/2 phosphorylation that was also PTX-sensitive and Gβγ-mediated. As observed for other Gβγ-mediated responses, T149A 5-HT1A receptor mutants (i2 and QM clones) were completely uncoupled from inhibition of ERK1/2 and MEK1/2, further indicating that

T149 is involved in coupling the receptor to Gβγ subunits and their subsequent effectors in raphe cells. However in hippocampal HN2-5 cells, 5-HT1A receptor activation enhanced phospho-MAPK involving a pathway that includes PLCβ-mediated release of intracellular calcium to activate the Ras/Raf/MEK cascade (Adayev *et al.*, 1999). Although in RN46A cells we observed 5-HT1A-mediated calcium increase, the inhibition of MAPK was the dominant effect. This represents a novel signaling pathway that is present in raphe cells. Previous studies have shown that intravenous DPAT administration reduced hippocampal MAPK phosphorylation *in vivo* (Chen *et al.*, 2002). However, the mechanism underlying the observed MAPK inhibition could reflect a direct inhibition of MAPK phosphorylation by hippocampal 5-HT1A receptors, or an indirect reduction of 5-HT-induced MAPK activation via reduced hippocampal 5-HT neurotransmission due to inhibitory activation of 5-HT1A autoreceptors. By examining RN46A cells in culture, we provide evidence that DPAT-induced inhibition MAPK phosphorylation is a direct consequence of 5-HT1A autoreceptor activation in raphe cells.

The precise signal transduction mechanisms linking G-protein coupled receptors to inhibition of MAPK phosphorylation have not yet been entirely elucidated. Stimulation of MAPK in fibroblasts may involve Gβγ-GRK-mediated internalization and signaling through β-arrestin/Src complex (Luttrell, 2002). However the mechanism by which Gβγ signaling inhibits MAPK activity in RN46A cells remains unclear. In pituitary cells, dopamine-D2S receptors inhibited MAPK by a Gβγ-insensitive pathway (Banihashemi & Albert, 2002), distinct from the Gβγ-sensitive 5-HT1A-mediated pathway in RN46A cells. In neuronal cells, activation of Gαi/o-coupled receptors may inhibit MAPK phosphorylation by decreasing cellular cAMP levels (Vossler *et al.*, 1997).

Consistent with this idea, activated 5-HT1A receptors produce a reduction of AMPA-evoked currents in cortical pyramidal neurons that is dependent on PKA inhibition (Cai *et al.*, 2002). However, inhibition of cAMP formation and PKA activation is Gβγ-independent and would not explain Gβγ-mediated inhibition of MAPK activation. The 5-HT1A-mediated inhibition of MAPK activation is too rapid to be accounted for by induction of a MAPK phosphatase, as previously described for signaling of 5-HT1B receptors to chronic inhibition of MAPK activity (Albert & Tiberi, 2001). However acute activation of a MAPK phosphatase, triggered by an unknown pathway could mediate the 5-HT1A-induced inhibition of MAPK phosphorylation. 5-HT1A-mediated inhibition of ERK1/2 increased dramatically in differentiated versus undifferentiated RN46A cells, suggesting an up-regulation of effectors required for this pathway. Differentiation of RN46A cells induces serotonergic genes, such as tryptophan hydroxylase, serotonin transporter, and 5-HT1A receptor genes (White *et al.*, 1994; Eaton *et al.*, 1995), and may induce an effector(s) for MAPK inhibition that is present in differentiated raphe neurons *in vivo*.

The 5-HT1A-mediated decrease in MAPK activity in RN46A cells fits with the inhibitory signaling of neuronal 5-HT1A receptors and is expected to inhibit gene expression. For example, inhibition of MAPK could participate in 5-HT1A-mediated inhibition of CREB-phosphorylation and may mediate down-regulation of 5-HT1A mRNA in hippocampal cultures (Nishi & Azmitia, 1999) and in the dentate gyrus (Sibug *et al.*, 1998; Huang & Azmitia, 1999). During development, inhibition of MAPK activity by the 5-HT1A autoreceptor could regulate the number and differentiation of 5-HT neurons *in vivo*. Interestingly, BDNF (which activates MAPK and Akt), depolarization

and cAMP synergistically induce serotonergic differentiation of RN46A (Eaton & Whittemore, 1995) and raphe neurons in culture (Galter & Unsicker, 2000; Rumajogee *et al.*, 2002). The number and neurite length of 5-HT neurons derived from E14 raphe from 5-HT1A$^{-/-}$ mice is greater than from wild-type mice, suggesting that 5-HT1A receptors may inhibit 5-HT neuron proliferation and differentiation (Rumajogee *et al.*, 2004). Thus, reductions in MAPK and cAMP induced by the 5-HT1A autoreceptor could reduce the number or differentiation of serotonergic cells, perhaps predisposing subjects to depression. Consistent with this idea, the C(-1019)G polymorphism that leads to autoreceptor upregulation is associated with suicide and depression (Lemonde *et al.*, 2003) and might cause reduced serotonin neuron number, as observed in one study of post-mortem tissue from depressed suicide victims (Arango *et al.*, 2001).

In summary, we have identified 5-HT1A signaling pathways in rat raphe RN46A cells. Of particular interest, the 5-HT1A autoreceptor inhibits MEK/MAPK via coupling that requires the receptor i2 domain and Gβγ subunits. These results indicate that signaling in presynaptic serotonergic neurons may be very different from that inferred by studies in non-neuronal cell lines.

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Chapter III.

Molecular determinants in the second intracellular loop of the 5-hydroxytryptamine1A (5-HT1A) receptor for G-protein coupling.

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Abstract

This study provides the first comprehensive evidence that the second intracellular loop C-terminal domain (Ci2) is critical for receptor–G protein coupling to multiple responses. Although Ci2 is weakly-conserved, its role in 5-HT1A receptor function was suggested by the selective loss of Gβγ-mediated signaling in the T149A-5-HT1A receptor mutant. Over sixty point mutant 5-HT1A receptors in the alpha-helical Ci2 sequence (^{143}DYVNKRTPRR^{152}) were generated. Most mutants retained agonist binding and were tested for Gβγ signaling to adenylyl cyclase II or phospholipase C and Gαi coupling, to detect constitutive and agonist-induced Gi/Go coupling. Remarkably, most point mutations markedly attenuated 5-HT1A signaling, indicating that the entire Ci2 domain is critical for receptor G-protein coupling. Six signaling phenotypes were observed: wild-type-like; Gαi-coupled/weak Gβγ-coupled; Gβγ-uncoupled; Gβγ-selective coupled; uncoupled; and inverse coupling. Our data elucidate specific roles of Ci2 residues consistent with predictions based on rhodopsin crystal structure. The absolute coupling requirement for lysine, arginine, and proline residues is consistent with a predicted amphipathic alpha-helical Ci2 domain that is kinked at Pro150. Polar residues (T149, N146) located in the externally-oriented positively-charged face were required for Gβγ but not Gαi coupling, suggesting a direct interface with Gβγ subunits. The hydrophobic face includes the critical Y144 that directs the specificity of coupling to both Gβγ and Gαi pathways. The key coupling residues Y144/K147 (Ci2) are predicted to orient internally, forming hydrogen and ionic bonds with D133/R134 (Ni2 DRY motif) and E340 (Ci3) to stabilize the G-protein coupling domain. Thus, the 5-HT1A receptor Ci2 domain determines Gβγ specificity and stabilizes Gαi-mediated signaling.

Introduction

The receptor domains that mediate coupling to Gα-induced responses have been intensively studied, but determinants of Gβγ signaling have yet to be addressed (Bourne, 1997; Meng and Bourne, 2001; Sakmar et al., 2002). Deletion and point mutagenesis studies have indicated that the N- and C-terminal portions of the i3 domain (Ni3, Ci3) as well as i2 and C-terminal regions play important roles in receptor coupling to G-proteins (Shapiro et al, 2002). Using a saturation mutagenesis approach to study the Gq-coupled m5-muscarinic receptor, the i2 and Ni3 domains were predicted to form an amphipathic alpha-helical structure with charged residues of Ni3 recruiting G-proteins to form interactions with embedded hydrophobic residues (Burstein et al., 1996; Burstein et al., 1998; Hill-Eubanks et al., 1996). Similarly, saturation and cysteine mutagenesis of the Ci3 domain (near TMIV) suggests an alpha-helical structure containing residues that interact with G-protein and intracellular receptor domains to regulate constitutive activity (Liu et al., 1995; Schmidt et al., 2003; Shi et al., 2002; Spalding et al., 1998; Zeng et al., 1999). The crystal structure of cis-retinal-bovine rhodopsin (inactive state) (Palczewski et al., 2000) has revealed some intramolecular interactions that are thought to lock the receptor in an inactive state, especially between the conserved (D/E)RY motif at TM3/Ni2 and a conserved D/E residue of Ci3/TM6 (Ballesteros et al., 2001; Meng and Bourne, 2001; Zeng et al., 1999; Shapiro et al, 2002; Shapiro et al., 2002). Although the importance of the conserved (D/E)RY motif in the Ni2 region in receptor activation has been examined extensively (Meng and Bourne, 2001; Sakmar et al., 2002), the structure and importance of the adjacent Ci2 domain in coupling has yet to be addressed. Random mutagenesis of the m5-muscarinic i2 domain yielded few functional mutants of the Ci2

region (Burstein et al., 1998), suggesting the potential importance this domain in coupling. However, their Gαq-mediated signaling screen did not select nonfunctional mutants, hence no firm conclusion could be reached regarding Ci2 function. Furthermore, previous receptor mutagenesis studies have not addressed Gβγ-mediated signaling, which increasing evidence suggests can be dissociated from Gα-mediated coupling (Albert and Robillard, 2002; Chidiac, 1998).

