STRUCTURAL DETERMINANTS OF 5-HT1A RECEPTOR INTERACTION WITH Gαi SUBUNITS

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

Abstract

The 5-hydroxytryptamine (5-HT) system modulates numerous physiological and behavioural processes, and dysfunction within this system underlies many behavioural disorders, such as major depression. The 5-HT_{1A} receptor is the primary somatodendritic autoreceptor that controls the firing rate of 5-HT neurons, but is also coupled to numerous signalling pathways. An understanding of 5-HT_{1A} receptor signalling may lead to the development of antidepressant drugs that selectively target therapeutic pathways in treating depression. The 5-HT_{1A} receptor is coupled to inhibitory G-proteins via its intracellular loops 2 and 3. Point mutations within these loops selectively uncouple receptor signalling pathways. In this thesis, I addressed whether mutant receptors' uncoupling from signalling pathways is associated with alteration in G-protein interaction and coupling. Using bioluminescence resonance energy transfer (BRET) to monitor receptor-G-protein interactions, we show that both wild-type and mutant receptors demonstrate a saturable interaction with Gαi protein in unstimulated conditions. Addition of 5-HT increased the BRET signal for the wild-type 5-HT_{1A} receptor, and this increase was blocked by a 5-HT_{1A} receptor antagonist and G-protein blocker (pertussis toxin). Mutant receptors that were deficient in Gαi signalling, but not those that still signalled to Gαi, failed to respond to receptor activation with increased receptor-Gαi interaction. Pull down studies verified the basal and agonist-induced interaction of 5-HT_{1A} receptors with Gαi proteins. In conclusion, we have shown that the 5-HT_{1A} receptor interacts with Gαi consistent with a pre-coupled model and that 5-HT-induced activation enhances this interaction and requires specific residues in the intracellular loops.

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Abbreviation List

5-HIAA:	5-hydroxyindoleacetic acid
5-HT:	5-hydroxtryptamine
5-HTP:	5-hydroxy-L-tryptophan
AC:	adenylyl cyclase
ACII:	adenylyl cyclase II
ADP:	adenosine diphosphate
BRET:	bioluminescence resonance energy transfer
C:	carboxyl
cAMP:	cyclic adenosine monophosphate
CNS:	central nervous system
co-IP:	co-immunoprecipitation
D2:	dopamine 2
DMEM:	Dulbecco's Modified Eagle's Medium
D-PBS:	Dulbecco's phosphate buffered saline
DTBP:	dimethyl 3,3´-dithiobispropionimidate - 2 HCl
DTT:	dithiolthreitol
ERK1/2:	extracellular signal-regulated kinase 1 and 2
FBS:	fetal bovine serum
FRET:	fluorescence resonance energy transfer
GDP:	guanosine diphosphate
GFP2:	green fluorescent protein 2
GI:	gastrointestinal
GIRK:	G-protein inward rectifying potassium channels
GPCR:	G-protein coupled receptors
G-protein:	guanine nucleotide-binding proteins
GTP:	guanosine triphosphate
HBS:	HEPES buffered saline
HBSS-EDTA:	Hank's Buffered Salt Solution- Ethylenediaminetetraacetic acid
HCN4:	potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 4
HEK293:	human embryonic kidney 293
HRP:	horseradish peroxidase
i2:	intracellular loop 2
i3:	intracellular loop 3
KO:	knockout
MAO-A:	monoamine oxidase A
MAPK:	mitogen-activated protein kinase
MEK1/2:	MAPK/ERK Kinase 1 and 2
NF-κB:	nuclear factor kappa-light-chain-enhancer of activated B cells
N:	amino
PCPA:	parachlorophenylalanine
PI3-K:	phosphoinositol 3-kinase
PLC:	phospholipase C
PKC:	protein kinase C
PKC-α:	protein kinase C-α

PNS:	peripheral nervous system
PTX:	pertussis toxin
PVDF:	polyvinylidene difluoride
RET:	resonance energy transfer
RLuc:	Renilla reniformis luciferase
SERT:	serotonin transporters
SSRI:	selective serotonin reuptake inhibitor
TCA:	tricyclic antidepressants
TPH:	tryptophan hydroxylase
VMAT2:	vesicular monoamine transporter type 2
YFP:	yellow fluorescence protein

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Acknowledgements

I would like to thank Dr. Paul Albert for giving me this wondrous opportunity to study in his lab and for always being there to provide insightful feedback and guidance during my period of study. I would also like to also thank my advisory committee members, Dr. Mario Tiberi and Dr. Charlie Thompson, for taking the time to review my progress and provide valuable instructions and much needed help along the way. I would also like to express my gratitude to our lab manager, Mireille Daigle, for all her technical advice and help and to Jon Urben who has since day one helped me gain the skills necessary to complete my research. A big thank you goes out to all my friends and colleagues in the lab, past and present, for all their encouragement, for helping me brainstorm ideas and troubleshoot experiments as well as providing a shoulder to cry on and helping me keep my sanity during difficult times throughout my study. I would like to thank in addition my friends and colleagues in other labs within the department for creating such an enriching environment to learn, grow, and have fun. It has been a valuable and rewarding experience. Finally I would like to express my gratitude to my parents and family for their constant love and support.

Introduction

Serotonin, also known by its chemical name, 5-hydroxytryptamine (5-HT), is an important neurotransmitter, neurocrine, and endocrine messenger within the body. Outside of the central nervous system, 5-HT plays a role in controlling gut motility and also serves as both a vasoconstrictor and vasodilator in the cardiovascular system. 5-HT, however, has a much more pronounced role in the CNS and is involved in numerous behavioural and mental disorders such as major depressive disorder, schizophrenia anxiety, and drug addiction. Major depressive disorder is characterized by prolonged feelings of sadness and worthlessness, change in appetite, anhedonia, insomnia, and poor concentration (Public Health Agency 2002). According to a 2006 publication by the Public Health Agency of Canada, about 10-14% of men will experience depression in their life time; the prevalence among women is higher, ranging from 13.5-18% (Public Health Agency, 2006). The World Health Organization ranks major depression as the 4th leading contributor to the global burden of disease, which currently affects 121 million people. It is estimated that depression will reach 2nd place by 2020. Dysfunctions within the 5-HT is one of the more often cited cause of major depression.

A brief history of the discovery and later studies of 5-HT and 5-HT receptors

5-HT is one of the oldest neurotransmitters and hormones. It is estimated to have first appeared 700-800 million years ago and is found today, well conserved, in a diverse group of species (Hannon and Hoyer, 2008). Research into 5-HT began nearly a century ago as a novel agent found in blood that caused vasoconstriction. Its name was derived from its ability to act as a serum tonic factor, hence the portmanteau term, serotonin (Sjoerdsma and Palfreyman, 1990). Throughout the first half of the twentieth century, many attempts were made in order to identify

this mysterious compound, but the search was confounded by other vasoconstricting factors within the blood. It was not until the mid twentieth century when 5-HT was first isolated and identified by Page's group from blood serum (Rapport et al, 1947). To say, however, this was the first instance of the "discovery" of 5-HT would be incorrect. In the 1930s, Erspamer and Vialli recorded a substance found within the enterochromaffin cells of the gastrointestinal (GI) tract (Sjoerdsma and Palfreyman, 1990, Erspamer and Vialli, 1937). This they named enteramine, which was later found to be same compound as the one that would be described as serotonin by Page's group decades later. The first synthetic 5-HT was described in 1951 by Hamlin and Fisher (Hamlin and Fisher 1951). These studies laid the ground work for our modern understanding of 5-HT.

The creation of synthetic 5-HT and the emergence of new analytical methods allowed scientists in the next couple of decades to identify the synthesis and degradation pathways, enzymes and receptors involved in the 5-HT system. It was not, however, until roughly the 1980s before 5-HT receptors were categorized into different families. 5-HT receptors were first categorized as either M or D type depending on whether their effects in the guinea pig ileum were blocked by morphine or dibenzyline respectively (Gaddum and Picarelli, 1957). Through the use of radioligands, Peroutka and Snyder distinguished two families of 5-HT receptors in brain membranes based on differential ligand binding: 5-HT1, which bound [^{3}H]5-HT and 5-HT$_2$, which bound [^{3}H]spiperone (Peroutka et al, 1979). Of these, 5-HT$_2$ corresponded to the D type receptor previously noted by Gaddum's group; however, the M receptor still remained distinct. 5-HT1 would further be categorized into subtypes A through D in the last half of the decade (Fozard, 1987). Another family of 5-HT receptors, 5-HT$_3$, which corresponds to the M type

receptor, was described in this decade based on the discovery of specific antagonist to 5-HT3 receptors (Fozard, 1984). Since then, studies using either radioligand binding, 5-HT stimulated adenylyl cyclase (AC), or electrophysiological studies further segregated the characteristics of the different receptor families and subtypes and found new drugs that would specifically target each (Pedigo et al, 1981). The motivational drive behind these studies is, of course, the fact that the 5-HT system is implicated in many modern day disorders as it exhibits a plethora of effect both in the CNS and in the periphery.

The 5-HT system

The 5-HT system originates from the raphe nuclei of the brain stem, and not surprisingly, it is where most of the serotonergic neurons are found (Kandel et al, 2000). Within the raphe, the system is further divided into the rostral nuclei, localized in the midbrain and rostral pons, and the caudal nuclei, localized in the medulla oblongata. The dorsal region of the rostral raphe nuclei contains the majority of 5-HT neurons in mammalian CNS. It sends projections throughout the brain and is the region underlying behavioural disorders (Descarries et al, 1982). On the other hand, the caudal raphe nuclei have projections to the cerebellum, autonomic motor system, and spinal chord and have greater implication in the regulation of the non-CNS organs such as the heart and gut (Törk, 1990). Through the use of immunohistochemical methods, the two subdivisions of the 5-HT system were found to be almost completely separate from each other, with either projections going up to the brain or down through the spinal cord (Aitken and Törk, 1988). Areas within the brainstem and the cerebellum, however, are dually innervated by both subdivisions. Figure 1 shows a schematic of the innervation of the 5-HT neurons within the CNS.

