uOttawa

L'Université canadienne
Canada's university

FACULTÉ DES ÉTUDES SUPÉRIEURES ET POSTDOCTORALES

uOttawa

L'Université canadienne
Canada's university

FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

Jonathan Paul Urben
AUTEUR DE LA THÈSE / AUTHOR OF THESIS

M.Sc. (Cellular and Molecular Medicine)
GRADE / DEGREE

Department of Cellular and Molecular Medicine
FACULTÉ, ÉCOLE, DÉPARTEMENT / FACULTY, SCHOOL, DEPARTMENT

Interaction of Protein Phosphatase 2A with the Serotonin-1A Receptor

TITRE DE LA THÈSE / TITLE OF THESIS

P. Albert
DIRECTEUR (DIRECTRICE) DE LA THÈSE / THESIS SUPERVISOR

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

EXAMINATEURS (EXAMINATRICES) DE LA THÈSE / THESIS EXAMINERS

C. Kennedy

M. Tiberi

Gary W. Slater
Le Doyen de la Faculté des études supérieures et postdoctorales / Dean of the Faculty of Graduate and Postdoctoral Studies

INTERACTION OF PROTEIN PHOSPHATASE 2A WITH THE SEROTONIN-1A RECEPTOR

JONATHAN P. URBEN

Department of Cellular and Molecular Medicine
Faculty of Medicine
University of Ottawa
Ottawa, Ontario
Canada

June 2009

This thesis is submitted in partial fulfillment of the requirements for the degree of Master of Science (M.Sc.) to the Department of Cellular and Molecular Medicine (Neuroscience Program)

Your file Votre référence
ISBN: 978-0-494-61345-0
Our file Notre référence
ISBN: 978-0-494-61345-0

ABSTRACT

Serotonin-specific reuptake inhibitor (SSRI) treatment of depressed patients displays a three week latency in amelioration of symptoms. Delayed desensitization of 5-hydroxytryptamine 1A (5-HT1A) autoreceptors is believed to be in part responsible for this latency and may involve receptor dephosphorylation. We hypothesized the 5-HT1A secondary intracellular (i2) loop interacts with receptor regulatory molecules. Yeast two-hybrid screen of a human cDNA library using 5-HT1A-i2 peptide as bait identified a novel 5-HT1A-i2 effector protein phosphatase 2A catalytic subunit (PP2AC). The 5-HT1A-i2-PP2AC interaction was quantified using yeast mating/β-galactosidase assay. Furthermore, co-immunoprecipitation of tagged recombinant 5-HT1A receptor and endogenous PP2AC verified the interaction. Treatment with 5-HT1A receptor agonist enhanced the interaction. Mutation of critical receptor residues reduced or eliminated binding of PP2AC. Our results indicate a novel interaction between the 5-HT1A receptor and PP2AC and future studies will provide new insight into the role of protein phosphatase-receptor interactions in regulating 5-HT1A receptor desensitization, and resensitization.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATONS

5-CT	5-carboxytryptamine
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	5-hydroxytryptophan transporter, serotonin transporter
8-OH-DPAT	8-hydroxy-2-(di-n-propylamino)tetralin
AC	adenylyl cyclase
Ala	alanine
ALDH-2	aldehyde dehydrogenase
AP-2	β-2 adaptin
Asn	asparagine
Asp	aspartate
β_2-AR	β_2-adrenergic receptor
βarr	β-arrestin
C5a	complement component 5a receptor
Ca^{2+}	calcium
CAMKII	Ca^{2+}/calmodulin-dependent protein kinase II
CAMKIV	Ca^{2+}/calmodulin-dependent protein kinase IV
CB2	cannaboid 2 receptor
cAMP	cyclic adenosine monophosphate
CCK	cholecystokinin receptor
cDNA	complimentary DNA
CHO	Chinese hamster ovarian cells
Ci2	carboxy-terminal of the second intracellular loop
Ci3	carboxy-terminal of the third intracellular loop
CNS	central nervous system
CO2	carbon dioxide
CSF	cerebrospinal fluid
CXCR2	CXC chemokine receptor 2
Cys	cysteine
DAG	diacylglycerol
DBD	DNA binding domain
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
DRN	dorsal raphe nucleus
DSP	dithiobis[succinimidylpropionate]
DTBP	dimethyl 3,3′-dithiobispropionimidate
DTT	dithiothreitol
e1	first extracellular
e2	second extracellular
e3	third extracellular
ECL	enhanced chemiluminescence

EDTA	ethylenediaminetetraacetic acid
ERK1/2	extracellular signal-regulated kinases 1 and 2
FBS	fetal bovine serum
GDP	guanosine diphosphate
GFP	green fluorescence protein
GI	gastrointestinal tract
GIRK	G protein-coupled inwardly rectifying potassium channel
GluR	metabotropic glutamate receptor
GPCR	G protein-coupled receptor
GRK	G protein-coupled receptor kinase
GRP	G protein-coupled phosphatase
GTP	guanosine triphosphate
H-bond	hydrogen bond
HBS	HEPES-buffered saline
HEK293	human embryonic kidney 293 cells
HEPES	N-2-hydroxyethylpiperazine-N9-2-ethanesulfonic acid
HL60	human promyelocytic leukemia cells
HRP	horseradish peroxidase
i1	first intracellular
i2	second intracellular
i3	third intracellular
IL-8	interleukin-8
IP_3	inositol triphosphate
kb	kilobases
KCl	potassium chloride
kDa	kilodalton
MAO-A	monoamine oxidase A
MAOI	monoamine oxidase inhibitors
MDD	major depressive disorder
mRNA	messenger RNA
NMDA	N-methyl-D-aspartic acid
Ni2	amino-terminal of the second intracellular loop
Ni3	amino-terminal of the third intracellular loop
NP-40	nonidet P-40
OA	okadaic acid
OPGN	O-nitrophenyl-β-D-galactosidase
PP2A	protein phosphatase 2A
PP2AC	protein phosphatase 2A catalytic subunit
PP2B	protein phosphatase 2B
PP2C	protein phosphatase 2C
PBS	phosphate buffered saline
PIP_2	phosphatidylinositol-4,5-biphosphate
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PM	plasma membrane

PNS	peripheral nervous system
PP1	protein phosphatase 1
PTX	pertussis toxin
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
Ser	serine
SERT	serotonin transporter, 5-HTT
SSRI	selective-serotonin reuptake inhibitor
TCA	tricyclic antidepressant
TBS	Tris-buffered saline
Thr	threonine
TM	transmembrane
TPA	12α-O-tetradecanyoyl phorbol 13β-acetate
TPH	tryptophan hydroxylase
TR	thromboxane receptor
VMAT	vehicular monoamine transporter

ACKNOWLEDGMENTS

Firstly, I would like to thank Dr. Paul Albert for providing me the guidance and opportunity to perform my research and write this thesis. Dr. Albert is an exceptional scientist and displays great enthusiasm and dedication to his work; he strives to inspire his students and provides encouragement along the way. Throughout my thesis-writing, he provided motivation, sound advice and focus. I would like to thank the members of my advisory committee: Dr. Mario Tiberi and Dr. David Park for imparting valuable instruction, encouraging words and for sacrificing their time to support me throughout my studies. To my friends and fellow colleagues, both past and present, in Dr. Albert's laboratory, thank you for providing a stimulating and enriching environment in which to learn and grow: notably, Dr. Kirsten Jacobsen, Dr. Houman Nafisi, Kimberley Galaraga, Margaret Csezak and Anne Millar. I am especially grateful to Mireille Daigle for her excellent technical assistance and advice; her contributions to the lab are enormous. On a personal note, I want to thank Dr. Brice Le François for becoming one of my closest friends – a constant pillar of support throughout my studies – and for introducing me to the exciting world of soccer.

I want to thank my parents, who have provided me with many wonderful opportunities and have inspired me to achieve my goals. Finally, I would like to recognize my wife, Michelle, who has been a constant source of support and who has made possible the opportunity to study and write this thesis. Together we have shared challenges and met them head on with resilience. Thank you for your love. To you, I dedicate this thesis.

INTRODUCTION

Serotonin System

Serotonin: Discovery, Synthesis and Metabolism

While testing the effects of amine substances on smooth muscle contraction during the 1930s, Vittorio Erspamer isolated a chemical he named enteramine from rabbit gastric mucosa. Erspamer believed the substance originated from enterochromaffin cells of the gastrointestinal (GI) tract (Erspamer and Vialli, 1937). Serotonin was 'discovered' in the next decade as Irvine Page, Maurice Rapport and Arda Green isolated from bovine blood serum, a potent vasoconstrictor substance they coined serotonin (Rapport et al., 1948). One year later, in 1949, Rapport elucidated the structure of serotonin as 5-hydroxytryptamine (5-HT) (Rapport, 1949), and three years later Erspamer and Biagio Asero determined the structure of enteramine was also that of 5-HT (Erspamer and Asero, 1952). 5-HT was found to be expressed in the mammalian brain (Twarog and Page, 1953) leading to the notion that it functions as a neurotransmitter in the central nervous system (CNS). Woolley and Shaw first suggested in 1954 that 5-HT plays a role in mental illness (Woolley and Shaw, 1954) and over the next two decades drugs targeting the serotonergic system were developed and were effective in the treatment of depression. These drugs included monoamine oxidase inhibitors (MAOI) and tricyclic antidepressants (TCA), and functioned to enhance serotonergic neurotransmission. Modern advancements lead to the development of selective serotonin reuptake inhibitors (SSRI) such as fluoxetine (Prozac), sertraline (Zoloft) and paroxetine (Paxil) and are now commonly prescribed worldwide for the treatment of depression.

Serotonin is found in three distinct areas in the adult human with the enterochromaffin cells of the GI tract being the most prominent area (90%). Platelets (8-9%) and the CNS (1-2%) account for the remainder of total serotonin in the human body (Lambert et al., 1995). Ironically, despite such a small fraction of serotonin present in the CNS, it affects a multitude of functions including appetite, sleep, pain response, cognitive awareness, temperature regulation, mood and behavior and endocrine functions (Buhot, 1997). Serotonin cannot cross the blood brain barrier and is therefore synthesized within the brain from the essential amino acid L-tryptophan. L-tryptophan cannot be synthesized *de novo* and therefore dietary uptake of L-tryptophan is necessary. L-tryptophan undergoes hydroxylation through the rate limiting enzyme tryptophan hydroxylase (TPH) to form 5-hydroxytryptophan (5-HTP). Two distinct isoforms of TPH have been identified; TPH2 is exclusive to the brain and is the major isomer located there, while TPH1 is expressed in the brain (as the minor isomer) and peripheral tissues (Walther et al., 2003). 5-HTP undergoes further decarboxylation through 5-hydroxytryptophan decarboxylase to form 5-HT. Vehicular monoamine transporter (VMAT) uptakes synthesized 5-HT into secretory vesicles which are stored for future exocytotic release. Non-vesicular sequestered 5-HT is inactivated by two mitochondrial enzymes monoamine oxidase A (MAO-A) and aldehyde dehydrogenase (ALDH-2) and converted to 5-hydroxyindole acetic acid (5-HIAA). 5-HIAA is excreted into the cerebrospinal fluid (CSF) and eventually leaves the body in the urine. Hence, levels of 5-HIAA in urine are a measure of 5-HT synthesis (Lambert et al., 1995).

Central Serotonergic System

The serotonergic system consists of an assorted group of neurons, and the brain stem raphe nuclei have been shown to contain the greatest number of these neurons (Tork, 1990; Jacobs and Azmitia, 1992). Rostral raphe nuclei contain the greatest abundance (85%) of all serotonergic neurons in the brain and the caudal raphe nuclei contain the remaining (15%) 5-HT neurons (Hornung, 2003). Serotonergic neurons emanating from the rostral raphe nuclei project and innervate the forebrain limbic system structures including the amygdala, thalamus, hypothalamus, basal ganglia, hippocampus and cingulate gyrus (Tork, 1990; Jacobs and Azmitia, 1992). The cortico-limbic system is widely innervated by 5-HT neurons originating from the raphe nuclei to regulate mood, neuroendocrine stress and sleep (Hoyer et al., 1994; Jacobs and Azmitia, 1992). The caudal raphe nuclei neurons have descending projections to the spinal cord and cerebellum.

Serotonin Receptors

5-HT receptors are conserved membrane-bound receptors and are located in the CNS, peripheral nervous system (PNS) and even non-neuronal tissues (GI, cardiovascular tissue and circulatory system). All 5-HT receptors, except for one subclass, couple to heterotrimeric G-proteins to carry out the functions of the serotonergic system. These receptors consist of seven transmembrane (TM) domains, with the exception of the ligand gated ion channel 5-HT3 receptor. Extensive studies involving pharmacological data, radioligand binding, signal pathways and structural analysis have identified seven classes of 5-HT receptors: 5-HT1 through 5-HT7. These receptors are sub classified into eighteen distinct subtypes: 5-HT1A/B/D/E/F, 5-HT2A/B/C, 5-HT3A/B/C/D/E, 5-HT4,

5-HT5A/B, 5-HT6 and 5-HT7 (Hoyer et al., 1994; Hoyer and Martin, 1997; Hoyer et al., 2002; Niesler et al., 2007).

Serotonin 1A Receptor

The 5-HT1A receptor is the most characterized receptor of these subtypes. The human gene encoding the 5-HT1A receptor is located on chromosome 5q11.2-q13 and was unexpectedly isolated using specific β_2-adrenergic (β_2-AR) receptor probes to screen a human genomic library (Kobilka et al., 1987). The orphan receptor was identified as the 5-HT1A receptor one year later (Fargin et al., 1988). The receptor, initially cloned as a 421 amino acid protein due to a sequencing error, was subsequently shown to consist of a 422 amino acid polypeptide with a molecular weight of 46 kilodaltons (kDa) (Albert et al., 1990; Raymond et al., 1999). Further characterization revealed that the receptor contains an extracellular amino terminus and cytoplasmic carboxy terminal domain as well as seven hydrophobic TM domains (TM1-TM7), each consisting of 20-24 amino acids. These hydrophobic TM domains are predicted to form membrane-spanning α helices and are connected via three extracellular and three intracellular loops. The receptor is constitutively palmitoylated at specific C-terminal cysteine residues; Cys417 and Cys420 (Papoucheva et al., 2004).

The intracellular loops are critical to coupling of the receptor to the heterotrimeric G-proteins. Four protein kinase C (PKC) sites implicated in receptor desensitization are found in the intracellular loops: Thr149 (i2 loop) and Thr229, Ser253, Thr343 (i3 loop) (Lembo and Albert, 1995; Lembo et al., 1997; Wu et al., 2002). The two i3 sites (Thr229 and Thr343) are putative protein kinase A (PKA) sites (Liu and Albert, 1991; Raymond and Olsen, 1994). Three consensus sites for N-linked glycosylation are located near the

amino terminus of the receptor and formation of glycosylated receptors can account for the heavier forms (55-60 kDa) of the receptor detected by immunoblotting. Serotonergic raphe neurons express presynaptic somatic and dendritic 5-HT1A receptors, which function as classical autoreceptors (Sotelo et al., 1990; Riad et al., 2000). These autoreceptors modulate neurotransmitter synthesis, terminal release and alter the rate of neuronal firing, which lead to controlled regulation of the release of 5-HT onto postsynaptic sites (Albert et al., 1996; Evrard et al., 1999). The 5-HT1A receptor is also expressed as a postsynaptic receptor in the hippocampus, raphe nuclei, cortex, hypothalamus and amygdala (Chalmers and Watson, 1991; Pompeiano et al., 1992).

5-HT action throughout the cortico-limbic system is mediated by post-synaptic 5-HT1A receptors (Tork, 1990), while presynaptic 5-HT1A autoreceptors mediate negative feedback of raphe firing (Bortolozzi et al., 2004; Evrard et al., 1999). The 5-HT1A receptor is a member of the G protein-coupled receptor (GPCR) superfamily that mediates the action of neurotransmitters, hormones and odorants and is the most frequent target of current drug therapies (Bockaert et al., 2002). The rhodopsin-11-cis retinal complex is the first solved GPCR crystal structure (Palczewski et al., 2000). The 5-HT1A receptor is also a prototypic GPCR consisting of seven predicted TM domains, three intracellular domains (i1, i2, and i3) and three extracellular domains (e1, e2, e3), as well as an intracellular C-terminal tail (Peroutka and Howell, 1994) (Figure 1). Relative to the TM and extracellular domains little is known about the G-protein-coupled-intracellular domains (Bourne, 1997; Meng and Bourne, 2001; Menon et al., 2001; Ostrowski et al., 1992). Deducing the roles of the intracellular domains involved in receptor-G protein coupling and desensitization could lead to development of ligands to

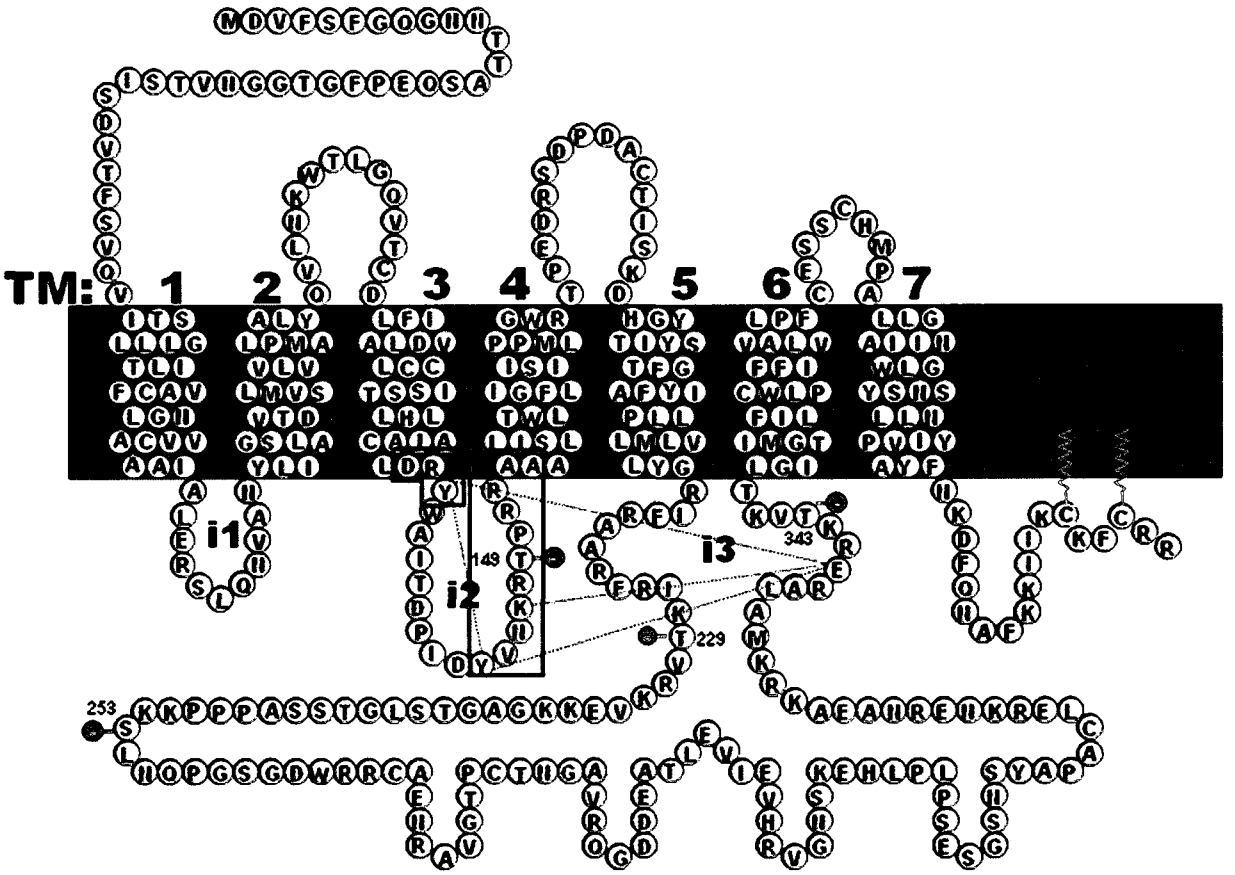


Figure 1: Model of the 5-HT1A receptor.

The amino acid sequence of the 5-HT1A receptor is shown with the predicted transmembrane domains (TM) and intracellular loops (i1, i2, i3) as indicated. Shaded residues are identical to those of the β2-adrenergic receptor, and the conserved DRY motif (Ni2) and the alpha-helical region mutated in this proposal (Ci2) are boxed. Potential ionic/hydrogen bonds (dashed lines) and palmitoylation sites (squiggle) are indicated. PKC sites discussed are numbered.

selectively block specific receptor pathways. Mutagenesis studies reveal that receptor-G-protein coupling involves the TM-proximal segments of the i2 and i3 loops as well as the C-terminal tail (Kushwaha and Albert, 2005; Lembo and Albert, 1995; Lembo et al., 1997). Random and site directed mutagenesis studies provide evidence in support of an amphipathic α-helical structure for the amino-terminal of the third intracellular loop (Ni3) and the carboxy-terminal of the third intracellular loop (Ci3) (Albert et al., 1998). These amphipathic helical structures and a specific threonine (T149) residue are conserved in a wide range of GPCR (Burstein et al., 1996; Burstein et al., 1998; Hill-Eubanks et al., 1996; Liu et al., 1996; Liu et al., 1995; Wess, 1997).

5-HT1A i2 and i3 loops

Previous studies identified phosphorylation of three i3 loop PKC sites and PKC site Thr149 of the i2 loop as a site of PKC-induced desensitization (Raymond, 1991). 12α-O-tetradecanyoyl phorbol 13β-acetate (TPA) induced activation of PKC phosphorylates the 5-HT1A receptor and uncouples Gβγ but not Gα signaling (Lembo and Albert, 1995; Liu and Albert, 1991; Raymond, 1991). The potency of TPA- and 5-HT-induced desensitization was reduced by mutation of Thr149 or mutation of three of the i3 PKC sites. These findings suggest a role for PKC in both TPA and agonist-induced desensitization (Lembo and Albert, 1995; Wu et al., 2002). 5-HT1A signaling to the same PKC-sensitive Gβγ pathways was uncoupled by mutation of Thr149 (Lembo et al., 1997; Wu et al., 2002; Wurch et al., 2003). Because of its importance in signaling and desensitization, yeast-two hybrid assay was performed using a long-i2 peptide to identify 5-HT1A-G-protein interactions; however, we initially believed we had identified a novel interaction of Ser/Thr protein phosphatase PP2A with

the 5-HT1A-i2 domain. Upon further examination an error in subcloning of the long-i2 peptide into the yeast vector pAS2-1 was found and I will address the significance of this in the results section.

Mental Illness and the Serotonin System

Major Depressive Disorder

Major depressive disorder (MDD) is becoming an area of increasing concern, as it affects between 15-20% of the general population (Holden, 2000). Relative to men, women are twice as likely to develop depression (Doris et al., 1999). Patients suffering from depression usually exhibit anxiety; comorbidity with anxiety occurs in 25-50% of depressed individuals (Kessler et al., 2002). Common symptoms of depression include abnormalities affecting mood, anhedonia, insomnia, changes in appetite, feelings of worthlessness, psychomotor activity such as agitation and suicidal thoughts (Fava and Kendler, 2000). Major depressive disorder symptoms are those that persist longer than two weeks and result in severe changes in the day to day function of an individual. According to the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) the criteria for major depressive disorder are numerous; however, the range of signs and symptoms among individuals varies (Fava and Kendler, 2000).

Serotonin System and Major Depressive Disorder

The indoleamine hypothesis of depression was proposed in 1969 and stated that depression or mania was related to reduced serotonergic activity. This reduction in activity was believed to be the result of decreased serotonin release or less available serotonin receptors; both are critical to serotonin signal transduction (Mann, 1999). As a

result, the serotonin system became the subject of numerous studies. Research suggests that hyperactivity of the 5-HT system is involved in anxiety disorders (Gross and Hen, 2004) and that decreased activity of the 5-HT system has the potential to lead to depression (Blier and de Montigny, 1999; Lesch and Heils, 2000). Studies measuring 5-HT metabolite 5-HIAA in cerebrospinal fluid (CSF) established a correlation between severity of depression and decreased levels of 5-HIAA. These findings suggest that a decrease in metabolite levels are directly related to reduced availability of 5-HT, which in turn leads to symptoms of depression (Risch and Nemeroff, 1992). Individuals treated with reserpine or parachlorophenylalanine, inhibitors of VMAT and TPH respectively, showed development of symptoms of depression. These findings support the indoleamine hypothesis as blockage of VMAT results in depletion of monoamine stores and inhibition of TPH function selectively depletes 5-HT levels (Nemeroff, 2002). Furthermore, patients in remission from depression where shown to suffer transient relapse when subjected to a dietary acute tryptophan depletion (Booij et al., 2005; Leyton et al., 2000) while normal healthy patients developed acute melancholia (Ellenbogen et al., 1996).

Analysis of post mortem brains from depressed suicide victims compared to non-suicide controls had elevated density of 5-HT1A autoreceptors in the dorsal raphe nucleus (DRN) and no changes in levels of postsynaptic receptor sites (Stockmeier et al., 1998). Interestingly, conflicting reports from positron emission topography (PET) studies showed a widespread reduction of postsynaptic 5-HT1A receptors in patients with major depression (Sargent et al., 2000) and bipolar disorders (Drevets et al., 2000). A reduction in the expression of postsynaptic 5-HT1A receptors suggests reduced post

synaptic response to 5-HT, while elevated levels of inhibitory presynaptic receptors would reduce 5-HT neuron firing and further reduce 5-HT release. These findings suggest that a reduction in serotonergic activity through either of these mechanisms may predispose individuals to major depression.

The 5-HT1A Autoreceptors Role in Antidepressant Treatment

Clinical drugs used to treat depression (MAOIs, TCA, SSRI) target the 5-HT system as dysregulation of 5-HT levels and/or 5-HT receptor function are implicated in neuronal psychiatric disorders. The 5-HT1A receptor has been implicated as an antidepressant target as it regulates the serotonin system. As most therapeutic drugs act to boost synaptic 5-HT levels, hypoactivity of the 5-HT system may predispose individuals to episodes of depression (Stahl, 1998). SSRIs in particular act to rapidly block 5-hydroxytryptamine transporter (5-HTT) preventing reuptake of 5-HT into the neuron. The result is increased levels of 5-HT in the synapse leading to increased serotonergic neurotransmission. Antidepressants display a three week latency in improving the clinical symptoms of depression and this is thought to be due to delayed desensitization of 5-HT1A autoreceptors (Albert et al., 1996; Artigas et al., 2001; Blier and de Montigny, 1994; Hjorth et al., 2000; Pineyro and Blier, 1999).