Hence, we have examined in detail the role of the Ci2 domain in 5-HT1A receptor coupling to both Gαi and Gβγ-mediated signaling pathways. The 5-HT1A receptor is a Gi/Go-coupled receptor that is a critical regulator of the brain serotonergic system and has been implicated in mental illnesses such as depression and anxiety (Albert and Lemonde, 2004; Gross and Hen, 2004; Pineyro and Blier, 1999). In previous studies we found that mutation of a single threonine residue (Thr149Ala) within the Ci2 region, reduced or blocked 5-HT1A receptors from coupling to several Gβγ-mediated pathways, but not to Gαi-mediated inhibition of forskolin or Gs-stimulated adenylyl cyclase activation (Albert et al., 1998; Lembo et al., 1997; Liu et al., 1999). The affected Gβγ pathways included: phospholipase C-mediated calcium mobilization (Kushwaha and Albert, 2005; Lembo et al., 1997; Wurch et al., 2003); inhibition of dihydropyridine-induced (L-type) and N-type calcium channel activation (Lembo et al., 1997; Wu et al., 2002); and constitutive activation of adenylyl cyclase II (Albert et al., 1999). The T149A mutation also blocked 5-HT1A-mediated MAPK inhibition in raphe RN46A cells (Kushwaha and Albert, 2005). Thus, the T149A residue of the Ci2 domain plays a crucial role in Gβγ-induced pathways of the 5-HT1A receptor. In addition to the i2 domain, the i3 and palmitoylated C-terminal domains of the 5-HT1A receptor have also

been implicated in G-protein coupling (Papoucheva et al., 2004; Sun and Dale, 1999; Turner et al., 2004; Varrault et al., 1994). Peptides derived from the Ni3 or Ci3 domains of the 5-HT1A receptor can mimic Gαi-mediated inhibition of adenylyl cyclase, while i2 peptides prevented this coupling (Ortiz et al., 2000; Varrault et al., 1994). This suggests that Ni3 and Ci3 domains may mediate activation of Gαi, while the i2 loop is involved in receptor interaction with Gαi, but not its activation.

Computer-based analysis predicts that the Ci2 domain has an amphipathic alpha-helical structure that is conserved among many G-protein coupled receptors (GPCRs) (Albert et al., 1998). In order to provide insight into the structural determinants of the Ci2 loop and empirical validation of their importance for 5-HT1A receptor coupling to G-protein signaling, we have generated a series of random targeted point mutants of nine critical i2 residues that are predicted to form the amphipathic α-helical domain. Based on the coupling of tolerated substitutions we have used computer modeling to identify critical amino acid side-chain interactions that are implicated in Gαi and Gβγ signaling of the receptor. Using this approach we have provided the first comprehensive insights into the role of the Ci2 domain in both Gα and Gβγ signaling.

Materials and Methods

Materials. All chemicals were reagent grade. Forskolin, IBMX, MUG, 5-HT, pertussis toxin, EGTA, and 8-OH-DPAT were purchased from Sigma (St. Louis, MO). Fura-2-AM was obtained from Molecular Probes (Eugene, OR). [^{125}I]-succinyl cAMP (2200 Ci/mmol was purchased from NEN Life Science Products (Boston, MA). Anti-3', 5'-cAMP antibody was obtained from ICN Biomedicals, Inc. (Aurora, Ohio). The QuikChange™ XL Site-Directed Mutagenesis Kit was obtained from Stratagene (La Jolla, CA).

Plasmids. To generate the 5-HT1A expression plasmids a 1.9 kb BamHI/XbaI fragment of the rat 5-HT1A receptor gene wild-type (1A) or receptor mutant (T149A-i2) were subcloned into BamHI/XbaI-digested pcDNA3 (Invitrogen). For construction of 5-HT1A-i2 mutant receptors, single point mutations of each amino acid in the 5-HT1A-i2 loop sequence DYVNKRTPRR were generated randomly using a primer-directed mutagenesis kit (QuikChange™ XL Site-Directed Mutagenesis Kit, Stratagene). For example, to generate 5-HT1A receptors mutated at T149 the following oligonucleotides were used, Sense: 5'-TATAGACTATGTGAACAAAAGG NNGCCCCGG-3'and Antisense: 5'-CGCGCCGGGGCNNCCTTTTGTTCACATAGTC-3'. Mutant primers used for each i2 loop residue are listed in the *Supplemental Table 1 (Appendix1)*. Using the wild-type 5-HT1A cDNA (described above) as template, we prepared the sample reactions according to the manufacturer's protocol and used the recommended cycling parameters to amplify PCR products. The receptor mutants were identified using Sanger

dideoxynucleotide termination DNA sequencing method with the following primer located 40-80 nucleotides adjacent to the sites of the mutation, 5'-CTGTGCTGCACCTCGTCCATCCTG-3'.

Cell Culture and Transfection. Human embryonic kidney (HEK) 293 cells were maintained in DMEM (Wisent) +7% fetal bovine serum (FBS) (Invitrogen) at 37°C in 5% CO_2. HEK 293 cells were plated at 7 X 10^6 cells/10-cm dish and incubated overnight. For measurement of constitutive 5-HT1A receptor activity, cells were transiently transfected by calcium phosphate co-precipitation with 12 μg each of the indicated plasmids (adenylyl cyclase II, Gαi2, and 5-HT1A-wt or 5-HT1A-i2 mutant receptor), and 6 μg of pCMV-βGAL in 7 ml/dish DMEM +10% FCS, 20 mM Hepes (pH 7.0) at 37°C (5% CO_2) for 4-6 h. Consistent basal cAMP levels were observed between wt and mutant receptors indicating similar levels of adenylyl cyclase II expression between transfections. To measure the inhibition of dopamine-stimulated cAMP formation by 5-HT1A receptor activation, HEK 293 cells were transiently transfected with 12 μg of both the human dopamine-D1 and rat 5-HT1A receptors. The cells were then plated onto 6-well plates for cAMP and β-galactosidase assays. Consistent dopamine responses were obtained amongst wt and mutant receptors indicating equivalent expression levels of the D1 receptor coupling between transfections. Ltk- cells were cultured in α-MEM (Wisent) supplemented with 5% FBS at 37°C (5% CO_2). For intracellular calcium measurements Ltk- cells were transiently transfected with 5 μg of either 5-HT1A-wt or 5-HT1A-i2 mutant receptors using Lipofectamine Plus reagents (Invitrogen) according to the manufacturer's protocol. Cells were harvested after 48 hrs

for intracellular calcium measurements. In parallel experiments, membranes were prepared from Ltk- cells transiently transfected with the indicated 5-HT1A mutant receptors and subjected to binding analysis with [^{3}H]-OH-DPAT.

cAMP Assay. Measurement of cAMP was performed as described previously (Albert et al., 1998). Briefly, 24 h after plating HEK cells into 6-well dishes, the cells were washed once with serum-free DMEM and incubated in 1 ml/well of DMEM, 20 mM N-2-hydroxyethylpiperazine-N9-2-ethanesulfonic acid (pH 7.0), 100 μM IBMX for 15-20 min at 37°C. Experimental compounds were added to triplicate wells as indicated. Media were collected, centrifuged at 14,000 X *g* (30 s) to remove floating cells, and the supernatant was stored at -20°C until assayed for cAMP using a specific radioimmunoassay (ICN) as described (Albert et al., 1998). Attached cells were harvested using reporter lysis buffer (Promega), centrifuged for 2 min at 4°C, and stored at -20°C. Transfection efficiency was monitored by cotransfection of β-galactosidase plasmid and cAMP values were normalized to β-gal activity (Albert et al., 1999). Pertussis toxin (50 ng/ml) was present overnight (16h) in indicated samples.

β-Galactosidase Assay. Transfection efficiencies were monitored by β-galactosidase assay. The transfected cells were rinsed with PBS, resuspended in 200 μl of reporter lysis buffer, incubated for 15 min at room temperature, scraped, frozen, and thawed to complete cell lysis. The lysates were centrifuged (14,000 rpm, 2 min, 4°C) and the supernatant was recovered for measurement of β-galactosidase activity. Equal volumes (30 μl each) of cell extract and 0.3 mM MUG substrate in 15 mM Tris (pH 8.8) were

mixed gently, incubated in the dark at 37°C for 30 min, and the reaction was terminated upon addition of 50 μl of Stop solution (300 nM glycine, 15 mM EDTA pH 11.2). The sample was transferred to 2 ml of Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$), and the fluorescence was measured at λ_{EX} =350nm, λ_{EM} =450 nm on a Perkin-Elmer LS-50 spectrofluorometer (Buckinghamshire, United Kingdom). The average β-galactosidase activity for transfection of mutant receptor plasmids in each experiment ranged from 0.8-1.3-fold of that for wild-type receptor.

Ligand Binding. Cell membranes were prepared from transfected cultures on 15-cm dishes by replacing the growth medium with ice-cold hypotonic buffer (15 mM Tris-HCl, pH 7.4, 2.5 mM $MgCl_2$, 0.2 mM EDTA). After swelling for 10-15 min at 4°C, the cells were scraped from the plates, sonicated on ice, centrifuged (14,000 rpm for 20 min) and resuspended in ice-cold TME buffer (75 mM Tris-HCl, pH 7.4, 12.5 mM $MgCl_2$, 1 mM EDTA). Aliquots of thawed and sonicated membrane preparation (100 μg/tube) were added to triplicate tubes containing 200 μl TME and 10 nM [^{3}H]-8-OH-DPAT (Amersham) without or with 5-HT (10 μM) to determine total vs. non-specific binding at room temperature (30 min). Reactions were terminated by filtration through GF/C (Whatman) filters, washing with 3 x 4 ml of ice-cold buffer (50 mM Tris-HCl, pH 7.4) and 3 ml of scintillation fluid added to filters to quantify radioactivity by liquid scintillation counting. Protein was assayed with the BCA protein assay kit (Pierce) with bovine serum albumin as a standard.

Measurement of Intracellular Calcium. As described previously (Liu and Albert, 1991), cells were grown to 80% confluence, harvested with trypsin/EDTA, resuspended in 3 ml of serum-free DMEM with the calcium indicator Fura-2 AM (3 μM), and incubated for 30 min at 37°C with shaking (100 rpm). The cells were washed once with HBBS-Ca^{2+} (pH 7.4), resuspended in 2 ml of buffer, and subjected to fluorometric measurement. Changes in fluorescence ratio was recorded on a Perkin-Elmer Cetus (Buckinghamshire, UK) LS-50 spectrofluorometer and analyzed by computer, based on a K_d value of 227 nM for the Fura-2/Ca^{2+} complex. Calibration of R_{max} was performed by the addition of 0.1% Triton X-100 and 20mM Tris base and of R_{min} by the addition of 10mM EGTA. Experimental compounds were added directly to cuvette at the specified concentrations at the indicated times.

Statistical Analysis. The data are presented as mean ± SEM of at least three independent experiments. Statistical analyses of the data were done using GraphPad Prism software (San Diego, CA).