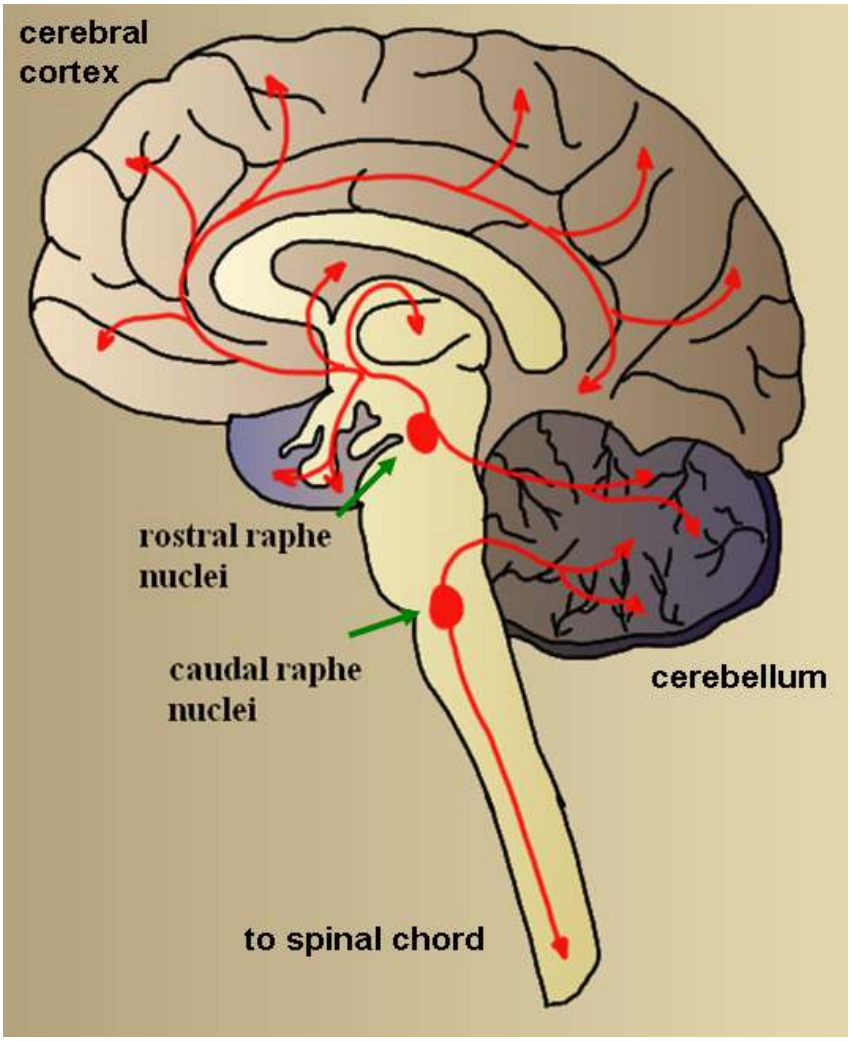


Figure 1. Projections of the 5-HT system. Projections from the two raphe nuclei to other regions in the CNS are indicated. The rostral raphe nuclei projects primarily to the cerebral cortex, cerebellum, hippocampus, and other limbic areas. The caudal raphe nuclei send projections to the cerebellum and down towards the spinal cord.

The 5-HT life cycle: synthesis, release, reuptake, and degradation

5-HT is synthesized from L-tryptophan through a two-enzyme mediated pathway. First L-tryptophan is converted into 5-hydroxy-L-tryptophan (5-HTP) by tryptophan hydroxylase (TPH). TPH has two isoforms which are designated TPH1 and TPH2. TPH2 is the predominant form found within the brain while TPH1 is present in the periphery such as gut, pineal gland, spleen, and thymus (Walther et al, 2003, Walther and Bader, 2003). This first conversion is the rate determining step (Albert and Lemonde, 2004). It is important to know that while 5-HT cannot cross the blood brain barrier, L-tryptophan and 5-HTP can, and thus the second enzymatic step must occur locally with regards to the CNS. 5-HTP is decarboxylated into 5-HT through the action of aromatic L-amino acid decarboxylase. Within neurons, 5-HT is loaded into presynaptic vesicles by the vesicular monoamine transporter type 2 (VMAT2), which also serves as a regulator for the amount of readily releasable 5-HT (Veenstra-VanderWeele et al, 2000). Upon release into the synaptic cleft, two eventual fates await the 5-HT: reuptake and degradation. 5-HT is rapidly taken back into the neuronal cells and recycled by serotonin transporters (SERT), which are found on the pre-synaptic nerve terminal or dendritic spines. SERT serves an important role in regulating the 5-HT signal and can transport, to a lesser degree, other endogenous amines such as dopamine and drugs that alter 5-HT release such as amphetamines (Murphy et al, 2004). Without the presence of SERT, neurons cannot keep up with the demand for 5-HT in 5-HT signalling (Kim et al, 1995). The alternative fate of 5-HT to reuptake is degradation. 5-HT is metabolized by monoamine oxidase A (MAO-A) and converted into 5-hydroxyindoleacetic acid (5-HIAA), which is excreted in the urine.

5-HT receptors

5-HT, being a transmitter, must exert its effects through receptors. Currently there are 7 families of 5-HT receptors, name 5-HT_1 through 5-HT_7, and 14 subtypes. 5-HT receptors are all membrane bound receptors that are found in the CNS, the peripheral nervous system (PNS) and non-neuronal organs such as the gastrointestinal and cardiovascular systems. All 5-HT receptors are metabotropic receptors with the exception of 5-HT_3, which are ligand-gated ion channels (Hannon and Hoyer, 2008). The metabotropic 5-HT receptors are G-protein coupled receptors (GPCR). They all have seven hydrophobic transmembrane domains with an extracellular amino (N) terminus and an intracellular carboxyl (C) terminus. They are coupled to heterotrimeric guanine nucleotide-binding proteins (G-proteins).

G-proteins

G-proteins are heterotrimeric proteins that transduce signals from GPCR and activate secondary messenger systems or membrane channels. The G-proteins are composed of a larger Gα subunit and two smaller Gβ and Gγ subunits, which exert their effect as a complex. Upon activation, the Gα subunit exchanges its bound guanosine diphosphate (GDP) for guanosine triphosphate (GTP), and the G-protein begins to mediate downstream signalling. The signal is terminated when the natural GTPase activity of the Gα subunit hydrolyzes GTP to GDP (Simon et al, 1991).

There are currently four known classes of G-proteins (Gs, Gi/o, Gq, and $G_{12/13}$) and numerous subtypes within each class. Conventionally, the G-proteins are named after their Gα subunits, which identifies, with regards to the Gα subunit, the receptor-specificity and the signalling effector-specificity of the G-protein subtype. Gs, couples directly to membrane bound AC and

increases cyclic adenosine monophosphate (cAMP); Gi/o inhibits AC; Gq stimulates membrane-bound phospholipase C (PLC); and $G_{12/13}$ interacts with GTPase in gene regulation pathways.

As mentioned previously, the Gi/o class of receptors is primarily characterized by its ability to inhibit AC to decrease intracellular cAMP. This class of inhibitory G-proteins is inactivated by pertussis toxin (PTX). PTX causes adenosine diphosphate (ADP)-ribosylation of the Gα subunit at the C terminus and prevents it from coupling with the receptor (Neves et al, 2002, Simon et al, 1991).

The C terminal amino acids of Gαi play a role in both conferring specificity to inhibitory G proteins in addition to dictating sensitivity to PTX. A previous study has shown that replacing the last 5 amino acids of the Gαq subunit with those from members of the Gi/o protein family resulted in a chimeric Gαq that displayed both Gi/o-like signalling activation and sensitivity to PTX (Conklin et al, 1993).

The interaction between GPCR and G-proteins has been classically described by the collision coupling model. In this model, following agonist treatment, GPCR and G-proteins diffuse laterally within the cell membrane and come together to elicit the signalling effects (Tolkovsky and Levitzki, 1978, Oldham and Hamm, 2008). Recent studies, however, have shown support for a pre-assembled GPCR-G-protein signalling complex model. In this model, the GPCR, G-protein, and signalling effects are constitutively bound, perhaps via scaffolding proteins, and the complex is activated via conformational changes of GPCR or G-protein (Galés et al, 2006). The

truth may lie somewhere in between where there are pre-assembled GPCR and G-protein complexes, but GPCR-activation also causes uncoupled GPCR and G-proteins to associate.

The 5-HT$_{1A}$ receptor

The 5-HT$_{1A}$ receptor was first discovered as a genomic clone that cross hybridized with a β_2 adrenergic receptor probe at reduced stringency (Kobilka et al, 1987). This clone was originally named G-21 and was later confirmed through ligand binding studies to be 5-HT$_{1A}$ (Fargin et al, 1988). The rat 5-HT$_{1A}$ receptor, which share 88% similarity with humans, was first cloned and characterized in 1990 (Albert et al, 1990). A schematic of the 5-HT$_{1A}$ receptor is presented in Figure 2.

5-HT$_{1A}$ is a 422 amino acid protein encoded by the intronless gene, *HTR1A*, on chromosome 5q11.2-q139 (Hannon and Hoyer, 2008, Albert et al, 2004). Like all 5-HT$_1$ receptors, 5-HT$_{1A}$ binds preferentially to Gi/o G-proteins. Through the G-protein subunits, the receptor is coupled to numerous different signalling pathways. Binding studies and mRNA detection have localized 5-HT$_{1A}$ receptors to the hippocampus, entorhinal cortex, raphe nuclei, neocortex, and thalamus (Chalmers and Watson, 1991). 5-HT$_{1A}$ receptors are not only found in the forebrain as post-synaptic receptor but are also found pre-synaptically on the soma and dendrites of the 5-HT neurons in the raphe nuclei.

The two populations of 5-HT$_{1A}$ receptor, though identical in amino acid sequences, show different signalling characteristics. In vitro 5-HT$_{1A}$ receptors couple to Gαi subtypes on the order of Gαi3 > Gαi2 > Gαi1 >> Gαo (Bertin et al, 1992); however a later study using tissues

from different regions of the brain show various preferences to the Gαi subtypes (Mannoury la Cour et al, 2006). While all forms of Gαi/o subunits are expressed ubiquitously, 5-HT_{1A} receptors bind preferentially to Gαi3 in the anterior raphe, Gαo in the hippocampus, Gαi3 and Gαo in the cerebral cortex, and Gαi1, Gαi3, Gαo, and Gαz in the hypothalamus. Lately a study has shown that variations in agonist activation of 5-HT_{1A} receptors can produce different patterns of Gαi/o subunit coupling (Valdizán et al, 2010). In addition, pre-synaptic and post-synaptic receptors can respond differently to ligands. For instance receptors in the dorsal raphe exhibit stronger inhibition following treatment from 5-HT_{1A} antagonists such as spiperone and pindolol compared to other regions of the brain such as the hippocampus (Blier et al, 1993, Artigas et al, 1994). Recently, a study has shown zinc ion's inducing allosteric modulation of agonist and antagonist binding for 5-HT_{1A} receptors found within cortical and hippocampal regions, which provides further support for the two pharmacologically different 5-HT_{1A} receptor populations (Barrondo and Sallés, 2008). There are currently many compounds that can target 5-HT_{1A} receptors, but many of these are non-specific. There are, however, a number of selective 5-HT_{1A} receptor agonists and antagonists, which are summarized in Table 1 (Barnes and Sharp, 1999)

5-HT_{1A} receptor specific agonists	Non-specific 5-HT_{1A} partial agonist	5-HT_{1A} receptor specific neutral antagonist	Non-specific 5-HT_{1A} antagonist
8-OH-DPAT Dipropyl-5-CT Gepirone	BMY 7378 NAN-190 MDL 73005 EF SDZ 216525 Buspirone Isipirone	WAY100635 (S)-UH-301 NAD-299	Propranolol Spiperone Pindolol