Acute SSRI treatment increases 5-HT levels within the midbrain raphe and post synaptic sites (Adell and Artigas, 1991). Increased dendrodendritic release of 5-HT in raphe nuclei results in activation of the 5-HT1A autoreceptors and inhibition of cell firing, reduction of terminal 5-HT release and attenuates the increase in extracellular release of 5-HT produced from inhibiting the 5-HTT (Artigas et al., 1996). Chronic antidepressant treatment results in desensitization of the raphe 5-HT1A autoreceptors,

thus eliminating the negative feedback inhibition and facilitating 5-HT neurotransmission (Blier and de Montigny, 1994). The desensitization is specific to 5-HT1A autoreceptors as postsynaptic receptors appear resistant to agonist induced desensitization (Blier and de Montigny, 1994; Pineyro and Blier, 1999). 5-HT1A autoreceptors are also targeted by pindolol to reduce antidepressant latency and 5-HT1A partial agonists like buspirone target post-synaptic sites to mediate anti-anxiety actions. The 5-HT1A agonist properties of atypical antipsychotic drugs clozapine and aripiprazole (Newman-Tancredi et al., 2005; Newman-Tancredi et al., 2001) are believed to alleviate the cognitive defects in schizophrenia (Sumiyoshi et al., 2001a; Sumiyoshi et al., 2001b). Thus, the development of new therapeutic remedies targeting the 5-HT1A receptor could likely benefit the future treatment of mental illness.

G protein-coupled Receptors

G protein-coupled Receptor Structure

The GPCR superfamily is a diverse group of proteins and is the largest evolutionary conserved family of proteins. GPCRs function as signal transduction molecules and convert external signals such as light, odour and neurotransmitters into cellular responses. Cellular responses are achieved through coupling to heterotrimeric G-proteins comprised of Gα and Gβγ subunits. As these receptors are involved in a wide spectrum of physiological responses they are the ideal targets for drug development. Twenty years ago information regarding the tertiary structure of GPCRs was derived from low resolution structures of retinal light sensing receptor rhodopsin (Schertler et al., 1993) and high resolution structures of the bacterial light driven proton pump, bacteriorhopdopsin (Henderson and Schertler, 1990). The first reported true tertiary

structural model of a GPCR was obtained when inactive bovine rhodopsin was first crystallized for X-ray crystallography. The resulting three-dimensional structure was at resolution 2.8 Å and confirmed our predicated structural and functional knowledge with respect to GPCRs (Palczewski et al., 2000). The serotonin receptor and other biogenic amine receptors (adrenergic, dopamine, histamine, muscarinic) belong to the Family A class; the largest and most studied family of GPCR (Gether, 2000). Conserved characteristics of Family A receptors include a short 40 amino acid extracellular amino terminus, palmitoylated cysteine residues in the carboxy-terminal (Gether, 2000; Palczewski et al., 2000) and highly conserved key residues: Asn-Pro-XX(X)-Tyr (NPXX(X)Y) motif in TM7 and Glu/Asp-Arg-Tyr (E/DRY) motif located near the boundary of TM3 and the i2 loop (Figure 1).

G-proteins

GPCR activation of effector molecules that carry out cellular responses involves heterotrimeric guanine-nucleotide binding proteins (G-proteins), consisting of α, β and γ subunits (Gilman, 1987). These subunits bind to the intracellular GPCR domains and the α subunit cycles through binding guanosine diphosphate (GDP) and guanosine triphosphate (GTP); this cycle is controlled by the receptor. The α subunit is bound to GDP and is in a complex with the βγ dimer whenever the receptor is inactive with no ligand bound. GPCR activation with ligand results in a conformational change in the α subunit and GDP dissociates and is replaced with GTP. The active α subunit-GTP conformation dissociates from the βγ dimer and goes on to bind and activate effectors. The βγ subunits regulate other functions perhaps more than the α subunit (Clapham and Neer, 1997). The α subunit contains intrinsic GTPase activity that results in hydrolysis of

GTP to GDP, leading to dissociation of the α subunit from its effector and promotes re-association with the βγ subunits.

5-HT1A Receptor Activation and Signal Transduction Pathways

The 5-HT1A receptor regulates effectors such as adenylyl cyclase (AC) and ion channels through coupling to heterotrimeric Gi/Go proteins composed of Gαi/o and Gβγ subunits. Gβγ subunits mediate cell-specific signaling pathways, while activation of Gαi subunits mediate ubiquitous inhibition of AC (Albert, 1994; Raymond et al., 1999). Second messenger PKC has been shown to desensitize 5-HT1A signaling to Gβγ pathways but not Gαi pathways; PKA shows no effect (Liu and Albert, 1991). Pertussis toxin (PTX) inhibits Gi1, Gi2, Gi3 and Go proteins and blocks almost all 5-HT1A-mediated actions (Figure 2). These results reveal that the 5-HT1A receptor is Gi/Go-coupled; however, identification of a remarkable receptor- and effector-specific G-protein specificity for Gi/Go-coupled receptors suggests specific 5-HT1A receptor-G-protein interactions (Albert and Morris, 1994; Albert and Robillard, 2002; Albert and Tiberi, 2001; Liu et al., 1999).

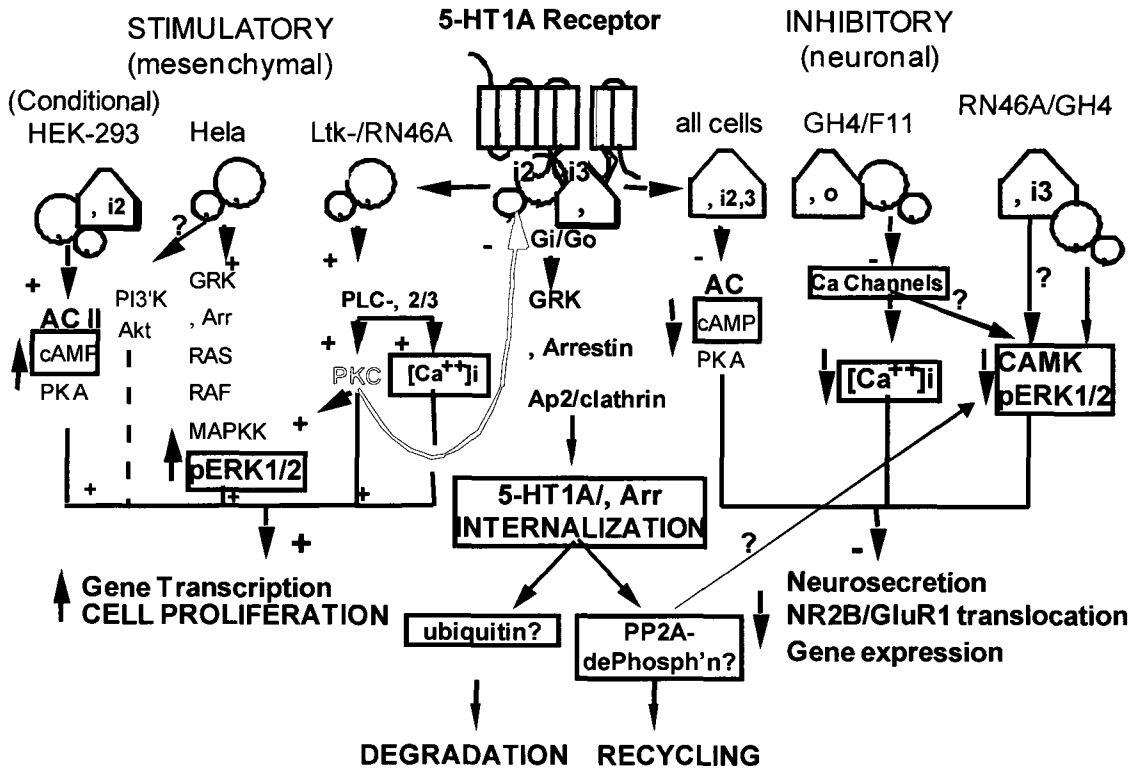


Figure 2: Signaling pathways of the 5-HT1A receptor.

Shown figuratively are stimulatory and inhibitory signaling pathways of the 5-HT1A receptor in both mesenchymal (fibroblast/immune) and neuronal cell types. The specific cell lines in which these signaling pathways are studied are as indicated. The G protein subunits involved are based on studies using the 5-HT1A receptor or closely related receptors. The predicted outcome on neurosecretion has been measured in GH4 cells (Albert, 2002), and on cell proliferation in Balb/c- and NIH-3T3 fibroblasts (Abdel-Baset et al., 1992; Varrault et al., 1994). In hippocampal neurons, 5-HT1A receptors have been shown to reduce translocation of NR2B and GluR1 receptors to synaptic membrane by inhibiting CAMKII/ERK signaling to microtubules (Cai et al., 2002; Schiapparelli et al., 2005; Yuen et al., 2005).

Gαi/o pathways

While other receptors differ in Gαi specificity, the 5-HT1A receptor specifically inhibits forskolin- or Gs-stimulated cyclic adenosine monophosphate (cAMP) formation via Gαi2 (Albert and Robillard, 2002; Albert and Tiberi, 2001; Liu et al., 1999; Liu et al., 1994). L-type calcium channels are inhibited by 5-HT1A receptor activation in GH4 pituitary cells. This is mediated by Gβγ and is dependent on Gαo, which does not couple to inhibition of AC in most cells (Albert, 2002; Banihashemi and Albert, 2002; Liu et al., 1999; Liu et al., 1994).

Cell-specific Gβγ pathways

Calcium channels (Ca^{2+}): Inhibition of N-type calcium channels via a direct interaction with Gβγ subunits have been reported in the literature (Herlitze et al., 1996; Ikeda, 1996; Jarvis and Zamponi, 2001). In vivo inhibition of N- and P/Q-type calcium currents is observed in raphe neurons and is mediated by 5-HT1A (Bayliss et al., 1997; Chen and Penington, 1996; McAllister-Williams and Kelly, 1995; Penington et al., 1991). 5-HT1A-induced inhibition of N-type calcium currents is observed in neuronal F11 cells while in pituitary GH4 cells L-type calcium influx was inhibited via Go. Fibroblast cells (Ltk-, Balb/c-3T3, NIH-3T3) had no detectable calcium currents (Albert, 1994; Liu et al., 1999; Wu et al., 2002). 5-HT1A coupling to L- and N-type channels is inhibited by activation of PKC and mutating Thr149 located in the second intracellular loop (i2) of the 5-HT1A receptor inhibited the action of PKC. This suggests that PKC may phosphorylate Thr149 to uncouple the receptor from Gβγ signaling to N-type channels.

Potassium (K^+) channels: Gβγ subunits have been shown to interact with and activate the G protein-coupled inwardly rectifying potassium channel (GIRK) family of K channels (Clapham and Neer, 1997; Ford et al., 1998; Huang et al., 1995; Yan and Gautam, 1996; Yan and Gautam, 1997). The 5-HT1A receptor increases potassium conductance via GIRK channels (Luscher et al., 1997) in hippocampal CA1, raphe, septal, or hypothalamic neurons and in turn decreases action potential frequency (Andrade et al., 1986; Colino and Halliwell, 1987; Katayama et al., 1997; Newberry, 1992; Penington et al., 1993).

ACII: AC has ten subtypes and Gβγ is shown to interact and activate just five: ACII, ACIV, ACV, ACVI and ACVII (Chen et al., 1995; Gao et al., 2007, Sunahara et al., 1996). Co-transfection of 5-HT1A receptor, Gαi2 and ACII induces a constitutive agonist-independent 5-HT1A-mediated stimulation of cAMP formation in human embryonic kidney 293 (HEK293) cells (Albert et al., 1999). Hippocampal membranes enriched in ACII (Baker et al., 1999) reveal 5-HT1A-induced ACII activation to increase cAMP (Cadogan et al., 1994; De Vivo and Maayani, 1990; Shenker et al., 1987).

Phospholipase C (PLC): PLCβ2 and -β3 are directly activated by Gβγ subunits (Chen et al., 1995; Exton, 1996; Ford et al., 1998; Katz et al., 1992) leading to phosphatidylinositol-4,5-biphosphate (PIP_2) hydrolysis, inositol triphosphate (IP_3) formation and release of calcium from internal stores raising the intracellular calcium concentration. We have shown that 5-HT1A receptors mediate PLC activation in fibroblasts and raphe RN46A cells, suggesting a potential role in raphe neurons; however,

not in pituitary cells (Kushwaha and Albert, 2005; Liu and Albert, 1991), indicating cell-specificity of this pathway.

G protein-coupled Receptor Desensitization

Homologous and Heterologous Desensitization

Desensitization involves the repeated or sustained agonist stimulation of a receptor and results in reduction in receptor responsiveness. Desensitization is a protective cellular mechanism to protect cells from the harmful effects of repeated or persistent agonist stimulation. The process involves three main steps including: uncoupling (seconds to minutes), internalization (minutes to hours) and down regulation or degradation (hours to days). Secondary messengers PKA and PKC, G-protein receptor kinases (GRKs) and arrestins are the regulatory proteins involved in desensitization (Lefkowitz, 1998; Perry and Lefkowitz, 2002).

Heterologous desensitization involves phosphorylation of GPCRs via secondary messenger kinases PKA and/or PKC on specific Thr/Ser residues found on the intracellular loops and/or C-terminal tail (Lefkowitz, 1998). The phosphorylation of the receptor induces conformational changes affecting the receptor/G-protein interactions (Lefkowitz, 1998). Heterologous desensitization is non-specific, as any stimulus can increase cAMP or diacylglycerol (DAG) levels, leading to activation of PKA and PKC respectively.

Homologous desensitization is agonist specific and is mediated by the GRK-arrestin system. Agonist stimulation of the receptor leads to recruitment of GRK to phosphorylate the receptor and promote the binding of an arrestin. Arrestin binding follows phosphorylation of agonist activated receptor by GRK and causes steric

inhibition of the receptor/G-protein interactions (Pierce et al., 2002). Concurrent with the desensitization (uncoupling) mechanism, arrestins are also involved in agonist-stimulated endocytosis of receptors. β-arrestin-1/2 (βarr) targets agonist stimulated receptors to clathrin coated vesicles through interaction with clathrin adaptor protein β2-adaptin (AP-2) and clathrin (Goodman et al., 1996; Laporte et al., 1999). The resulting receptor containing vesicles are pinched off the PM by GTPase dynamin and the internalized receptor can be recycled rapidly, targeted to endosomes for slow recycling or targeted to lysosomes for degradation. Endocytosis has been shown to promote receptor resensitization and signaling (Daaka et al., 1998; Ferguson, 2001).

Desensitization of 5-HT1A Receptor

5-HT1A receptor agonist treatment acts directly on 5-HT1A autoreceptors to induce desensitization and internalization (Riad et al., 2001). Desensitization of these receptors results in disinhibition of the serotonergic neuron, enhancing the action potential firing rate and releasing more 5-HT to augment serotonergic neurotransmission. Autoreceptor desensitization is crucial to the therapeutic action of antidepressant drugs. The clinically observed three week latency during chronic antidepressant treatment is thought to be due to delayed desensitization of autoreceptors (Albert et al., 1996; Artigas et al., 2001; Blier and de Montigny, 1994; Hjorth et al., 2000; Blier and de Montigny, 1999) (Figure 3). Evidence suggests that desensitization of the 5-HT1A receptor is mediated by PKC and PKA; however, recent evidence suggests that GRKs are also involved. The 5-HT1A receptor has four PKC sites, Thr149A (i2 loop) and Thr229, Ser253 and Thr343 (i3 loop) and these are implicated in receptor desensitization (Lembo and Albert, 1995; Lembo et al., 1997; Wu et al., 2002). J. Raymond showed that in cells

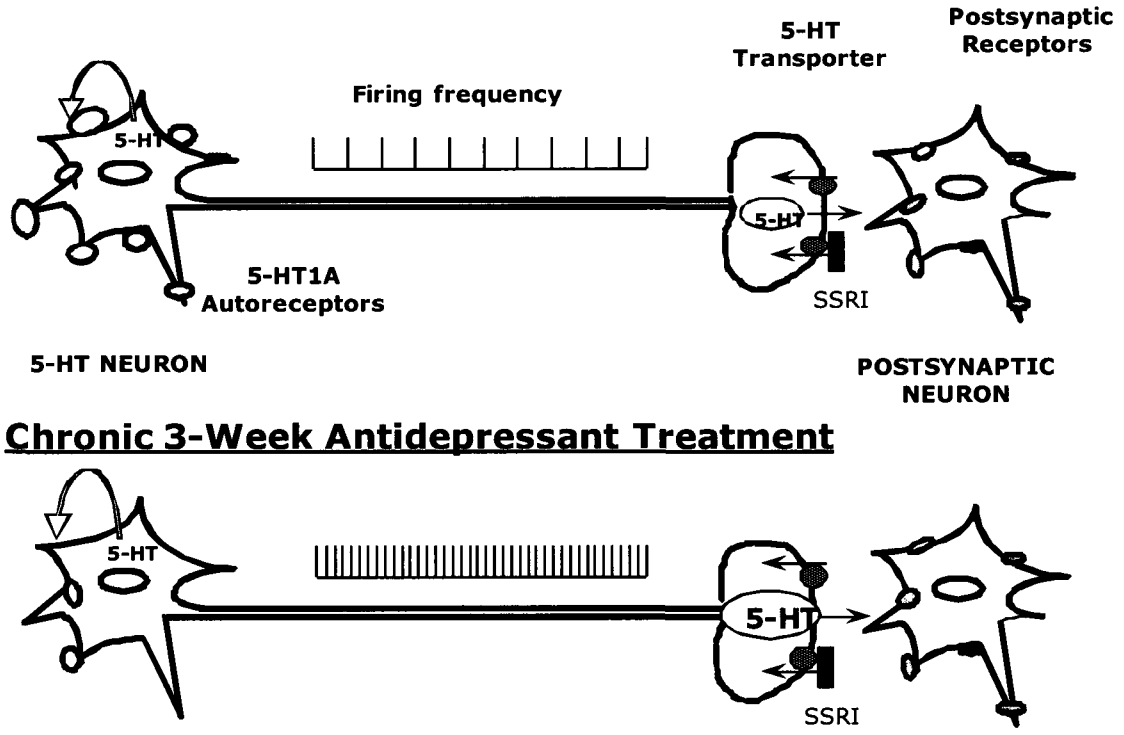


Figure 3: 5-HT1A autoreceptor desensitization upon chronic antidepressant treatment.

Acutely, anti-depressants like selective serotonin reuptake inhibitors (SSRI) reduce serotonergic firing by augmenting 5-HT at cell body, leading to activation of 5-HT1A autoreceptors. After three weeks, a reduction in the number of 5-HT1A autoreceptors via homologous desensitization is observed: this disinhibits the serotonergic neuron, enhancing action potential firing rate, and releasing more 5-HT, correlating with clinical amelioration of depression. Reprinted by permission from Macmillan Publishers Ltd: [Nature], Neuropsychopharmacology, (Albert et al., 1996), copyright 1996.

expressing 5-HT1A receptor, phorbol ester PKC stimulation results in: (1) rapid phosphorylation on at least two of the four PKC sites and (2) desensitizes several 5-HT1A receptor mediated signaling pathways (Raymond, 1991). Pretreatment of 5-HT1A receptors expressed in HeLa cells with phorbol esters undergo rapid uncoupling from G-proteins and the uncoupling is reversed when treated with PKC inhibitors (Harrington et al., 1994). The Thr149 site is implicated in coupling to N-type calcium channels and mutation of T149 to alanine (Ala) confers resistance to the receptor to low concentrations of TPA. These results suggest that this residue is important for receptor uncoupling where PKC is weakly activated (Wu et al., 2002). PKA also plays a role in 5-HT1A receptor phosphorylation and desensitization in multiple cell types. Activation of PKA potentiates PKC mediated desensitization of 5-HT1A receptors expressed in fibroblast Ltk^- cells (Liu and Albert, 1991). These findings are consistent with those of Raymond and Olsen who showed that stimulation of PKA increased PKC actions on 5-HT1A-mediated AC inhibition in Chinese hamster ovarian (CHO) cells (Raymond and Olsen, 1994). In HeLa cells, forskolin induced activation of PKA resulted in loss of high affinity 5-HT1A receptor binding sites and desensitization of receptor-mediated AC inhibition (Harrington et al., 1994). Treatment of 5-HT1A receptors with agonist (5-HT, 8-OH-DPAT and 5-CT) can also lead to desensitization and rapid uncoupling of receptor from G-protein and downstream signaling pathways (van Huizen et al., 1993; Harrington et al., 1994; Hensler et al., 1996).

Hyperphosphorylation of the receptor via Gβγ-dependent recruitment of G-protein receptor kinase (GRK) has been shown to mediate uncoupling and internalization (Ferguson, 2001; Riad et al., 2001). The phosphorylated receptor is

internalized via β-arrestin-1/2 (βarr) and recruitment of a β2-adapatin (AP-2)/dynamin/clathrin mediated internalization process. Our preliminary data (Figure 4) show that upon agonist treatment, the 5-HT1A receptor rapidly recruits GFP-β-arrestin2 within minutes and internalizes in vesicles. The fate of the internalized receptor is thought to be mediated by ubiquitinylation of the receptor or βarr (Shenoy and Lefkowitz, 2005). The vesicles are then targeted to lysosomal fusion leading to degradation of the receptor (Marchese and Benovic, 2001; Marchese et al., 2003; Martin et al., 2003; Perroy et al., 2004; Shenoy and Lefkowitz, 2003; Shenoy and Lefkowitz, 2005; Shenoy et al., 2001). The 5-HT1A receptor is internalized as previously described (Riad et al., 2004); however, the roles of receptor dephosphorylation or ubiquitinylation in directing receptor trafficking are unknown (Figure 5).

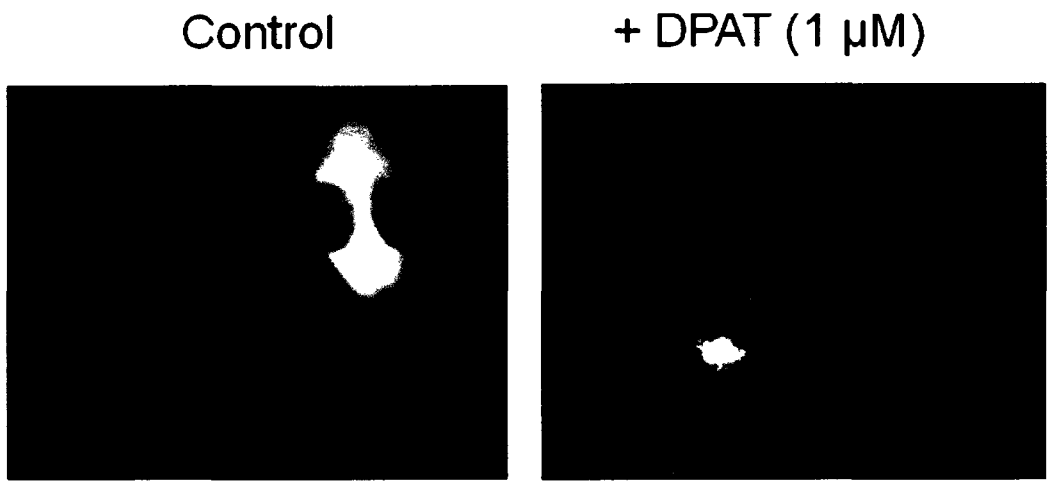


Figure 4: 5-HT1A agonist induced translocation of *β*-arrestin2-GFP in LZD7 cells.

LZD-7 (mouse Ltk$^-$ fibroblasts stably transfected with the rat 5-HT1A receptor cDNA) were transiently transfected with β-arrestin2-GFP, and the response to 5-HT1A agonist DPAT (1 μM) observed after 5 minutes of treatment. The control cell was not treated. Representative cells for each condition are shown. Data courtesy of Dr. Paul Albert.

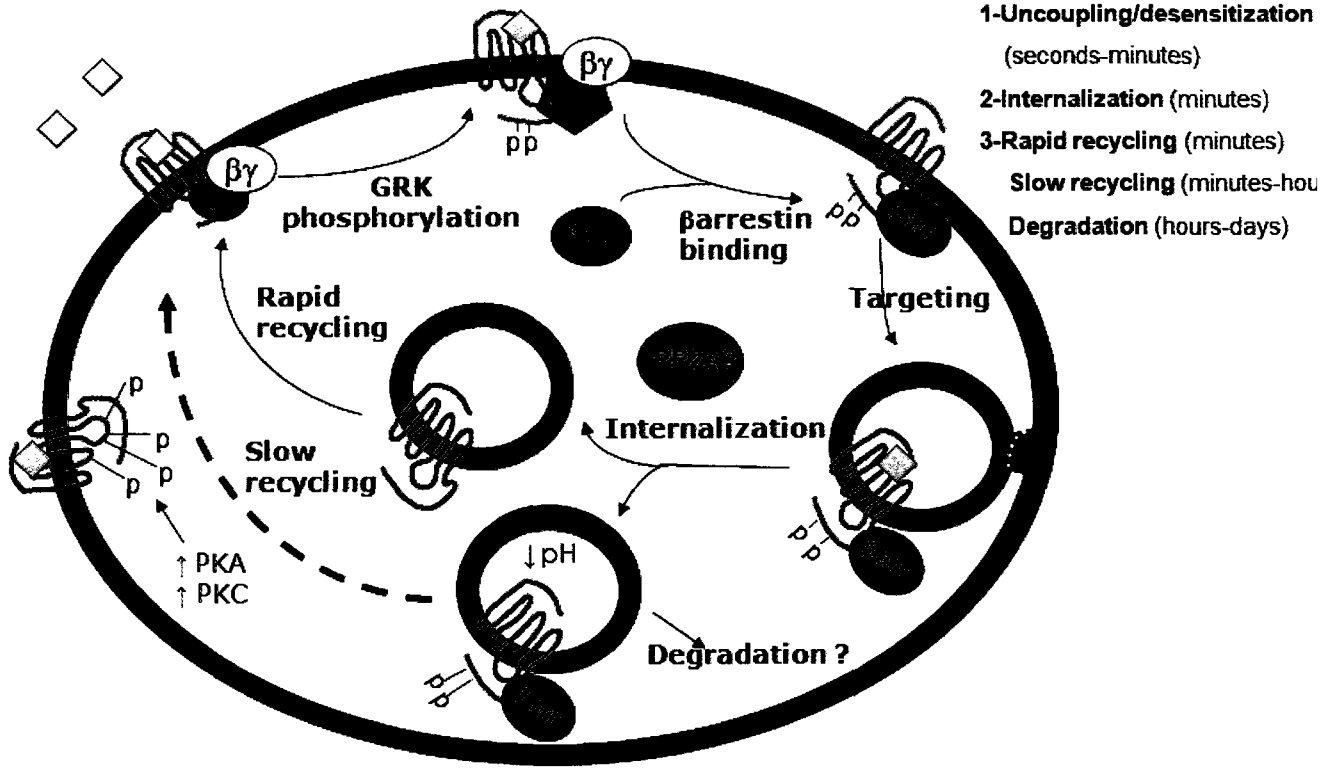


Figure 5: Second messenger/GRK-phosphorylation induced receptor internalization.