Modeling. A preliminary model of the rat 5-HT1A receptor (inactive state) was prepared using bovine rhodopsin as template using methodology similar to that described previously (Setola et al, 2005; Shapiro et al, 2002). The full details of the model will be described in a subsequent publication.

Results

Mutagenesis strategy. We employed a random primer-based mutagenesis approach to incorporate point mutations targeted at the 5-HT1A-i2 loop sequence '^{143}DYVNKRTPRR^{152}', a region implicated in G-protein coupling of the receptor (Albert et al., 1998). The function of mutant receptors was examined using three rapid functional assays in transiently transfected cells: coupling to adenylyl cyclase II or to phospholipase Cβ (both Gβγ-mediated); or inhibition of Gs-stimulated adenylyl cyclase activation (Gαi-mediated). Each of these 5-HT1A receptor-mediated signals is blocked by pertussis toxin treatment indicating an obligatory role for Gi/Go proteins. Constitutive coupling to adenylyl cyclase II was assessed in HEK-293 cells cotransfected with 5-HT1A receptor, adenylyl cyclase II, and Gαi2, which results in an agonist-independent increase in cAMP that is mediated by Gβγ signaling and is reduced in the T149A 5-HT1A receptor mutant (Albert et al., 1999) (Fig. 1A). In this model addition of agonist does not increase cAMP any further, hence constitutive receptor coupling is measured. Agonist-mediated 5-HT1A receptor signaling to phospholipase Cβ was assayed by measuring DPAT-induced calcium mobilization in transfected Ltk- fibroblast cells, a Gβγ-mediated pathway that is reduced by the T149A mutation (Lembo et al., 1997) (Fig. 2). Coupling of 5-HT1A receptors to Gαi (Liu et al., 1999) was examined by assaying agonist-mediated inhibition of Gs-stimulated (via D1 receptor) cAMP accumulation in transfected HEK-293 cells (Albert et al., 1999) (Fig. 1B).

Figure 1. Inverse agonist function of 5-HT1A-i2 mutant receptors on Gαi and Gβγ responses.

A, Agonist independent Gβγ-mediated activation of adenylyl cyclase II. HEK 293 cells were transiently transfected with expression plasmids for adenylyl cyclase II, Gαi2, and the indicated wild-type (1A) or i2 mutant 5-HT1A receptors and agonist-independent cAMP production was measured. Constitutive receptor activity was determined using the average difference between pertussis toxin-treated and non-treated cells, normalized to wild-type 5-HT1A activity (100%) for 3-6 independent experiments. Significant difference of receptor activity from zero (pertussis toxin-treated) was calculated using paired t-test, $^{**}p<0.01$, $^{*}p<0.05$. *B*, Agonist-induced inhibition of Gs-stimulated cAMP accumulation. HEK 293 cells were transiently co-transfected with expression plasmids for dopamine-D1 receptor and wild-type or mutant 5-HT1A receptor. Cells were treated with dopamine agonist apomorphine (10 μM) (control) or apomorphine and 5-HT1A agonist 8-OH-DPAT (1 μM) and cAMP was measured. Values were normalized to apomorphine-stimulated cAMP accumulation (100%). Significant differences from 100% were calculated using paired t-test, $^{**}p<0.01$, $^{*}p<0.05$.

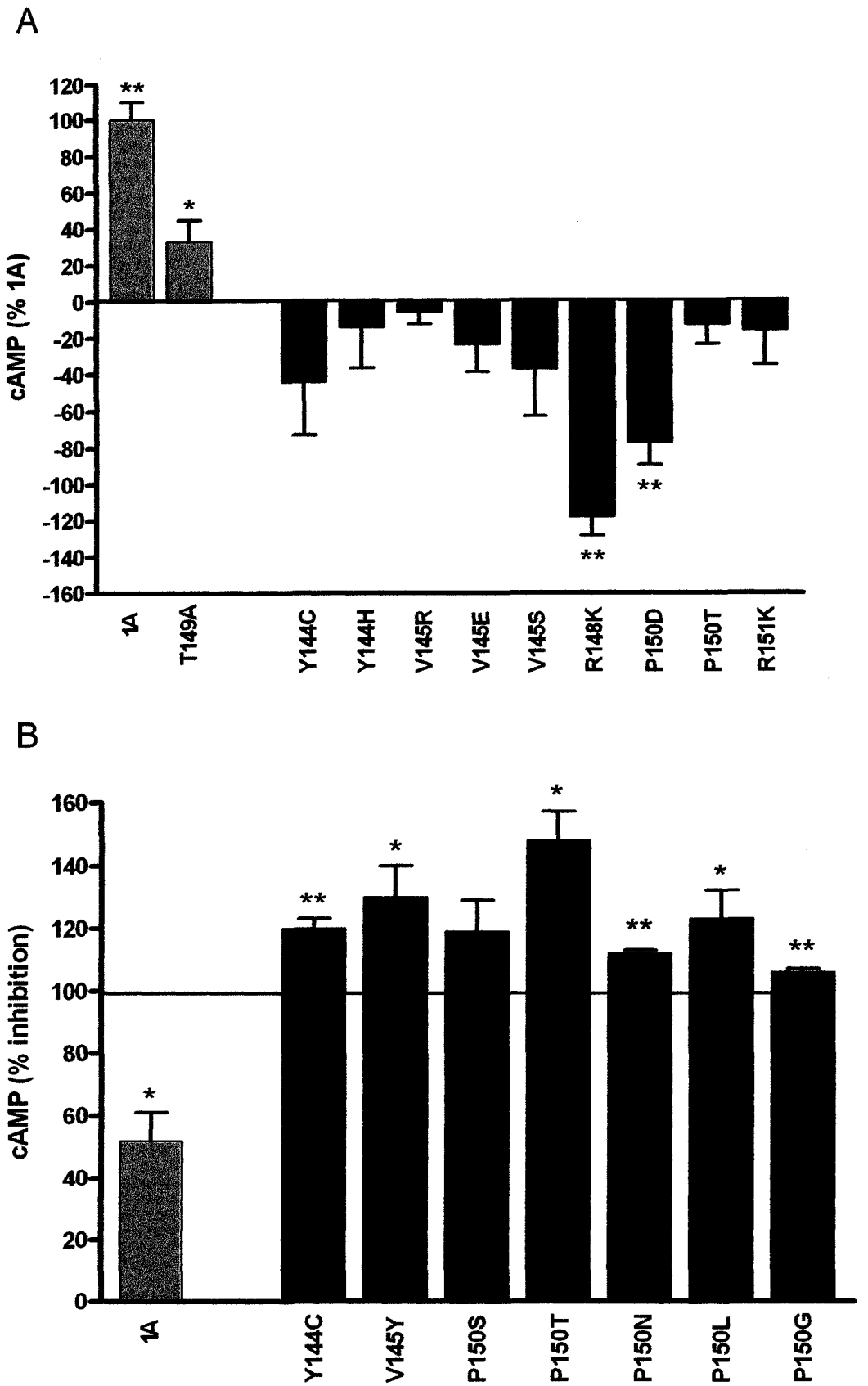
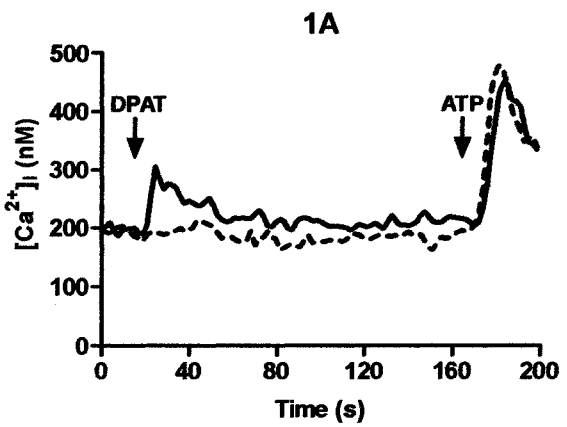
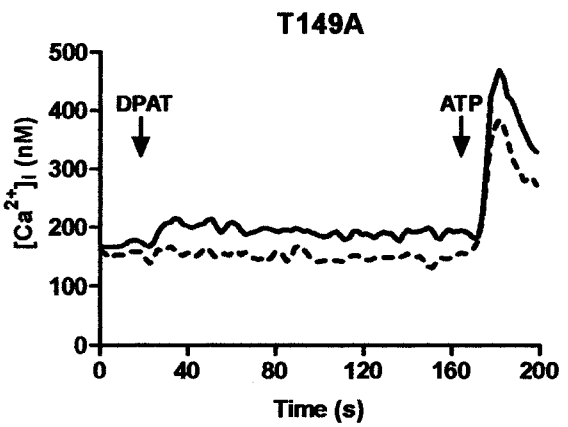
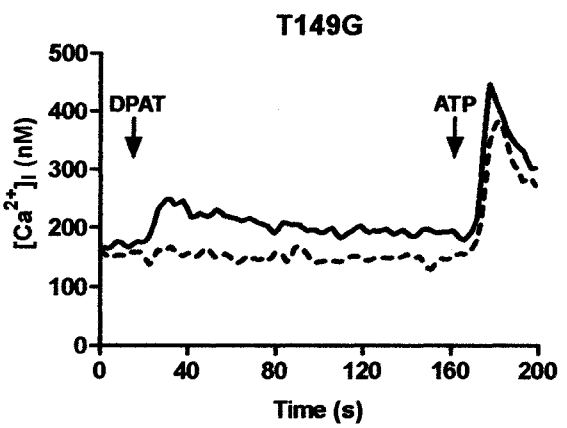
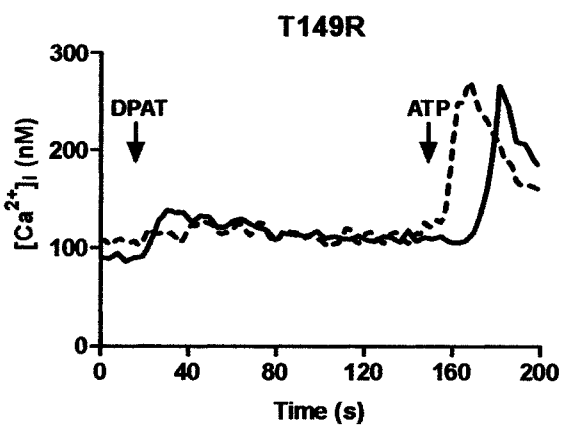


Figure 2. Coupling of 5-HT1A-i2 mutants to calcium mobilization is Gi/Go-specific. Fibroblast Ltk- cells were transiently transfected with either 5 μg of wild-type 5-HT1A (1A) or 5-HT1A-i2 T149 mutant receptors (i2 (T149A), Gly (T149G), Arg (T149R)), and changes in intracellular free calcium concentration in response to 5-HT1A agonist DPAT (1 μM; solid line) were measured (*see Experimental Methods*). Calcium mobilization of the wild-type (1A) and mutant 5-HT1A receptors was blocked by pre-treatment with pertussis toxin (50 ng/ml, 16h; dashed line). Response to ATP (10 μM; solid line) was used as a positive control. Traces shown are representative; similar results were obtained from at least three independent experiments.