Table 1. Commonly used agonist and antagonist both specific and non-specific directed towards the 5-HT1A receptor

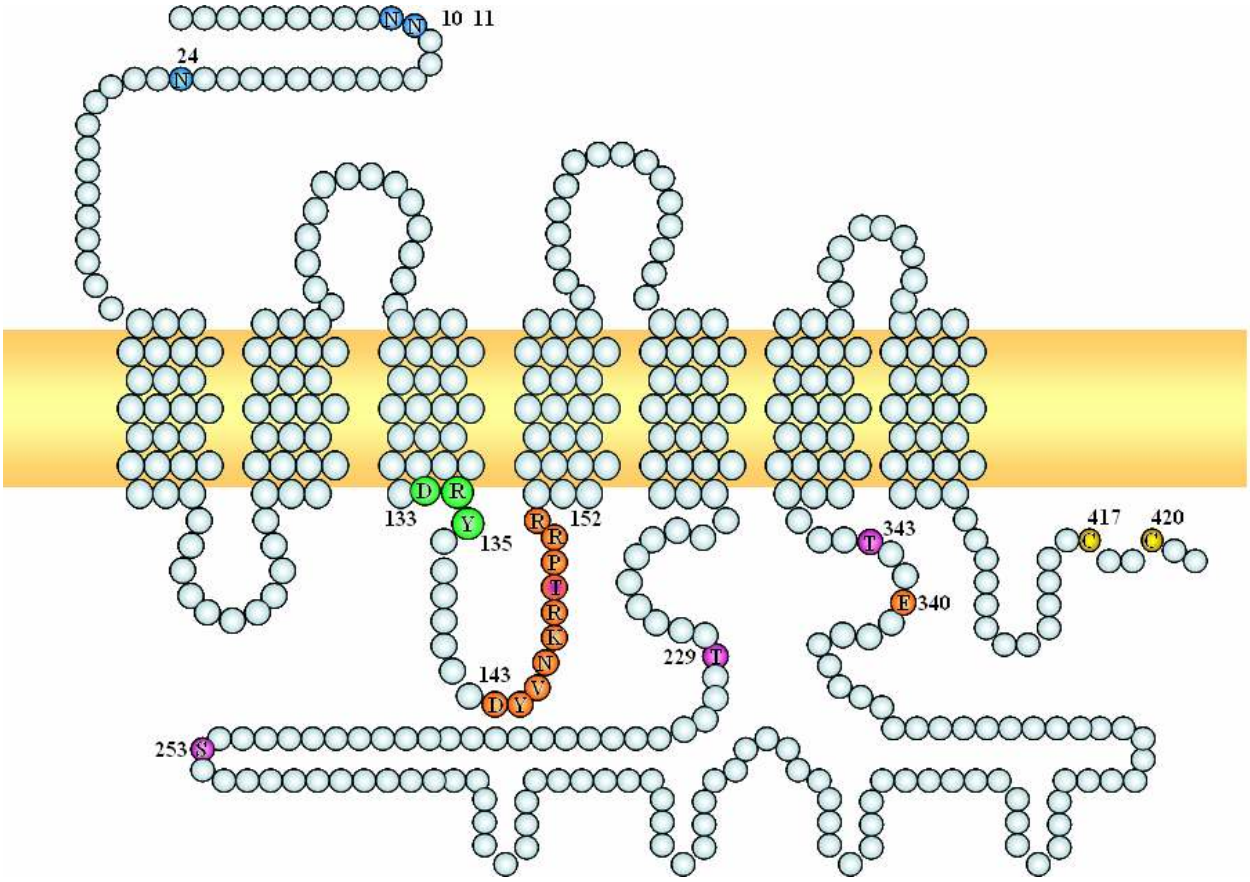


Figure 2. Model of the 5-HT$_{1A}$ receptor. The 5-HT$_{1A}$ receptor is a GPCR with seven transmembrane domains. Here, important residues are identified and highlighted. Glycosylation sites are noted in blue, the conserved DRY sequence in green, the 10 i2 residues and E340 of the i3 loop that putatively mediate G-protein coupling are indicated in orange, phosphorylation sites in purple, and palmitoylation sites in yellow.

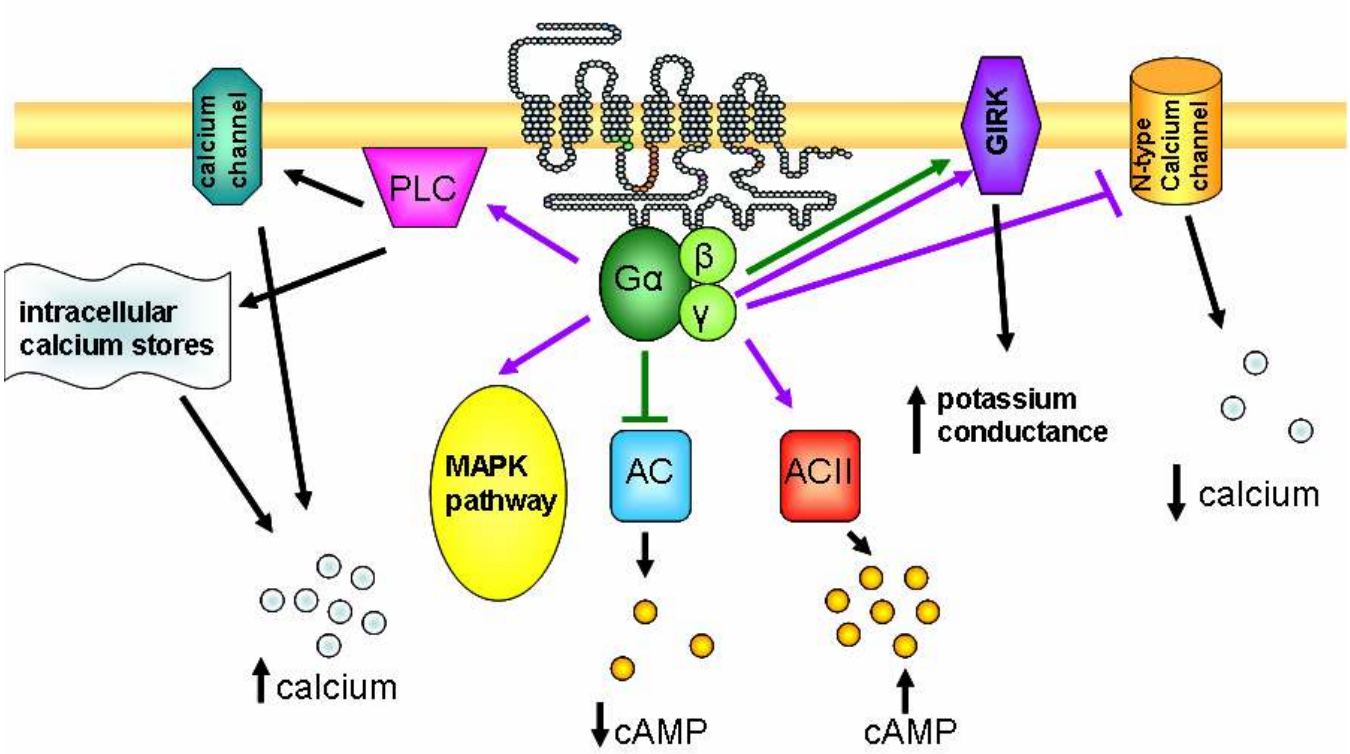


Figure 3. **5-HT$_{1A}$ signalling pathways.** Signalling pathways of the 5-HT$_{1A}$ are shown in this simplified cell representation. Arrows indicate activation of a signalling effector and barred lines indicate inhibition. Purple arrows and lines indicate signalling effectors activated by Gβγ subunits and green arrows and lines indicate activation by the Gα subunit.

G-protein mediated 5-HT_{1A} pathways

The 5-HT_{1A} receptor is known to couple to a wide range of signalling pathways, but many of these couplings were studied only in vitro and appeared to vary depending on the cell line in which the receptor was expressed. 5-HT_{1A} signalling pathways are summarized in Figure 3.

Inhibition of adenylyl cyclase

One of the main effects of 5-HT_{1A} activation, characteristic of Gi/o coupled GPCR, is the reduction of cellular cAMP through inhibition of AC (Albert et al, 1990). This inhibition is primarily attributed to Gαi subunits. In the pituitary cells, GH4C1, when any one of the three subtypes of Gαi (Gαi1, Gαi2, and Gαi3) was knocked out, 5-HT_{1A} mediated decreases in basal cAMP levels was altered (Liu et al, 1999). It has been postulated that cells require all three forms of Gαi to mediate inhibition of basal cAMP levels, because this effect is not present in other non-neuronal cell lines, such as fibroblasts and ovary cells, that do not have the full complement of the three Gαi subunits. In non-neuronal cells lines, however, activation of 5-HT_{1A} receptors can still inhibit stimulatory G-protein increases in cAMP. This has been attributed to altered signalling behaviour based on the availability of the Gαi subtypes to which the receptors can couple. For example, in the GH4C1 cells, both Gαi2 and Gαi3 are required to inhibit Gs stimulated cAMP increase (Liu et al, 1999). Although generally 5-HT_{1A} receptors serve to decrease cAMP, this effect was found to be absent from 5-HT1A receptors found on the 5-HT neurons of the dorsal raphe (Clarke et al, 1996). While this result can be attributed heterogeneity of the cell sample used in the experiment, a likely possibility is that the 5-HT_{1A} receptor couples to another complement of Gαi compared to elsewhere in the brain, thus producing the altered signalling profile.

Neuronal hyperpolarization

Within the CNS, the 5-HT_{1A} receptor is best known as the major somatodendritic autoreceptor that regulates the firing rate of 5-HT neurons (Sprouse and Aghajanian, 1987, Sotelo et al, 1990). Upon activation, the 5-HT_{1A} receptor is responsible for activating a calcium-independent potassium current via G-protein inward rectifying potassium channels (GIRK) resulting in neuron hyperpolarization (Colino and Halliwell, 1987, Williams et al, 1988, Araneda and Andrade, 1991). This effect was found to be mediated by Gβγ as the primary activator of GIRK, however experiments in xenopus oocytes using the fluorescence resonance energy transfer have shown interaction between Gβγ, GIRK, and the Gαi3 subunit (Kovoor and Lester, 2002, Berlin et al 2010). Recently there has also been evidence that 5-HT neurons are also inhibited by post-synaptic 5-HT_{1A} receptors in the medial prefrontal cortex, through an indirect feedback loop (Bambico et al, 2008). Whether through direct or indirect means, neuronal hyperpolarization results in less neurotransmitter release and by extension causes a decrease in the influx of calcium in to the neuron.