Acute desensitization by receptor uncoupling following phosphorylation by PKA, PKC or GRK (seconds), internalization via targeting to clathrin coated pits (seconds-minutes) or degradation (hours) occurs too rapidly to explain the 2-3 week time course for 5-HT1A autoreceptor down regulation in vivo. The hypothetical role of PP2A would be to dephosphorylate the internalized 5-HT1A receptor, promoting dissociation of βarr and recycling to the plasma membrane (resensitization). Reprinted by permission from Aspet: [Pharmacological Reviews] (Ferguson, S.S., 2001), copyright 2001.

Protein Phosphatase 2A

Phosphatases

Protein phosphatases are critical molecules in biological systems responsible for dephosphorylation of a wide range of substrates. The four major classes of serine/threonine-specific protein phosphatases include protein phosphatase 1 (PP1), protein phosphatase 2 (PP2A), protein phosphatase 2B (PP2B), and protein phosphatase 2C (PP2C). PP1 and PP2A are calcium-independent while PP2B and PP2C depend on intracellular calcium.

Structure

The holoenzyme of PP2A consists of a dimer composed of the 36-kDa catalytic subunit (PP2AC) and a 65-kDa regulatory subunit A (PR65). Substrate specificity and dephosphorylation activity are linked to regulatory subunits (B subunits) that bind to PR65 (Fan et al., 2001). There are two ubiquitously expressed mammalian isoforms of the protein phosphatase catalytic subunit, PP2AC-α and PP2AC-β and they share 97% sequence identity (Stone et al., 1987; Arino et al., 1988). The alpha isoform is ten times more abundant and high levels of the alpha isoform are found in the brain and heart (Khew-Goodall and Hemmings, 1988). The regulatory A subunit has two isoforms (PR65α and PR65β) which have 86% sequence identity. The A subunits remain tightly associated with the PP2AC subunit and act as a scaffold to which the appropriate regulatory B subunit can bind (Ruediger et al., 1992). Regulatory B subunits have the largest family consisting of PR53/B, PR56/61/B', PR48/59/72/130/B'' and PR93/PR110/B''' subunit families (Lechward et al., 2001). The PR55/B family consists of a 55 kDa protein encoded by four genes giving rise to four isoforms (PR55α, PR55β,

PR55γ and PR55δ). PR55β and PR55γ are highly expressed in the brain while PR55α, PR55β have distinct localization patterns within neurons. The alpha isoform is expressed in the cell body and nucleus of GABAergic purkinje cells in the cerebellar cortex, while PR55β is found in the dendrites but not the nucleus (Strack et al., 1998).

Function

Protein phosphatases are responsible for dephosphorylation and regulation of a wide range of substrates. One key functions of PP2A is to control and regulate the actions of stimulus activated protein kinases. Stimulation of a cell results in phosphorylation and activation of specific kinases that act as second messengers to phosphorylate effector molecules. PP2A has been identified as the major phosphatase that regulates and inactivates activated protein kinases through dephosphorylation (Millward et al., 1999). In particular the PP2ACα isoform has been shown to be critical during mouse development, as PP2ACα knockout mice are embryonic lethal (Gotz et al., 2000). PP2AC is also involved in carcinogenesis as inhibition of PP2AC by OA, calyculin A and other inhibitors have been shown to induce cancer (MacKintosh and MacKintosh, 1994). Reversible phosphorylation of cyclin-dependent protein kinases (Cdk)-cyclin complexes are involved in progression of cell cycle from G2 to M phase. Studies have reported that PP2A plays a role in this transition by negatively regulating Cdc2 protein kinase that forms a complex with cyclin B. PP2A is involved in the dephosphorylation of a Thr161 phosphorylation site in Cdc2, thus inhibiting the kinase and preventing the phase transition (Karaiskou et al., 1999).

PP2A has previously been shown to dephosphorylate several different types of non-GPCR and GPCR receptors. The catalytic subunit of PP2A (PP2AC) was shown to

associate with the carboxy domain of the NR3A subunit of the N-methyl-D-aspartic acid (NMDA) receptor and this association resulted in an increase in the phosphatase activity and dephosphorylation of serine 897 of the NMDA receptor subunit NR1 (Chan and Sucher, 2001). The PP2A core enzyme (PP2AC and PR65) has been shown to interact with the CXC chemokine receptor 2 (CXCR2). Treatment of stable CXCR2 expressing HEK293 cells with agonist interleukin-8 (IL8) results in a robust phosphorylation of the receptor and PP2A was found to dephosphorylate the receptor (Fan et al., 2001).

Pitcher and colleagues identified a phosphatase coined G protein-coupled phosphatase (GRP) that was associated with both the plasma and vesicular membrane. GRP was shown to dephosphorylate GRK phosphorylated β_2-AR receptor and was identified as a subclass of PP2A (Pitcher et al., 1995). In 1999, Shih and colleagues later found that both PP2A and PP2B form complexes with the β2-AR receptor and inhibition of either prevents recovery of the β2-AR receptor responsiveness in cells (Shih et al., 1999). β2-AR in vesicular fractions are less phosphorylated relative to receptors found in the plasma membrane (Sibley et al., 1986). Suppression of receptor internalization through blocking agents of mutation of internalization motifs impairs receptor dephosphorylation and subsequent resensitization (Yu et al., 1993; Barak et al., 1994).

Additionally, *in vitro* it has been shown that the serine/threonine protein phosphatases PPl and PP2A act on the cholecystokinin (CCK) receptor. PP2A activity on the phosphorylated receptor is stimulated by agonist binding to CCK receptor (Lutz et al., 1993). The thromboxane (TR) receptor is also rapidly dephosphorylated by protein phosphatases in HEK293 cells and this is completely blocked by combined treatment with PP1 and PP2A inhibitors. Interestingly, dephosphorylation of the receptor is

partially blocked when treated with specific inhibitors of either PP1 or PP2A, which suggests that the receptor is dephosphorylated by PP1- and PP2A- like protein phosphatases (Spurney, 2001). The phosphorylation state of the immune system expressed GPCR cannaboid 2 (CB2) receptor is regulated by the action of PP2A. Treatment of CB2 stably transfected CHO cells with agonist CP-55,940 for one hour leads to a robust phosphorylation of the receptor and upon treatment with receptor antagonist SR 144528 a decrease in phosphorylation is seen. Introduction of PP2A inhibitor OA inhibited the dephosphorylation of the receptor suggesting PP2A is the major phosphatase involved (Bouaboula et al., 1999). OA pretreatment has also been shown to effect the state of phosphorylation of the complement component 5a (C5a) receptor in human promyelocytic leukemia (HL60) cells. Treatment of cells expressing agonist C5a increases phosphorylation of the C5a receptor relative to untreated cells; however, pretreatment of these cells with OA increases basal phosphorylation of C5a receptors in the absence of C5a stimulation and inhibits or retards dephosphorylation of agonist-stimulated receptor (Giannini and Boulay, 1995).

Recent studies suggest that activation of dopamine (D2 class) receptor leads to the formation of scaffolding complex involving β-arrestin 2, Akt and PP2A. Mice knockout studies showed that lacking functional β-arrestin 2 had reduced dopamine-dependent behaviors, loss of Akt regulation by dopamine in the striatum and disruption of the dopamine-dependent interaction of Akt with PP2A. PP2A is implicated in dephosphorylation of Akt on residue Thr308 leading to inactivation of Akt (Beaulieu et al., 2005).

Rationale for Proposed Studies

The specific system under investigation in this report is the serotonin (5-HT) system. The 5-HT system is associated with several mental illnesses, which include depression, anxiety and other related mood disorders (Charney et al., 1990; Mann et al., 2001; Murphy et al., 1999; Pineyro and Blier, 1999). The 5-hydroxytryptamine 1A (5-HT1A) receptor is a crucial regulator of this system (Albert and Lemonde, 2004). Thus, an understanding of the regulation of this receptor has implications for regulation of the entire serotonin system. Our initial hypothesis, derived from i2 loop mutagenesis studies, was that the 5-HT1A-i2 interacts with proteins involved in coupling, and from yeast hybrid screening we identified potential 5-HT1A regulatory proteins, one of which was PP2AC. To this end we have identified by yeast two hybrid screening and verified by co-immunoprecipitation a novel interaction between protein phosphatase 2A (PP2A) catalytic subunit and the 5-HT1A receptor, which could have implications for the desensitization and resensitization of the receptor.

Hypothesis and Aims

Protein phosphatase 2A (PP2A) interacts with the 5-HT1A second intracellular loop (5-HT1A-i2) to potentially dephosphorylate i2 or i3 PKC or GRK phosphorylation sites to re-sensitize the receptor.

i. To verify that protein phosphatase 2A (PP2A) interacts with the 5-HT1A receptor in mammalian cells.

ii. To examine the effect of 5-HT treatment on the interaction between protein phosphatase 2A (PP2A) and the 5-HT1A receptor.

MATERIALS AND METHODS

Materials

All chemicals were reagent grade. Restriction enzymes were purchased from New England Biolabs (Mississauga, ON). 12α-O-tetradecanyoyl phorbol 13β-acetate (TPA), O-nitrophenyl-β-D-galactosidase (ONPG), anti-FLAG M1 antibody, anti-FLAG M2 agarose beads, anti-phosphothreonine and anti-phosphoserine antibodies were purchased from Sigma-Aldrich (St. Louis, MO). Anti-PP2AC antibody was purchased from Cell Signaling Technology Inc. (Danvers, MA). S-protein horseradish peroxidase (HRP) conjugate antibody and S-protein agarose beads were obtained from Novagen-Merck. pEGFP vector and yeast cDNA library were purchased from Clontech (Mountainview, CA). Coomassie (Bradford) protein assay kit and chemical cross linkers dithiobis[succinimidylpropionate] (DSP) and dimethyl 3,3′-dithiobispropionimidate (DTBP) were obtained from Pierce Biotechnology (Rockford, IL). The QuikChange XL II site directed mutagenesis kit was obtained from Strategene (La Jolla, CA). Polyvinylidene difluoride membranes were purchased from PerkinElmer Life Sciences; enhanced chemiluminescence (ECL) detection kits were obtained from Roche Applied Sciences; sera and media were obtained from Wisent, Inc. (St.-Bruno, Canada). Lipofectamine was purchased from Invitrogen. The human PP2AC gene was obtained from Origene (Rockville, MD).

Plasmids

To generate FLAG 5-HT1A receptor expression plasmids a 1.9 kb EcoRI/XhoI fragment of the rat 5-HT1A receptor gene wild-type or receptor mutant were subcloned

into EcoRI/XhoI-digested pcDNA3-FLAG vector. Three point mutations were needed to correct the original pAS2-1 5-HT1A-long-i2 yeast construct, done using a primer-directed mutagenesis kit (QuikChange XL II Site-Directed Mutagenesis Kit, Stratagene). The first mutation corrected the frame of the construct, placing the i2 loop in frame with the Gal4 DNA binding domain (DBD); the second mutation converted an incorrect methionine residue with the correct isoleucine residue; last the third mutation placed a stop codon at the end of the i2 loop. To generate the His/S-tagged PP2AC expression plasmid a 1.0 kb EcoRI fragment of the human PP2AC gene (Origene) was subcloned into EcoRI-digested pTriex-4 vector. A single base insertion using a primer-directed mutagenesis kit (QuikChange XL II Site-Directed Mutagenesis Kit, Stratagene) was necessary to correct the original His/S-tagged 5-HT1A receptor construct. The insertion mutation corrected the frame of the construct, placing the receptor in frame with the His/S-tag.

Cell Culture and Transient Transfection

HEK293 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Wisent, St.-Bruno, QC), plus 5% fetal bovine serum (FSB) (Invitrogen) at 37°C in 5% CO_2. HEK293 cells were plated at 2×10^6 cells/10 cm dish and incubated overnight. For determination of PKC mediated phosphorylation of the 5-HT1A receptor, cells were transfected by calcium phosphate co-precipitation with 5 μg of pcDNA3-FLAG-5-HT1A (wild-type or Ci2 mutants) in 9 ml/dish DMEM + 5% FBS, and 2X HEPES-buffered saline (HBS), pH 7.0, at 37°C in 5% CO_2 for 24 h. To detect the interaction between the 5-HT1A receptor and PP2A catalytic subunit, 5 μg of pcDNA3-FLAG-5-HT1A (wild-type or Ci2 mutants) or wild type His/S-tagged 5-HT1A constructs were transiently

transfected into HEK293 cells by either calcium phosphate co-precipitation (in 9 ml/dish DMEM + 5% FBS, and 2X HBS, pH 7.0, at 37°C in 5% CO_2 for 24 h) or Lipofectamine Plus reagent (Invitrogen) according to the manufacturer's protocol.

Protein Kinase C mediated phosphorylation Assay

The pcDNA3-FLAG-5-HT1A (wild-type or Ci2 mutants) constructs were transiently transfected into HEK293 cells by calcium phosphate co-precipitation. Forty-eight hours after transfection, the cells were pretreated (5 minutes) with 100 nM TPA and then scraped in RIPA lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS and Complete Mini protease inhibitor cocktail (Roche Applied Sciences)) and left at 4°C on shaker for 3 hours to lyse. They were lysed further by passing through 25 gauge needle. The cell lysate was centrifuged at 14,000 rpm for 20 minutes at 4°C. The supernatants were incubated with 40 µl of anti-FLAG M2-agarose affinity gel (Sigma) at 4°C overnight. The gel was washed three times with RIPA lysis buffer at 4°C, resuspended in 1X SDS-loading buffer and boiled for 5 minutes to elute the proteins. The eluted supernatants were resolved on an SDS-10% polyacrylamide gel that was transferred onto a polyvinylidene difluoride membrane (PDVF) and blocked at room temperature for 1 hour in 5% skimmed milk in Tris-buffered saline (TBS) buffer. Phosphorylation was analyzed by Western blot, incubating the membranes with rabbit polyclonal anti-phosphothreonine (1:2500), anti-phosphoserine (1:2500) and anti-phosphoT149-5-HT1A (1:2500) antibodies overnight at 4°C. Secondary antibody was anti-rabbit horseradish peroxidase (1:5000) and was incubated for 1 hour at room temperature. Western blot was visualized using enhanced chemiluminescence (ECL)

detection kit (Roche Applied Sciences), exposed to Kodak BioMax MR film and developed.

Co-immunoprecipitation/Co-precipitation Assay

The pcDNA3-FLAG-5-HT1A (wild-type or Ci2 mutants), wild type His/S-tagged 5-HT1A receptor and His/S-tagged PP2AC constructs were transiently transfected into HEK293 cells by either calcium phosphate precipitation or Lipofectamine Plus reagent (Invitrogen). Forty-eight hours after transfection, cells were treated with 10 μM 5-HT (10-15 minutes) and proteins were cross-linked with 2.5 mM dithiobis[succinimidylpropionate] (DSP) at 4°C or 5.0 mM dimethyl 3,3′-dithiobispropionimidate (DTBP) at 4°C for 30 minutes prior to harvesting. The cells were scraped in RIPA lysis buffer and left at 4°C on shaker for 3 hours to lyse. They were lysed further by passing 3 to 4 times through 25 G needle at room temperature. The cell lysate was centrifuged at 14,000 rpm for 20 minutes at 4°C. The supernatants containing approximately 800 μg of total protein were incubated with 40 μl of anti-FLAG M2-agarose affinity gel (Sigma Aldrich) or 40 μl of S-protein agarose beads (Novagen) at 4°C overnight. The agarose beads were washed three to five times with RIPA lysis buffer (4°C), resuspended in 1X SDS-loading buffer with 100 mM DTT to cleave the cross linker and boiled for 5 minutes to elute the proteins. The eluted supernatants were resolved on an SDS-10% polyacrylamide gel that was transferred onto a polyvinylidene difluoride membrane (PDVF) and blocked at room temperature for 1 hour in 1% commercial blocking solution (Invitrogen) in Tris-buffered saline (TBS) buffer and analyzed by Western blotting using a mouse monoclonal anti-PP2A catalytic subunit (1:5000) (Cell Signaling), anti-FLAG M1 antibody (1:2500) (Sigma Aldrich) and

S-protein conjugated HRP (1:5000) antibodies to detect immunoprecipitated phosphatase subunit and 5-HT1A receptor. Secondary antibody was anti-mouse horseradish peroxidase (1:5000) and was incubated for 1 hour at room temperature. Western blot was visualized using BM Chemiluminescence Blotting Substrate detection kit (Roche Applied Sciences), exposed to Kodak BioMax MR film and developed.

Chemical Cross linking

Dithio-bis(succinimidylpropionate reagent

We used the cleavable homobifunctional, amine-reactive reagent DSP dithio-bis(succinimidylpropionate) to covalently link the transient interaction between the 5-HT1A receptor and the PP2A catalytic subunit. DSP was solubilized in DMSO to a final concentration of 25 mM (stock solution) prior to use and added to a final concentration of 2.5 mM and incubated with the cells for 30 minutes on ice at 4°C. The reaction was stopped by adding from a stock solution of 1 M Tris, pH 7.5 to a final concentration of 15 mM and incubated on ice for 15 minutes. Cross linker was cleaved using 100 mM DTT in the SDS loading buffer prior to separation on a gel.

Dimethyl 3,3′-dithiobispropionimidate reagent

We used the cleavable homobifunctional, imidoester cross linking reagent DTBP (dimethyl 3,3′-dithiobispropionimidate) to covalently link the transient interaction between the 5-HT1A receptor and the PP2A catalytic subunit. DTBP was solubilized in phosphate buffered saline (PBS) to a final concentration of 50 mM (stock solution) prior to use and added to a final concentration of 5.0 mM and incubated with the cells for 30 minutes on ice at 4°C. The reaction was stopped by adding from a stock solution of 1 M

Tris, pH 7.5 to a final concentration of 20 mM and incubated on ice for 15 minutes. Cross linker was cleaved using 100 mM DTT in the SDS loading buffer prior to separation on a gel.

Yeast mating assay and β-galactosidase Assay between 5-HT1A long i2 and PP2AC, Nedl-1 and dynein

5-HT1A-long i2 was subcloned in pAS2-1 (leu+) while PP2AC, Nedl-1 and dynein constructs were cloned into the pACT2 (trp+) yeast vector as part of a yeast cDNA screening library (Clontech). For β-galactosidase assays, pAS2-5-HT1A long i2 loop peptide and pACT2 constructs (dynein, Nedl1, and PP2ACα clones) were transformed into Y187 and AH109 strains and plated onto S.D.-Trp- and S.D.-Leu- plates, respectively. Resultant colonies were mated in S.D.-Leu-Trp- medium at 30°C overnight and were diluted to A_{600} = 0.7, from which 10 μl of the yeast cell medium was spotted on S.D.-Leu-Trp- and S.D.-Leu-Trp-His plates. Liquid β-galactosidase assays were performed using mated yeast cells described above. Briefly, yeast cells subcultured in S.D.-Leu-Trp-His- medium (30°C, overnight), were lysed by three freeze-thaw cycles and re-suspended in Z buffer (60 mM Na_2HPO_4, 40mM NaH_2PO_4, 10mM KCl, 1 mM $MgSO_4$, pH 7.0). For quantitative assay, O-nitrophenyl-β-D-galactopyranoside (Sigma) and yeast lysate were incubated at 30°C; absorbance at 420 nm was measured to determine β-galactosidase activity units, which was normalized by the β-galactosidase unit of the positive control vector pCL1 in each assay.

Re-optimization of transfection and protein detection

HEK293 cells were transfected with various amounts of empty pEGFP vector (Clontech) via calcium phosphate precipitation or Lipofectamine Plus reagent (Invitrogen). Cells were analyzed for fluorescence under a fluorescence microscope. Once optimal transfection conditions were determined, HEK293 cells were transfected with 6 μg of FLAG-5-HT1A and 1 μg of empty pEGFP (positive transfection control) or 5 μg of FLAG-RASA3 (positive control) and 1 μg of empty pEGFP (positive transfection control) using Lipofectamine Plus for 48 hours (1:3 ratio of Lipofectamine to Lipofectamine Plus reagent per μg of plasmid). Cells were analyzed for fluorescence under a fluorescence microscope prior to harvest. The cells were washed twice with PBS and harvested. The cells were then lysed with 800 μl of lysis buffer containing 50 mM Tris, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS and Complete Mini protease inhibitor cocktail (Roche). After gentle rotation for 3 hours, samples were centrifuged for 15 minutes at 14 000 rpm and the supernatant was collected. 20 μl of supernatant was combined with 20 μl of 2 X SDS-PAGE loading buffer, and proteins separated on a 10% SDS-PAGE gel, and visualized by immunoblotting using anti-FLAG M1 antibody (Sigma Aldrich).

Statistical Analysis

The data are presented as mean ± SEM of at least three independent experiments. The data were analyzed by the student's t-test (two tailed, paired) as indicated using the GraphPad Prism program (San Diego, CA). The corresponding bands for co-precipitated PP2AC were digitally quantified using Adobe Photoshop and the results were normalized to the amount of tagged 5-HT1A receptor precipitated.

RESULTS

Solubilization of FLAG-tagged 5-HT1A receptors for phosphorylation and co-immunoprecipitation studies

Wild-type and mutant 5-HT1A receptors were successfully transfected into HEK293 cells and detected after receptor solublization and subsequent immunoprecipitation using anti-FLAG M2 Affinity Gel (Sigma) (Figure 6). The wild-type FLAG tagged receptor is functional and has been characterized relative to the wild-typed untagged form with respect to ligand binding, G-protein coupling and receptor number (Kushwaha, unpublished data). Two specific FLAG-5-HT1A receptor species of molecular weight (M.W.) 48 and 60 kDa were detected in cells transfected with the wild-type or T149A mutant receptor plasmid, but not observed in non-transfected cells. Treatment of cells with 5-HT has no effect on the amount of receptor immunoprecipitated. Further studies using another tagged variant of the 5-HT1A receptor showed similar results.

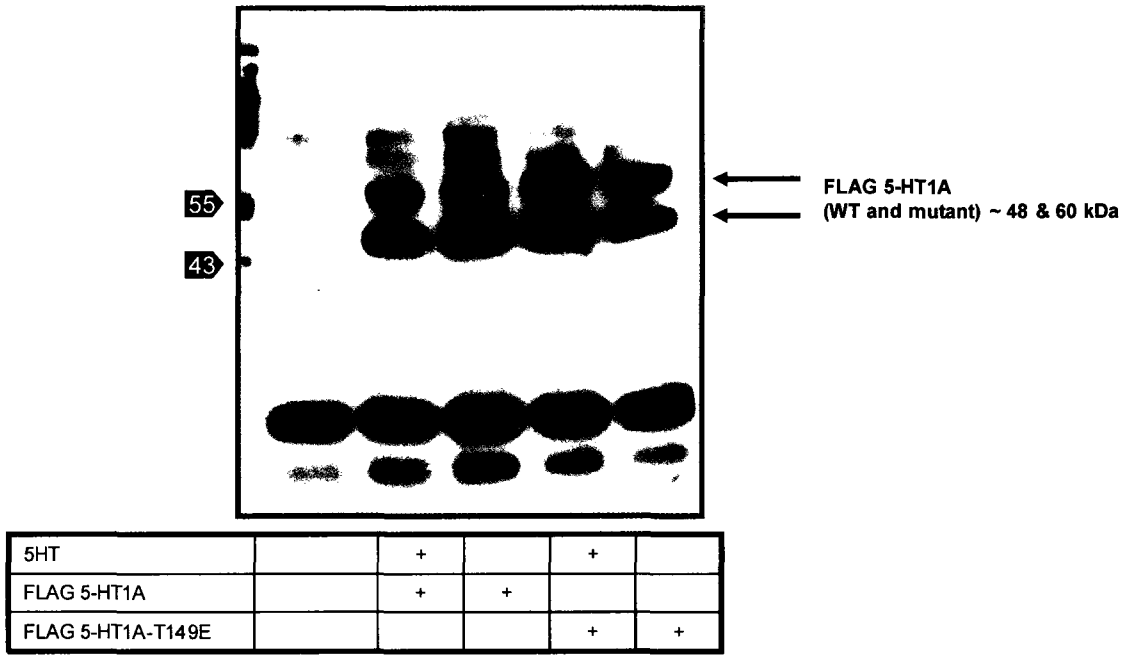


Figure 6: Solubilization of FLAG-tagged 5-HT1A receptors for phosphorylation and co-immunoprecipitation studies.

Plasmids encoding FLAG 5-HT1A receptor and mutant FLAG 5-HT1A-T149E receptor were transiently transfected in HEK293 cells and compared to non-transfected HEK293 cells. After 48 hours, the cells were treated or not with 5-HT (10 μM) for 10-15 minutes as indicated. The protein levels of cell lysates were quantified and equal amounts of protein incubated with anti-FLAG M2 agarose beads. Proteins were eluted and separated on a 10% SDS-PAGE gel, and visualized by immunoblotting using anti-FLAG M1 antibody. Shown at left is the migration of molecular weight markers (kDa). Two specific bands were detected in 5-HT1A-transfected cells but not non-transfected cells (arrows). Data courtesy of Jin Lu.

5-HT1A receptor phosphorylation via protein kinase C

John Raymond has previously shown that activation of PKC via phorbol ester TPA leads to phosphorylation and eventual desensitization of the 5-HT1A receptor (Raymond, 1991). Examination of total wild-type, mutant 5-HT1A-T149A and mutant 5-HT1A-T149E phosphorylation following immunoprecipitation with anti-FLAG M2 agarose and Western blot using anti-phosphoserine (Sigma Aldrich) or anti-phosphothreonine (Sigma Aldrich) commercial antibodies was done. Western blot with anti-FLAG M1 antibody revealed a major band for 5-HT1A transfected cells at 44 kDa. Weak bands corresponding to the molecular weight of the 5-HT1A receptor (48 and 60 kDa) were detected using anti-phosphoserine antibody, particularly in the TPA-treated wild-type 5-HT1A lanes, but not in non-transfected cells (Figure 7). A very weak signal was present in the TPA-treated T149A and T149E mutants. These data suggest that the 5-HT1A receptor is phosphorylated by PKC at Ser sites, and that this phosphorylation may be affected by the T149A mutation. Using the anti-phosphothreonine antibody, no bands were detected at 48 or 60 kDa, but a band at 70 kDa was present in the TPA-treated 5-HT1A and T149A lanes but not untreated lanes (Figure 7). Because it migrates at a different size than the 5-HT1A receptor, the identity of this protein is unclear but it could be a receptor-associated protein that is phosphorylated by PKC, or a low abundance glycosylated form of the receptor that was not detected using the anti-FLAG antibody.