Mutant phenotypes. Overall, 61 mutant receptors were generated and examined using the screening assays described above and these data are assembled in Table I. Most of the mutants displayed significant levels of specific binding (^{3}H-DPAT) that was comparable to that of non-mutated 5-HT1A receptors (Table I). Some mutants displayed variation in binding levels due to transfection efficiency, but all displayed at least one functional response except for one case (K147Q), indicating that these mutant receptors folded correctly and were functional. In addition the level of basal and dopamine-stimulated cAMP was similar in transfections of different mutants (data not shown), indicating consistent levels of adenylyl cyclase II or D1 receptor coupling among transfections. The majority (44/61) of the mutant receptors remained significantly coupled to Gαi-mediated inhibition of cAMP accumulation (Table I). Gβγ-signaling of mutant receptors, such as phospholipase Cβ–mediated calcium mobilization, was blocked by pertussis toxin (Fig. 2), indicating mediation by Gi/Go proteins. The mutant 5-HT1A receptors were classified into six main signaling phenotypes (Table II): wild-type-like (3/61, T149R,G; N146T); Gαi-coupled/weak Gβγ-coupled (8/61, T149AEQ; V145LK; R148G; R151AT); Gβγ-uncoupled (16/61); Gβγ-selective coupled (16/61); uncoupled (17/61); and a few mutants displayed inverse basal activity to inhibit adenylyl cyclase II (Fig. 1A) or mediated inverse agonist activity to potentiate D1-induced adenylyl cyclase activation (Fig. 1B). Only two mutants were identified (R152N/D) that lacked Gαi coupling and retained minimal Gβγ coupling, indicating an important role for the Ci2 domain in Gβγ coupling. Within the Gβγ-coupled group of receptors, signaling to adenylyl cyclase II or phospholipase C was selectively preserved depending on the mutation, and in several cases Gαi signaling was also preserved (Table II). At other sites in the i2 domain most

TABLE I. Summary of binding, Gαi- and Gβγ-coupling properties of 5-HT1A-i2 mutants.

Agonist-induced changes in intracellular free calcium concentration in Ltk- cells transiently transfected with wild-type 5-HT1A (1A) or 5-HT1A-i2 mutant receptors were measured and quantified as percent control over basal $[Ca^{2+}]_i$ levels (*Column 1*), *NR: no response to treatments. In parallel experiments, membranes were prepared from Ltk- cells transiently transfected with the indicated 5-HT1A mutant receptors and subjected to binding analysis with [^{3}H]-OH-DPAT (*Column 2*). Agonist independent Gβγ-mediated activation of adenylyl cyclase II was determined using HEK 293 cells transiently transfected with expression plasmids for adenylyl cyclase II, Gαi2, and the indicated wild-type (1A) or i2 mutant 5-HT1A receptor. Agonist-independent cAMP production was determined using the average difference between pertussis toxin-treated and non-treated cells for 3-6 independent experiments, where (+++) indicates ≥80%, (++) ≥ 50%, and (+) between 19-27% increase in cAMP production compared to wild-type 5-HT1A receptor and (-), no detectable activity (*Column 3*). For Gαi-mediated inhibition of adenylyl cyclase, HEK cells were transiently co-transfected with 12 μg of D1 receptor cDNA and an equal amount of wild-type (1A) or 5-HT1A-i2 mutant receptors. Inhibition of D1/Gαs-stimulated (10 μM Apomorphine) cAMP accumulation by 5-HT1A agonist 8-OH-DPAT (1 μM) was measured by specific RIA and data were presented as percent inhibition of D1-stimulated cAMP levels (*Column 4*). In all cases, data are expressed as mean ±SEM of at least three independent experiments, and values that are significantly different from zero were identified using Student's paired t-test and are indicated, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

SUBSTITUTION	DPAT-INDUCED ↑ $[Ca^{2+}]i$ (% over basal $[Ca^{2+}]i$)	RECEPTOR NUMBER (pmol/transfection)	CONSTITUTIVE ACTIVITY (Level of coupling to βγ-mediated stimulation of ACII)	% INHIBITION OF D1-STIMULATED cAMP ACCUMULATION
WT (1A)	$101 \pm 8^{**}$	$1.9 \pm 0.4^{*}$	+++	$48 \pm 9^{*}$
T149A (i2)	$27 \pm 9^{*}$	$2.3 \pm 0.7^{*}$	+	$53 \pm 10^{*}$
D143F	$15 \pm 3^{*}$	1.5 ± 0.9	-	$54 \pm 8^{**}$
D143V	$22 \pm 4^{*}$	$2.5 \pm 0.2^{**}$	-	$56 \pm 9^{**}$
D143N	$26 \pm 7^{*}$	$0.9 \pm 0.1^{**}$	-	$45 \pm 9^{*}$
D143L	$29 \pm 7^{*}$	0.7 ± 0.4	-	$52 \pm 3^{**}$
D143C	NR	$2.9 \pm 0.8^{*}$	-	$44 \pm 12^{*}$
Y144H	NR	$1.9 \pm 0.2^{**}$	-	$22 \pm 3^{**}$
Y144A	4 ± 2	$1.7 \pm 0.5^{*}$	+++	11 ± 8
Y144N	$25 \pm 7^{*}$	$2.4 \pm 0.6^{*}$	-	$44 \pm 5^{**}$
Y144F	NR	$2.3 \pm 0.6^{*}$	++	$43 \pm 7^{**}$
Y144V	10 ± 6	$2.3 \pm 0.2^{**}$	-	$7 \pm 2^{*}$
Y144I	NR	1.4 ± 0.7	-	$26 \pm 5^{*}$
Y144C	NR	2.1 ± 1.1	-	$-20 \pm 3^{**}$
V145L	$26 \pm 3^{**}$	2.2 ± 0.9	+	$34 \pm 11^{*}$
V145K	$23 \pm 9^{*}$	$1.4 \pm 0.2^{**}$	+	$41 \pm 13^{*}$
V145Y	4 ± 3	1.3 ± 0.7	-	$-30 \pm 10^{*}$
V145R	NR	$1.6 \pm 0.6^{*}$	-	15 ± 9
V145Q	NR	$1.9 \pm 0.2^{**}$	-	$27 \pm 7^{*}$
V145E	12 ± 5	$1.8 \pm 0.5^{*}$	-	$32 \pm 9^{*}$
V145S	NR	$1.5 \pm 0.6^{*}$	-	-12 ± 7
V145W	NR	$1.2 \pm 0.2^{**}$	-	$17 \pm 6^{*}$
N146T	$56 \pm 17^{*}$	$1.8 \pm 0.3^{**}$	++	$31 \pm 8^{*}$
N146F	$14 \pm 5^{*}$	$1.9 \pm 0.7^{*}$	-	$42 \pm 10^{*}$
N146A	20 ± 13	$1.0 \pm 0.2^{*}$	-	$53 \pm 10^{*}$
K147R	NR	$2.3 \pm 0.6^{*}$	-	-3 ± 6
K147Q	NR	2.5 ± 1.2	-	-11 ± 9
R148L	$17 \pm 5^{*}$	$2.5 \pm 0.4^{**}$	-	$13 \pm 4^{*}$
R148Q	$16 \pm 2^{**}$	$1.3 \pm 0.2^{**}$	-	$20 \pm 8^{*}$
R148K	12 ± 6	$2.1 \pm 0.1^{***}$	-	4 ± 10
R148E	$28 \pm 4^{**}$	$1.5 \pm 0.1^{**}$	-	$20 \pm 3^{**}$

R148P	NR	$3.2 \pm 0.9^{*}$	+	$21 \pm 4^{**}$
R148G	$19 \pm 6^{*}$	$2.8 \pm 1.1^{*}$	+	$53 \pm 10^{*}$
R148V	20 ± 9	$1.2 \pm 0.4^{*}$	-	$45 \pm 8^{*}$
T149E	$20 \pm 4^{*}$	$1.0 \pm 0.4^{*}$	+	$50 \pm 3^{**}$
T149V	10 ± 5	1.7 ± 1.0	-	$46 \pm 14^{*}$
T149G	$80 \pm 8^{**}$	$3.3 \pm 0.6^{*}$	++	$52 \pm 8^{**}$
T149R	$50 \pm 3^{**}$	1.5 ± 0.9	+++	$60 \pm 8^{**}$
T149Q	$22 \pm 2^{**}$	1.8 ± 0.8	+	$39 \pm 4^{**}$
T149M	15 ± 7	$1.9 \pm 0.2^{**}$	-	$42 \pm 4^{**}$
T149W	9 ± 7	$2.0 \pm 0.7^{*}$	-	$44 \pm 5^{**}$
T149P	6 ± 9	$2.6 \pm 0.4^{**}$	++	$27 \pm 9^{*}$
P150R	2 ± 5	$2.3 \pm 0.1^{***}$	-	-6 ± 3
P150N	NR	1.3 ± 0.8	-	$-12 \pm 1^{**}$
P150D	NR	$1.3 \pm 0.2^{**}$	-	4 ± 2
P150G	NR	2.0 ± 1.0	-	$-6 \pm 1^{**}$
P150I	7 ± 8	$1.7 \pm 0.2^{**}$	-	$4 \pm 1^{*}$
P150L	NR	0.9 ± 0.6	-	$-23 \pm 9^{*}$
P150F	NR	$1.9 \pm 0.5^{*}$	-	15 ± 11
P150S	NR	$0.8 \pm 0.1^{**}$	-	-19 ± 10
P150T	NR	$2.1 \pm 0.8^{*}$	-	$-48 \pm 9^{*}$
R151Q	4 ± 6	1.7 ± 0.8	-	$22 \pm 8^{*}$
R151A	$26 \pm 4^{**}$	$2.3 \pm 0.9^{*}$	+	$24 \pm 8^{*}$
R151M	$8 \pm 1^{**}$	2.1 ± 1.0	-	$20 \pm 4^{*}$
R151T	$27 \pm 3^{**}$	$1.0 \pm 0.1^{**}$	+	$38 \pm 7^{*}$
R151L	2 ± 5	$0.8 \pm 0.2^{*}$	-	$30 \pm 6^{*}$
R151K	2 ± 4	1.5 ± 1.1	-	$26 \pm 6^{*}$
R152N	$16 \pm 4^{*}$	1.4 ± 0.6	-	10 ± 7
R152V	8 ± 7	$1.3 \pm 0.5^{*}$	-	$37 \pm 8^{*}$
R152A	13 ± 7	$2.2 \pm 0.9^{*}$	-	$56 \pm 7^{**}$
R152P	$26 \pm 3^{**}$	1.1 ± 0.5	-	12 ± 11
R152D	$27 \pm 6^{*}$	1.5 ± 0.8	-	-11 ± 2

TABLE II. Signaling phenotypes of mutant 5-HT1A receptors.