Regulator of intracellular calcium levels

Depending on the cell type in which the receptors were expressed, 5-HT_{1A} receptors have been found to be able to both increase or decrease intracellular calcium levels. In Ltk- cells, which are of the fibroblast lineage, activation of the receptor was found to cause a 2.6-fold increase in intracellular calcium via activation of the PLC pathway. This effect was also found to be present in the rat raphe cell line, RN46A (Kushwaha and Albert, 2005). In contrast, when the receptors were expressed in a neuroendocrine cell line, the pituitary GH4C1 cells, 5-HT_{1A} receptors were able to inhibit the calcium influx by 40% following induction by the calcium channel agonist,

(+/-)BAY K8644 (Lembo et al, 1997). This effect is believed to be caused by the 5-HT_{1A} receptor's ability to reduce whole cell calcium current through N-type calcium channels, which serves to further inhibit the firing rate of neurons (Penington and Kelly, 1990).

Activation of adenylyl cyclase II (ACII)

The Gβγ subunits coupled to Gi/o G-proteins have the added ability to activate ACII in HEK293 cells. Although this may seem to contradict the normal actions of Gi/o proteins of inhibiting AC, there appears to be different levels of activation depending on the cell and receptor type. In one study, HEK293 cells were co-transfected with a Gs receptor, the α_2 adenosine receptor, and an inhibitory dopamine-2 (D2) receptor. Activation of D2 caused a decrease in intracellular cAMP. On the other hand, when ACII DNA was also transfected into the cell, activation of D2 caused an increase in the cAMP (Federman et al, 1992). In contrast, Gβγ activation of ACII was not seen under normal conditions in GH4C1 cells. It has been postulated that normally, the effects of Gβγ mediated ACII activation is masked by the inhibitory actions of the Gαi subunits. Only when specific Gαi subunits are removed, is the cAMP increase mediated by Gβγ apparent (Liu et al, 1999). Thus there appears to be a dynamic interaction between AC activation and inhibition that are mediated by the 5-HT_{1A} receptor, and the Gβγ activation of ACII may help the Gαi subunits in regulating the levels of cAMP within the cells.

5-HT_{1A} and the MAPK pathway

The Gβγ subunits of the 5-HT_{1A} receptor have been additionally implicated in activation of the mitogen-activated protein kinase (MAPK) pathway in non-neuronal cell lines. The MAPK pathway is a multi-step process that is involved in cell growth and survival through the inhibition

of pro-apoptotic genes (Bonni et al, 1999). The classical initiators for the MAPK pathway are the growth factor tyrosine kinase receptors. These receptors commence protein signalling through the phosphorylation of Shc, which binds to Grb2 to activate GTPase Ras. GTPase Ras then triggers a phosphorylation or activation of a cascade of proteins such as Raf1, MAPK/ERK Kinase 1 and 2 (MEK1/2), and extracellular signal-regulated kinase 1 and 2 (ERK1/2). The resulting ERK1/2 activates various protein kinases downstream that lead to ion channel activation or gene transcription (Polter and Li, 2010). The Gβγ subunits of Gi/o coupled GPCR have been found to act upon Shc-Grb2 complex and mediate MAPK activity through the common tyrosine kinase receptor pathway (van Biesen et al, 1995). A later study demonstrated that stimulation of Gi/o coupled GPCR, and more specifically 5-HT_{1A} receptors, also activated phosphoinositol 3-kinase (PI3-K) and PLC. These signalling effectors activate nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), a DNA transcription regulator that leads to increase in cell survival (Cowen et al, 1999, Hsiung et al, 2005). Studies into MAPK pathway activation in cells of neuronal lineage have resulted in a mixture of results. For instance, activation of 5-HT_{1A} receptors in the hippocampus-derived HN2-5 cells resulted in inhibition of capase-3 and apoptosis through an ERK1/2 and protein kinase C-α (PKCα) mediated pathway (Adayev et al, 2003). This pathway was also implied to be the underlying mechanism through which 5-HT_{1A} receptor activation can provide neuroprotection against ethanol-induced death in embryonic rhombencephalon neurons (Druse et al, 2004). In contrast, 5-HT_{1A} receptors have also been found to cause decreases in the levels of phosphorylated ERK1/2 and inhibition of the MAPK pathway in rat raphe RN46A cells (Kushwaha et al, 2005). A later study in vivo demonstrated a decrease in phosphorylated ERK1/2 in the hippocampus at 5 and 15 minutes following treatment with 8-OH-DPAT, whereas the hypothalamus showed an

increase in phosphorylated ERK1/2 at 5 minutes that was followed by a decrease to basal levels at 15 minutes (Crane et al, 2007). The apparent time dependency of the MAPK pathway activation may explain the discrepancy with the previous MAPK activation results found in cultured HN2-5 cells. Thus taken together, these studies demonstrate that there is great heterogeneity in the pattern of MAPK activation across different cell lines and conditions, much like the other pathways mediated by the 5-HT_{1A} receptor. This large variation in effect of the 5-HT_{1A} receptor's signalling likely accounts for how a single receptor can mediate cell specific effects in a variety of cell types in the human body.

Interaction between 5-HT1A receptor and G-protein subunits

The second and third intracellular loops (i2 and i3) of the 5-HT_{1A} receptor are implicated in coupling to G-protein subunits (Albert et al, 1998, Kushwaha and Albert, 2005). Four protein kinase C (PKC) phosphorylation sites are present within the 5-HT_{1A} receptor's i2 and i3 loops: T149 in the i2 loop and T229, S253, and T343 in the i3 loop. These four residues have implications in coupling of the receptor to G-proteins, because phosphorylation of these PKC sites causes uncoupling from Gβγ induced mobilization of calcium (Lembo and Albert, 1995). The T149 residue is of particular interest because it is conserved among several GPCR. Point mutations at this residue caused uncoupling from the Gβγ mediated pathways in neuronal F11, Ltk-, RN46A, and GH4C1 cells (Lembo et al, 1997, Wu et al, 2002, Kuswaha and Albert, 2005). Because of the mutational effects of the threonine, the flanking residues (DYVNKRTPRR from amino acid position 143 to 152) were postulated to also affect G-protein coupling. While point mutations at the various amino acids in the DVNKRTPRR region did not have significant effects in ligand binding compared to the wild type in the majority of the mutants, there were profound

changes in the activation of signalling pathways. A large portion of the i2 mutants, displayed signalling profiles changes comparable to Gβγ-uncoupled, Gβγ selectively coupled, or Gαi and Gβγ uncoupled phenotypes. A few mutants even displayed inverse basal activity such as inhibiting constitutive activity of ACII. (Kushwaha et al, 2006).

Studies into the 3rd and 6th transmembrane domain of the rhodopsin receptor have also suggested at the possible role of the i3 loop in mediating GPCR coupling to G-proteins (Sheikh et al, 1996, Farrens et al, 1996, Albert et al, 1998). Peptides derived from the i3 domain have been found to activate Gαi inhibition of AC, and subsequent alterations to the peptides length or sequence produced different levels of AC inhibition. (Ortiz et al, 2000). Within the i3 loop, the glutamate residue at position 340 (E340) has been identified as a possible mediator of G protein coupling because of its interaction with the E/DRY motif of the i2 loop (Seeber et al, 2003). The E/DRY motif is a conserved sequence of three amino acids among GPCR that is commonly associated with dictating receptor conformation and G-protein recognition and coupling (Rovati et al, 2007)

Bioluminescence Resonance Energy Transfer (BRET)

One of the techniques that is commonly used to study GPCR interactions is the BRET assay. The BRET assay is a technique that allows for the monitoring of protein-protein interactions in live cells.

The BRET assay was first described in 1999 as a variation on the fluorescence resonance energy transfer (FRET). Both assays rely on the naturally occurring phenomenon of the Förster resonance energy transfer between an energy donor protein, and an acceptor fluorophore (Xu et

al, 1999). The transfer of energy occurs through the direct transfer of energy from the excited donor to the acceptor through a non-radiative (resonance) process (Ward and Cormier, 1978). The maximum distance of energy transfer is 10 nm, which correlates with the distance between interacting proteins (Hamdan et al, 2006). In the BRET assay, the donor protein is the *Renilla reniformis* luciferase (RLuc).

The technique involves the fusion of the RLuc or acceptor fluorophore to one of the proteins in a putative protein interaction pair. Usually the RLuc or fluorophore are attached to the N-terminus or the C-terminus so that they have minimal disruption to the protein's normal function. There are, however, exceptions to this general rule as certain proteins, such as the Gαi, lost function following attachment of the RLuc to the N-terminus (Galés et al, 2005). Thus it is important to test for functionality of the fusion proteins. When the donor RLuc is activated by its substrate, it will emit blue light at around 400nm wavelength. Concurrently, if the acceptor fluorophore is within the 10 nm distance, RLuc will transfer energy to the acceptor. This causes the acceptor to also emit light that has a longer wavelength the extent of which is determined by the fluorophore's identity.

Currently there are three types of BRET assay, which are known as BRET1, BRET2, and BRET3. The three types are distinguished based on the acceptor fluorophore used in conjunction with the donor, RLuc. The first two methods are the most commonly used. In the BRET1 assay, yellow fluorescence protein (YFP) is used as the acceptor, and RLuc is activated by the substrate coelenterazine h. This process produces donor emission of ~480nm and an acceptor emission of ~530 nm (Xu et al, 1999). In the BRET2 assay, YFP is replaced with a variant of the green

fluorescent protein (GFP) known as GFP2. The substrate is also a modified form of the coelenterazine h known as coelenterazine 400a or DeepBlueC™ (Biosignal Packard). The donor emission peak in BRET2 is ~407 nm and the acceptor emission peak is ~515 nm (Hamdan et al, 2006). The BRET2 assay is summarized in Figure 4.

BRET3 is a more recently described method where the acceptor protein is a variant on mutant red fluorescent protein known as mOrange. This system results in a more red-shifted light emission of 564 nm. The advantage of this method is that the red shifted light is less attenuated by biological tissue, and thus BRET3 can image signals from superficial and deep tissues of small animal models such as mice (De et al, 2009).

The BRET assay has been shown to allow for further optimization of its signal. For instance, improved signals have been obtained using mutants of the RLuc that improves quantum efficiency and stability (De et al, 2007). In addition, whereas normally, coelenterazine activated BRET signals last normally up to 30 minutes, a study has shown that through usage of a protective form of coelenterazine, known as EnduRen™, the signal can be recorded for up to six hours (Pfleger et al, 2006).

Although FRET and BRET are both resonance energy transfer based assays, they each possess unique characteristics that make one of the assays better than the other for different experiments. BRET is initiated by a single dose of substrate, and thus it shows various time frames of signal decay as mentioned previously, and often times, the proper variant of the BRET assay needs to be selected for optimal result. FRET on the other hand is initiated by light stimulation, and thus

there is greater control when monitoring kinetics between protein interactions. On the other hand, FRET possesses the risk of photobleaching the samples, and a greater chance of emission contamination between the donor signal and the acceptor signal. FRET's ability to record time-resolved changes within the cells allows for the study of receptor dynamics within the cell such as expression, internalization, co-localization, and oligomerization using cell microscopy (Pfleger and Eidne, 2005).