A second experiment was inconclusive as only non-specific bands were visualized utilizing anti-phosphoserine (Sigma Aldrich) and anti-phosphothreonine (Sigma Aldrich) antibodies, despite that probing with anti-FLAG M1 antibody (Sigma Aldrich) verified

the presence of 5-HT1A receptor (Figure 8). Probing with a specific anti-phospho-5-HT1A-T149 antibody (Sigma) revealed faint bands approximately near the size of the 5-HT1A receptor and treatment with TPA yielded a stronger band relative to non-treatment conditions (Figure 8). Mutation of residue T149 in the receptor to the non-phosphorylatable alanine (T149A) seems to have blocked the effect (Figure 8). These results are consistent with phosphorylation of the 5-HT1A receptor by PKC at Ser and Thr residues, particularly T149.

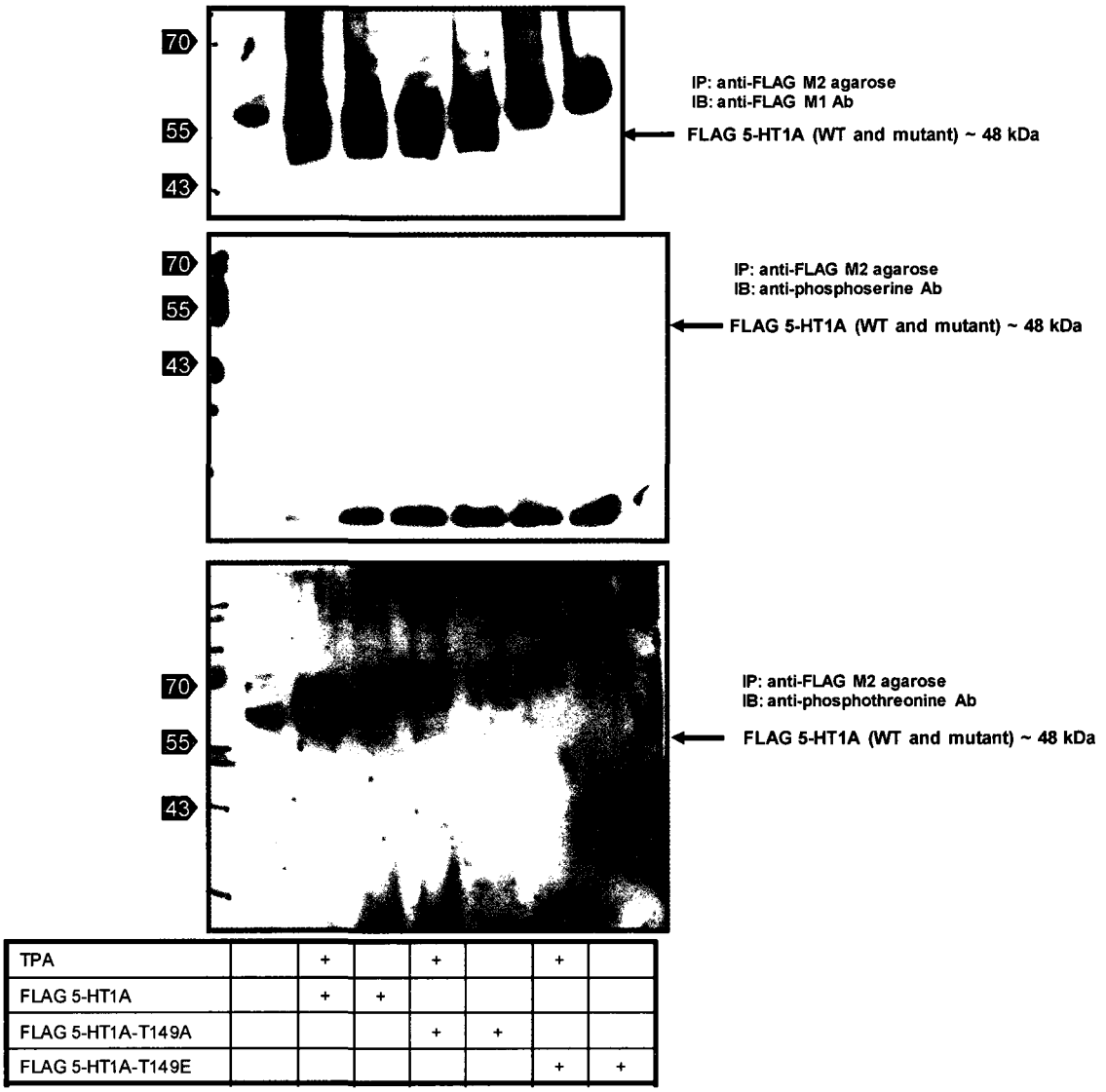


Figure 7: PKC mediated phosphorylation of wild-type and mutant 5-HT1A receptors.

Plasmids encoding FLAG 5-HT1A receptor, mutant FLAG 5-HT1A-T149A receptor and mutant FLAG 5-HT1A-T149E receptor were transiently transfected in HEK293 cells. The transfected cells were treated with the PKC activator TPA (100 nM) or control (ddH_2O) for 5 minutes and the cells lysed. Cell lysates were quantified and incubated with anti-FLAG M2 agarose beads. Proteins were eluted and separated on a 10% SDS-PAGE gel, and visualized by immunoblotting using anti-FLAG M1 (above), anti-phosphoserine (middle) and anti-phosphothreonine (below) antibodies as indicated. Molecular weight markers are shown at left (kDa). Bands specific for 5-HT1A receptor-transfected cells are shown (arrows).

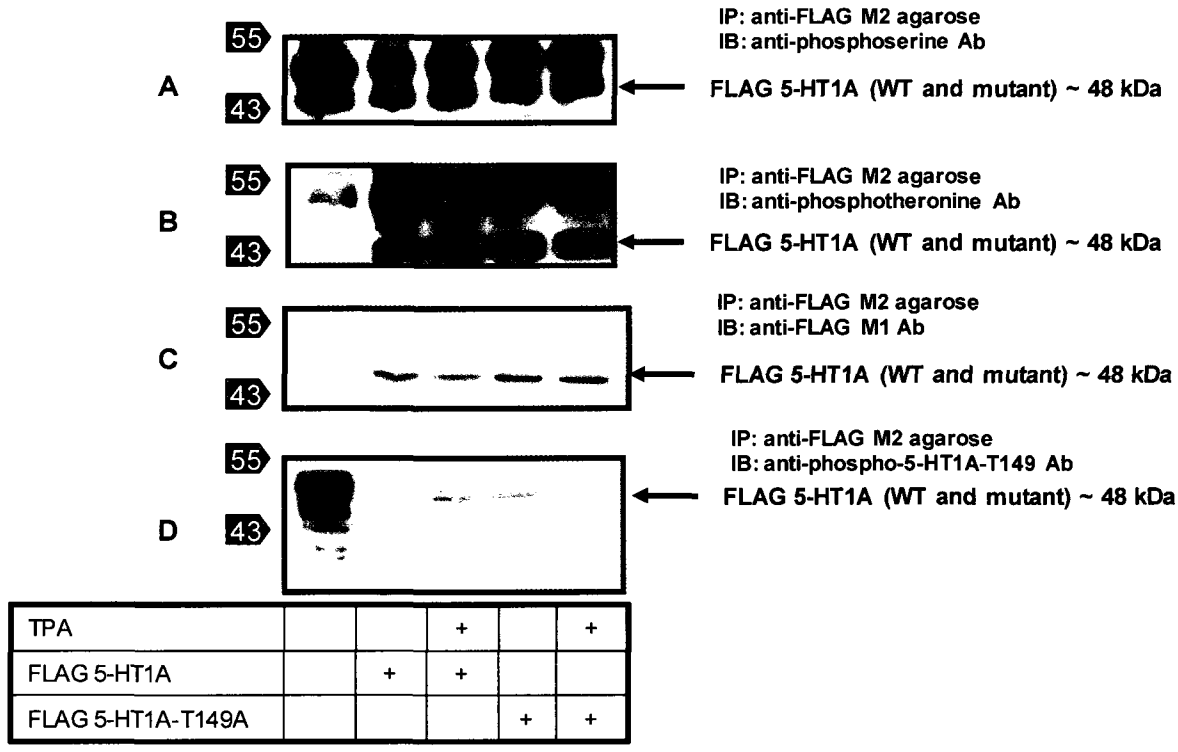


Figure 8: PKC mediated phosphorylation of wild-type and mutant 5-HT1A receptors.

Plasmids encoding FLAG 5-HT1A receptor, mutant FLAG 5-HT1A-T149A receptor and mutant FLAG 5-HT1A-T149E receptor were transiently transfected in HEK293 cells. The transfected cells were treated with the PKC activator TPA (100 nM) or control (ddH_2O) for 5 minutes and the cells lysed. Cell lysates were quantified and incubated with anti-FLAG M2 agarose beads. Proteins were eluted and separated on a 10% SDS-PAGE gel, and visualized by immunoblotting using anti-phosphothreonine (A), anti-phosphothreonine (B) anti-FLAG M1 (C) and anti-phospho-5-HT1A-T149 (D) antibodies as indicated. Molecular weight markers are shown at left (kDa). Bands specific for 5-HT1A receptor-transfected cells are shown (arrows).

5-HT1A i2/regulatory protein interactions

Yeast mating/β-galactosidase assay

Dr. Kushwaha's initial yeast mating/B-galactosidase assays using an assumed wild-type 5-HT1A long i2 construct and one with a mutated PKC site (T149A) showed decreased interaction with the 5-HT1A-long i2 relative to the wild type receptor in yeast-two hybrid assay (data not shown). However, upon sending the long i2 construct for automated sequencing an error in subcloning was discovered. Dr. Kushwaha's construct used in the yeast/mating and B-galactosidase assays were one nucleotide base out of frame with the Gal4 DNA binding domain and is predicted to encode a non-sense protein (Figure 9A, upper). Yeast mating/ B-galactosidase assays were re-tested using a corrected construct in which the extra base was deleted through mutagenesis. Interactions with the correct long i2 construct were re-confirmed with PP2AC and dynein but not Nedl-1 (Figure 9B).

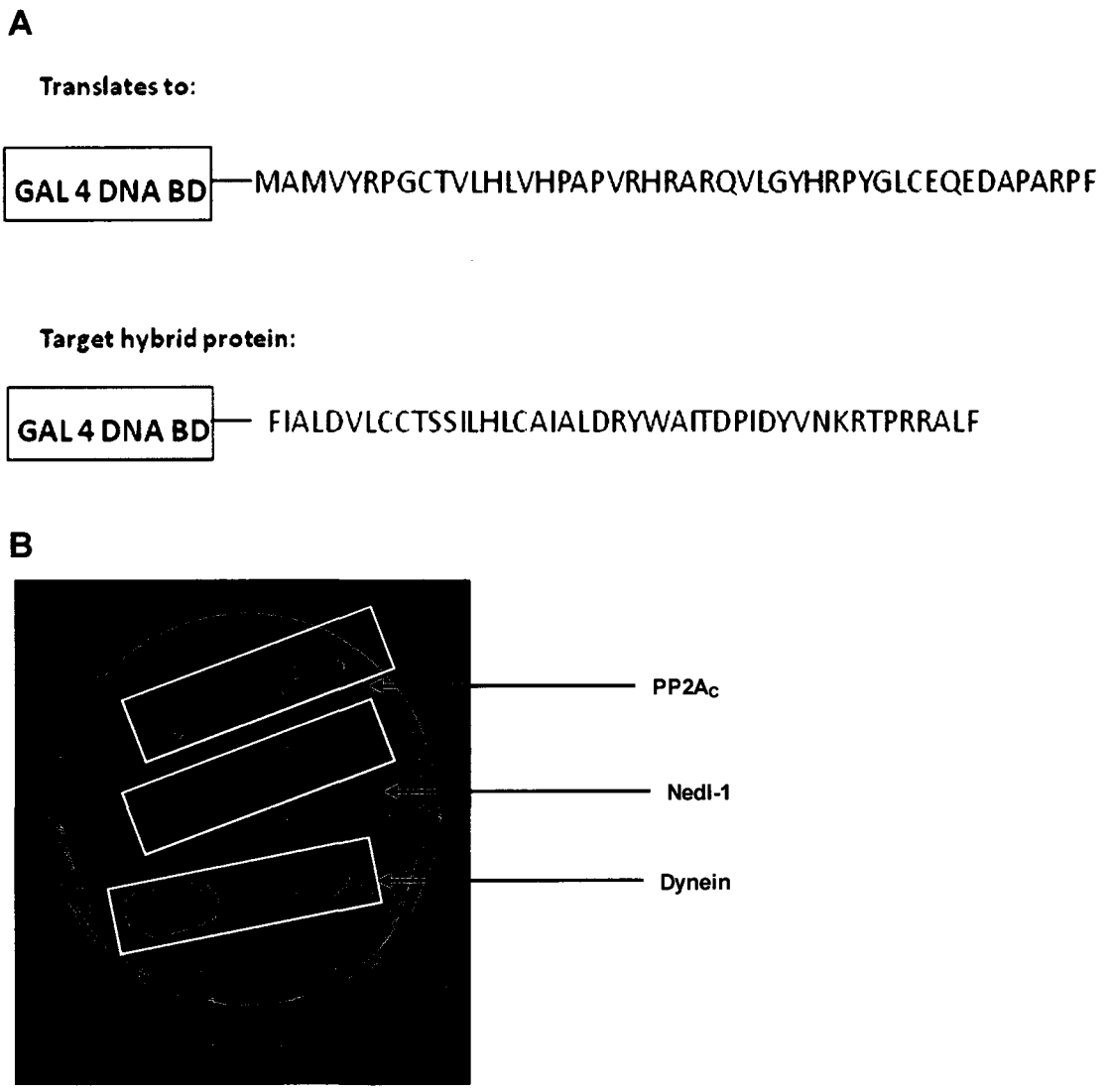


Figure 9: Yeast mating assay with 5-HT1A long i2 and PP2AC, dynein and Nedl-1.

(A) Upper schematic refers to Gal4 DNA binding domain fusion protein encoded by the erroneous subcloned long i2 into the yeast vector pAS2-1. The lower schematic refers to the Gal4 DNA binding domain fusion protein encoded by the corrected long i2 in the yeast vector pAS2-1. (B) Interactions with proper long-i2 construct were re-confirmed with PP2AC and dynein clones. No interactions were observed with Nedl-1.

It is unclear whether this construct was used in the initial screening, or became altered in subsequent preparations. The original non-sense yeast hybrid construct yielded a positive interaction with the PP2AC subunit clone and this could be due to several reasons. First, the non-sense target construct contained a threonine residue which is the target of PP2A, a threonine- and serine-specific phosphatase. Second, the yeast transcriptional machinery could have skipped over a single base, and in turn, it inadvertently corrected the frame of the construct and resulted in translation of the correct i2 loop sequence. Yeast-two hybrid studies were repeated using the corrected 5-HT1A-i2 construct and suggested a potential interaction between the i2 domain of the 5-HT1A receptor and PP2AC subunit and dynein. The interaction with the PP2AC subunit and dynein was re-confirmed using β-galactosidase assay (Figure 10).

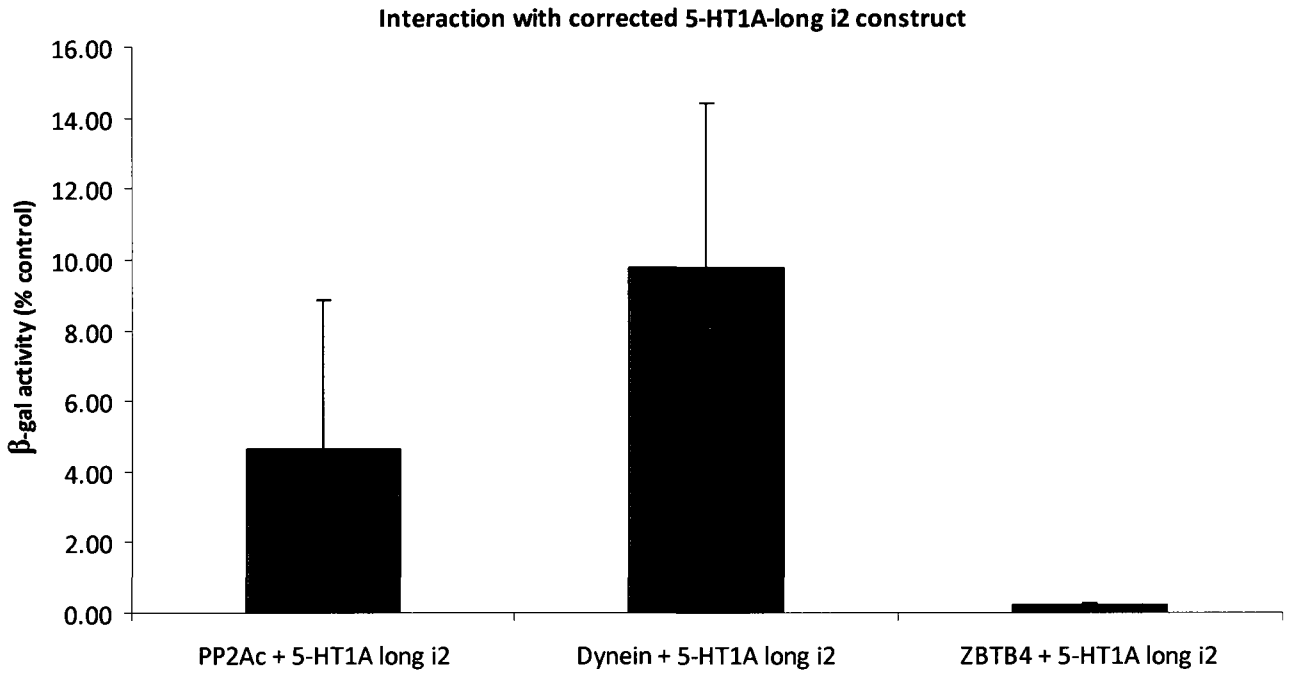


Figure 10: β-galactosidase quantification of 5-HT1A-long i2 yeast interactions.

Liquid β-galactosidase assays were performed using mated yeast cells described above. Briefly, yeast cells subcultured in S.D.-Leu-Trp-His- medium (30°C, overnight), were lysed by three freeze-thaw cycles and re-suspended in Z buffer (60 mM Na_2HPO_4, 40mM NaH_2PO_4, 10mM KCl, 1 mM $MgSO_4$, pH 7.0). For quantitative assay, O-nitrophenyl-β-D-galactopyranoside (Sigma) and yeast lysate were incubated at 30°C; absorbance at 420 nm was measured to determine β-galactosidase activity units, which was normalized by the β-galactosidase unit of the positive control vector pCL1 in each assay.

FLAG-tagged 5-HT1A Co-Immunoprecipitation assay

Wild-type 5-HT1A receptor and PP2AC subunit were found to co-immunoprecipitate together with and without 5-HT treatment in the presence of dithiobis[succinimidylpropionate] (DSP) chemical cross linking agent. Previous experiments to show the interaction without the addition of a chemical cross linker were unsuccessful, as no specific bands corresponding to PP2AC were detected in the wild type and mutant receptor transfected cells (Figure 11). The interaction between the receptor and phosphatase appears to be weak and transient, owing to the dynamic nature of the G-protein coupled receptor-protein interactions, as the addition of a cross linker was needed to capture the interaction for analysis (Figure 12). As expected, the non transfected cell extract showed no band corresponding to the PP2AC subunit after immunoprecipitation with anti-FLAG M2 antibody. By contrast, in cells transfected with Flag-5-HT1A receptor, PP2A catalytic subunit was present in the immunoprecipitate, and was consistently increased in the presence of 5-HT. The non-specific bands correspond to the molecular weight of the heavy and light IgG chains. These initial data suggested that the 5-HT1A-PP2A interaction does occur in cells, and hence it could regulate receptor phosphorylation and sensitization. The interaction appears to be enhanced following 5-HT treatment, providing evidence that the interaction may play a role in homologous desensitization to enhance de-phosphorylation of the GRK-phosphorylated receptor. Co-transfection with exogenous PP2AC did not increase the interaction, presumably because overall PP2A levels were not increased, and this is consistent with past studies noting the notorious difficulties involved in overexpresion of PP2AC. PP2AC levels are regulated under a strict autoregulatory mechanism at the translational

level and transfection of PP2AC constructs leads to decreases in production of endogenous PP2AC making overexpression a challenge (Baharians and Schonthal, 1998). While FLAG-5-HT1A wild type and PP2AC co-immunoprecipitate together initial data suggest FLAG 5-HT1A-E340K does not co- immunoprecipitate with PP2AC subunit possibly due to disruption of i2 loop structure (Figure 13). These results are consistent with Dr. Kushwaha's previous cell signaling studies involving the 5-HT1A receptor third intracellular (i3) loop E340 5-HT1A mutant receptors (data unpublished). Relative to wild type 5-HT1A receptor, mutant E340K showed inverse activity promoting dopamine-stimulated cAMP accumulation (Gαi response) and decreased 8-OH-DPAT induced intracellular calcium levels (Gβγ response) (data not shown). Dr. Kushwaha et. al., have predicted that residue E340 plays a critical role in facilitating interactions between the N-terminal i2 loop (Ni2) DRY motif and the C-terminal (Ci2) YXXKR motif. The residue Glu340 located on the C-terminal side of the third intracellular loop (Ci3) provides ionic or other types of interactions with the residues K147, Y144 and R134 (Figure 1), which in turn provides the i2 loop the proper conformation of the Gαi and Gβγ subunit binding sites in the second and third intracellular loops respectively (Kushwaha et al., 2006).

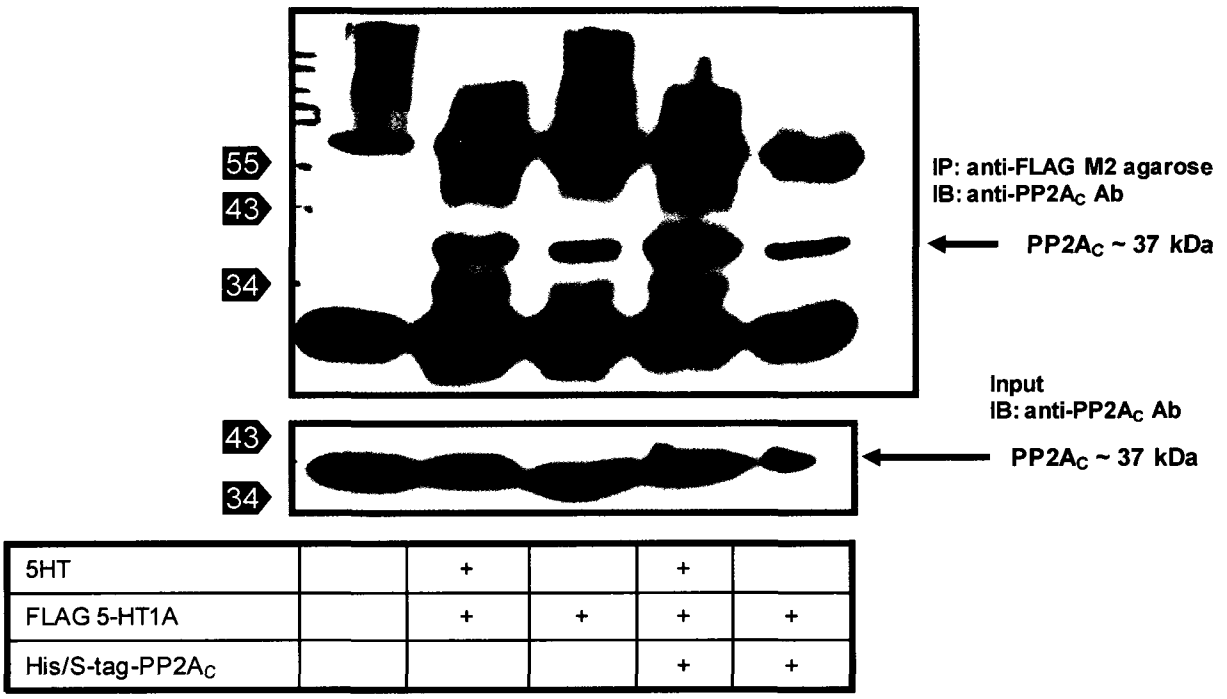
The PP2AC subunit co-immunoprecipitated with the wild-type FLAG 5-HT1A, mutant FLAG-5-HT1A-T149A and mutant FLAG 5-HT1A-T149E receptors whether treated with 5-HT or not in the presence of DSP chemical cross linker, again 5-HT treatment led to increased association of PP2AC with the wild-type 5-HT1A receptor, but only background levels for the T149A mutant, while the T149E data were not interpretable due to incomplete transfer (Figure 14). These data further support yeast studies that the 5-HT1A receptor-PP2A interaction does occur in cells. Interestingly, the

interaction is enhanced in the presence of 5-HT, suggesting the recruitment of PP2A to the receptor upon activation. The 5-HT1A-PP2A interaction was not observed upon mutation of T149 or E340, suggesting that the i2 loop structure is required for correct interaction, which is consistent with the interaction of PP2A with the i2 loop in yeast studies.

Input (cell lysate)					+
pCDNA3 FLAG		+			
FLAG 5-HT1A			+		
FLAG 5-HT1A-T149A				+	

Figure 11: Co-immunoprecipitation of wild type and i2-mutant FLAG-5-HT1A receptor and PP2A catalytic subunit without chemical cross linking.