Mutant 5-HT1A receptors were grouped according to their responses in the Gαi-mediated (% inhibition of DA-stimulated cAMP accumulation) and Gβγ-mediated (DPAT-induced increase in intracellular calcium, and Gβγ-mediated stimulation of adenylyl cyclase II) assays. For Gβγ assays the maximal response produced by wild-type 5-HT1A receptor was designated as 100%. Wild-type-like receptors, include mutants with adenylyl cyclase II coupling of ≥ 50% and calcium mobilization ≥ 60% of wild-type 5-HT1A receptor, and inhibition of cAMP accumulation ≥ 30%. Gαi-coupled/weak Gβγ-coupled mutants, include receptors that behave like the 5-HT1A-T149A receptor. These mutants had adenylyl cyclase II coupling of ≤ 30% and calcium mobilization 19-27% of wild-type 5-HT1A receptor, and inhibition of cAMP accumulation 24-53%. Uncoupled mutants had undetectable responses. Gβγ-uncoupled mutants include receptors that were uncoupled from both Gβγ responses (calcium mobilization and adenylyl cyclase II), but still retained Gαi function (inhibition of cAMP). Gβγ-selective coupled, include receptors that possess at least one intact Gβγ response. Receptors have been separated into two categories: i) mutants that couple to adenylyl cyclase II stimulation and ii) mutants that couple to phospholipase Cβ-mediated calcium mobilization. Several receptor mutants also displayed inverse activity. Some mutants inhibited the basal activity of adenylyl cyclase II, and others potentiated DPAT-induced D1 receptor stimulation to increase cAMP accumulation.

Ci2 Residue	Wild-type-like	Gαi-coupled/ weak Gβγ-coupled	Uncoupled	Gβγ-uncoupled	Gβγ-selective coupled		Inverse basal activity	
					ACII	PLCβ	ACII	Potentiation of DA-stimulated [cAMP]
D143				D143C		D143F D143V D143N D143L		
Y144			Y144V Y144C	Y144H Y144I	Y144A Y144F	Y144N		Y144C
V145		V145L V145K	V145R V145S	V145Q V145E V145W				V145Y
N146	N146T			N146A		N146F		
K147			K147R K147Q					
R148		R148G	R148K	R148V	R148P	R148L R148Q R148E	R148K	
T149	T149G T149R	T149A T149E T149Q		T149V T149M T149W	T149P			
P150			P150R P150N P150D P150G P150I P150L P150F P150S P150T				P150D	P150T P150N P150L P150G
R151		R151A R151T		R151Q R151M R151L R151K				
R152				R152V R152A		R152N R152P R152D		

mutants were non-functional, suggesting that very few mutations preserve i2 structure required for proper G-protein coupling.

Predicted structures. Because 5-HT1A-i2 peptides mimic 5-HT1A receptor coupling to inhibition of adenylyl cyclase (Thiagaraj et al., 2002; Varrault et al., 1994) and trigger Gβγ-mediated calcium mobilization (Kushwaha and Albert, unpublished) we examined the predicted peptide secondary structures of the mutant Ci2 domains. The presence of a predicted amphipathic alpha-helical structure (Albert et al., 1998) was not altered by any of the point mutations of the Ci2 domain. Garnier-Robson analysis (*Supplemental Table 2, Appendix 1*) revealed a predicted coil at T149/P150. Any mutant predicted to be disrupted, displaced or even reduced at the T149/P150 coil domain (T149AQWVM; all P150 mutants; V145KYES; N146A; R148LQV; R151AMLK; R152VA) displayed impaired coupling to Gβγ or both Gαi and Gβγ signaling (Table I). Strikingly, any substitution at residue Pro150 (Table I) completely disrupted receptor function and was predicted to disrupt the predicted coil structure in the i2 peptide (*Supplemental Table 2, Appendix 1*). Conversely, addition of a proline residue (e.g., R148P, T149P, R152P) resulted in weakly coupled receptors and an expanded coil domain. By contrast, the predicted hydrophobic alpha-helix at the start of TMIV (from R151-A155) was dispensable for coupling in the T149G/R mutants, while several mutants that lacked receptor signaling appeared to retain the TMIV alpha-helix. Thus the predicted coil structure at T149/P150 of the Ci2 domain appears to play a critical role 5-HT1A receptor signaling.

Inverse Activity: Several mutants displayed inverse activity (Fig. 1). The greatest inverse basal activity towards adenylyl cyclase II was observed for the R148K mutant receptor, which reduced cAMP levels to below that of pertussis toxin-treated samples, suggesting that the receptor inhibits the basal activity of adenylyl cyclase II (Fig. 1A). In HEK cells transfected with the R148K mutant, DPAT treatment did not alter basal or D1-induced cAMP, indicating deficient coupling to Gαi and lack of effect on Gs-stimulation. Hence the R148K mutant may prevent mobilization of Gβγ, perhaps by binding free Gβγ subunits. Other mutants appeared to convert DPAT agonism to inverse or biased agonism, as suggested by DPAT-induced potentiation of dopamine-D1 receptor stimulation (Fig. 1B). For these mutants and wild-type 5-HT1A receptors, DPAT had no detectable effect on basal cAMP levels. Thus co-activation of Gs by D1 receptor activation was required to observe DPAT-induced potentiation. This enhancement of D1 response was blocked by pertussis toxin treatment indicating that the potentiation is mediated by Gi/Go proteins and is not due to weak agonist-mediated stimulation of Gs as observed for mutants of 5-HT1A Ci3 domain (Malmberg and Strange, 2000). DPAT-induced potentiation was greatest for the P150T-5-HT1A receptor, which lacked coupling to adenylyl cyclase II. Oppositely the R148K mutant that inhibited adenylyl cyclase II had no effect on D1-stimulated cAMP. Thus, distinct mutations of the Ci2 domain of the 5-HT1A receptor reduced basal activity or altered agonist function.

Functional 5-HT1A mutants: Mutations at the T149 and Y144 sites, the most conserved residues in the Ci2 domain (Albert et al., 1998), resulted in receptors that retained the most complete coupling. The T149 mutants all coupled to Gαi and several retained Gβγ

coupling, indicating that the polar hydroxyl residue of T149 is dispensable for coupling (Table I, Fig. 2). The T149G mutant coupled better to phospholipase C while T149R coupled better to adenylyl cyclase II, indicating that Gβγ effector selectivity was affected by mutations at this site. The negatively-charged T149E mutant, which mimics phospho-threonine, displayed weak Gβγ coupling, but normal Gαi coupling. Similarly, activation of protein kinase C, which phosphorylates the 5-HT1A receptor (Raymond, 1991) at several sites, including T149 (Kushwaha and Albert, unpublished), selectively uncouples Gβγ signaling, with little effect on Gαi signaling (Kushwaha and Albert, 2005; Lembo and Albert, 1995; Wu et al., 2002). The results from T149 mutants displayed selective impairments of Gβγ signaling indicating that the T149 site is critical for selective coupling of Gβγ subunits. Mutational analysis of the Y144 residue revealed that this is the most important Ci2 site in directing coupling of 5-HT1A receptors to both Gαi and Gβγ signaling. For example, the conservative Y144F mutation retained coupling to Gβγ/adenylyl cyclase II and Gαi, but not to Gβγ/phospholipase C, indicating a key role for the polar hydroxyl moiety of Y144 in coupling. The Y144A mutant coupled effectively to Gβγ/adenylyl cyclase II, but not to Gαi or Gβγ/phospholipase C, while the Y144N mutant coupled to Gαi and Gβγ/phospholipase C, but not to Gβγ/adenylyl cyclase II. This diversity of Y144 mutant signaling phenotypes indicates a critical role for the Y144 residue as a signaling terminus that is critical for the specificity of receptor coupling to Gαi and Gβγ.

Discussion

Structure-function studies of GPCR coupling to G-proteins have implicated the receptor i2 and i3 loops in G-protein activation, particularly the highly-conserved N-terminal E/DRY i2 motif (Wess et al., 1997; Shapiro et al, 2002), but the role of the Ci2 domain is undetermined. Previous studies have examined primarily Gα-mediated signaling, but the structural determinants of Gβγ-mediated signaling remain to be addressed (Bourne, 1997). Given that specific receptor- and effector-dependent Gβγ combinations mediate Gβγ signaling, accumulating data suggest that direct receptor-Gβγ interactions may underlie this specificity (Albert and Robillard, 2002; Chidiac, 1998; Clapham and Neer, 1997). We have examined the coupling of point mutants at each residue of the 5-HT1A Ci2 domain to Gαi- or Gβγ-mediated pathways. The majority of 5-HT1A-Ci2 mutant receptors displayed ligand binding that was comparable to wild-type receptors. However most point mutants were uncoupled, either selectively from Gβγ or from both Gβγ and GαI signaling. This represents the first detailed evidence that the entire Ci2 domain plays a key role in receptor signaling to both Gβγ and Gα, which was unexpected given the limited primary sequence homology of this domain (Albert et al., 1998). The sequence divergence of Ci2 domains may determine receptor-dependent Gβγ specificity.