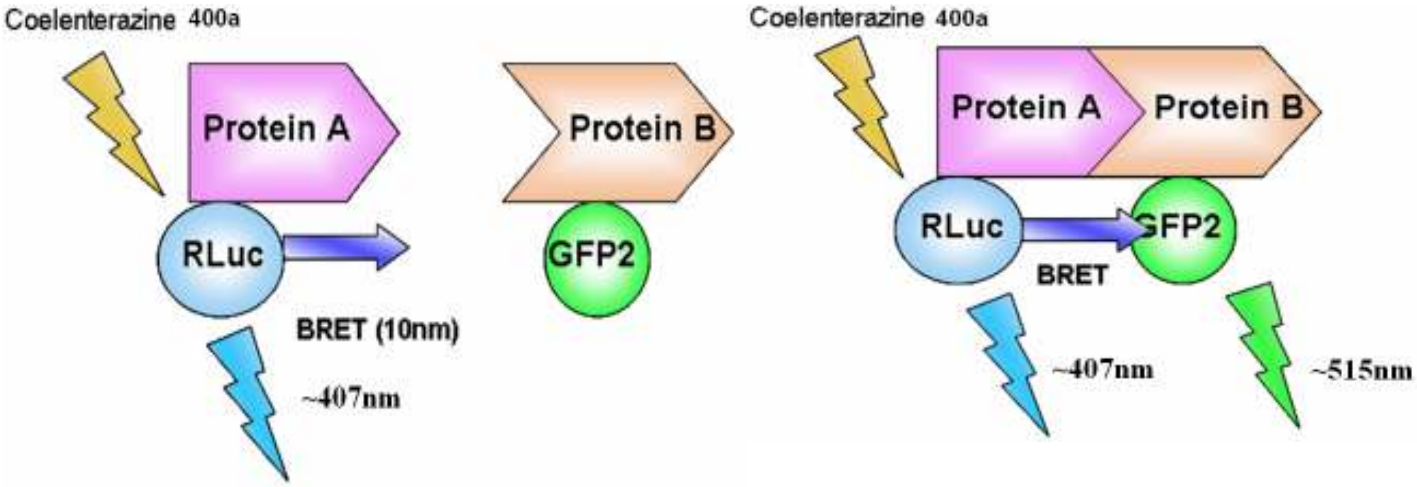


Figure 4. Mechanism of Bioluminescence Resonance Energy Transfer. Protein A and Protein B represent two proteins of interest that have been fused to the donor protein, RLuc, and the acceptor protein, GFP2 respectively. Upon excitation by the substrate, coelenterazine 400a, RLuc will give off an emission at ~407 nm wavelength, which overlaps with the excitation spectrum of the GFP2 protein. If the proteins are interacting, the donor and acceptor proteins would be close enough for the emission to stimulate GFP2, which will fluoresce at ~515 nm wavelength.

The role of the 5-HT system and 5-HT_{1A} receptors in major depressive disorder

As mentioned previously, dysfunctions within the 5-HT system is the most often cited cause of major depressive disorder. Despite there being one study that found only a mild level of dysphoria in healthy individuals depleted of 5-HT, there are, nevertheless, evidences that the 5-HT system is implicated in the treatment of depressed individuals (Young et al, 1985). Patients with bipolar or unipolar depression treated with antidepressants were found to relapse when given small doses of parachlorophenylalanine (PCPA), which inhibits the synthesis of 5-HT (Shopsin et al, 1976). In addition, treatments for depression often fall along two streams: increase the availability of the 5-HT or increase the response to them. The first methods can be achieved by inhibiting either reuptake receptors or enzymes that would normally metabolize them. The second method is to sensitize the receptors via drugs to elicit a stronger signal response (Gardier et al, 1996, Blier and de Montigny, 1999).

The class of antidepressants known as tricyclic antidepressants (TCA) has a dual effect of not only blocking reuptake of 5-HT but has also been shown to sensitize post-synaptic 5-HT_{1A} receptors in various regions of the brain (Blier and de Montigny, 1999). Although TCA works to both increase the availability of 5-HT and sensitize post-synaptic receptors, it is not commonly used anymore due to its effect on other neurotransmitter systems such as noradrenaline, which leads to increased adverse effects.

Selective serotonin reuptake inhibitors (SSRI) are currently the most prescribed class of antidepressants due to their fewer side effects compared to another class of reuptake inhibitor, TCA (Montgomery 1994). As the name indicates, the SSRI exert its effects by selectively

blocking the 5-HT transporter to increase the availability of 5-HT in the synaptic cleft. This is not the sole action of SSRI because therapeutic effects are not seen until 2-4 weeks following SSRI treatment (Charney et al, 1990). In fact, treatments that promote increase 5-HT in the synaptic cleft actually cause initial decrease in the firing rate of the 5-HT neurons via activation of the 5-HT_{1A} autoreceptors. It is believed that the latent therapeutic actions of antidepressant arise only upon the desensitization and internalization of the 5-HT_{1A} autoreceptors, which release their inhibitory effects on the neuron and leading to a net increase in 5-HT transmission. There are however, various studies that hint at more complex changes other than simple internalization of the receptors. Autoradiography has shown internalization of the 5-HT_{1A} receptor following acute treatment of 8-OH-DPAT; however, after chronic treatment with SSRI, although 5-HT1A RNA levels were reduced in the raphe area, there was no difference in the ligand binding and density of the 5-HT_{1A} receptor of the dorsal raphe nuclei (Le Poul et al, 2000, Riad et al, 2004, Le Poul et al, 1995). Despite this, there is a functional desensitization of the receptors, which can be most likely explained by gradual uncoupling of the 5-HT_{1A} receptor following rounds of internalization and retargeting to the membrane (Newman et al, 2004, Riad et al, 2008). Furthermore, neurogenesis in the hippocampus following chronic SSRI treatment has been shown to be important in eliciting SSRI's therapeutic effects, which may also explain the latency before the therapeutic effects are seen (Santarelli et al, 2003). Therefore the simple act of increasing the amount of 5-HT using SSRI leads to profound changes within the 5-HT neuron via the 5-HT_{1A} receptors.

Clinical approaches that try to improve the efficacy of SSRI treatment have found that treatment can be improved when the SSRI is co-administered with a 5-HT_{1A} receptor antagonist, such as

pindolol (Artigas et al, 1996). This at first may seem paradoxical because the addition of the antagonist can silence the 5-HT_{1A} autoreceptors at the same time as the post-synaptic ones. Experiments have shown, however, that pindolol appears to inhibit to a greater degree the pre-synaptic receptors compared to the post-synaptic ones, again reinforcing differences between the pre-synaptic and post-synaptic 5-HT_{1A} receptors. Thus through a better understanding of the 5-HT_{1A} receptor signalling pathway, perhaps new treatments options can be created that better target the causes of depression, shorten their period of therapeutic onset, and reduce the presence of adverse side effects (Blier and Bergeron, 1995, Beïque et al, 2000).

Animal models for depression

Knockout (KO) mice lacking the 5-HT_{1A} receptor have been generated using the BALB/c and the C57BL6/J mice strains (Parks et al, 1998, Ramboz et al, 1998, Heisler et al, 1998). While growth and reproductive capabilities of the KO animals are similar to wild type, the KO mice demonstrated increased anxiety that appeared to be geared more specifically towards novel, threatening stimuli, which have parallels in human panic or post traumatic stress disorders (Klemenhagen et al, 2006). The anxiety-like behaviours can be rescued through the expression of 5-HT_{1A} receptors in the post-synaptic areas or at the early post natal days 5-21 (Gross et al, 2002). Treatment with either agonist or antagonist for the 5-HT_{1A} receptor generally resulted in anxiolytic and anxiogenic effects respectively (Parks et al, 1998). In the forced swim test, a classical test for depression, the KO animals exhibited increased mobility, which may have resulted from increased 5-HT's effect on locomotion or resistance to behaviours associated with depression. Surprisingly, basal levels of 5-HT, depolarization-evoked 5-HT, or the concentration of 5-HT transporters were later found to be similar between wt and KO animals (He et al, 2001).

The KO animals did show, however, 2 fold increase in 5-HT concentration following acute administration of the SSRI, fluoxetine. It is believed that the lack of 5-HT concentration profile in the KO mice is due to compensatory mechanisms from other 5-HT receptors. Among these the 5-HT_{1B} receptor has been postulated to be a likely candidate, and its sensitization may explain in part the similar basal levels of 5-HT in KO and wild type mice (Ramboz et la, 1998, He et al, 2001). A recent study has demonstrated that mice over expressing the 5-HT_{1A} autoreceptor did not respond to antidepressants and possessed a blunted response towards stress. In contrast decreasing 5-HT_{1A} autoreceptors prior to antidepressant treatment yielded greater response (Richardson-Jones et al, 2010).

Although it is impossible to assess depression in animals the same way as humans, certain laboratory tests have been designed to approximately assess depression within animals based on performance improvement following chronic but not acute antidepressant treatment. The forced swim test, measuring how long an animal struggles to stay afloat before giving up, and the novelty suppressed feeding tests, how long an animal takes to venture into an aversive environment to get food, are two tests that are commonly used as a model for depression (Santarelli et al, 2003, Albert et al, 2004).

Goal

Because the 5-HT_{1A} receptor is involved in numerous pathways within the body and underlies many behavioural disorders, especially major depressive disorder as observed from previous studies in both humans and animal models, insights into the signalling characteristics of the 5-HT_{1A} receptor may shed light on how better treatment regimes can be made to treat these disorders. To this end, a greater understanding of the regions that the 5-HT_{1A} receptor uses to interact with G-proteins and mediate its effect is needed. Both the i2 and i3 loops within the receptor was found to play important roles in G-protein coupling, and mutations within these loops resulted in uncoupling of the receptor from various pathways. These changes include uncoupling from Gαi mediated pathways, selective uncoupling from Gβγ mediated pathways, inverse Gβγ pathway activation, and complete uncoupling from all pathways (Kushawaha et al, 2006).

This study sets out to verify that the changes in signalling pathway activation found in the 5-HT_{1A} receptor mutants are caused by changes to their G-protein coupling. This study will focus specifically on the Gαi1 subunit. In addition, this study will also study the nature of the interaction between the receptor and the Gαi subunit by studying the spatial relationship between the two proteins following agonist and antagonist treatment.

<u>Approach:</u>

Of the i2 and i3 mutants previously generated by Dr. Kushwaha, seven mutants, each representing a specific signalling profile, were selected for study. They are summarized in Table 2. Verification of Gαi coupling changes was performed using the BRET2 assay using green

fluorescent protein 2 (GFP2) tagged wild type and mutant 5-HT_{1A} receptors and Renilla reniformis luciferase (RLuc)-tagged Gαi1 subunits. Spatial information regarding the interaction between the 5-HT_{1A} receptor and Gαi1 subunit was primarily studied using the BRET2 assay and further explored with the pull down assay.