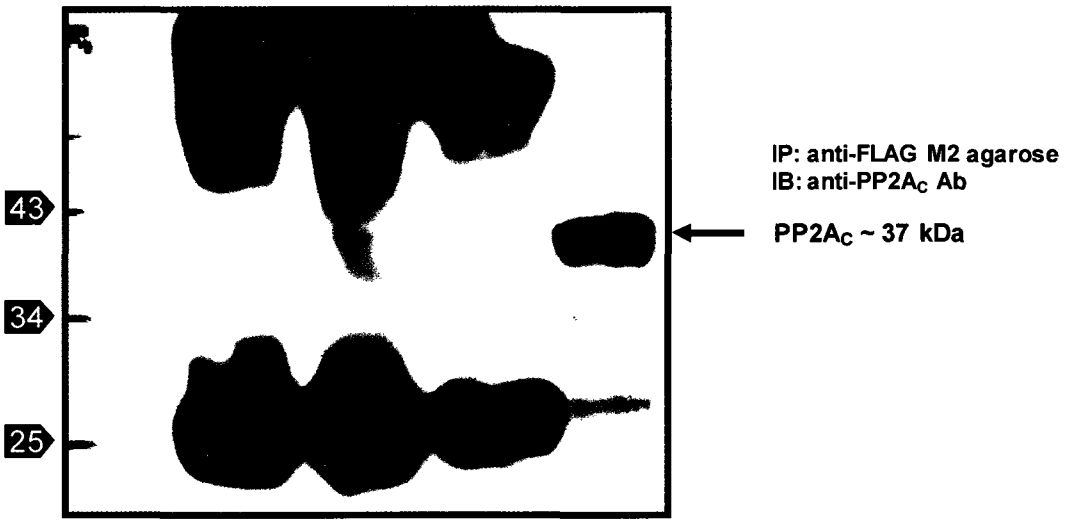
Plasmids encoding FLAG 5-HT1A receptor, mutant FLAG 5-HT1A-T149A receptor and empty pcDNA3 FLAG vector were transiently transfected in HEK293 cells and compared to non-transfected HEK293 cells. After 48 hours, the cells were harvested and the protein levels of cell lysates were quantified and equal amounts of protein incubated with anti-FLAG M2 agarose beads. Proteins were eluted and separated on a 10% SDS-PAGE gel, and visualized by immunoblotting using anti-PP2AC antibody as indicated. Shown at left is the migration of molecular weight markers (kDa). Without treatment with chemical cross linker no specific band for PP2AC was detected in the tagged 5-HT1A-transfected (wild type or mutant) cells (arrows).



5HT		+		+	
FLAG 5-HT1A		+	+	+	+
His/S-tag-PP2Ac				+	+

Figure 12: Co-immunoprecipitation of FLAG-tagged 5-HT1A receptor and PP2A catalytic subunit with DSP chemical cross linking.

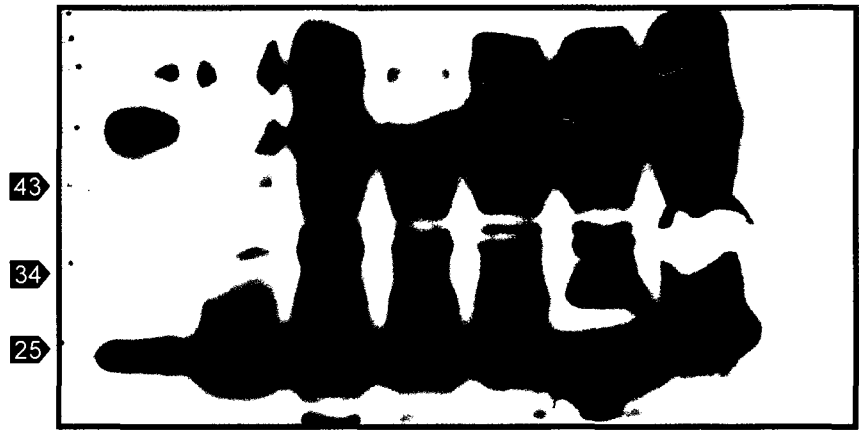
Plasmids encoding FLAG 5-HT1A receptor and PP2A catalytic subunit (PP2AC) were transiently transfected in HEK293 cells and compared to non-transfected HEK293 cells. After 48 hours, the cells were treated with or without 5-HT (10 μM) for 10-15 minutes. and proteins were cross-linked with 2.5 mM dithiobis[succinimidylpropionate] (DSP) prior to harvesting as indicated. The protein levels of cell lysates were quantified and equal amounts of protein incubated with anti-FLAG M2 agarose beads. Proteins were eluted and separated on a 10% SDS-PAGE gel, and visualized by immunoblotting using anti-PP2AC antibody as indicated. Shown at left is the migration of molecular weight markers (kDa). A specific band was detected in 5-HT1A-transfected cells but not non-transfected cells (arrows).



10% input				+
FLAG 5-HT1A		+		
FLAG 5-HT1A-E340K			+	

Figure 13: Co-immunoprecipitation of wild type and i3-mutant FLAG-5-HT1A receptor and PP2A catalytic subunit with DSP chemical cross linking.

Plasmids encoding FLAG 5-HT1A receptor and mutant FLAG 5-HT1A-E340K were transiently transfected in HEK293 cells and compared to non-transfected HEK293 cells. After 48 hours, the cells were lysed and proteins cross linked with 2.5 mM dithiobis[succinimidylpropionate] (DSP) prior to harvesting. The protein levels of cell lysates were quantified and equal amounts of protein incubated with anti-FLAG M2 agarose beads. Proteins were eluted and separated on a 10% SDS-PAGE gel, and visualized by immunoblotting using anti-PP2AC antibody as indicated. Shown at left is the migration of molecular weight markers (kDa). A specific band was detected in wild type 5-HT1A-transfected cells but not mutant 5-HT1A-E340K and non-transfected cells (arrows).



5HT			+		+		+
FLAG 5-HT1A		+	+				
FLAG 5-HT1A-T149A				+	+		
FLAG 5-HT1A-T194E						+	+

Figure 14: Co-immunoprecipitation of wild type and i3-mutant FLAG-5-HT1A receptor and PP2A catalytic subunit with DSP chemical cross linking.

Plasmids encoding FLAG 5-HT1A receptor, mutant FLAG 5-HT1A-T149A receptor and mutant FLAG 5-HT1A-T149E receptor were transiently transfected in HEK293 cells and compared to non-transfected HEK293 cells. After 48 hours, the cells were treated or not with 5-HT (10 μM) for 10-15 minutes and proteins were cross-linked with 2.5 mM dithiobis[succinimidylpropionate] (DSP) prior to harvesting as indicated. The protein levels of cell lysates were quantified and equal amounts of protein incubated with anti-FLAG M2 agarose beads. Proteins were eluted and separated on a 10% SDS-PAGE gel, and visualized by immunoblotting using anti-PP2AC antibody as indicated. Shown at left is the migration of molecular weight markers (kDa). A specific band was detected in 5-HT1A-transfected cells (wild type and mutant) but not non-transfected cells (arrows).

Re-optimization of plasmid transfection and protein detection

Eventually, technical difficulties arose in detecting the FLAG-tagged receptors in either co-immunoprecipitated samples or even input lysates of these co-immunoprecipitation experiments. Troubleshooting pointed to poor transfection efficiency as transfection of 4 μg of empty GFP vector using the calcium phosphate precipitation method yielded no fluorescence cells when observed with the fluorescence microscope (Figure 15A). Switching to Lipofectamine Plus transfection reagent provided better transfection efficiency (Figures 15B & 15C) and transfection of 6 μg of empty GFP vector gave the best efficiency (Figure 15D). However, when 6 μg of FLAG-5-HT1A or 5 μg of FLAG-RASA3 (positive control) were co-transfected with 1 μg of empty GFP vector (positive transfection control) into HEK293 cells using Lipofectamine Plus reagent, no bands corresponding to the molecular weight of the 5-HT1A receptor (48 kDa) or RASA3 (~ 100 kDa) were detected using anti-FLAG M1 antibody (Figure 16), despite visualizing fluorescence under the microscope. Furthermore, no specific band for the positive control FLAG-RASA3 was detected. Therefore, we decided to label the 5-HT1A receptor with a different tag using the pTriex-4 vector that contains both His- and S-antigen tags.

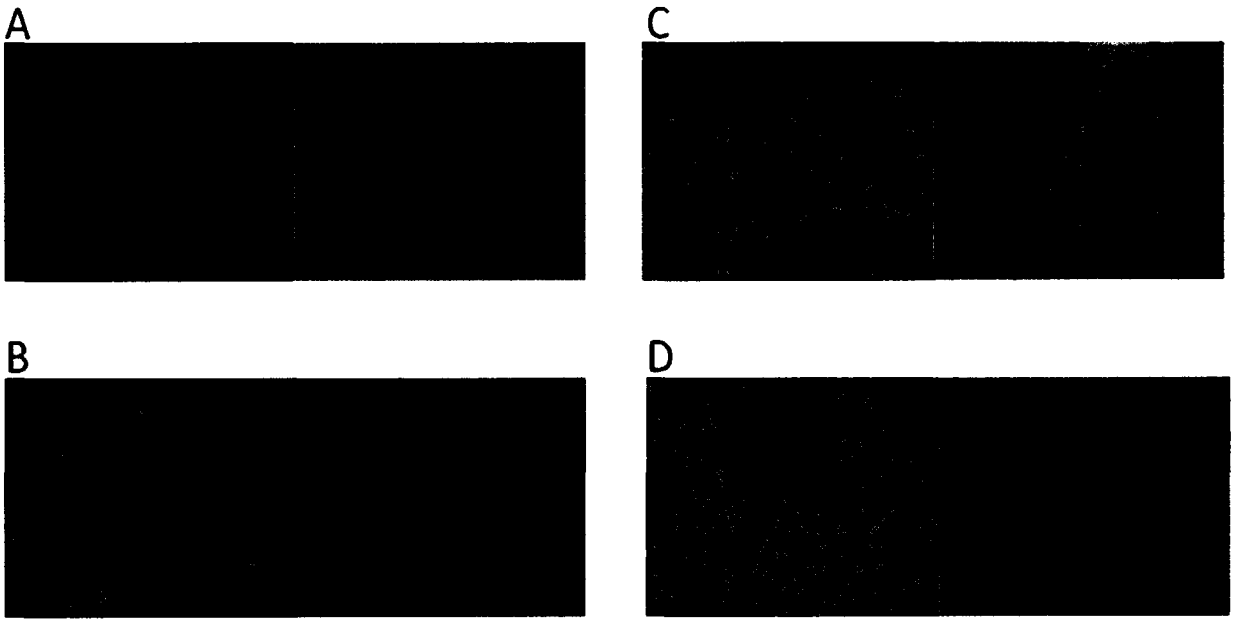


Figure 15: Re-optimization of transfection using empty pEGFP2 vector.

(A) Calcium phosphate precipitation transfection of 4 μg of GFP vector in HEK293 cells (24 hours post-transfection). (B) Lipofectamine transfection of 4 μg of GFP vector in HEK293 cells (24 hours post-transfection). (C) Lipofectamine transfection of 6 μg of GFP vector in HEK293 cells (24 hours post-transfection). (D) Lipofectamine transfection of 6 μg of GFP vector in HEK293 cells (48 hours post-transfection).

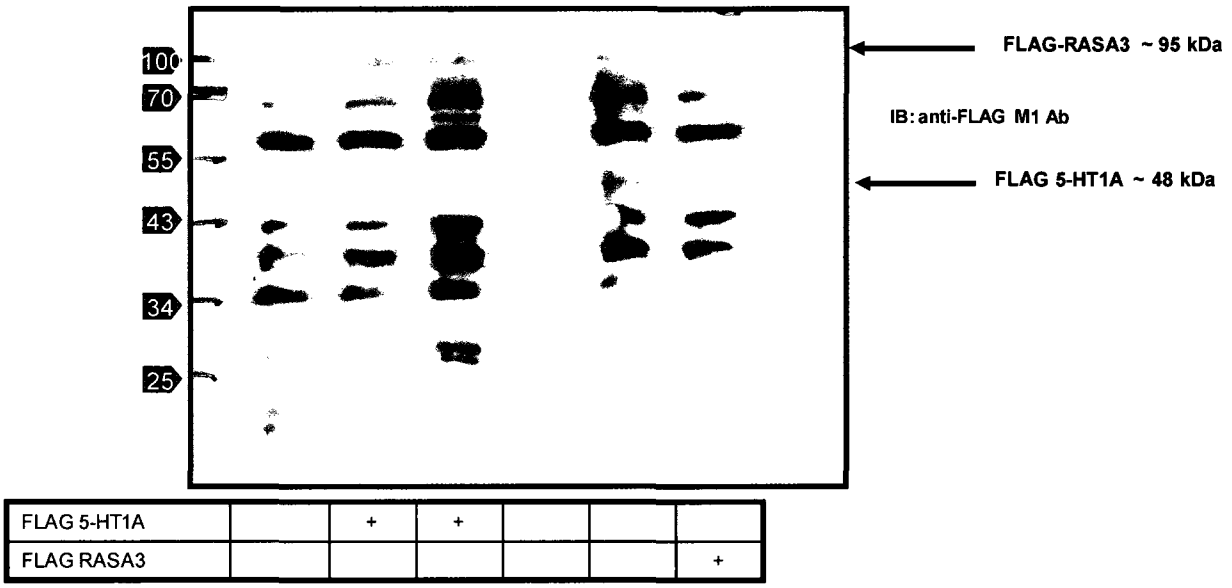


Figure 16: Re-optimization of protein expression and detection.

Plasmids encoding FLAG 5-HT1A receptor and FLAG RASA3 were transiently transfected in HEK293 cells and compared to non-transfected HEK293 cells. After 48 hours, the cells were harvested and the protein levels of cell lysates were quantified and equal amounts of protein incubated with anti-FLAG M2 agarose beads. Proteins were eluted and separated on a 10% SDS-PAGE gel, and visualized by immunoblotting using anti-FLAG M1 antibody. Shown at left is the migration of molecular weight markers (kDa). No specific bands for the 5-HT1A receptor or the positive control FLAG-RASA3 were detected.

Solubilization of His/S-tagged 5-HT1A receptors for phosphorylation and co-precipitation studies

Wild-type His/S-tagged 5-HT1A receptors were successfully transfected into HEK293 cells and detected after receptor solublization and subsequent precipitation using S-protein agarose beads (Novagen) (Figures 17, 18). His/S-tagged 5-HT1A receptor function has not been examined; however, similar N-terminal tagged FLAG tagged receptors are functional and have been characterized relative to the wild-type untagged form with respect to ligand binding, G-protein coupling and receptor number (Kushwaha, unpublished data). Western blot of the precipitates using S-protein HRP conjugate revealed a single specific band of 53 kDa was detected in cells transfected with the tagged, but not the non-tagged, 5-HT1A receptor. The greater size of the His/S-tag could account for the larger M.W. observed compared to the native non-glycosylated FLAG-tagged 5-HT1A receptor in previous figures (48 kDa). Treatment of cells with 5-HT had no effect on the amount of receptor precipitated (Figure 18), as observed previously with the FLAG-tagged 5-HT1A receptor variant.

5-HT1A i2/regulatory protein interactions

His/S-tagged 5-HT1A Co-precipitation assay

As with the FLAG tagged receptor variant, S-tagged 5-HT1A receptor and PP2AC subunit were found to co-precipitate together with and without 5-HT treatment, only in the presence of dithiobis[succinimidylpropionate] (DSP) or a similar chemical cross-linking agent. Previous experiments to show the interaction without the addition of chemical cross linker were unsuccessful, as no specific bands corresponding

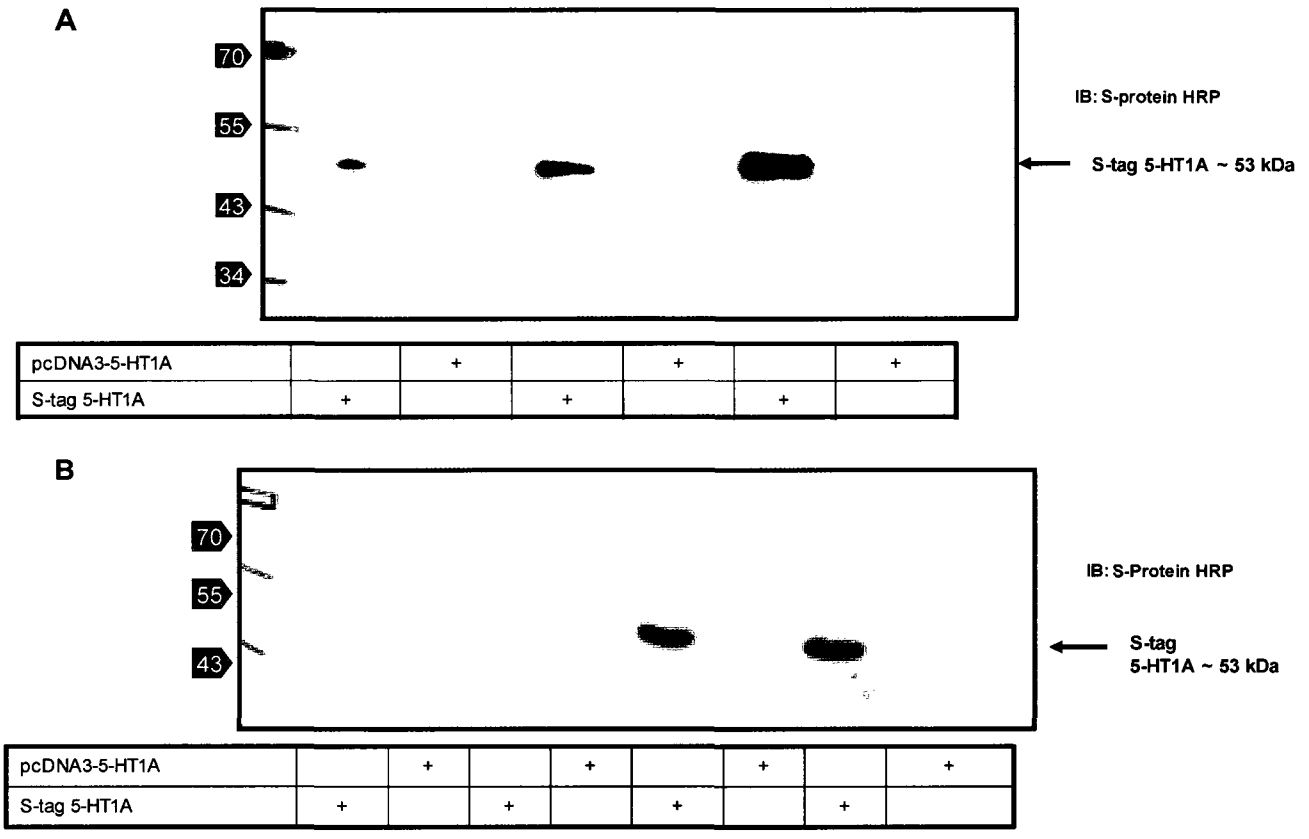


Figure 17: Solubilization of His/S-tagged 5-HT1A wild type receptors for phosphorylation and co-precipitation studies.

Plasmids encoding His/S-tag 5-HT1A receptor and untagged 5-HT1A receptor (pcDNA3-5-HT1A) were transiently transfected in HEK293 cells and tagged receptor compared to non-tagged receptor transfected HEK293 cells. (A) After 48 hours, the cells were harvested and the protein levels of cell lysates were quantified and 15, 30 and 50 μg of total protein were separated on a 10% SDS-PAGE gel, and visualized by immunoblotting using S-protein conjugated HRP. Shown at left is the migration of molecular weight markers (kDa). A specific band was detected in tagged 5-HT1A transfected cells but not untagged 5-HT1A transfected cells (arrows). (B) After 48 hours, the cells were harvested and the protein levels of cell lysates were quantified and 10, 20, 30 and 40 μg of total protein were separated on a 10% SDS-PAGE gel, and visualized by immunoblotting using S-protein conjugated HRP. Shown at left is the migration of molecular weight markers (kDa). A specific band was detected in tagged 5-HT1A transfected cells but not untagged 5-HT1A transfected cells (arrows).

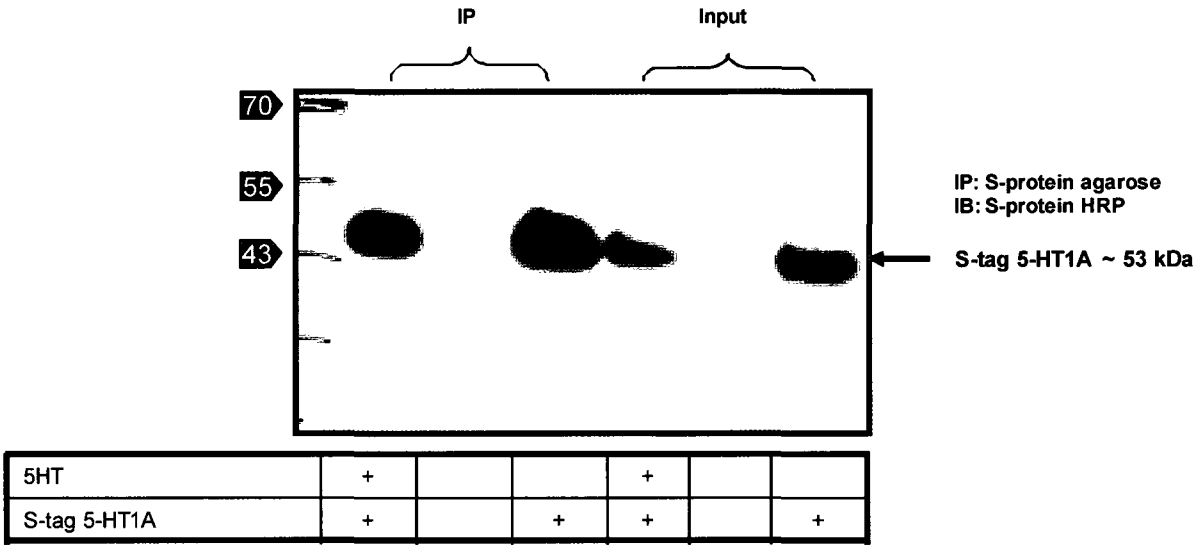


Figure 18: Solubilization and precipitation of His/S-tagged 5-HT1A receptors for phosphorylation and co-precipitation studies.

Plasmid encoding His/S-tag 5-HT1A receptor was transiently transfected in HEK293 cells and compared to non-transfected HEK293 cells. After 48 hours, the cells were treated or not with 5-HT (10 μM) for 10-15 minutes as indicated. The protein levels of cell lysates were quantified and equal amounts of protein incubated with S-protein agarose beads. Proteins were eluted and separated on a 10% SDS-PAGE gel, and visualized by immunoblotting using S-protein conjugated HRP. Shown at left is the migration of molecular weight markers (kDa). A specific band was detected in 5-HT1A-transfected cells but not non-transfected cells (arrows) and treatment with 5-HT had no effect on the level of receptor precipitated.

to PP2AC were detected in the receptor transfected cells (Figure 19). The transient or weak nature of the interaction necessitates the use of the chemical cross linker. Co-precipitation studies were performed using HEK293 cells transfected with His/S-tagged 5-HT1A receptor or His/S-tagged-Freud1, a transcription factor not known to interact with PP2A. The 5-HT1A receptor was detected using S-protein HRP conjugate as a single band of 53 kDa but upon addition of 5-HT, two bands (53 and 90-100 kDa) were detected, suggesting the formation of a dimer, while no bands were present in the non-transfected control (Figure 20). The presence of these putative dimers is likely due to the inability of dithiothreitol (DTT) to cleave the cross linker prior to separation on an SDS PAGE gel. Overall, the loading of 5-HT1A receptor protein appeared similar. For Freud-1, a major 90-100 kDa band was detected, and some smaller bands that may represent degradation products. Wild-type His/S-tagged 5-HT1A receptor and PP2AC subunit were found to co-precipitate together with more PP2A detected in the 5-HT treatment lane vs. untreated, in the presence of DSP chemical cross linker (Figure 20). Surprisingly, His/S-tagged hFreud-1 (short isoform) and the PP2AC subunit co-precipitated together in the absence of DSP cross linker, suggesting that these proteins have a strong interaction. In a separate experiment (Figure 21), a specific band for the 5-HT1A receptor (53 kDa) was detected upon transfection of the 5-HT1A receptor (without or with 5-HT treatment), but not with the vector pTriex-4. As shown in the input, similar amounts of 5-HT1A receptor and PP2A were loaded, and in the precipitation the 5-HT1A receptor and PP2AC subunit were again found to co-precipitate together in the presence of DSP chemical cross linker and 5-HT treatment enhanced this

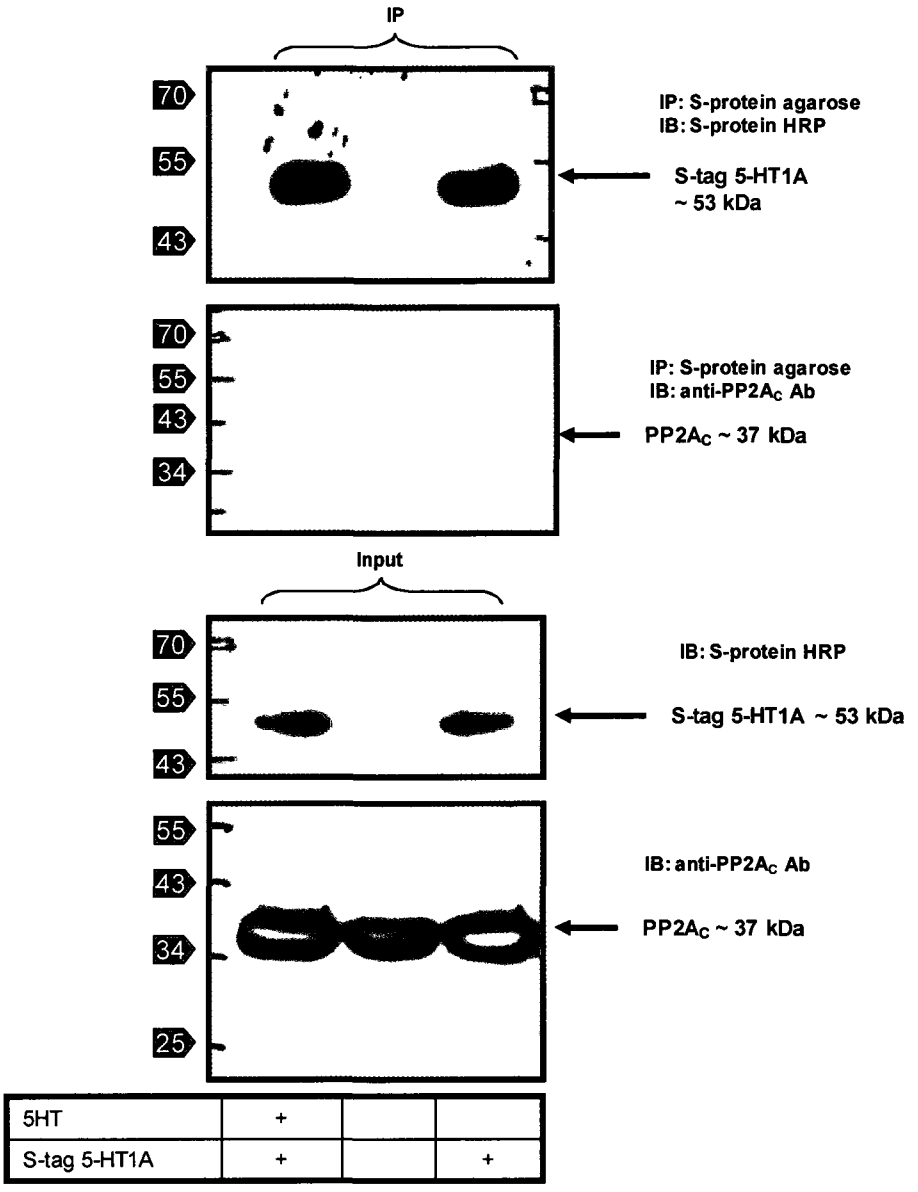


Figure 19: Co-precipitation of His/S-tagged 5-HT1A receptor and PP2A catalytic subunit without chemical cross linking.