Although mutations in the Ci2 domain did not affect the predicted amphipathic α-helical secondary structure, mutations that altered the predicted coil localized at P150 resulted in completely uncoupled or inversely-coupled receptors (Supplemental Table 2). The ACII assay provides a sensitive measure of ligand-independent 5-HT1A receptor activity that is sensitive to PTX, and Gβγ scavengers, allowing determination constitutive

activity (Albert et al., 1999). Two mutants displayed inverse basal activity towards ACII (R148K, P150D) while other mutants switched coupling of the full agonist DPAT to inverse agonism (Fig. 1B). Notably, substitutions at P150 generated receptors with inverse agonist properties at Gαi or inverse basal coupling to Gβγ responses. The loss of the coil induced by mutations at P150 may transmit a general perturbation to completely inhibit weak basal activity the receptor.

In order to visualize the potential roles of the Ci2 domain residues in tertiary receptor structure, a computer model of the 5-HT1A receptor based on the X-ray crystal structure of bovine rhodopsin (Palczewski et al., 2000) was generated (Fig. 3). This model predicts that the Ci2 domain lies in close proximity to the Ni2, Ni3 and Ci3 portions of intracellular loops. In particular, the model predicts that Ci2 residues Y144 and K147 lie in close proximity to Ci3 residue E340, possibly forming hydrogen, van der Waals or ionic bonds. Viewed from a rotated angle (Fig. 3C), it becomes apparent that K147 and R148 are in close proximity to D133 and could interact with the conserved DRY motif-perhaps via ionic or other types of interactions. The R134 site is also predicted to be near enough to E340 (Ci3) to facilitate intramolecular interactions between Ni2 (DRY motif), Ci2 (YXXKR motif) and Ci3 (E340). A similar network of interactions between i2 and Ci3 via the DRY motif and the homologous E318 residue of the 5-HT2A receptor has been noted previously (Shapiro et al., 2002). The lack of coupling of the K147R- and R148K-5-HT1A receptors suggests that the ε-amino or guanidinium side-chains of these residues make specific, spatially restricted interactions. Mutations of Y144 showed the greatest diversity of coupling phenotypes (depending on the substitution), suggesting that this site not only stabilizes receptor coupling domains,

Figure. 3. Predicted structural model of the wild-type rat 5-HT1A receptor.

The depicted model of the rat 5-HT1A receptor was based on the high resolution crystal structure of bovine rhodopsin in its inactive state and homology with previously published 5-HT receptor models (Shapiro et al, 2002; Setola et al, 2005; see *Methods*) and visualized using Protein Explorer (PE, http:// www.proteinexplorer.org). *A*, Side view of the wild-type 5-HT1A receptor. The α-helices are shown in pink, β-sheets are labeled with orange arrows, and the i2 and i3 loops are indicated. *B*, Amino acids of the N- and C-terminal regions of the i2 and i3 loops (Ni2, Ni3, Ci2, and Ci3) are indicated. Potential predicted inter-helical ionic and/or hydrogen bonding between side-chains of Ci3 residue Glu340 (E340) and Ci2 residues Tyr144/Lys147 (Y144/K147) are indicated by a dashed green line. *C*, Detailed view of the i2 loop domain showing predicted secondary structure, alpha-helices are colored *pink* and beta-sheets are in *gray*. Predicted coordinate hydrogen and ionic bonding of Tyr144/Lys147 (Ci2), Asp133/Arg134 (Ni2 DRY motif) and Glu340 (Ci3) are indicated as a green dashed line.

but could also have direct interactions with Gβγ subunits to determine signaling selectivity. The YXXKR motif is absolutely conserved in all 5-HT1A receptor homologues (to *C. elegans*), as well as in several 5-HT1, muscarinic (M1-5), and adrenergic (α2) receptors and the Y144 residue is highly conserved among Class I GPCRs (Albert et al., 1998), suggesting a conserved role in these receptors. Our data support the importance of the YXXKR residues in determining the efficiency and selectivity 5-HT1A receptor coupling.

Our previous studies had implicated T149 in Gβγ coupling of the 5-HT1A receptor (Albert et al., 1999; Kushwaha and Albert, 2005; Lembo et al., 1997; Wu et al., 2002)and Thr149 mutants all retained coupling to Gαi, but were selectively uncoupled from Gβγ. For example, the T149G mutant coupled better to PLCβ-mediated calcium mobilization than to stimulation of ACII, whereas the T149R mutant coupled better to ACII than to PLCβ. The polar hydroxyl group of Thr149 is predicted to project outward from hydrophilic face of the receptor (Fig. 3C) and could stabilize Gβγ binding to the receptor. Similarly, N146 and D143 mutations also retained coupling to Gαi but had selective impairments in Gβγ signaling. Like Thr149, the side-chains of these residues are polar and are also predicted to project outwards into the cytoplasmic face (Fig. 3B, C) and may stabilize Gβγ interactions. In summary our data are consistent with a model of positively charged residues in the 5-HT1A-Ci2 domain that create a hydrophilic face that permits specific interactions of polar side-chains of D143, N146 and T149 to mediate Gβγ coupling.

In contrast to the 5-HT1A Ci2 domain, the i3 loop (Ni3 and Ci3) has been implicated in G-protein signaling, but mainly to coupling to Gαi. Peptides corresponding

to the 5-HT1A Ci3 domain mimic Gαi-mediated inhibition of forskolin-stimulated AC and attenuate inhibitory coupling of 5-HT1A receptors to AC (Malmberg and Strange, 2000; Ortiz et al., 2000). Similarly, 5-HT1A-i2 loop peptides mediate direct coupling to inhibition of cAMP (Varrault et al., 1994) Mutation of Ci3 residues (V344E and T343A/V344E) enhanced receptor coupling to Gs over Gi, consistent with its role in determining Gαi specificity (Malmberg and Strange, 2000). Thus, while Ci3 appears to mainly dictate Gαi specificity, our results indicate that Ci2 is mainly implicated in Gβγ signaling but also contributes to Gαi signaling. The C-terminal domain of the 5-HT1A receptor also appears to be critical for coupling to both Gαi and Gβγ pathways. Unlike other receptors, the 5-HT1A receptor is constitutively palmitoylated at C-terminal cysteine residues (417 and 420) and palmitoylation is required for coupling to Gi (i.e. loss of inhibition of AC activity) and Gβγ-mediated activation of ERKs (Papoucheva et al., 2004). Thus, Ci2, Ni3, Ci3 and palmitoylated 5-HT1A C-terminal domains appear critical for G-protein coupling, and may directly interact to form a G-protein coupling interface.

In summary, our mutagenesis studies show that multiple residues in the Ci2 sequence ^{143}DYVNKRTPRR^{152} are implicated in 5-HT1A receptor coupling to Gβγ and Gαi. The pattern of mutations predicts that the positively-charged face of the predicted amphipathic Ci2 alpha-helix is absolutely required for coupling and that uncharged residues in this face (T149, N146) direct Gβγ but not Gαi coupling. The opposite inwardly-oriented face includes a critical Y144 residue that directs the specificity of coupling to both Gβγ and Gαi pathways, and interacts with the Ci3 or Ni2 loops forming the G-protein coupling interface.

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Chapter IV. Summary and General Discussion

Findings from animal models, human imaging and postmortem studies indicate a key role for 5-HT1A autoreceptors in the etiology and treatment of mood disorders such as depression and anxiety. There is strong evidence that desensitization of raphe 5-HT1A autoreceptors is an essential component of the antidepressant effect. Chronic, but not acute, treatment with antidepressants selectively desensitizes 5-HT1A autoreceptors, whereas postsynaptic receptors are relatively resistant to agonist-induced desensitization (Blier and de Montigny, 1994; Albert et al., 1996; Pineyro and Blier, 1999). Desensitization of these presynaptic 5-HT1A receptors increases the firing frequency of raphe 5-HT neurons and enhances serotonergic neurotransmission which correlates with improvement of depressive symptoms (Blier and de Montigny, 1994; Le Poul et al., 1995; Kreiss and Lucki, 1997; Blier and de Montigny, 1999). Unfortunately, current antidepressant compounds require two to four weeks to demonstrate their effectiveness and are not always successful. Acceleration and enhancement of the antidepressant response can be achieved by the use of antagonists (e.g. pindolol) to block 5-HT1A autoreceptor function (Artigas et al., 2001), or treatment with full or partial 5-HT1A receptor agonists to desensitize the receptor (Blier and de Montigny, 1994). However, these treatments have limited specificity since they act on both pre- and post-synaptic 5-HT1A receptors which may result in undesirable side-effects. Recent studies in 5-HT1A knockout mice have implicated the post-synaptic receptor in anxiety and depression (Gross et al., 2002), the antidepressant response and antidepressant-induced neurogenesis (Santarelli et al., 2003).

Characterization of 5-HT1A receptor PKC site mutants in raphe RN46A cells

We wanted to better understand the structural basis for 5-HT1A receptor-G-protein coupling and desensitization. Previous studies have implicated PKC as being a key molecule and perhaps even an initiating factor, in 5-HT1A receptor desensitization. Initially, it was shown that acute activation of PKC by phorbol esters could phosphorylate and desensitize the 5-HT1A receptor (Raymond, 1991). In Ltk- fibroblast cells, activation of PKC selectively blocked 5-HT1A receptor-induced PLC activation and ensuing calcium mobilization (a Gβγ-mediated response), but had no effect on Gαi-mediated inhibition of AC activity (Liu and Albert, 1991). Further mutational analysis revealed that Ltk- cells expressing a triple mutant 5-HT1A receptor lacking PKC sites in the i3 loop (T229A/ S253G/ T343A) were almost completely resistant to PKC action (Lembo and Albert, 1995). Unlike the i3-triple mutant, the i2 PKC site (T149A) mutant receptor failed to couple to Gβγ-mediated PLC activation, but did couple via Gαi to inhibit cAMP production (Lembo et al., 1997; Wurch et al., 2003). In contrast, PKC activation uncoupled the 5-HT1A receptor from inhibition of N-type calcium channels in F11 neuronal cells (Wu et al., 2002). This effect was blocked by mutation of the i2-PKC site implying a role for T149 phosphorylation in PKC-induced uncoupling. The three i3 PKC sites appeared to be more important for agonist-induced uncoupling of the 5-HT1A receptor from N-type calcium channels (Wu et al., 2002).