Mutant	Effect
T149E	weak Gβγ coupled, Gα coupled
T149V	Gβγ uncoupled, Gα coupled
Y144A	Gβγ-ACII stimulation, no coupling to Gβγ-PLC; Gα uncoupled
R152D	Gβγ-PLC coupled, Gβγ-ACII uncoupled, Gα uncoupled
R148K	uncoupled with constitutive inverse Gβγ activity
E340G	Gα uncoupled, Gβγ coupled
E340K	completely uncoupled

Table 2. Select mutants of the 5-HT_{1A} receptor with altered signalling pathway activation. The table lists the seven mutants used in this study. T149E, T149V, Y144A, R152D, and R148K all represent i2 loop mutants while E340G and E340K are i3 loop mutants.

Materials and Methods:

Plasmids

The 5-HT_{1A}-GFP2 wild type and mutant receptors were generated through the insertion of the rat HTR1A gene into the pGFP2 plasmid digested with BamHI and BglII. The S-tagged 5-HT_{1A} plasmid was generated by inserting the rat HTR_{1A} gene into EcoRI and XhoI digested pTriex-4 plasmid. Mutant receptors with their respective tags were subsequently made through point mutagenesis of the wild type receptors using primer-directed mutagenesis kit (QuikChange XL II Site-Directed Mutagenesis Kit, Stratagene). RLuc-tagged Gαi1 subunits with RLuc inserted at amino acid positions 60, 91, and 122 were received from the lab of Dr. Bouvier (Galés et al, 2006). HCN4-GFP2 plasmids were received from Dr. Accili's lab (Whitaker and Accili, 2008).

Cell culture:

Human embryonic kidney cells (HEK293) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Wisent) supplemented with 9%-10% fetal bovine serum (FBS) (Invitrogen). Cells were grown at 37^{o}C with 5% CO_2.

Calcium phosphate transfection protocol:

2.5 μg of each plasmid DNA was added to 1.5 ml micro-centrifuge tubes. 450 μl of ddH_2O and 50 μl of 2.5M $CaCl_2$ were then added to each tube, and the tubes were gently swirled. Following that, 500 μl of 2X HEPES buffered saline (HBS) was added slowly, drop by drop, into the mixture, and the mixture was allowed to incubate for a short moment while the cell plates were prepared for transfection. It is important to note that HBS but must be added slowly or else too

much precipitate will form, reducing transfection efficiency. 8 ml of fresh DMEM was added to the cell plates followed by 1 ml of the transfection reagent mixture.

On the following day, the media within the cell plates were changed with 10ml of fresh DMEM and allowed to grow for another night. The cells were ready to be collected the next day for assays.

Lipofectamine-Plus transfection protocol

HEK293 cells were grown to a density of $4x10^4$ cells per cm^2 in 6 well plates. The following day, the cells were transfected with 2 μg of the GFP2-tagged wild type 5-HT_{1A} receptor or HCN4 plasmid and an increasing amount (0.01 μg, 0.05 ug, 0.1 μg and 0. 2ug) of RLuc-Gαi1-91 plasmid. The plasmids were incubated in 750 μl of serum-free DMEM along with 2 μl of Plus Solution (Invitrogen). In a separate tube, 2 μl of Lipofectamine (Invitrogen) was prepared in 750 μl of serum-free DMEM. After 15 minutes, the two plasmid and plus solution was mixed with the Lipofectamine and allowed to incubate for a further 30 minutes. The media within the cell plates were removed and replaced with 1.5 ml of serum-free media. 1.5 ml of transfection reagent-plasmid mixture was added to each well. The cells were returned to the incubator for 4 hours. Following four hours, the serum-free reagent was replaced with normal DMEM supplemented with 10% FBS. The cells were allowed to grow for two days.

BRET

The BRET protocols are adapted from Dr. Accili's published study (Whitaker and Accili, 2008).

HEK293 cells were used in the experiment. Cells were grown in DMEM supplemented with 9-10% FBS to a density of ~$1.6x10^5$ cells per cm^2 in a 10 cm culture plate. The cells were subsequently split using a 1:10 dilution factor into new plates, using HBBS-EDTA to detach the cells. The following day, the cells were transfected using either the calcium phosphate method or the lipofectamine-plus method in the case of the titration experiments.

Cells were washed twice with 2 ml of Dulbecco's phosphate buffered saline (D-PBS) (Wisent) and then detached using 6-8 ml of HBBS-EDTA. The amount of HBBS-EDTA used depended on the design of the BRET experiment where 1 ml of HBBS-EDTA cells would make one duplicate sample. The cells were centrifuged at 750 g for 5 minutes. The HBBS-EDTA was aspirated from each tube, and the cells resuspended in 150 µl or 100 µl of BRET buffer for agonist treated samples and non-treated samples respectively. The contents of each tube were then added equally to two wells of a 96-well opaque white optic plate. At this point, antagonist such as WAY100635 was added to the designated antagonist treated wells. The cells were left to incubate in the dark for 30 minutes. Afterwards, the GFP signal (515 nm wavelength) for the cells were read using the Victor ^{3}V optic plate reader (Perkin Elmer) as an indicator of transfection efficiency. Subsequently, 25 µl of 4 µM of 5-HT (to make a 1 µM 5-HT final concentration) was added to each agonist treated wells, and then 25 µl of 20 µM of coelenterazine 400a (Biosignal Packard) previously prepared in BRET buffer was added to each well. The plate was then read at two different wavelengths, ~407 nm for RLuc and ~515 nm for GFP2.

The BRET ratio is a measure of interaction between the RLuc and GFP2 and is calculated by the following formula:

(GFP2 of sample – GFP2 of control wells) / (RLuc of sample – RLuc of control wells)

Because the strength of the GFP2 signal is dependent upon the strength of the RLuc and the reading is provided in the form of a ratio, this provides some normalization for variations in transfection efficiency and cell quantity.

Pertussis Toxin assay

PTX functional assay was performed using the normal BRET protocol. 50 ng/ml of PTX was incubated with the cells 16 hours prior to cell harvesting for the BRET assay

cAMP functional assay

cAMP was measured using the cAMP Glo™ assay kit (Promega), and followed the protocol outlined in the kit. In brief, HEK293 cells were transfected with 2.5 μg of either wild type 5-HT1A-GFP2, S-5-HT_{1A}, or untagged 5-HT_{1A} plasmid along with 2.5 μg of RLuc-Gαi1-91 using the calcium phosphate transfection method. Cells were washed twice with D-PBS and detached with HBBS-EDTA. Cells were transferred to a 1.5 ml micro-centrifuge tube and spun at 1000x g for 10 minutes. Cells were resuspended in 100 ml of buffer and transferred to a 96-well plate. Cells were treated briefly with either buffer or 2 μM of 5-HT followed by 2 μM forskolin. Cells were subsequently lysed, and incubated with protein kinase A and detection solution followed by

cAMP reagent solution to activate the luciferase. The luciferase was subsequently read using the Victor ^{3}V multilabel counter (Perkin Elmer).

Pull down protocol

Cells were transfected with S-tagged wild type and T149E mutant 5-HT_{1A} receptors and FLAG-tagged Gαi3-Q204L using the $CaCl_2$ transfection protocol. Prior to harvesting, cells were placed on ice and washed twice with 2 ml of D-PBS. Cells were then divided into either the untreated control group or groups treated with 4 μM 5-HT or 50 μM WAY100635 and 5-HT and treated with the respective chemical agents. Immediately following treatment, cells were cross-linked using 5 mM dimethyl 3,3´-dithiobispropionimidate - 2 HCl (DTBP) (Thermo Scientific) for 15 minutes. The DTBP was subsequently quenched using 50 μl of 1M Tris-HCl (pH 7.4). Cells were washed again two times with 2 ml of D-PBS and lysed using 800 μl of RIPA lysis buffer supplemented with 1X Complete protease inhibitor cocktail (Roche). Cells were scraped into 1.5 μl micro-centrifuge tubes and passed through 25G 5/8 needles to break apart remaining cell membrane debris. The tubes were rotated for two hours at 4^{o}C to solubilize the proteins. Afterwards, cells were spun down at 4^{o}C at 14,000 rpm for 15 minutes. 100 μl of the cell lysate was retained for protein concentration measurement and pull down inputs. The rest of the cell lysate (900 μl) was incubated with 40μl of S-protein agarose (Novagen) overnight.

The following day, pull-down tubes were spun at 4^{o}C at 7000 rpm for 1 minute. The supernatant was removed and the agarose beads were washed with 800 μl of S-protein wash buffer. The process was repeated 3-4 more times. The beads were then incubated overnight with a mixture

of 100 mM dithiolthreitol (DTT), 6X SDS loading dye, and RIPA lysis buffer at a ratio of 1:1:2 respectively.

Subsequently the pull-down tubes were boiled for 5 minutes before being loaded on a 10% SDS-PAGE gel and resolved. Proteins were transferred onto polyvinylidene difluoride (PVDF) membrane and were probed with S-protein horseradish peroxidase (HRP) conjugate (1:5000 dilution) for the 5-HT1A receptor or FLAG antibody (1:1000 dilution) followed by anti-rabbit HRP conjugate (1:5000 dilution). Antibody binding was detected using immobilon western chemiluminescent HRP substrate (Millipore) and resolved on film.

Statistical Analysis

The mean and standard errors of experiments were calculated, and statistical significance was determined using the student's t test (two tailed, paired) with significance being present when $p<0.05$.

Recipes for reagents:

BRET buffer:
Dulbecco's phosphate buffered saline (D-PBS) with 2 μg/ml of aprotinin

2.5M $CaCl_2$ (filter sterilized using 0.22 μm filter)
9.19 g $CaCl_2$ dihydrate
250 ml ddH2O

2.5mM Coelenterazine 400a (DeepBlueC™)
2 ml 100% Ethanol (incubated overnight with molecular sieve 4 Å –Sigma Aldritch)
1 mg DeepBlueC

10X HBBS (pH 7.2)

1.18 M	NaCl
46 mM	KCl

100 mM	D-glucose
200 mM	HEPES buffer

Make up to 1 L with ddH_2O

HBBS+EDTA

450 ml	sterile 5mM EDTA
50 ml	10X HBBS

1 bottle/1000 ml HBBS+EDTA	lyophilized trypsin (5g) (Sigma)

2X HEPES Buffered Saline (HBS) pH 7.1 recipe (filter sterilized using 0.22 μm filters)

1 4ml	5 M NaCl
12.5 ml	1 M HEPES
0.375 ml	1 M Na_3PO_4 or Na_2HPO_4 or 1M NaH_2PO_4
250 ml	ddH_2O

RIPA lysis buffer recipe (pH 7.4):

25 ml	1 M Tris-HCl (pH 8.0)
15ml	5 M NaCl
5 ml	10% SDS
5 mM	0.5 M EDTA
4.8 ml	NP-40 (nonidet P-40 deoxycholic acid)
2.5 g	Deoxycholate Sodium Salt
0.21 g	Sodium Fluoride
1.11 g	Disodium Pyrophosphate

Add ddH_2O to make 500 ml and adjust to pH 7.4

S-protein wash buffer recipe (pH 7.4)

50 mM	Tris-HCl
150 mM	NaCl
1 mM	EDTA
1%	TritonX-100

Results:

Bioluminescence resonance energy transfer

The BRET assay is a technique that allows for real time measurement of protein-protein interaction in vivo. The principle of the assay relies upon activation of a bioluminescent protein attached to a protein of interest. The bioluminescent protein would subsequently transfer energy through resonance to an acceptor fluorophore attached to the putative interacting protein, causing the fluorophore to emit light. The maximum distance of transfer is 10nm, and thus only two proteins that are within close proximity would fluoresce. The BRET2 assay is used in this study, and it employs RLuc as the donor and GFP2 as the acceptor. Further information and background on the BRET assay is provided in Appendix B.