Plasmid encoding His/S-tag 5-HT1A receptor was transiently transfected in HEK293 cells and compared to non-transfected HEK293 cells. After 48 hours, the cells were treated or not with 5-HT (10 μM) for 10-15 minutes and the protein levels of cell lysates were quantified and equal amounts of protein incubated with S-protein agarose beads. Proteins were eluted and separated on a 10% SDS-PAGE gel, and visualized by immunoblotting using S-protein conjugated HRP (above) and anti-PP2AC antibody (below). Shown at left is the migration of molecular weight markers (kDa). Without treatment with chemical cross linker no specific band for PP2AC was detected in the tagged 5-HT1A-transfected cells (arrows).

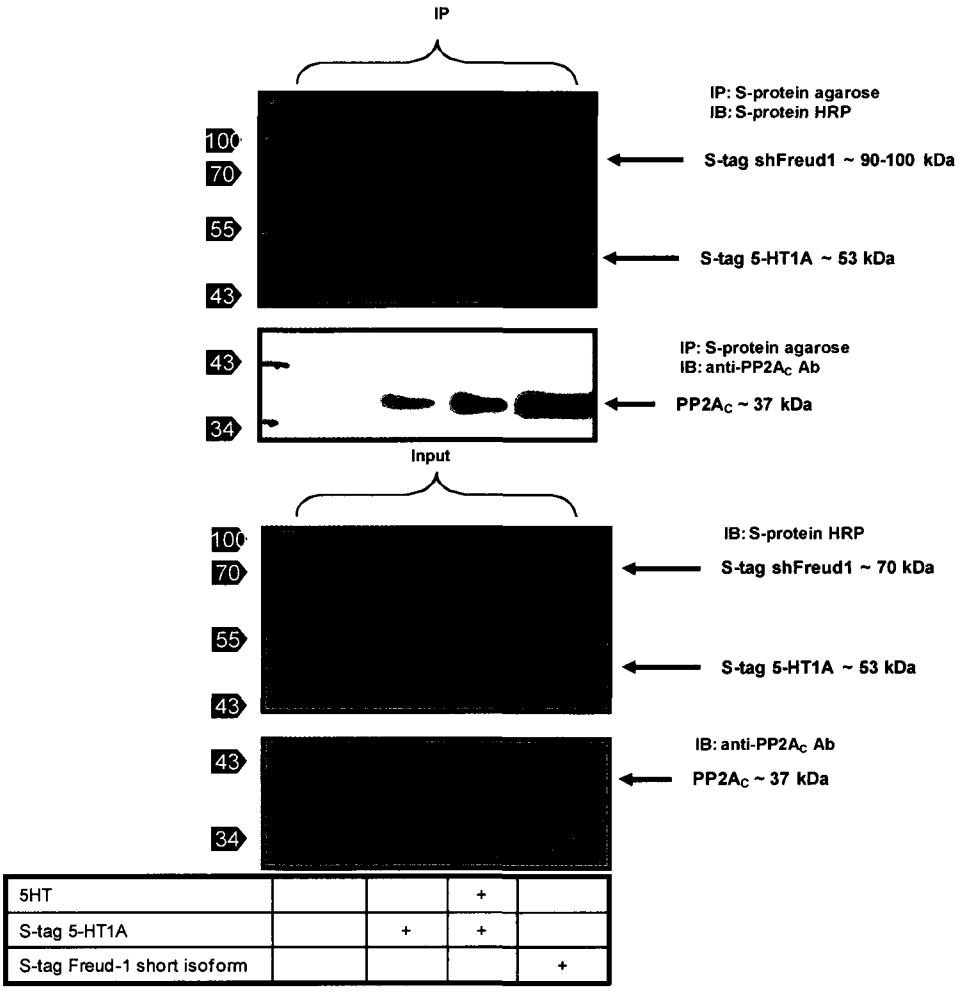


Figure 20: Co-precipitation of His/S-tagged 5-HT1A receptor and PP2A catalytic subunit with DSP chemical cross linking.

Plasmids encoding His/S-tag 5-HT1A receptor and His/S-tag hFreud-1 (short isoform) were transiently transfected in HEK293 cells and compared to non-transfected HEK293 cells. After 48 hours, the 5-HT1A transfected cells were treated or not with 5-HT (10 μM) for 10-15 minutes as indicated and proteins were cross-linked with 2.5 mM dithiobis[succinimidylpropionate] (DSP) prior to harvesting. Freud-1 transfected cells were untreated. The protein levels of cell lysates were quantified and equal amounts of protein incubated with S-protein agarose beads. Proteins were eluted and separated on a 10% SDS-PAGE gel, and visualized by immunoblotting using S-protein conjugated HRP (above) and anti-PP2AC antibody (below). Shown at left is the migration of molecular weight markers (kDa). Two specific bands for the 5-HT1A receptor were detected (53 kDa, 100 kDa), while a specific band was present for Freud-1 (100 kDa), while no bands were detected in non transfected cells. Specific bands for PP2AC was detected in 5-HT1A-transfected and Freud-1 transfected cells but not empty vector and non-transfected cells (arrows).

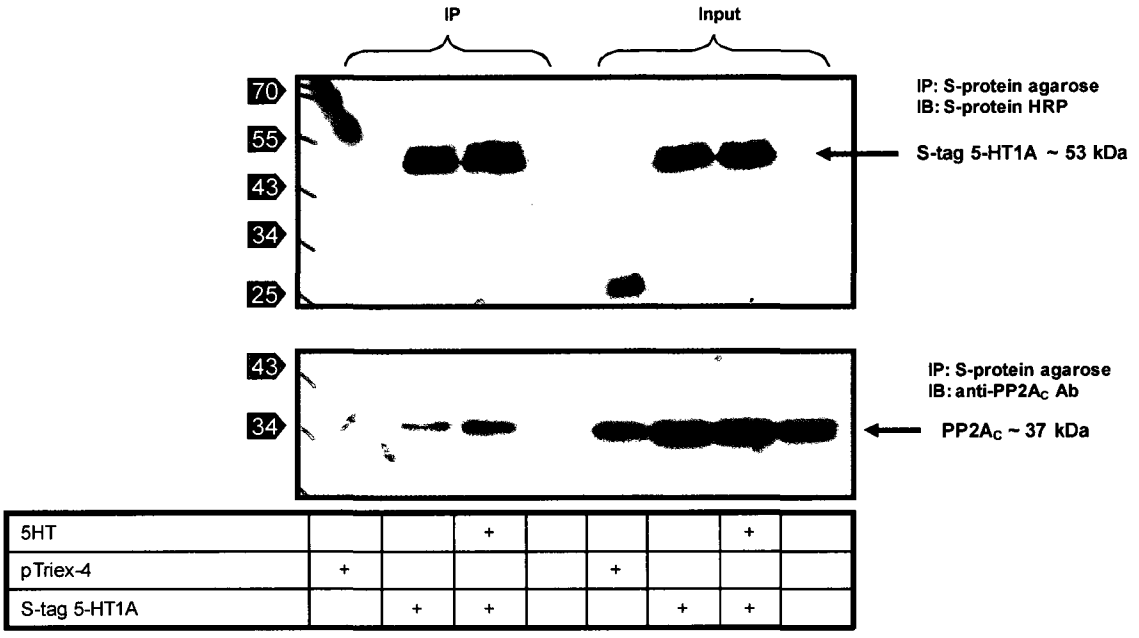


Figure 21: Co-precipitation of His/S-tagged 5-HT1A receptor and PP2A catalytic subunit with DSP chemical cross linking.

Plasmids encoding empty His/S-tag vector (pTriex-4) and His/S-tag 5-HT1A receptor were transiently transfected in HEK293 cells and compared to non-transfected HEK293 cells. After 48 hours, the cells were treated or not with 5-HT (10 μM) for 10-15 minutes and proteins were cross-linked with 2.5 mM dithiobis[succinimidylpropionate] (DSP) prior to harvesting. The protein levels of cell lysates were quantified and equal amounts of protein incubated with S-protein agarose beads. Proteins were eluted and separated on a 10% SDS-PAGE gel, and visualized by immunoblotting using S-protein conjugated HRP (above) and anti-PP2AC antibody (below). Shown at left is the migration of molecular weight markers (kDa). A specific band for PP2AC was detected in 5-HT1A-transfected cells but not empty vector and non-transfected cells (arrows).

association (Figure 21). The empty His/S-tagged vector pTriex-4 (lane 1) and non-transfected (lane 4) cell extracts produced no co-precipitation of PP2AC (Figure 22), despite treatment with DSP cross linker, suggesting the interaction between the 5-HT1A receptor and the phosphatase subunit is specific. The results were reproducible, as co-precipitation of the PP2AC subunit is observed in the presence of His/S-tagged 5-HT1A receptor positive transfected cells with and without 5-HT treatment in the presence of a second chemical cross linker, dimethyl 3,3′-dithiobispropionimidate (DTBP) (Figure 22). The empty His/S-tagged vector pTriex-4 (lane 1) and non-transfected (lane 4) and cell extracts produced no co-precipitation of PP2AC (Figure 22) despite treatment with DTBP cross linker, again suggesting a specific interaction between the receptor and phosphatase. Interestingly, the agonist specific enhancement of the interaction was not seen using DTBP cross linker. This could be due to the inability of DTBP to permeate the cell membrane as it is a more hydrophilic molecule relative to DSP.

A third and fourth replicate experiment utilizing DSP chemical cross linker showed again that the receptor and the phosphatase catalytic subunit co-precipitate together with and without 5-HT treatment (Figure 23, 24). The data for PP2AC co-precipitated with 5-HT1A receptor from four independent DSP cross linking experiments were quantified by densitometry and normalized to control untreated samples and demonstrated a statistically significant 2-fold increase in the level of 5-HT1A-PP2A interaction (Figure 25). The finding that the interaction between the receptor and the phosphatase catalytic subunit appears to be enhanced following 5-HT treatment provides evidence that the interaction may play a role in receptor resensitization following

homologous desensitization to enhance dephosphorylation of the GRK- or PKC-phosphorylated receptor.

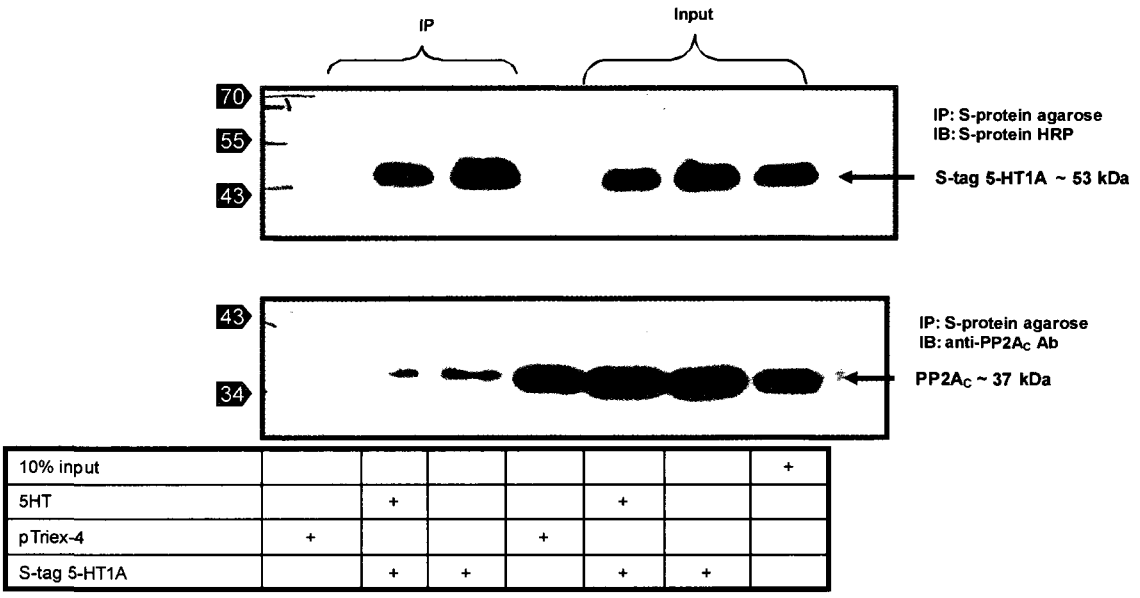


Figure 22: Co-precipitation of His/S-tagged 5-HT1A receptor and PP2A catalytic subunit with DTBP chemical cross linking.

Plasmids encoding empty His/S-tag vector (pTriex-4) and His/S-tag 5-HT1A receptor were transiently transfected in HEK293 cells and compared to non-transfected HEK293 cells. After 48 hours, the cells were treated or not with 5-HT (10 μM) for 10-15 minutes and proteins were cross-linked with 5.0 mM dimethyl 3,3′-dithiobispropionimidate (DTBP) for 30 minutes on ice prior to harvesting. The protein levels of cell lysates were quantified and equal amounts of protein incubated with S-protein agarose beads. Proteins were eluted and separated on a 10% SDS-PAGE gel, and visualized by immunoblotting using S-protein conjugated HRP (above) and anti-PP2AC antibody (below). Shown at left is the migration of molecular weight markers (kDa). A specific band for PP2AC was detected in 5-HT1A-transfected cells but not empty vector and non-transfected cells (arrows).

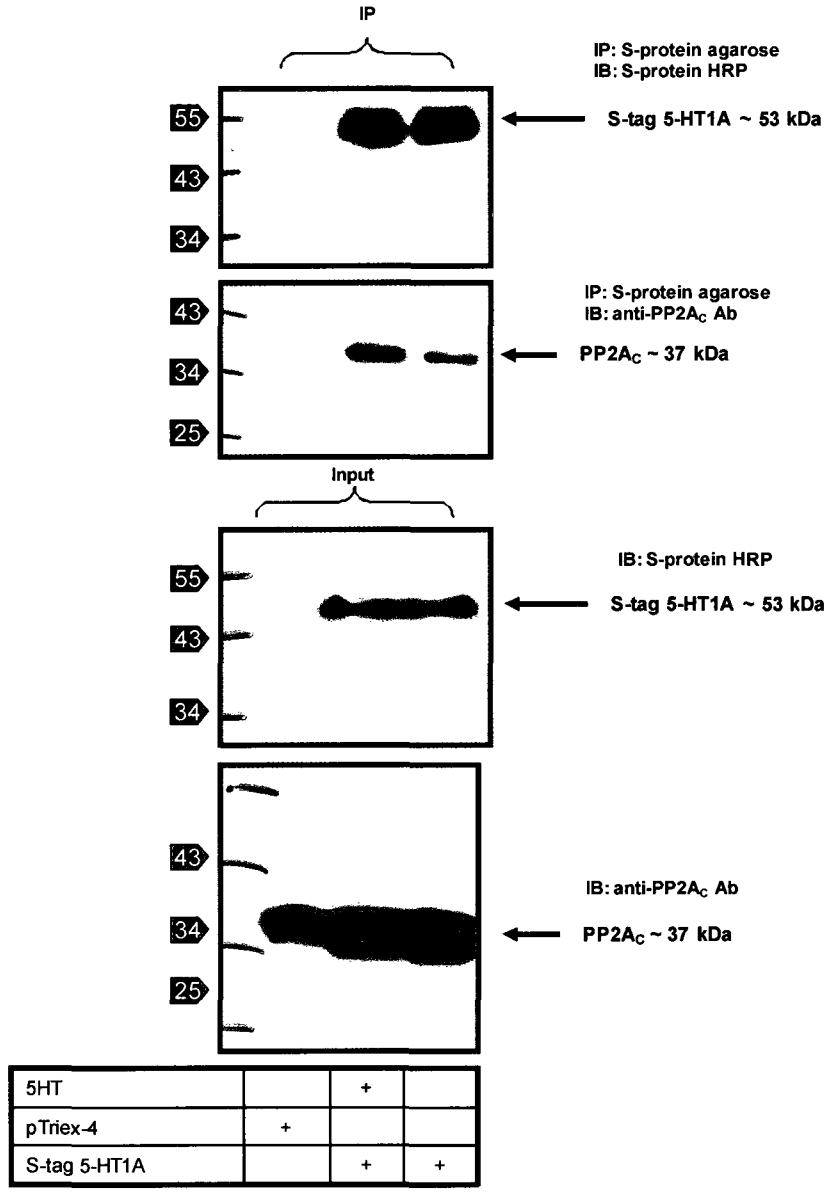


Figure 23: Co-precipitation of His/S-tagged 5-HT1A receptor and PP2A catalytic subunit with DSP chemical cross linking.

Plasmids encoding empty His/S-tag vector (pTriex-4) and His/S-tag 5-HT1A receptor were transiently transfected in HEK293 cells and compared to non-transfected HEK293 cells. After 48 hours, the cells were treated or not with 5-HT (10 μM) for 10-15 minutes and proteins were cross-linked with 2.5 mM dithiobis[succinimidylpropionate] (DSP) prior to harvesting. The protein levels of cell lysates were quantified and equal amounts of protein incubated with S-protein agarose beads. Proteins were eluted and separated on a 10% SDS-PAGE gel, and visualized by immunoblotting using S-protein conjugated HRP (above) and anti-PP2AC antibody (below). Shown at left is the migration of molecular weight markers (kDa). A specific band for PP2AC was detected in 5-HT1A-transfected cells but not empty vector and non-transfected cells (arrows).

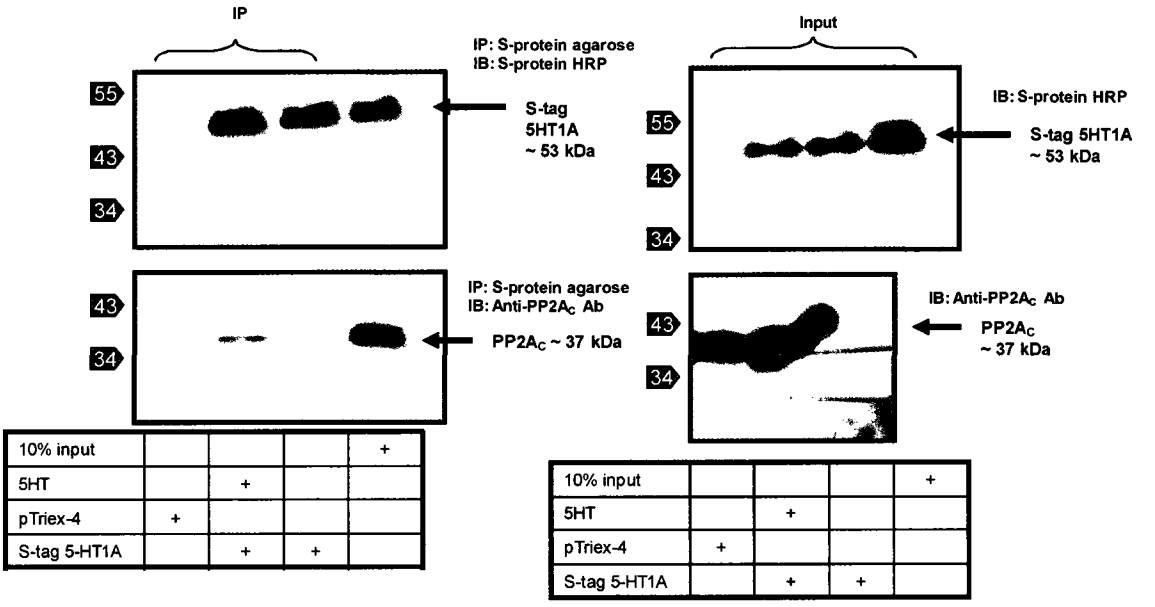


Figure 24: Co-precipitation of His/S-tagged 5-HT1A receptor and PP2A catalytic subunit with DSP chemical cross linking.

Plasmids encoding empty His/S-tag vector (pTriex-4) and His/S-tag 5-HT1A receptor were transiently transfected in HEK293 cells and compared to non-transfected HEK293 cells. After 48 hours, the cells were treated or not with 5-HT (10 μM) for 10-15 minutes and proteins were cross-linked with 2.5 mM dithiobis[succinimidylpropionate] (DSP) prior to harvesting. The protein levels of cell lysates were quantified and equal amounts of protein incubated with S-protein agarose beads. Proteins were eluted and separated on a 10% SDS-PAGE gel, and visualized by immunoblotting using S-protein conjugated HRP (above) and anti-PP2AC antibody (below). Shown at left is the migration of molecular weight markers (kDa). A specific band for PP2AC was detected in 5-HT1A-transfected cells but not empty vector and non-transfected cells (arrows).

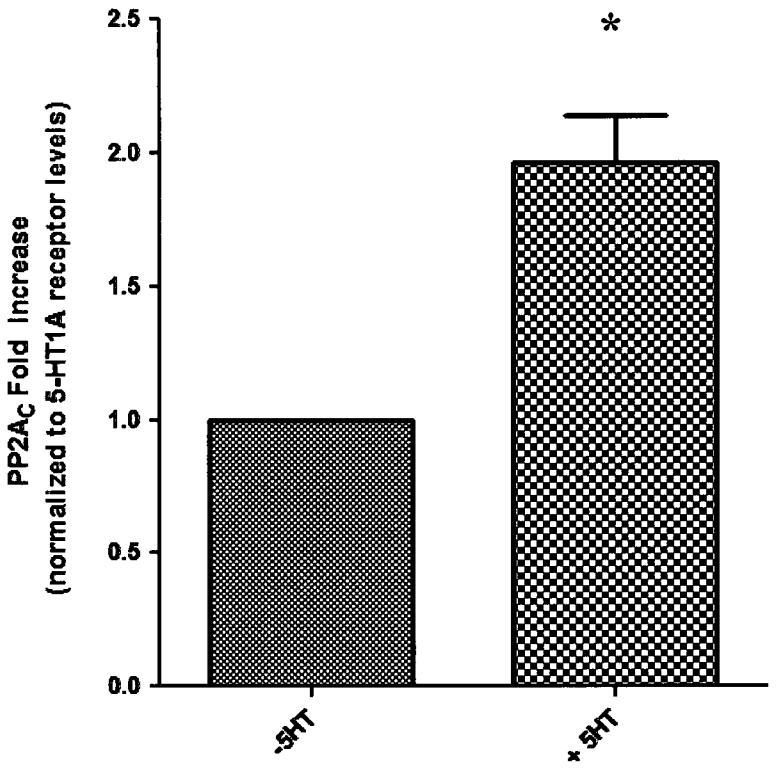


Figure 25: Enhancement of the interaction between PP2AC and the 5-HT1A receptor following treatment with 5-HT.

5-HT stimulation enhances the interaction between the protein phosphatase 2A catalytic subunit and the 5-HT1A receptor in transiently transfected HEK293 cells. Cells were treated with 10μM 5-HT or control (ddH_2O) for 10-15 minutes prior to assay. Data was from four independent co-precipitation experiments utilizing DSP chemical cross linker. Immunoblots were quantified digitally using Adobe Photoshop and PP2AC levels were normalized first to amount of 5-HT1A receptor protein precipitated and were then calculated as fold of control treatment and expressed as mean ± SEM for +5-HT. *$P<0.05$, versus control.

DISCUSSION

Novel Interaction between 5-HT1A Receptor and Protein Phosphatase catalytic subunit

Substantial evidence has implicated regulation of 5-HT1A receptor expression in association to mental illnesses, including anxiety and depression (Charney et al., 1990; Mann et al., 2001; Murphy et al., 1999; Pineyro and Blier, 1999). Rapid receptor desensitization cannot account for the three week latency in antidepressant treatment of depressed patients and, hence, other regulatory mechanisms must be occurring. The novel interaction between the 5-HT1A receptor and PP2AC identified here could account for one of these mechanisms. PP2A could act to dephosphorylate and allow resensitization of internalized agonist-stimulated 5-HT1A receptors. PP2A and PP2A-like phosphatases have previously been shown to interact and dephosphorylate several different GPCRs. Treatment of stable CXCR2 transfected cells with CXCL8 agonist increases the association of CXCR2 with PP2A. Stimulation of these cells with CXCL8 results in robust phosphorylation of the receptor and this is reversed after removal of the agonist after one hour. OA treatment was shown to inhibit this dephosphorylation of the receptor in a concentration dependent manner and even increased basal phosphorylation levels of CXCR2 in the absence of CXCL8 stimulation (Fan et al., 2001). In addition studies of the CXCR2 receptor showed inhibition of CXCR2 internalization has been shown to block receptor dephosphorylation (Yang et al., 1999). These results suggest that CXCR2 receptor internalization is required for association of the receptor with PP2A. The interaction found here suggests that PP2A could potentially dephosphorylate second messenger/GRK hyperphosphorylated-

internalized receptor to promote dissociation of βarr and recycle the receptor to the membrane (resensitization). The β2-AR receptor also undergoes dephosphorylation by both PP2A and PP2B, which form complexes with the receptor and inhibition of either phosphatase prevents β2-AR receptor resensitization (Pitcher et al., 1995; Shih et al., 1999). Further evidence supporting the argument that PP2A is responsible for dephosphorylation of internalized receptors is that β2-AR receptors in vesicular fractions are less phosphorylated relative to receptors found in the plasma membrane (Sibley et al., 1986). As well, suppression of receptor internalization through blocking agents or mutation of internalization motifs impairs receptor dephosphorylation and subsequent resensitization (Yu et al., 1993; Barak et al., 1994).

PP2A was shown to interact with the CB2 receptor as OA treatment inhibited SR 144528-induced dephosphorylation of CB2 receptor. In contrast inhibition of PP2B with cyclosporin A failed to alter dephosphorylation of the receptor. A strong inhibition was also observed using a potent specific PP2A inhibitor calyculin A, suggesting that PP2A is the major phosphatase responsible for dephosphorylation of CB2 (Bouaboula et al., 1999). Yeast two hybrid studies indicate PP2AC and the 5-HT1A receptor interact directly; however, it is possible that the two proteins are part of a large scaffolding complex.