We wanted to examine these PKC site mutants in raphe RN46A cells, a neuronal cell line that endogenously express the receptor and, moreover, is a model of presynaptic 5-HT1A receptor function. We identified and characterized several important 5-HT1A signaling pathways in these raphe neurons: i) in RN46A cells, as in most cell types, the 5-HT1A receptor couples to inhibition of AC activity; ii) like in fibroblast-derived cell

lines, the 5-HT1A receptor couples to Gβγ-dependent activation of PLCβ resulting in calcium mobilization; and iii) the 5-HT1A receptor couples to a novel signaling pathway in raphe cells that is PTX-sensitive and Gβγ-mediated, to inhibit ERK1/2 activity via inhibition of MEK1/2 phosphorylation.

It appears that PKC-mediated signaling is differentially regulated in raphe cells compared to fibroblasts or F11 DRG neurons. For example, the i3 mutant that was resistant to PKC action in Ltk- fibroblasts, was just as sensitive as the wild-type 5-HT1A receptor to TPA treatment when expressed in RN46A cells. These responses may be due to the presence of specific PKC isoforms within each cell type. Although the contribution of different isoforms to PKC-mediated uncoupling was not addressed in this study, it warrants further investigation.

Another novel finding was 5-HT1A-mediated inhibition of MAPK phosphorylation in raphe neurons. This appeared to be a major pathway in these cells as the observed inhibition was greatly enhanced upon differentiation. This effect was shown to be mediated by Gβγ subunits released from PTX-sensitive G-proteins that couple to inhibition of MEK1/2 activity. However, the factors involved in linking Gβγ and MEK1/2 remain unknown. It has been shown in previous studies performed in fibroblast cells that 5-HT1A-mediated MAPK *activation* involves a complicated PTX-sensitive pathway comprising Gβγ subunits, PI3K, Src, Shc, Grb2, mSos, Ras and Raf with possible cross-talk to receptor tyrosine kinase signaling (Cowen et al., 1996; Garnovskaya et al., 1996). Our lab has demonstrated inhibition of MAPK activity by the dopamine D2S receptor in neuroendocrine GH4 cells. In contrast to the results presented here, this inhibitory pathway was shown to be dependent on Gαi3/Gαo subunits and was

mediated by c-Raf and B-Raf kinase inhibition (Banihashemi and Albert, 2002). Future experiments raphe RN46A cells could be aimed at determining whether or not the 5-HT1A-initiated inhibitory MAPK pathway shown here is mediated by inhibition of the Ras/Raf pathway. For example, the actions of 5-HT1A receptor stimulation on c-Raf and B-Raf, which phosphorylate MEK1/2, may be examined in RN46A cells expressing the wild-type receptor using an immune complex-coupled assay in which recombinant glutathione-*S*-transferase (GST)-MEK1 activates and phosphorylates GST-p42 MAPK (Banihashemi and Albert, 2002). In addition, the effect of PKC activation and the structural determinants of the i2 loop on 5-HT1A mediated inhibition of MAPK could be examined using RN46A cells stably transfected with select 5-HT1A-i2 loop mutant receptors (generated in the study described below).

Structural determinants of the 5-HT1A i2 loop for G-protein coupling

The study described in Chapter 2 provided more substantive evidence for the importance of the T149 residue in not only PKC-mediated uncoupling of the 5-HT1A receptor, but also in coupling of the receptor to Gβγ signaling. In raphe cells, the T149A mutant receptors (i2 and quadruple mutant) uncoupled the 5-HT1A receptor from Gβγ-mediated calcium mobilization and MAPK inhibition. The i2 mutant, when expressed in neuroendocrine-derived GH4C1 cells, failed to mediate inhibition of dihydropyridine-induced calcium channel activation, another pathway thought to be mediated by Gβγ subunits (Chen and Penington, 1997; Clapham and Neer, 1997). In HEK 293 cells, Gβγ-dependent activation of ACII is abrogated when co-expressed with the 5-HT1A-T149A receptor mutant and Gαi2 (Albert et al., 1999). Taken together with the studies

performed in Ltk- and F11 cells described above, it appears that each of the pathways affected by the i2 mutant receptor is mediated by Gβγ subunits, implying that the T149 residue plays a crucial role in Gβγ signaling of the 5-HT1A receptor. Our initial hypothesis postulated that the T149 residue was an interaction site or within an interaction domain for the Gβγ subunit.

This proposal led us to investigate the role of T149 and surrounding C-terminal i2 loop residues in 5-HT1A receptor coupling to G-proteins at a structural level. Computer-based structural analysis shows that Thr149 is located in the hydrophilic face of a predicted amphipathic α-helix that is conserved in the structure among several Gi/Go-coupled receptors (Albert et al., 1998). Using a random mutagenesis approach we generated over 65 different point mutations of the rat 5-HT1A-Ci2 loop sequence ^{143}DYVNKRTPRR^{152}. Although site-directed mutagenesis is a convenient method to use to identify important i2 loop residues involved in 5-HT1A receptor/G-protein coupling, this technique suffers from the potential limitation that changes to single amino acids are designed and not random, and potentially important sites of mutation may be missed. To offset this limitation we generated multiple mutations at each of the Ci2 residues listed above and analyzed each mutant receptor using several biochemical assays. Both Gαi and Gβγ-mediated pathways were tested since our initial data with the T149A mutant indicated that Gαi signaling was intact, while Gβγ signaling was blocked by the mutation. In the absence of detailed structural data (i.e. crystal structure of a GPCR bound to its G-protein) the techniques employed here have provided key information to better understand receptor/G-protein interaction sites.

Our mutagenesis experiments are the first to show that the C-terminal region of the i2 loop is crucial for 5-HT1A receptor signaling to G-proteins, especially to Gβγ subunits. Specifically, we demonstrated the absolute requirement for lysine, arginine and proline residues for G-protein coupling. Positively charged lysine and arginine residues are believed to form the hydrophilic face of a predicted amphipathic α-helix that is kinked at P150. Within this hydrophilic face are the polar, uncharged residues, T149 and N146, which were shown to be required for specific coupling to Gβγ, but not Gαi subunits. Consistent with out initial hypothesis, this data suggests that T149 may be one of the residues involved in direct interaction with Gβγ subunits. The hydrophobic face of the α-helix comprises key residues, Y144 and K147. Y144 was shown to direct coupling specificity to both Gβγ and Gαi proteins. Both Y144 and K147 are predicted to form intramolecular interactions with other intracellular domains including the Ni2 DRY motif and Ci3 residue E340 to stabilize the G-protein coupling domain. From these results we concluded that the 5-H1A receptor Ci2 domain determines Gβγ specificity and stabilizes Gαi-mediated signaling.

Our experiments are consistent with the idea that the G-protein binding domain on a receptor is the result of coordinated interactions between the intracellular loops. It follows that disruption of these putative intermolecular interactions could lead to impairments in Gαi and/or Gβγ signaling. Preliminary data from E340 mutant receptors (see *Appendix 2*) indicates that this residue is more important for stabilizing 5-HT1A receptor coupling to Gαi than to Gβγ subunits, which is consistent with the role of Ci3 in Gα signaling (Malmberg and Strange, 2000). The conservative E340D substitution, which maintains a negatively charged moiety, remained coupled to G-proteins, whereas

the E340G mutant receptor was uncoupled from Gαi, but not Gβγ signaling. Substitution of E340 with the positively charged Lys resulted in a mutant receptor that is inversely coupled to Gαi-mediated inhibition of AC and has impaired Gβγ coupling to PLC. It appears that the Lys mutation, perhaps due to its positive charge, interferes with Gαi coupling to the Ci3 domain, which could in turn affect the structure of the Ci2 and its ability to couple to Gβγ subunits.

A recent study characterized a naturally occurring 5-HT1A receptor variant, originally discovered in a patient with Tourette's syndrome, which comprises an Arg219Leu mutation (R220 in the rat receptor) (Bruss et al., 2005). This mutant receptor exhibited reduced coupling to G-proteins and a 60-90% decrease in Gαi-mediated inhibition of AC activity. Interestingly, this R219L mutation is located in the Ni3 region of the 5-HT1A receptor which, according to our model based on rhodopsin crystal structure, is in close proximity to both the Ci2 Y144 residue and the Ni2 DRY region. Although it was not addressed in this study, it would be very interesting to see if R219L would also affect Gβγ signaling. This mutant receptor could confer 5-HT1A signaling deficits in patients carrying the R219L mutation and may even result in decreased response or no response to drugs acting at this site.

Future Directions

These mutational studies have provided us with a strong basis to understand 5-HT1A receptor coupling to individual G-protein subunits at a structural level. We began examining the structure-activity relations of the i2 domain with the ultimate goal of developing receptor- and effector-selective inhibitors of 5-HT1A signaling. With the

information derived from our studies we would like to design Ci2 peptides that can selectively block separate Gα and Gβγ mediated signals. Blocking the receptor signal at the level of the G-protein affords a more specific effect than that achieved by blocking all of the receptor's functions as is the case with antagonist treatment. For example, a Ci2 peptide that could specifically block Gβγ inhibition of K^+channels or Ca^{2+}channels, but keep Gαi inhibition of AC intact may result in a molecule with more potent and efficacious antidepressant activity.

These studies, although extensive, do leave other avenues to be explored. For example, to demonstrate whether the 5-HT1A-i2 domain and in particular the Ci2 region, directly interacts with specific G protein subunits, yeast two-hybrid mating assays could be performed with regions of the i2 loop (e.g. Ci2 sequence) and specific Gα, Gβ, and Gγ subunits. Similarly, to verify receptor/G-protein interaction, co-immunoprecipitation studies may be performed with tagged 5-HT1A wild-type and mutant receptors and G protein subunits.

The Ci2 mutants could be further characterized with respect to their coupling to other 5-HT1A Gβγ-mediated responses such as coupling to ion channels, GIRKs, N-type calcium channels, and inhibition of MAPK.

Other experiments could further investigate the predicted intramolecular interactions between the Ci2 domain, Ni2 DRY motif and the Ci3 E340 residue. We hypothesized that residues Y144 and K147 comprising the hydrophobic face of the amphipathic α-helix are oriented into a G-protein coupling pocket where they may form hydrogen and ionic bonds with D133/R134 and E340. Indeed, if this is the case, then a double mutant comprising mutations at potential intramolecular interaction sites (e.g.

K147E and E340K), should retain receptor function and would lend validity to our hypothesis.