BRET profile wild type 5-HT_{1A} with Gαi1 subunits

HEK293 cells were used in determining the interaction between wild type and mutant 5-HT_{1A} receptors with the Gαi subunit using the BRET method. Initially, the BRET signal between the wild type 5-HT_{1A}-GFP2 and RLuc-Gαi1 constructs, received from Dr. Bouvier's lab, was assessed. The 5-HT_{1A} receptor was tagged with GFP2 at the C-terminal domain, which allows an intracellular localization, and has minimal impact on receptor function. The three Gαi1, Gαi1-60, Gαi1-91, and Gαi1-122, have the RLuc sequence inserted at inter-domain loops found at the amino acid positions indicated in their names. By having the RLuc inserted at the inter-domain loops instead of at the N-terminus, the chimeric Gαi1 subunits retained their function (Galés et al, 2005). BRET signals obtained from the three Gαi1 subunits, following agonist and antagonist treatments showed different results as presented in Figure 5. Using HEK293 cells co-transfected with 5-HT_{1A}-GFP2 and the Gαi1-60 constructs, upon treatment with the agonist, 1μM

5-HT, the BRET ratio showed a slight but non-significant increase. A similar ratio was obtained following 1 μM 5-HT and antagonist, 100 μM WAY100635. Thus the Gαi1-60 did not show response to either agonist or antagonist treatment. Co-transfection of 5-HT_{1A}-GFP2 and the Gαi1-91 construct exhibited a classical responsiveness to agonist treatment, whereby the BRET signal increased significantly following agonist treatment. With this subunit, the addition of the antagonist was able to return the signal back to basal untreated levels. Surprising results were obtained using the Gα1-122 subunit. The basal BRET signal level was higher than that of the other two subunits. With agonist addition, the signal increased still. Strangely, however, the addition of the antagonist appeared to potentiate the BRET interaction even further, whereas one would expect that the signal would decrease back to basal levels. Taken together, the BRET profiles indicate a possible spatial or conformational change between the 5-HT_{1A} receptor and the Gαi1 subunit following agonist and antagonist treatment, which may bring certain regions of the Gαi1 closer to the C-terminus of the receptors and others away. Nonetheless, because the Gαi1-91 demonstrated the classical agonist-responsive characteristic, it was chosen for further study into the nature of the 5-HT_{1A} receptor and Gαi1 interaction.

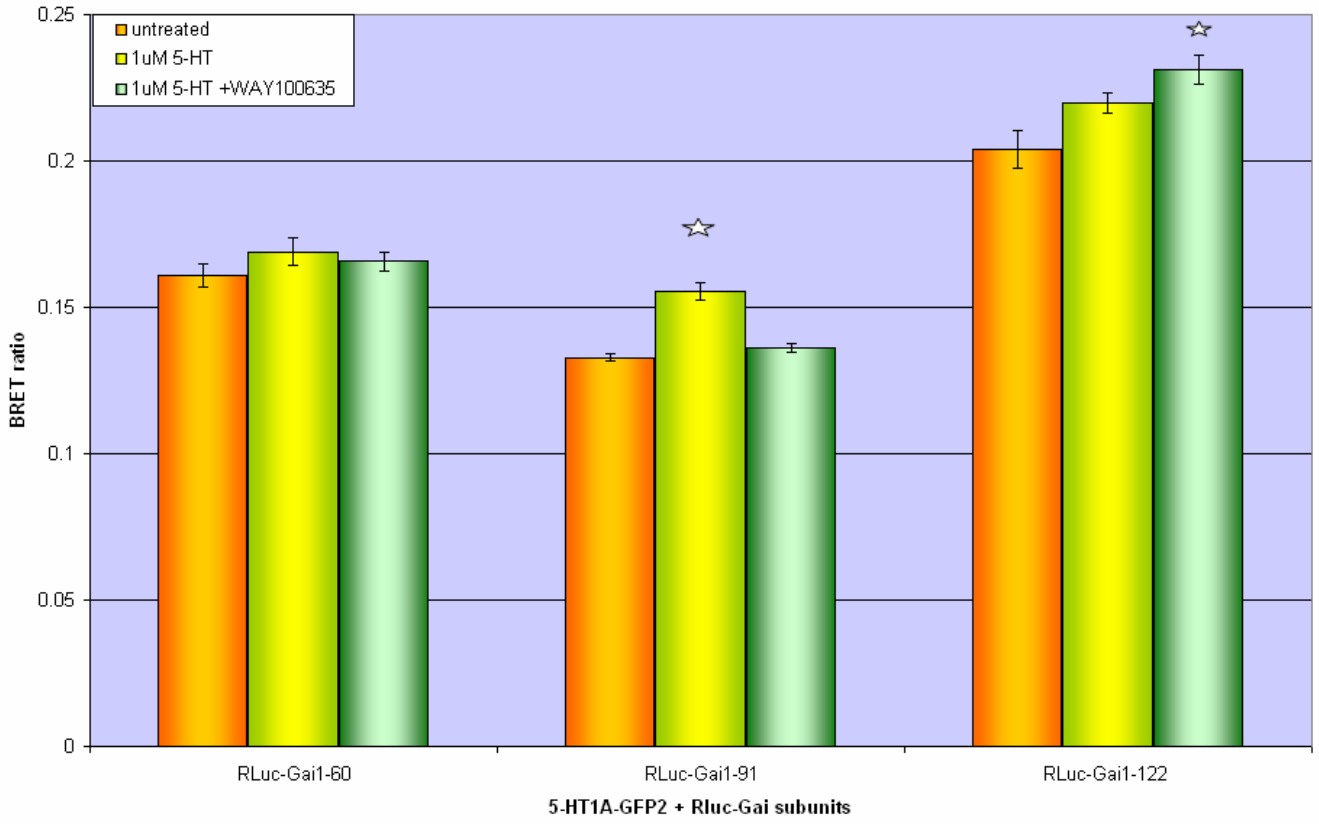


Figure 5. BRET signal between wild type GFP2-tagged 5-HT$_{1A}$ receptor and three variations of RLuc-tagged Gαi1. 5-HT$_{1A}$-GFP2 and Gαi1 with RLuc inserted at amino acid positions 60, 91, or 122 were co-transfected into HEK293 cells. BRET interactions were monitored under untreated, agonist (1μM 5-HT), and agonist (1μM 5-HT) and antagonist (100μM WAY100635) conditions. Stars indicate significant increases in BRET ratio compared to their respective untreated controls ($p<0.05$) (n=8).

Assessment of non-specific BRET interactions

Since receptors and G-protein subunits were being introduced into the cells, there was the possibility of over expression of the proteins, which may cause non-specific BRET signals via random diffusion or collision. Therefore, a negative control was introduced to assess non-specific BRET interactions. A GFP2-tagged potassium channel, potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 4 (HCN4), was obtained from Dr. Eric Accili. The channel is a cyclic nucleotide-gated channel, and therefore, does not directly interact with G-proteins. It was chosen as a negative control because, like the 5-HT_{1A} receptor, it has a membrane localization appropriate to control for background BRET signal with Gαi subunits. BRET interaction profiles, the interaction under both agonist and antagonist conditions, between the wild type 5-HT_{1A} receptor or HCN4 and Gαi1-91 were constructed and presented in Figure 6. HEK293 cells were transfected with 5 μg of each plasmid construct. The BRET signal between the HCN4 and Gαi1-91 was unresponsive to agonist or antagonist conditions and remained at a lower BRET ratio of around 0.06. In contrast, the wild type 5-HT_{1A} receptor had not only a higher basal BRET signal, averaging around ~1.11, but also exhibited an even higher increase, ~1.25, following agonist treatment. The lack of increase in BRET signal following HCN4's treatment with 5-HT not only demonstrated that it does not respond to the agonist, but also that there is the potential of a background BRET signal from non-interacting BRET pairs. In contrast, the BRET signals for the interaction between the 5-HT_{1A} and Gαi1-91 under untreated conditions suggest that there is a basal level of interaction between the two proteins.