Novel Interaction between Freud-1 transcription factor and Protein Phosphatase catalytic subunit

Our lab has previously identified and characterized a novel transcriptional repressor Freud-1 and its function to repress 5-HT1A receptor expression (Ou et al., 2003; Lemonde et al., 2004; Rogaeva and Albert, 2007). Calcium-regulated transcription

factor, Freud-1 has been shown to repress 5-HT1A receptor expression in neurons (Ou et al., 2003). Recent studies have shown that phosphorylation of Freud-1 by calcium/calmodulin-dependent protein kinase IV (CaMKIV) inactivates the repressor function of Freud-1 (Albert et al., 2009). Expression of the Thr/Ser specific CaMKIV is localized in the brain to the thymus, T-lymphocytes, spleen and male germ cells (Ohmstede et al., 1989; Frangakis et al., 1991; Means et al., 1991). CaMKIV induces serine and threonine phosphorylation at sites Ser644 and Thr780 of Freud-1 leading to its inactivation. The novel interaction identified between PP2AC and the Freud-1 suggests that the phosphatase is responsible for dephosphorylation of inactive phosphorylated Freud-1 to re-activate it. Re-activation of Freud-1 would allow for the repression of 5-HT1A receptor expression. Previous studies have shown that PP2A and CaMKIV exist in a stable complex and PP2A can dephosphorylate CaMKIV on Thr200 to negatively regulate CaMKIV transcriptional function (Chow et al., 2005) and it is possible that CaMKIV, PP2A and Freud-1 exist in a complex to regulate 5-HT1A receptor expression.

5-HT stimulation enhances the PP2AC and the 5-HT1A receptor interaction

Agonist stimulation of 5-HT1A receptor transfected HEK293 cells had a two fold increase in the amount of PP2AC co-precipitated with the receptor. These findings suggest that the 5-HT stimulation promotes recruitment of PP2A to the receptor above basal levels and are consistent with our hypothesis that the phosphatase is responsible for dephosphorylation of the receptor. Agonist treatment would lead to homologous desensitization of the receptor and GRK-induced phosphorylation and subsequent βarr-mediated internalization (Riad et al., 2001). Furthermore, we would expect

hyperphosphorylated internalized receptor or plasma membrane associated receptor to recruit more PP2A relative to non-stimulated receptor.

Future Studies

Pull down and in vitro phosphorylation

Pull-down studies will verify the 5-HT1A-i2-PP2AC interaction. The following experiments will indicate a direct interaction in vitro. Pull-down assays using GST-tagged short and long-i2 recombinant proteins bacterially expressed His/S-tagged PP2A, will verify the 5-HT1A-i2/PP2A interaction. These experiments will test the interaction in vitro, indicating a direct interaction. The effect of PKC and PP2A will be examined in vitro by phosphorylation of long-i2 using purified PKC subtypes shown to phosphorylate the receptor and by performing pull down with His, GST or S-antigen columns. Phosphorylation will be monitored by 32P incorporation while PP2A activity measured by loss of ^{32}P-i2 loop. Phosphorylation at the T149 site will be quantified by Western blot using anti-phosphoT149.

Bioluminescence Resonance Energy Transfer (BRET)

BRET studies will be used to verify the interaction between the phosphatase and the 5-HT1A receptor. BRET2 involves the expression of recombinant green fluorescent protein 2 (GFP2) and renilla luciferase (Rluc) tagged proteins. An interaction between the two proteins is verified through the transfer of energy from the donor (Rluc tagged protein) to the acceptor (GFP2-tagged protein). These studies can be done in the presence or absence of PP2A inhibitors (okadaic acid, calyculin A) to block the interaction or with selective i2 and i3 mutants known to disrupt the i2 loop of the receptor

thus inhibiting binding of the phosphatase. As well, pepducin (a synthetic lipidated peptide with the i2 sequence of the receptor) or HSV-TAT i2 peptides could be used to block the interaction selectively; however, since i2 also interacts with Gβγ subunit, this might be a complication.

Receptor biotinylation

The role of PP2A in 5-HT1A receptor internalization will be examined through receptor biotinylation studies. Cells expressing 5-HT1A receptor will be treated with 5-HT for 10 minutes and biotinylated at certain time courses to examine re-surfacing of 5-HT1A receptors. This will be done with and without pre-treatment with non-selective (okadaic acid) and selective (fostriecin or calyculin A) protein phosphatase inhibitors to examine whether PP2A plays a role in recycling of internalized receptor back to the membrane.

Function of the 5-HT1A/PP2A interaction

The function of the 5-HT1A/PP2A interactions will be addressed in the following manner. As PKC activation phosphorylates the 5-HT1A-Ci2 Thr149 site, cells expressing 5-HT1A receptor will be treated with non-selective (okadaic acid) and selective (fostriecin or calyculin A) protein phosphatase inhibitors and basal, TPA- or agonist-mediated T149 or 5-HT1A-Ser phosphorylation detected by Western blot. Using wild-type and selected 5-HT1A-Ci2 mutants in interaction, phosphorylation and signaling studies we will address the importance of the i2 domain in PKC and PP2A action. Through transfection of cells with siRNA of PP2A, the function of these interactions in 5-HT1A receptor localization, signaling or desensitization will be examined. It should be

noted that anti-depressants display a three-week latency period believed to be the result of desensitization of the 5-HT1A autoreceptors. These experiments will test the hypothesis that PP2A interaction with 5-HT1A-i2 domain might target dephosphorylation of i2 or i3 PKC or GRK sites to re-sensitize the 5-HT1A receptor. Receptor biotinylation studies could be used to measure the effect of PP2A dephosphorylation of the receptor on receptor resensitization in the presence and absence of PP2A inhibitors.

CONCLUSIONS

In summary, these studies provide new insight into the role of protein phosphatase-receptor interactions in regulating 5-HT1A receptor desensitization and resensitization. The data obtained identified a novel interaction between the 5-HT1A receptor and the PP2A catalytic subunit, and is likely to regulate receptor phosphorylation and sensitization. The interaction appears relatively transient or weak and to see the interaction within the cell, chemical cross-linking agents were necessary to prevent the transient or weak interaction from dissociating. However, similar transient or weak interactions have been observed between receptors and G-proteins requiring cross linking for observation observe by immunoprecipitation. The finding that the interaction is enhanced following 5-HT treatment suggests that this interaction may play a role in homologous desensitization to enhance dephosphorylation of the GRK-phosphorylated receptor. In addition, the 5-HT1A-PP2A interaction may also affect heterologous desensitization by PKC. The experiments outlined above will provide additional evidence to strengthen the experimental results shown here to verify the interaction and identify the function of the interaction.

REFERENCES

Abdel-Baset, H., Bozovic, V., Szyf, M., and Albert, P. R. (1992) Conditional transformation mediated via a pertussis toxin-sensitive receptor signalling pathway. *Molecular endocrinology (Baltimore, Md,* **6**(5), p. 730-740.

Adell, A., and Artigas, F. (1991) Differential effects of clomipramine given locally or systemically on extracellular 5-hydroxytryptamine in raphe nuclei and frontal cortex. An in vivo brain microdialysis study. *Naunyn-Schmiedeberg's archives of pharmacology,* **343**(3), p. 237-244.

Albert, P. R. (1994) Heterologous expression of G protein-linked receptors in pituitary and fibroblast cell lines. *Vitamins and hormones,* **48**, p. 59-109.

Albert, P. R. (2002) G protein preferences for dopamine D2 inhibition of prolactin secretion and DNA synthesis in GH4 pituitary cells. *Molecular endocrinology (Baltimore, Md,* **16**(8), p. 1903-1911.

Albert, P.R., Galaraga, K., Rogaeva, A. (2009) Calcium/calmodulin-dependent protein kinase IV phosphorylation of Freud-1/CC2D1A de-represses the 5-HT1A receptor gene. Society for Neuroscience, Chicago, IL.

Albert, P. R., Lembo, P., Storring, J. M., Charest, A., and Saucier, C. (1996) The 5-HT1A receptor: signaling, desensitization, and gene transcription. *Neuropsychopharmacology,* **14**(1), p. 19-25.

Albert, P. R., and Lemonde, S. (2004) 5-HT1A receptors, gene repression, and depression: guilt by association. *Neuroscientist,* **10**(6), p. 575-593.

Albert, P. R., and Morris, S. J. (1994) Antisense knockouts: molecular scalpels for the dissection of signal transduction. *Trends in pharmacological sciences,* **15**(7), p. 250-254.

Albert, P. R., Morris, S. J., Ghahremani, M. H., Storring, J. M., and Lembo, P. M. (1998) A putative alpha-helical G beta gamma-coupling domain in the second intracellular loop of the 5-HT1A receptor. *Annals of the New York Academy of Sciences,* **861**, p. 146-161.

Albert, P. R., and Robillard, L. (2002) G protein specificity: traffic direction required. *Cellular signalling,* **14**(5), p. 407-418.

Albert, P. R., Sajedi, N., Lemonde, S., and Ghahremani, M. H. (1999) Constitutive G(i2)-dependent activation of adenylyl cyclase type II by the 5-HT1A receptor. Inhibition by anxiolytic partial agonists. *The Journal of biological chemistry,* **274**(50), p. 35469-35474.

Albert, P. R., and Tiberi, M. (2001) Receptor signaling and structure: insights from serotonin-1 receptors. *Trends in endocrinology and metabolism: TEM,* **12**(10), p. 453-460.

Albert, P. R., Zhou, Q. Y., Van Tol, H. H., Bunzow, J. R., and Civelli, O. (1990) Cloning, functional expression, and mRNA tissue distribution of the rat 5-hydroxytryptamine1A receptor gene. *The Journal of biological chemistry,* **265**(10), p. 5825-5832.

Andrade, R., Malenka, R. C., and Nicoll, R. A. (1986) A G protein couples serotonin and GABAB receptors to the same channels in hippocampus. *Science (New York, N.Y,* **234**(4781), p. 1261-1265.

Arino, J., Woon, C. W., Brautigan, D. L., Miller, T. B., Jr., and Johnson, G. L. (1988) Human liver phosphatase 2A: cDNA and amino acid sequence of two catalytic subunit isotypes. *Proceedings of the National Academy of Sciences of the United States of America,* **85**(12), p. 4252-4256.

Artigas, F., Celada, P., Laruelle, M., and Adell, A. (2001) How does pindolol improve antidepressant action? *Trends in pharmacological sciences,* **22**(5), p. 224-228.

Artigas, F., Romero, L., de Montigny, C., and Blier, P. (1996) Acceleration of the effect of selected antidepressant drugs in major depression by 5-HT1A antagonists. *Trends in neurosciences,* **19**(9), p. 378-383.

Baharians, Z., and Schonthal, A. H. (1998) Autoregulation of protein phosphatase type 2A expression. *The Journal of biological chemistry,* **273**(30), p. 19019-19024.

Baker, L. P., Nielsen, M. D., Impey, S., Hacker, B. M., Poser, S. W., Chan, M. Y., and Storm, D. R. (1999) Regulation and immunohistochemical localization of betagamma-stimulated adenylyl cyclases in mouse hippocampus. *J Neurosci,* **19**(1), p. 180-192.

Banihashemi, B., and Albert, P. R. (2002) Dopamine-D2S receptor inhibition of calcium influx, adenylyl cyclase, and mitogen-activated protein kinase in pituitary cells: distinct Galpha and Gbetagamma requirements. *Molecular endocrinology (Baltimore, Md,* **16**(10), p. 2393-2404.

Barak, L. S., Tiberi, M., Freedman, N. J., Kwatra, M. M., Lefkowitz, R. J., and Caron, M. G. (1994) A highly conserved tyrosine residue in G protein-coupled receptors is required for agonist-mediated beta 2-adrenergic receptor sequestration. *The Journal of biological chemistry,* **269**(4), p. 2790-2795.

Bayliss, D. A., Li, Y. W., and Talley, E. M. (1997) Effects of serotonin on caudal raphe neurons: inhibition of N- and P/Q-type calcium channels and the afterhyperpolarization. *Journal of neurophysiology,* **77**(3), p. 1362-1374.

Beaulieu, J. M., Sotnikova, T. D., Marion, S., Lefkowitz, R. J., Gainetdinov, R. R., and Caron, M. G. (2005) An Akt/beta-arrestin 2/PP2A signaling complex mediates dopaminergic neurotransmission and behavior. *Cell,* **122**(2), p. 261-273.

Blier, P., and de Montigny, C. (1994) Current advances and trends in the treatment of depression. *Trends in pharmacological sciences,* **15**(7), p. 220-226.

Blier, P., and de Montigny, C. (1999) Serotonin and drug-induced therapeutic responses in major depression, obsessive-compulsive and panic disorders. *Neuropsychopharmacology,* **21**(2 Suppl), p. 91S-98S.

Bockaert, J., Claeysen, S., Becamel, C., Pinloche, S., and Dumuis, A. (2002) G protein-coupled receptors: dominant players in cell-cell communication. *International review of cytology,* **212**, p. 63-132.

Booij, L., Van der Does, A. J., Haffmans, P. M., Riedel, W. J., Fekkes, D., and Blom, M. J. (2005) The effects of high-dose and low-dose tryptophan depletion on mood and cognitive functions of remitted depressed patients. *Journal of psychopharmacology (Oxford, England),* **19**(3), p. 267-275.

Bortolozzi, A., Amargos-Bosch, M., Toth, M., Artigas, F., and Adell, A. (2004) In vivo efflux of serotonin in the dorsal raphe nucleus of 5-HT1A receptor knockout mice. *Journal of neurochemistry,* **88**(6), p. 1373-1379.

Bouaboula, M., Dussossoy, D., and Casellas, P. (1999) Regulation of peripheral cannabinoid receptor CB2 phosphorylation by the inverse agonist SR 144528.

Implications for receptor biological responses. *The Journal of biological chemistry,* **274**(29), p. 20397-20405.

Bourne, H. R. (1997) How receptors talk to trimeric G proteins. *Current opinion in cell biology,* **9**(2), p. 134-142.

Buhot, M. C. (1997) Serotonin receptors in cognitive behaviors. *Current opinion in neurobiology,* **7**(2), p. 243-254.

Burstein, E. S., Spalding, T. A., and Brann, M. R. (1996) Amino acid side chains that define muscarinic receptor/G-protein coupling. Studies of the third intracellular loop. *The Journal of biological chemistry,* **271**(6), p. 2882-2885.

Burstein, E. S., Spalding, T. A., and Brann, M. R. (1998) The second intracellular loop of the m5 muscarinic receptor is the switch which enables G-protein coupling. *The Journal of biological chemistry,* **273**(38), p. 24322-24327.

Cadogan, A. K., Kendall, D. A., and Marsden, C. A. (1994) Serotonin 5-HT1A receptor activation increases cyclic AMP formation in the rat hippocampus in vivo. *Journal of neurochemistry,* **62**(5), p. 1816-1821.

Cai, X., Gu, Z., Zhong, P., Ren, Y., and Yan, Z. (2002) Serotonin 5-HT1A receptors regulate AMPA receptor channels through inhibiting Ca2+/calmodulin-dependent kinase II in prefrontal cortical pyramidal neurons. *The Journal of biological chemistry,* **277**(39), p. 36553-36562.

Chalmers, D. T., and Watson, S. J. (1991) Comparative anatomical distribution of 5-HT1A receptor mRNA and 5-HT1A binding in rat brain--a combined in situ hybridisation/in vitro receptor autoradiographic study. *Brain research,* **561**(1), p. 51-60.

Chan, S. F., and Sucher, N. J. (2001) An NMDA receptor signaling complex with protein phosphatase 2A. *J Neurosci,* **21**(20), p. 7985-7992.

Charney, D. S., Krystal, J. H., Delgado, P. L., and Heninger, G. R. (1990) Serotonin-specific drugs for anxiety and depressive disorders. *Annual review of medicine,* **41**, p. 437-446.

Chen, J., DeVivo, M., Dingus, J., Harry, A., Li, J., Sui, J., Carty, D. J., Blank, J. L., Exton, J. H., Stoffel, R. H., and et al. (1995) A region of adenylyl cyclase 2 critical for regulation by G protein beta gamma subunits. *Science (New York, N.Y,* **268**(5214), p. 1166-1169.

Chen, Y., and Penington, N. J. (1996) Differential effects of protein kinase C activation on 5-HT1A receptor coupling to Ca2+ and K+ currents in rat serotonergic neurones. *The Journal of physiology,* **496 (Pt 1)**, p. 129-137.

Chow, F. A., Anderson, K. A., Noeldner, P. K., and Means, A. R. (2005) The autonomous activity of calcium/calmodulin-dependent protein kinase IV is required for its role in transcription. *The Journal of biological chemistry,* **280**(21), p. 20530-20538.

Clapham, D. E., and Neer, E. J. (1997) G protein beta gamma subunits. *Annual review of pharmacology and toxicology,* **37**, p. 167-203.

Colino, A., and Halliwell, J. V. (1987) Differential modulation of three separate K-conductances in hippocampal CA1 neurons by serotonin. *Nature,* **328**(6125), p. 73-77.

Daaka, Y., Luttrell, L. M., Ahn, S., Della Rocca, G. J., Ferguson, S. S., Caron, M. G., and Lefkowitz, R. J. (1998) Essential role for G protein-coupled receptor endocytosis

in the activation of mitogen-activated protein kinase. *The Journal of biological chemistry,* **273**(2), p. 685-688.

De Vivo, M., and Maayani, S. (1990) Stimulation and inhibition of adenylyl cyclase by distinct 5-hydroxytryptamine receptors. *Biochemical pharmacology,* **40**(7), p. 1551-1558.

Doris, A., Ebmeier, K., and Shajahan, P. (1999) Depressive illness. *Lancet,* **354**(9187), p. 1369-1375.

Drevets, W. C., Frank, E., Price, J. C., Kupfer, D. J., Greer, P. J., and Mathis, C. (2000) Serotonin type-1A receptor imaging in depression. *Nuclear medicine and biology,* **27**(5), p. 499-507.

Ellenbogen, M. A., Young, S. N., Dean, P., Palmour, R. M., and Benkelfat, C. (1996) Mood response to acute tryptophan depletion in healthy volunteers: sex differences and temporal stability. *Neuropsychopharmacology,* **15**(5), p. 465-474.

Erspamer, V., and Asero, B. (1952) Identification of enteramine, the specific hormone of the enterochromaffin cell system, as 5-hydroxytryptamine. *Nature,* **169**(4306), p. 800-801.

Erspamer, V., and Vialli, M. (1937) Ricerche sul secreto delle cellue enterocromaffini. *Boll d Soc Med-chir Pavia,* **51**, p. 357-363.

Evrard, A., Laporte, A. M., Chastanet, M., Hen, R., Hamon, M., and Adrien, J. (1999) 5-HT1A and 5-HT1B receptors control the firing of serotoninergic neurons in the dorsal raphe nucleus of the mouse: studies in 5-HT1B knock-out mice. *The European journal of neuroscience,* **11**(11), p. 3823-3831.

Exton, J. H. (1996) Regulation of phosphoinositide phospholipases by hormones, neurotransmitters, and other agonists linked to G proteins. *Annual review of pharmacology and toxicology,* **36**, p. 481-509.

Fan, G. H., Yang, W., Sai, J., and Richmond, A. (2001) Phosphorylation-independent association of CXCR2 with the protein phosphatase 2A core enzyme. *The Journal of biological chemistry,* **276**(20), p. 16960-16968.

Fargin, A., Raymond, J. R., Lohse, M. J., Kobilka, B. K., Caron, M. G., and Lefkowitz, R. J. (1988) The genomic clone G-21 which resembles a beta-adrenergic receptor sequence encodes the 5-HT1A receptor. *Nature,* **335**(6188), p. 358-360.

Fava, M., and Kendler, K. S. (2000) Major depressive disorder. *Neuron,* **28**(2), p. 335-341.

Ferguson, S. S. (2001) Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling. *Pharmacological reviews,* **53**(1), p. 1-24.

Ford, C. E., Skiba, N. P., Bae, H., Daaka, Y., Reuveny, E., Shekter, L. R., Rosal, R., Weng, G., Yang, C. S., Iyengar, R., Miller, R. J., Jan, L. Y., Lefkowitz, R. J., and Hamm, H. E. (1998) Molecular basis for interactions of G protein betagamma subunits with effectors. *Science (New York, N.Y,* **280**(5367), p. 1271-1274.

Frangakis, M. V., Ohmstede, C. A., and Sahyoun, N. (1991) A brain-specific Ca2+/calmodulin-dependent protein kinase (CaM kinase-Gr) is regulated by autophosphorylation. Relevance to neuronal Ca2+ signaling. *The Journal of biological chemistry,* **266**(17), p. 11309-11316.

Gao, X., Sadana, R., Dessauer, C. W., and Patel, T. B. (2007) Conditional stimulation of type V and VI adenylyl cyclases by G protein betagamma subunits. *The Journal of biological chemistry,* **282**(1), p. 294-302.

Gether, U. (2000) Uncovering molecular mechanisms involved in activation of G protein-coupled receptors. *Endocrine reviews,* **21**(1), p. 90-113.

Giannini, E., and Boulay, F. (1995) Phosphorylation, dephosphorylation, and recycling of the C5a receptor in differentiated HL60 cells. *J Immunol,* **154**(8), p. 4055-4064.

Gilman, A. G. (1987) G proteins: transducers of receptor-generated signals. *Annual review of biochemistry,* **56**, p. 615-649.

Goodman, O. B., Jr., Krupnick, J. G., Santini, F., Gurevich, V. V., Penn, R. B., Gagnon, A. W., Keen, J. H., and Benovic, J. L. (1996) Beta-arrestin acts as a clathrin adaptor in endocytosis of the beta2-adrenergic receptor. *Nature,* **383**(6599), p. 447-450.

Gotz, J., Probst, A., Mistl, C., Nitsch, R. M., and Ehler, E. (2000) Distinct role of protein phosphatase 2A subunit Calpha in the regulation of E-cadherin and beta-catenin during development. *Mechanisms of development,* **93**(1-2), p. 83-93.

Gross, C., and Hen, R. (2004) The developmental origins of anxiety. *Nature reviews,* **5**(7), p. 545-552.

Harrington, M. A., Shaw, K., Zhong, P., and Ciaranello, R. D. (1994) Agonist-induced desensitization and loss of high-affinity binding sites of stably expressed human 5-HT1A receptors. *The Journal of pharmacology and experimental therapeutics,* **268**(3), p. 1098-1106.

Henderson, R., and Schertler, G. F. (1990) The structure of bacteriorhodopsin and its relevance to the visual opsins and other seven-helix G-protein coupled receptors. *Philosophical transactions of the Royal Society of London,* **326**(1236), p. 379-389.

Hensler, J. G., Cervera, L. S., Miller, H. A., and Corbitt, J. (1996) Expression and modulation of 5-hydroxytryptamine1A receptors in P11 cells. *The Journal of pharmacology and experimental therapeutics,* **278**(3), p. 1138-1145.

Herlitze, S., Garcia, D. E., Mackie, K., Hille, B., Scheuer, T., and Catterall, W. A. (1996) Modulation of Ca2+ channels by G-protein beta gamma subunits. *Nature,* **380**(6571), p. 258-262.

Hill-Eubanks, D., Burstein, E. S., Spalding, T. A., Brauner-Osborne, H., and Brann, M. R. (1996) Structure of a G-protein-coupling domain of a muscarinic receptor predicted by random saturation mutagenesis. *The Journal of biological chemistry,* **271**(6), p. 3058-3065.

Hjorth, S., Bengtsson, H. J., Kullberg, A., Carlzon, D., Peilot, H., and Auerbach, S. B. (2000) Serotonin autoreceptor function and antidepressant drug action. *Journal of psychopharmacology (Oxford, England),* **14**(2), p. 177-185.

Holden, C. (2000) Mental health. Global survey examines impact of depression. *Science (New York, N.Y,* **288**(5463), p. 39-40.

Hornung, J. P. (2003) The human raphe nuclei and the serotonergic system. *Journal of chemical neuroanatomy,* **26**(4), p. 331-343.

Hoyer, D., Clarke, D. E., Fozard, J. R., Hartig, P. R., Martin, G. R., Mylecharane, E. J., Saxena, P. R., and Humphrey, P. P. (1994) International Union of Pharmacology

classification of receptors for 5-hydroxytryptamine (Serotonin). *Pharmacological reviews,* **46**(2), p. 157-203.

Hoyer, D., Hannon, J. P., and Martin, G. R. (2002) Molecular, pharmacological and functional diversity of 5-HT receptors. *Pharmacology, biochemistry, and behavior,* **71**(4), p. 533-554.

Hoyer, D., and Martin, G. (1997) 5-HT receptor classification and nomenclature: towards a harmonization with the human genome. *Neuropharmacology,* **36**(4-5), p. 419-428.

Huang, C. L., Slesinger, P. A., Casey, P. J., Jan, Y. N., and Jan, L. Y. (1995) Evidence that direct binding of G beta gamma to the GIRK1 G protein-gated inwardly rectifying K+ channel is important for channel activation. *Neuron,* **15**(5), p. 1133-1143.

Ikeda, S. R. (1996) Voltage-dependent modulation of N-type calcium channels by G-protein beta gamma subunits. *Nature,* **380**(6571), p. 255-258.

Jacobs, B. L., and Azmitia, E. C. (1992) Structure and function of the brain serotonin system. *Physiological reviews,* **72**(1), p. 165-229.

Jarvis, S. E., and Zamponi, G. W. (2001) Interactions between presynaptic Ca2+ channels, cytoplasmic messengers and proteins of the synaptic vesicle release complex. *Trends in pharmacological sciences,* **22**(10), p. 519-525.

Karaiskou, A., Jessus, C., Brassac, T., and Ozon, R. (1999) Phosphatase 2A and polo kinase, two antagonistic regulators of cdc25 activation and MPF auto-amplification. *Journal of cell science,* **112 (Pt 21)**, p. 3747-3756.

Katayama, J., Yakushiji, T., and Akaike, N. (1997) Characterization of the K+ current mediated by 5-HT1A receptor in the acutely dissociated rat dorsal raphe neurons. *Brain research,* **745**(1-2), p. 283-292.

Katz, A., Wu, D., and Simon, M. I. (1992) Subunits beta gamma of heterotrimeric G protein activate beta 2 isoform of phospholipase C. *Nature,* **360**(6405), p. 686-689.