Significance

Our results strongly suggest that 5-HT1A-mediated signaling can be separated into distinct Gαi-, Gαo- and Gβγ-mediated pathways. Based on previous findings we focused our attention on the contribution of the 5-HT1A i2 domain to Gβγ-mediated signaling. We have generated further evidence for coupling of the i2 domain to multiple Gβγ pathways including PLCβ, ACII activation, inhibition of N-type Ca^{2+} channels and a novel MAPK inhibition pathway that is specific to raphe cells. We have identified for the first time key amino acids in Ci2 for coupling to Gβγ and stabilizing coupling to Gα. Finally, we have identified key residues (Y144, N146, T149) that direct the specificity of Gβγ signaling. This information provides valuable insight into GPCR/G-protein coupling and may lead to the development of Ci2 peptides that specifically block Gβγ signaling to be used as potential antidepressant agents.

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Appendix 1. Supplemental Data to Chapter III

Supplementary Table 1. Oligonucleotides used to generate 5-HT1A-i2 mutant receptors. The nucleotides sequences for sense and antisense oligonucleotides used to incorporate random mutations at the indicated amino acid of the 5-HT1A receptor are shown.

Residue	Sense Oligo	Anti-sense Oligo
D143	5'-GGGCTATCACCGACCCTATA**NNC**TATGTGAACA AGAGGACGCCCC-3'	5'-GGGGCGTCCTCTTGTTCACATA**GNN**TATAGGGT CGGTGATAGCCC-3'
Y144	5'-CTATCACCGACCCTATAGAC**NNT**GTGAACAAAG GACGCCCCGG-3'	5'-CCGGGGCGTCCTCTTGTTCAC**ANN**GTCTATAGG GTCGGTGATAG-3'
V145	5'-CCGACCCTATAGACTAT**NNG**AACAAGAGGACG CCCCGGCG-3'	5'-CGCCGGGGCGTCCTCTTGTT**CNN**ATAGTCTATA GGGTCGG-3'
N146	5'-CCGACCCTATAGACTATGTG**NNC**AAGAGGACGC CCCGGCG-3'	5'-CGCCGGGGCGTCCTCTT**GNN**CACATAGTCTATA GGGTCGG-3'
K147	5'-CTATAGACTATGTGAAC**NNG**AGGACGCCCCGGC GCGCCGC-3'	5'-GCGGCGCGCCGGGGCGTCCT**CNN**GTTCACATAG TCTATAG-3'
R148	5'-CTATGTGAACAAG**NNG**ACGCCCCGGCGCGCCG CTGC-3'	5'-GCAGCGGCGCGCCGGGGCGT**CNN**CTTGTTCACA TAG-3'
T149	5'-GACTATGTGAACAAGAGG**NNG**CCCCGGCGCGC CGCTGC-3'	5'-GCAGCGGCGCGCCGGGG**CNN**CCTCTTGTTCACA TAGTC-3'
P150	5'-GTGAACAAGAGGACG**NNC**CGGCGCGCCGCTG-3'	5'-CAGCGGCGCGCCG**GNN**CGTCCTCTTGTTCAC-3'
R151	5'-CAAGAGGACGCCC**NNG**CGCGCCGCTGCGCTGAT CTC-3'	5'-GAGATCAGCGCAGCGGCGCG**CNN**GGGCGTCCT CTTG-3'
R152	5'-CAAGAGGACGCCCCGG**NNC**GCCGCTGCGCTGAT CTC-3'	5'-GAGATCAGCGCAGCGGC**GNN**CCGGGGCGTCCT CTTG-3'

Supplementary Table 2. Predicted secondary structures of the i2 domains of wild-type and mutant 5-HT1A receptors. The sequence of the i2 loop/TM4 junction, ITDPI**DYVNKRTPRR**AAALI, was examined in the wild-type and mutant 5-HT1A receptors using the Protean program (DNASTAR, Madison, WI) for secondary structure using Garnier-Robson analysis. Alpha-helical structure is identified in *red*, beta-sheet in *green*, turns in *blue*, and coils in *yellow*. Amino acid residues are identified by single letter code. Alongside the predicted structural profiles are responses of mutant 5-HT1A receptors in the Gαi and Gβγ functional assays. *Column 1*) phospholipase C: DPAT-induced increase in $[Ca^{2+}]i$ (Gβγ-mediated), (+++) indicates calcium mobilization ≥ 80% of wild-type control, (++) ≥ 50%, and (+) between 19-27% increase in $[Ca^{2+}]i$. *Column 2*) adenylyl cyclase II: constitutive activity of the receptor as measured by level of coupling to βγ-mediated stimulation of adenylyl cyclase II. The constitutive activities are indicated as in Table I. *Column 3*) cAMP: % inhibition of DA-stimulated cAMP accumulation (Gαi), (+) indicates cAMP inhibition ≥ 26% of control, (+/-) between 20-26% inhibition, and (-) indicates ≤ 20% inhibition.

SUBSTITUTION	PLC	ACII	cAMP	PREDICTED STRUCTURE ---DYVNKRTPRR---
WT (1A)	+++	+++	+	
T149A (i2)	+	+	+	
D143F	-	-	+	
D143V	+	-	+	
D143N	+	-	+	
D143L	+	-	+	
D143C	-	-	+	
Y144H	-	-	+/-	
Y144A	-	+++	-	
Y144N	+	-	+	
Y144F	-	++	+	
Y144V	-	-	-	
Y144I	-	-	+/-	
Y144C	-	-	-	
V145L	+	+	+	
V145K	+	+	+	
V145Y	-	-	-	
V145R	-	-	-	
V145Q	-	-	+/-	
V145E	-	-	+	
V145S	-	-	-	
V145W	-	-	-	
N146T	++	++	+	
N146F	-	-	+	
N146A	+	-	+	
K147R	-	-	-	
K147Q	-	-	-	
R148L	-	-	-	
R148Q	-	-	+/-	
R148K	-	-	-	
R148E	+	-	+/-	

R148P	-	+	+/-	
R148G	-	+	+	
R148V	+	-	+	
T149E	+	+	+	
T149V	-	-	+	
T149G	+++	++	+	
T149R	++	+++	+	
T149Q	+	+	+	
T149M	-	-	+	
T149W	-	-	+	
T149P	-	++	+	
P150R	-	-	-	
P150N	-	-	-	
P150D	-	-	-	
P150G	-	-	-	
P150I	-	-	-	
P150L	-	-	-	
P150F	-	-	-	
P150S	-	-	-	
P150T	-	-	-	
R151Q	-	-	+/-	
R151A	+	+	+/-	
R151M	-	-	+/-	
R151T	+	+	+	
R151L	-	-	+	
R151K	-	-	+/-	
R152N	-	-	-	
R152V	-	-	+	
R152A	-	-	+	
R152P	+	-	-	
R152D	+	-	-	

Appendix 2: Preliminary data for 5-HT1A receptor E340 mutant

Summary of binding, Gαi- and Gβγ-coupling properties of E340 mutants.

Agonist-induced changes in intracellular free calcium concentration in Ltk- cells transiently transfected with wild-type 5-HT1A (1A) or 5-HT1A-i2 mutant receptors were measured and quantified as percent control over basal $[Ca^{2+}]_i$ levels (*Column 1*), *NR: no response to treatments. In parallel experiments, membranes were prepared from Ltk- cells transiently transfected with the indicated 5-HT1A mutant receptors and subjected to binding analysis with [^{3}H]-OH-DPAT (*Column 2*). Agonist independent Gβγ-mediated activation of ACII was determined using HEK 293 cells transiently transfected with expression plasmids for ACII, Gαi2, and the indicated wild-type (1A) or i2 mutant 5-HT1A receptor. Agonist-independent cAMP production was determined using the average difference between PTX-treated and non-treated cells for 3-6 independent experiments. Coupling of the wild-type 5-HT1A receptor (positive control) was arbitrarily set at +++ (high constitutive activity) and the i2 (T149A) mutant (negative control) at + (low constitutive activity); (++), moderate activity, and (-), no detectable activity (*Column 3*). For Gαi-mediated inhibition of AC, HEK cells were transiently co-transfected with 12 μg of D1 receptor cDNA and an equal amount of wild-type (1A) or 5-HT1A-i2 mutant receptors. Inhibition of D1/Gαs-stimulated (10 μM Apomorphine) cAMP accumulation by 5-HT1A agonist 8-OH-DPAT (1 μM) was measured by specific RIA and data were presented as percent inhibition of D1-stimulated cAMP levels (*Column 4*). In all cases, data are expressed as mean ± SEM of three independent experiments, and values that are significantly different from zero were identified using Student's paired t-test and are indicated, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

SUBSTITUTION	DPAT-INDUCED ↑$[Ca^{2+}]i$ (% over basal $[Ca^{2+}]i$)	RECEPTOR NUMBER (pmol/transfection)	CONSTITUTIVE ACTIVITY (Level of coupling to βγ-mediated stimulation of ACII)	% INHIBITION OF DA-STIMULATED cAMP ACCUMULATION
WT 1A	$101 \pm 6^{**}$	$2.1 \pm 0.6^{*}$	+++	$52 \pm 4^{**}$
E340D	$100 \pm 12^{**}$	$1.9 \pm 0.7^{*}$	+++	$61 \pm 1^{***}$
E340G	$104 \pm 23^{*}$	$1.6 \pm 0.2^{**}$	+++	$-2 \pm 6^{**}$
E340K	$20 \pm 1^{***}$	$2.0 \pm 0.8^{*}$	++	$-36 \pm 11^{**}$

Appendix 3: Permission to Reprint Published Material

Appendix 4. Additional Co-Author Publications

O'Hare, M.J., Kushwaha, N., Zhang, Y., Aleyasin, H., Slack, R.S., Callaghan, S.M., Albert, P.R., Vincent, I., Park, D.S. (2005) Differential Roles of Nuclear and Cytoplasmic Cdk5 in Apoptotic and Excitotoxic Neuronal Death. J. Neurosci., 25(39):8954-8966.

Fortin, A., MacLaurin, J.G., Arbour, N., Cregan, S.P., Kushwaha, N., Callaghan, S.M., Park, D.S., Albert, P.R., Slack, R.S. (2004) The proapoptotic gene SIVA is a direct transcriptional target for the tumor suppressors p53 and E2F1. J. Biol. Chem., 279(27):28706-28714.

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Wu, X., Kushwaha, N., Albert, P. R., and Penington, N. J. (2002) A critical protein kinase C phosphorylation site on the 5-HT1A receptor controlling coupling to N-type calcium channels. J. Physiol. (Lond.), 538 (Pt 1): 41-51.

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