Kessler, R. C., Berglund, P. A., Dewit, D. J., Ustun, T. B., Wang, P. S., and Wittchen, H. U. (2002) Distinguishing generalized anxiety disorder from major depression: prevalence and impairment from current pure and comorbid disorders in the US and Ontario. *International journal of methods in psychiatric research,* **11**(3), p. 99-111.

Khew-Goodall, Y., and Hemmings, B. A. (1988) Tissue-specific expression of mRNAs encoding alpha- and beta-catalytic subunits of protein phosphatase 2A. *FEBS letters,* **238**(2), p. 265-268.

Kobilka, B. K., Frielle, T., Collins, S., Yang-Feng, T., Kobilka, T. S., Francke, U., Lefkowitz, R. J., and Caron, M. G. (1987) An intronless gene encoding a potential member of the family of receptors coupled to guanine nucleotide regulatory proteins. *Nature,* **329**(6134), p. 75-79.

Kushwaha, N., and Albert, P. R. (2005) Coupling of 5-HT1A autoreceptors to inhibition of mitogen-activated protein kinase activation via G beta gamma subunit signaling. *The European journal of neuroscience,* **21**(3), p. 721-732.

Kushwaha, N., Harwood, S. C., Wilson, A. M., Berger, M., Tecott, L. H., Roth, B. L., and Albert, P. R. (2006) Molecular determinants in the second intracellular loop

of the 5-hydroxytryptamine-1A receptor for G-protein coupling. *Molecular pharmacology,* **69**(5), p. 1518-1526.

Lambert, G. W., Kaye, D. M., Cox, H. S., Vaz, M., Turner, A. G., Jennings, G. L., and Esler, M. D. (1995) Regional 5-hydroxyindoleacetic acid production in humans. *Life sciences,* **57**(3), p. 255-267.

Laporte, S. A., Oakley, R. H., Zhang, J., Holt, J. A., Ferguson, S. S., Caron, M. G., and Barak, L. S. (1999) The beta2-adrenergic receptor/betaarrestin complex recruits the clathrin adaptor AP-2 during endocytosis. *Proceedings of the National Academy of Sciences of the United States of America,* **96**(7), p. 3712-3717.

Lechward, K., Awotunde, O. S., Swiatek, W., and Muszynska, G. (2001) Protein phosphatase 2A: variety of forms and diversity of functions. *Acta biochimica Polonica,* **48**(4), p. 921-933.

Lefkowitz, R. J. (1998) G protein-coupled receptors. III. New roles for receptor kinases and beta-arrestins in receptor signaling and desensitization. *The Journal of biological chemistry,* **273**(30), p. 18677-18680.

Lembo, P. M., and Albert, P. R. (1995) Multiple phosphorylation sites are required for pathway-selective uncoupling of the 5-hydroxytryptamine1A receptor by protein kinase C. *Molecular pharmacology,* **48**(6), p. 1024-1029.

Lembo, P. M., Ghahremani, M. H., Morris, S. J., and Albert, P. R. (1997) A conserved threonine residue in the second intracellular loop of the 5-hydroxytryptamine 1A receptor directs signaling specificity. *Molecular pharmacology,* **52**(1), p. 164-171.

Lemonde, S., Rogaeva, A., and Albert, P. R. (2004) Cell type-dependent recruitment of trichostatin A-sensitive repression of the human 5-HT1A receptor gene. *Journal of neurochemistry,* **88**(4), p. 857-868.

Lesch, K. P., and Heils, A. (2000) Serotonergic gene transcriptional control regions: targets for antidepressant drug development? *The international journal of neuropsychopharmacology / official scientific journal of the Collegium Internationale Neuropsychopharmacologicum (CINP),* **3**(1), p. 67-79.

Leyton, M., Ghadirian, A. M., Young, S. N., Palmour, R. M., Blier, P., Helmers, K. F., and Benkelfat, C. (2000) Depressive relapse following acute tryptophan depletion in patients with major depressive disorder. *Journal of psychopharmacology (Oxford, England),* **14**(3), p. 284-287.

Liu, J., Blin, N., Conklin, B. R., and Wess, J. (1996) Molecular mechanisms involved in muscarinic acetylcholine receptor-mediated G protein activation studied by insertion mutagenesis. *The Journal of biological chemistry,* **271**(11), p. 6172-6178.

Liu, J., Conklin, B. R., Blin, N., Yun, J., and Wess, J. (1995) Identification of a receptor/G-protein contact site critical for signaling specificity and G-protein activation. *Proceedings of the National Academy of Sciences of the United States of America,* **92**(25), p. 11642-11646.

Liu, Y. F., and Albert, P. R. (1991) Cell-specific signaling of the 5-HT1A receptor. Modulation by protein kinases C and A. *The Journal of biological chemistry,* **266**(35), p. 23689-23697.

Liu, Y. F., Ghahremani, M. H., Rasenick, M. M., Jakobs, K. H., and Albert, P. R. (1999) Stimulation of cAMP synthesis by Gi-coupled receptors upon ablation of distinct Galphai protein expression. Gi subtype specificity of the 5-HT1A receptor. *The Journal of biological chemistry,* **274**(23), p. 16444-16450.

Liu, Y. F., Jakobs, K. H., Rasenick, M. M., and Albert, P. R. (1994) G protein specificity in receptor-effector coupling. Analysis of the roles of G0 and Gi2 in GH4C1 pituitary cells. *The Journal of biological chemistry,* **269**(19), p. 13880-13886.

Luscher, C., Jan, L. Y., Stoffel, M., Malenka, R. C., and Nicoll, R. A. (1997) G protein-coupled inwardly rectifying K+ channels (GIRKs) mediate postsynaptic but not presynaptic transmitter actions in hippocampal neurons. *Neuron,* **19**(3), p. 687-695.

Lutz, M. P., Pinon, D. I., Gates, L. K., Shenolikar, S., and Miller, L. J. (1993) Control of cholecystokinin receptor dephosphorylation in pancreatic acinar cells. *The Journal of biological chemistry,* **268**(16), p. 12136-12142.

MacKintosh, C., and MacKintosh, R. W. (1994) Inhibitors of protein kinases and phosphatases. *Trends in biochemical sciences,* **19**(11), p. 444-448.

Mann, J. J. (1999) Role of the serotonergic system in the pathogenesis of major depression and suicidal behavior. *Neuropsychopharmacology,* **21**(2 Suppl), p. 99S-105S.

Mann, J. J., Brent, D. A., and Arango, V. (2001) The neurobiology and genetics of suicide and attempted suicide: a focus on the serotonergic system. *Neuropsychopharmacology,* **24**(5), p. 467-477.

Marchese, A., and Benovic, J. L. (2001) Agonist-promoted ubiquitination of the G protein-coupled receptor CXCR4 mediates lysosomal sorting. *The Journal of biological chemistry,* **276**(49), p. 45509-45512.

Marchese, A., Raiborg, C., Santini, F., Keen, J. H., Stenmark, H., and Benovic, J. L. (2003) The E3 ubiquitin ligase AIP4 mediates ubiquitination and sorting of the G protein-coupled receptor CXCR4. *Developmental cell,* **5**(5), p. 709-722.

Martin, N. P., Lefkowitz, R. J., and Shenoy, S. K. (2003) Regulation of V2 vasopressin receptor degradation by agonist-promoted ubiquitination. *The Journal of biological chemistry,* **278**(46), p. 45954-45959.

McAllister-Williams, R. H., and Kelly, J. S. (1995) The modulation of calcium channel currents recorded from adult rat dorsal raphe neurones by 5-HT1A receptor or direct G-protein activation. *Neuropharmacology,* **34**(11), p. 1491-1506.

Means, A. R., Bagchi, I. C., VanBerkum, M. F., and Kemp, B. E. (1991) Regulation of smooth muscle myosin light chain kinase by calmodulin. *Advances in experimental medicine and biology,* **304**, p. 11-24.

Meng, E. C., and Bourne, H. R. (2001) Receptor activation: what does the rhodopsin structure tell us? *Trends in pharmacological sciences,* **22**(11), p. 587-593.

Menon, S. T., Han, M., and Sakmar, T. P. (2001) Rhodopsin: structural basis of molecular physiology. *Physiological reviews,* **81**(4), p. 1659-1688.

Millward, T. A., Zolnierowicz, S., and Hemmings, B. A. (1999) Regulation of protein kinase cascades by protein phosphatase 2A. *Trends in biochemical sciences,* **24**(5), p. 186-191.

Murphy, D. L., Wichems, C., Li, Q., and Heils, A. (1999) Molecular manipulations as tools for enhancing our understanding of 5-HT neurotransmission. *Trends in pharmacological sciences,* **20**(6), p. 246-252.

Nemeroff, C. B. (2002) Recent advances in the neurobiology of depression. *Psychopharmacology bulletin,* **36 Suppl 2**, p. 6-23.

Newberry, N. R. (1992) 5-HT1A receptors activate a potassium conductance in rat ventromedial hypothalamic neurones. *European journal of pharmacology,* **210**(2), p. 209-212.

Newman-Tancredi, A., Assie, M. B., Leduc, N., Ormiere, A. M., Danty, N., and Cosi, C. (2005) Novel antipsychotics activate recombinant human and native rat serotonin 5-HT1A receptors: affinity, efficacy and potential implications for treatment of schizophrenia. *The international journal of neuropsychopharmacology / official scientific journal of the Collegium Internationale Neuropsychopharmacologicum (CINP),* **8**(3), p. 341-356.

Newman-Tancredi, A., Verriele, L., Touzard, M., and Millan, M. J. (2001) Efficacy of antipsychotic agents at human 5-HT(1A) receptors determined by [3H]WAY100,635 binding affinity ratios: relationship to efficacy for G-protein activation. *European journal of pharmacology,* **428**(2), p. 177-184.

Niesler, B., Walstab, J., Combrink, S., Moller, D., Kapeller, J., Rietdorf, J., Bonisch, H., Gothert, M., Rappold, G., and Bruss, M. (2007) Characterization of the novel human serotonin receptor subunits 5-HT3C,5-HT3D, and 5-HT3E. *Molecular pharmacology,* **72**(1), p. 8-17.

Ohmstede, C. A., Jensen, K. F., and Sahyoun, N. E. (1989) Ca2+/calmodulin-dependent protein kinase enriched in cerebellar granule cells. Identification of a novel neuronal calmodulin-dependent protein kinase. *The Journal of biological chemistry,* **264**(10), p. 5866-5875.

Ostrowski, J., Kjelsberg, M. A., Caron, M. G., and Lefkowitz, R. J. (1992) Mutagenesis of the beta 2-adrenergic receptor: how structure elucidates function. *Annual review of pharmacology and toxicology,* **32**, p. 167-183.

Ou, X. M., Lemonde, S., Jafar-Nejad, H., Bown, C. D., Goto, A., Rogaeva, A., and Albert, P. R. (2003) Freud-1: A neuronal calcium-regulated repressor of the 5-HT1A receptor gene. *J Neurosci,* **23**(19), p. 7415-7425.

Palczewski, K., Kumasaka, T., Hori, T., Behnke, C. A., Motoshima, H., Fox, B. A., Le Trong, I., Teller, D. C., Okada, T., Stenkamp, R. E., Yamamoto, M., and Miyano, M. (2000) Crystal structure of rhodopsin: A G protein-coupled receptor. *Science (New York, N.Y,* **289**(5480), p. 739-745.

Papoucheva, E., Dumuis, A., Sebben, M., Richter, D. W., and Ponimaskin, E. G. (2004) The 5-hydroxytryptamine(1A) receptor is stably palmitoylated, and acylation is critical for communication of receptor with Gi protein. *The Journal of biological chemistry,* **279**(5), p. 3280-3291.

Penington, N. J., Kelly, J. S., and Fox, A. P. (1991) A study of the mechanism of Ca2+ current inhibition produced by serotonin in rat dorsal raphe neurons. *J Neurosci,* **11**(11), p. 3594-3609.

Penington, N. J., Kelly, J. S., and Fox, A. P. (1993) Whole-cell recordings of inwardly rectifying K+ currents activated by 5-HT1A receptors on dorsal raphe neurones of the adult rat. *The Journal of physiology,* **469**, p. 387-405.

Peroutka, S. J., and Howell, T. A. (1994) The molecular evolution of G protein-coupled receptors: focus on 5-hydroxytryptamine receptors. *Neuropharmacology,* **33**(3-4), p. 319-324.

Perroy, J., Pontier, S., Charest, P. G., Aubry, M., and Bouvier, M. (2004) Real-time monitoring of ubiquitination in living cells by BRET. *Nature methods,* **1**(3), p. 203-208.

Perry, S. J., and Lefkowitz, R. J. (2002) Arresting developments in heptahelical receptor signaling and regulation. *Trends in cell biology,* **12**(3), p. 130-138.

Pierce, K. L., Premont, R. T., and Lefkowitz, R. J. (2002) Seven-transmembrane receptors. *Nat Rev Mol Cell Biol,* **3**(9), p. 639-650.

Pineyro, G., and Blier, P. (1999) Autoregulation of serotonin neurons: role in antidepressant drug action. *Pharmacological reviews,* **51**(3), p. 533-591.

Pitcher, J. A., Payne, E. S., Csortos, C., DePaoli-Roach, A. A., and Lefkowitz, R. J. (1995) The G-protein-coupled receptor phosphatase: a protein phosphatase type 2A with a distinct subcellular distribution and substrate specificity. *Proceedings of the National Academy of Sciences of the United States of America,* **92**(18), p. 8343-8347.

Pompeiano, M., Palacios, J. M., and Mengod, G. (1992) Distribution and cellular localization of mRNA coding for 5-HT1A receptor in the rat brain: correlation with receptor binding. *J Neurosci,* **12**(2), p. 440-453.

Rapport, M. M. (1949) Serum vasoconstrictor (serotonin) the presence of creatinine in the complex; a proposed structure of the vasoconstrictor principle. *The Journal of biological chemistry,* **180**(3), p. 961-969.

Rapport, M. M., Green, A. A., and Page, I. H. (1948) Partial purification of the vasoconstrictor in beef serum. *The Journal of biological chemistry,* **174**(2), p. 735-741.

Raymond, J. R. (1991) Protein kinase C induces phosphorylation and desensitization of the human 5-HT1A receptor. *The Journal of biological chemistry,* **266**(22), p. 14747-14753.

Raymond, J. R., Mukhin, Y. V., Gettys, T. W., and Garnovskaya, M. N. (1999) The recombinant 5-HT1A receptor: G protein coupling and signalling pathways. *British journal of pharmacology,* **127**(8), p. 1751-1764.

Raymond, J. R., and Olsen, C. L. (1994) Protein kinase A induces phosphorylation of the human 5-HT1A receptor and augments its desensitization by protein kinase C in CHO-K1 cells. *Biochemistry,* **33**(37), p. 11264-11269.

Riad, M., Garcia, S., Watkins, K. C., Jodoin, N., Doucet, E., Langlois, X., el Mestikawy, S., Hamon, M., and Descarries, L. (2000) Somatodendritic localization of 5-HT1A and preterminal axonal localization of 5-HT1B serotonin receptors in adult rat brain. *The Journal of comparative neurology,* **417**(2), p. 181-194.

Riad, M., Watkins, K. C., Doucet, E., Hamon, M., and Descarries, L. (2001) Agonist-induced internalization of serotonin-1a receptors in the dorsal raphe nucleus (autoreceptors) but not hippocampus (heteroreceptors). *J Neurosci,* **21**(21), p. 8378-8386.

Riad, M., Zimmer, L., Rbah, L., Watkins, K. C., Hamon, M., and Descarries, L. (2004) Acute treatment with the antidepressant fluoxetine internalizes 5-HT1A autoreceptors and reduces the in vivo binding of the PET radioligand [18F]MPPF in the nucleus raphe dorsalis of rat. *J Neurosci,* **24**(23), p. 5420-5426.

Risch, S. C., and Nemeroff, C. B. (1992) Neurochemical alterations of serotonergic neuronal systems in depression. *The Journal of clinical psychiatry,* **53 Suppl**, p. 3-7.

Rogaeva, A., and Albert, P. R. (2007) The mental retardation gene CC2D1A/Freud-1 encodes a long isoform that binds conserved DNA elements to repress gene transcription. *The European journal of neuroscience,* **26**(4), p. 965-974.

Ruediger, R., Roeckel, D., Fait, J., Bergqvist, A., Magnusson, G., and Walter, G. (1992) Identification of binding sites on the regulatory A subunit of protein phosphatase 2A for the catalytic C subunit and for tumor antigens of simian virus 40 and polyomavirus. *Molecular and cellular biology,* **12**(11), p. 4872-4882.

Sargent, P. A., Kjaer, K. H., Bench, C. J., Rabiner, E. A., Messa, C., Meyer, J., Gunn, R. N., Grasby, P. M., and Cowen, P. J. (2000) Brain serotonin1A receptor binding measured by positron emission tomography with [11C]WAY-100635: effects of depression and antidepressant treatment. *Archives of general psychiatry,* **57**(2), p. 174-180.

Schertler, G. F., Villa, C., and Henderson, R. (1993) Projection structure of rhodopsin. *Nature,* **362**(6422), p. 770-772.

Schiapparelli, L., Del Rio, J., and Frechilla, D. (2005) Serotonin 5-HT receptor blockade enhances Ca(2+)/calmodulin-dependent protein kinase II function and membrane

expression of AMPA receptor subunits in the rat hippocampus: implications for memory formation. *Journal of neurochemistry,* **94**(4), p. 884-895.

Shenker, A., Maayani, S., Weinstein, H., and Green, J. P. (1987) Pharmacological characterization of two 5-hydroxytryptamine receptors coupled to adenylate cyclase in guinea pig hippocampal membranes. *Molecular pharmacology,* **31**(4), p. 357-367.

Shenoy, S. K., and Lefkowitz, R. J. (2003) Trafficking patterns of beta-arrestin and G protein-coupled receptors determined by the kinetics of beta-arrestin deubiquitination. *The Journal of biological chemistry,* **278**(16), p. 14498-14506.

Shenoy, S. K., and Lefkowitz, R. J. (2005) Receptor-specific ubiquitination of beta-arrestin directs assembly and targeting of seven-transmembrane receptor signalosomes. *The Journal of biological chemistry,* **280**(15), p. 15315-15324.

Shenoy, S. K., McDonald, P. H., Kohout, T. A., and Lefkowitz, R. J. (2001) Regulation of receptor fate by ubiquitination of activated beta 2-adrenergic receptor and beta-arrestin. *Science (New York, N.Y,* **294**(5545), p. 1307-1313.

Shih, M., Lin, F., Scott, J. D., Wang, H. Y., and Malbon, C. C. (1999) Dynamic complexes of beta2-adrenergic receptors with protein kinases and phosphatases and the role of gravin. *The Journal of biological chemistry,* **274**(3), p. 1588-1595.

Sibley, D. R., Strasser, R. H., Benovic, J. L., Daniel, K., and Lefkowitz, R. J. (1986) Phosphorylation/dephosphorylation of the beta-adrenergic receptor regulates its functional coupling to adenylate cyclase and subcellular distribution. *Proceedings of the National Academy of Sciences of the United States of America,* **83**(24), p. 9408-9412.

Sotelo, C., Cholley, B., El Mestikawy, S., Gozlan, H., and Hamon, M. (1990) Direct Immunohistochemical Evidence of the Existence of 5-HT1A Autoreceptors on Serotoninergic Neurons in the Midbrain Raphe Nuclei. *The European journal of neuroscience,* **2**(12), p. 1144-1154.

Spurney, R. F. (2001) Regulation of thromboxane receptor (TP) phosphorylation by protein phosphatase 1 (PP1) and PP2A. *The Journal of pharmacology and experimental therapeutics,* **296**(2), p. 592-599.

Stahl, S. M. (1998) Basic psychopharmacology of antidepressants, part 1: Antidepressants have seven distinct mechanisms of action. *The Journal of clinical psychiatry,* **59 Suppl 4**, p. 5-14.

Stockmeier, C. A., Shapiro, L. A., Dilley, G. E., Kolli, T. N., Friedman, L., and Rajkowska, G. (1998) Increase in serotonin-1A autoreceptors in the midbrain of suicide victims with major depression-postmortem evidence for decreased serotonin activity. *J Neurosci,* **18**(18), p. 7394-7401.

Stone, S. R., Hofsteenge, J., and Hemmings, B. A. (1987) Molecular cloning of cDNAs encoding two isoforms of the catalytic subunit of protein phosphatase 2A. *Biochemistry,* **26**(23), p. 7215-7220.

Strack, S., Zaucha, J. A., Ebner, F. F., Colbran, R. J., and Wadzinski, B. E. (1998) Brain protein phosphatase 2A: developmental regulation and distinct cellular and subcellular localization by B subunits. *The Journal of comparative neurology,* **392**(4), p. 515-527.

Sumiyoshi, T., Matsui, M., Nohara, S., Yamashita, I., Kurachi, M., Sumiyoshi, C., Jayathilake, K., and Meltzer, H. Y. (2001) Enhancement of cognitive performance

in schizophrenia by addition of tandospirone to neuroleptic treatment. *The American journal of psychiatry,* **158**(10), p. 1722-1725.

Sumiyoshi, T., Matsui, M., Yamashita, I., Nohara, S., Kurachi, M., Uehara, T., Sumiyoshi, S., Sumiyoshi, C., and Meltzer, H. Y. (2001) The effect of tandospirone, a serotonin(1A) agonist, on memory function in schizophrenia. *Biological psychiatry,* **49**(10), p. 861-868.

Sunahara, R. K., Dessauer, C. W., and Gilman, A. G. (1996) Complexity and diversity of mammalian adenylyl cyclases. *Annual review of pharmacology and toxicology,* **36**, p. 461-480.

Tork, I. (1990) Anatomy of the serotonergic system. *Annals of the New York Academy of Sciences,* **600**, p. 9-34; discussion 34-35.

Twarog, B. M., and Page, I. H. (1953) Serotonin content of some mammalian tissues and urine and a method for its determination. *The American journal of physiology,* **175**(1), p. 157-161.

van Huizen, F., Bansse, M. T., and Stam, N. J. (1993) Agonist-induced down-regulation of human 5-HT1A and 5-HT2 receptors in Swiss 3T3 cells. *Neuroreport,* **4**(12), p. 1327-1330.

Varrault, A., Le Nguyen, D., McClue, S., Harris, B., Jouin, P., and Bockaert, J. (1994) 5-Hydroxytryptamine1A receptor synthetic peptides. Mechanisms of adenylyl cyclase inhibition. *The Journal of biological chemistry,* **269**(24), p. 16720-16725.

Walther, D. J., Peter, J. U., Bashammakh, S., Hortnagl, H., Voits, M., Fink, H., and Bader, M. (2003) Synthesis of serotonin by a second tryptophan hydroxylase isoform. *Science (New York, N.Y,* **299**(5603), p. 76.

Wess, J. (1997) G-protein-coupled receptors: molecular mechanisms involved in receptor activation and selectivity of G-protein recognition. *Faseb J,* **11**(5), p. 346-354.

Woolley, D. W., and Shaw, E. (1954) A Biochemical and Pharmacological Suggestion About Certain Mental Disorders. *Proceedings of the National Academy of Sciences of the United States of America,* **40**(4), p. 228-231.

Wu, X., Kushwaha, N., Albert, P. R., and Penington, N. J. (2002) A critical protein kinase C phosphorylation site on the 5-HT(1A) receptor controlling coupling to N-type calcium channels. *The Journal of physiology,* **538**(Pt 1), p. 41-51.

Wurch, T., Colpaert, F. C., and Pauwels, P. J. (2003) Mutation in a protein kinase C phosphorylation site of the 5-HT1A receptor preferentially attenuates Ca2+ responses to partial as opposed to higher-efficacy 5-HT1A agonists. *Neuropharmacology,* **44**(7), p. 873-881.

Yan, K., and Gautam, N. (1996) A domain on the G protein beta subunit interacts with both adenylyl cyclase 2 and the muscarinic atrial potassium channel. *The Journal of biological chemistry,* **271**(30), p. 17597-17600.

Yan, K., and Gautam, N. (1997) Structural determinants for interaction with three different effectors on the G protein beta subunit. *The Journal of biological chemistry,* **272**(4), p. 2056-2059.

Yang, W., Wang, D., and Richmond, A. (1999) Role of clathrin-mediated endocytosis in CXCR2 sequestration, resensitization, and signal transduction. *The Journal of biological chemistry,* **274**(16), p. 11328-11333.

Yu, S. S., Lefkowitz, R. J., and Hausdorff, W. P. (1993) Beta-adrenergic receptor sequestration. A potential mechanism of receptor resensitization. *The Journal of biological chemistry,* **268**(1), p. 337-341.

Yuen, E. Y., Jiang, Q., Chen, P., Gu, Z., Feng, J., and Yan, Z. (2005) Serotonin 5-HT1A receptors regulate NMDA receptor channels through a microtubule-dependent mechanism. *J Neurosci,* **25**(23), p. 5488-5501.

APPENDIX

Permission from Journals for republishing figures

From: ajpermissions
To: Jonathan Paul Urben;
Subject: RE: Permission for republishing
Date: Tuesday, June 09, 2009 10:52:25 AM

Dear Mr. Jonathan Paul Urben,

Thank you for your *Nature* permissions enquiry. Please accept this email as confirmation permission is granted free of charge for this use. Here is the following credit line you can use to acknowledge Nature Publishing Group:

Reprinted by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication)

Kind regards,

Charlotte Kinnah

Permissions Assistant
nature **p**ublishing **g**roup
The Macmillan Building
4-6 Crinan Street
London N1 9XW
e:permissions@nature.com
p: + 44 207 014 4129
f: + 44 207 843 4998

Please consider the environment before printing this email or its attachments

-----Original Message-----
From: Jonathan Paul Urben [mailto:jurbe045@uottawa.ca]
Sent: 07 June 2009 16:13
To: ajpermissions
Subject: Permission for republishing
Importance: High

Hello,

I am completing my M.Sc. degree at the University of Ottawa, and I am seeking permission to republish a figure published in Neuropsychopharmacology for my thesis.

The journal article is entitled 'The 5-HT1A receptor: signaling, desensitization, and gene transcription.' Paul R. Albert, Paola Lembo, John M. Storring, Alain Charest and Caroline Saucier . Volume 14 Issue 1 Pages 19-25 - January 1996.

The figure I wish to republish is Figure 1.

Thanking you in advance,

Jonathan P. Urben

University of Ottawa & Ottawa Health Research Institute
Dept. of Cellular & Molecular Medicine
451 Smyth Road, Ottawa, Ontario, K1H 8M5
Tel: (613) 562-5800 ext. 8315

e-mail: jurbe045@uottawa.ca