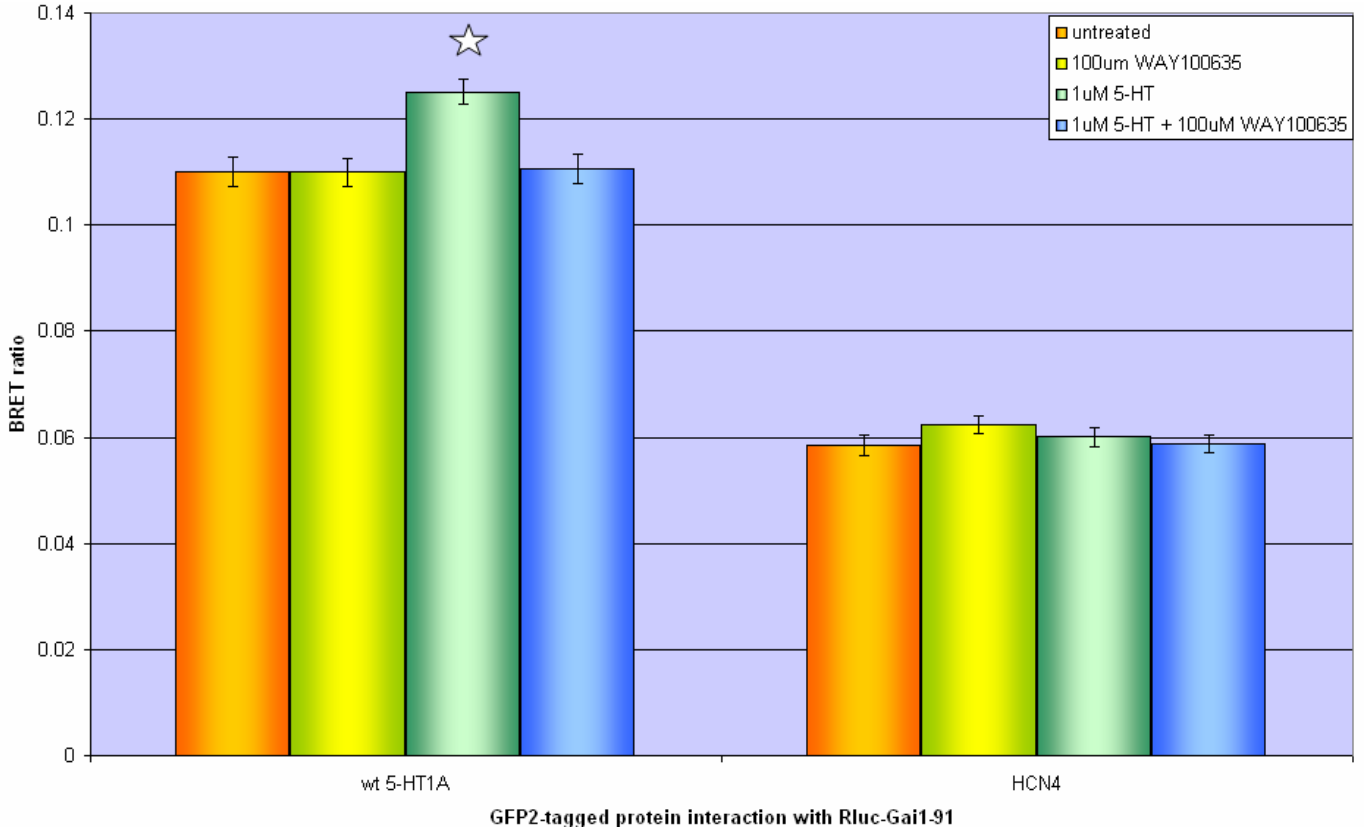


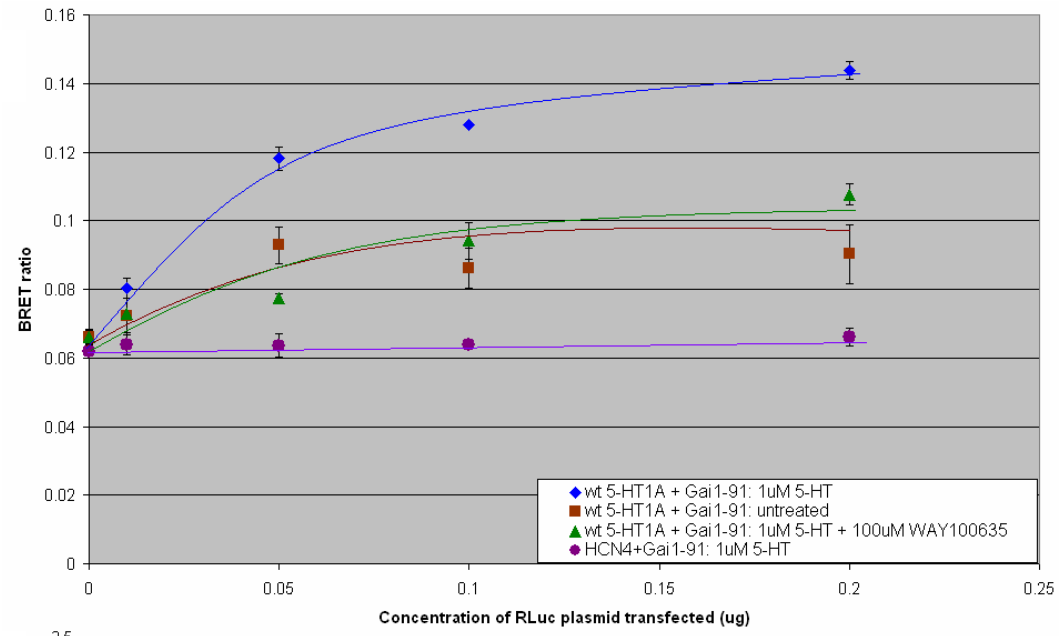
Figure 6. BRET profile comparison between wild type 5-HT$_{1A}$-GFP2 receptor's and HCN4-GFP2's interaction with RLuc-Gαi1-91. HEK293 cells were co-transfected with either wild type 5-HT$_{1A}$-GFP2 or HCN4-GFP2 and RLuc-Gαi1-91. BRET readings were taken under untreated control conditions, treatment with 100μM WAY100635, 1μM 5-HT, and 1μM 5-HT with 100μM WAY100635. Stars indicate significant BRET signal increase compared to the untreated controls ($p<0.05$) (n = 12-27)

BRET titration through variations in RLuc plasmid to GFP2 plasmid transfection ratio

To further elucidate the specificity of the BRET interaction between the 5-HT_{1A} receptor and Gαi1 subunit, BRET titration experiments were performed. In the titration experiment, the 5-HT_{1A}-GFP2, was held at the constant amount of 2 μg while the RLuc-Gαi1-91 concentration was increased gradually from 0 μg to 0.2 μg. The BRET signal for each successive increase in RLuc-Gαi1-91 concentration was observed under normal conditions, treated with 5-HT, and treated with 5-HT and WAY100635. In addition to the titration between 5-HT_{1A} and Gαi1-91, the experiment was repeated using the HCN4 in the place of 5-HT_{1A} as a negative control to further discern the background BRET signal levels (Figure 7A). With gradual increases in RLuc plasmid, there was a steady rise in the signal for all 5-HT_{1A} and Gαi1-91 interacting pairs. In agreement with what was previously observed, both the untreated and the antagonist treated groups exhibited neither as high an increase in BRET signal, nor as of a maximum BRET signal compared to the agonist treated group. The maximum BRET signal appeared to occur either at 0.2 μg of RLuc-plasmid or a little higher as the curves began to plateau indicating that the plasmid concentration ratio of 1:1 GFP-2 to RLuc plasmid, used in following BRET experiments, was enough to get maximal BRET response. An interesting point to take notice is that the BRET ratio did not show any significant increase at all for the interaction between HCN4 and Gαi1-91, as expected from non-specific interaction signal. Scatchard plots were constructed from the titration BRET signals after normalizing against the background signal presented by the HCN4 (Figure 7B). The 5-HT treated and 5-HT and WAY100635 treated groups demonstrated a very similar $BRET_{50}$ of 0.456 and 0.0462 respectively. In contrast, the untreated group had a much small $BRET_{50}$ of 0.0241, which may indicate better efficiency in BRET signal transduction but can also be an artefact caused by the small amount of data points

used. The $BRET_{max}$, however, were more in agreement with what was expected as the untreated and 5-HT and WAY100635 treated groups showed similar $BRET_{max}$ (0.0393 and 0.0537 respectively) that were both lower than that of the 5-HT treated group (0.1461). The fact that there was a BRET signal of around 0.06 even in the absence of RLuc plasmid indicated that the background signal noted previously was most likely a spill over signal caused by the intrinsic absorption spectrum of the GFP2.

A)



B)

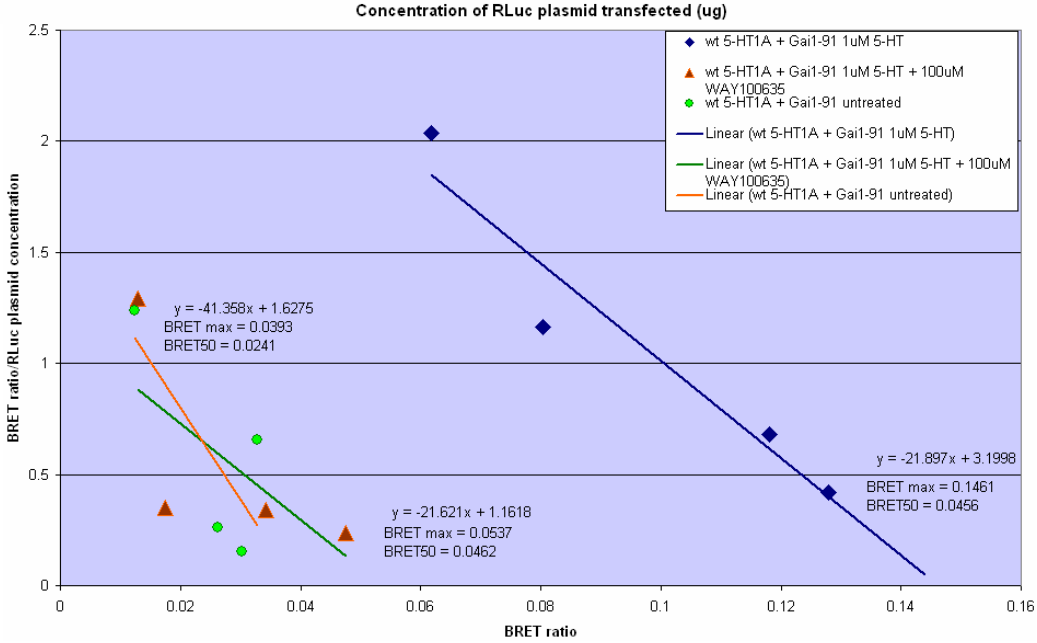


Figure 7. BRET titration graph and scatchard plot of wild type 5-HT$_{1A}$-GFP2 or HCN4-GFP2 and RLuc-Gαi1-91. A) HEK293 cells were transfected with 2 µg of 5-HT$_{1A}$-GFP2 or HCN4-GFP2 and increasing amounts of RLuc-Gαi1-91. The BRET interaction between the 5-HT$_{1A}$ and Gαi1-91 were observed under conditions of untreated controls, treated with 1 µM 5-HT, and treated with 1 µM 5-HT and 100 µM WAY100635. The interaction between HCN4 and Gαi1-91 was noted under treatment with 1 µM of 5-HT. B) The graph represents the Scatchard plot used to calculate maximal binding (BRET$_{max}$) and RLuc-plasmid concentration at half maximum BRET signal (BRET$_{50}$) through the use of linear regression. Titration data from Figure 7a was normalized against the background signal demonstrated by the HCN4. The linear equation, BRET$_{max}$ and BRET$_{50}$ values for each treatment condition are indicated on the chart.

Functional assay of the tagged 5-HT1A receptor constructs

With the BRET interaction characteristic established, the next step was to determine whether the GFP2 addition at the C terminus of the receptor impacted receptor function. Because the C terminus are sites of palmitoylation and are required for targeting and potential signalling, functional assays were needed for the 5-HT_{1A}-GFP2. Two ways of assessing the function of the 5-HT_{1A}-GFP2 receptor were used: assessment of the receptor sensitivity to PTX and the receptor's ability to decrease cAMP upon stimulation.

PTX sensitivity was assessed by pre-incubating the cells with 50 ng/ml of PTX 16 hours prior to cell harvesting and the BRET assay. Cells were divided into four treatment groups: untreated, PTX treated, 1 μM 5-HT treated, or 1 μM 5-HT and PTX treated. Pre-treatment with PTX abolished the 5-HT induced BRET ration increase as shown in Figure 8, in agreement with the uncoupling effects of PTX on Gi/o coupled GPCR. In addition, treatment with PTX alone did not cause any significant changes to the BRET signal indicating that the PTX did not affect the spatial relationship between the 5-HT_{1A} receptor and the Gαi1 protein as detected using the RLuc-Gαi1-91 construct.

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The cAMP assays were performed using the cAMP Glo™ assay protocol from Promega. Cells were transfected with both receptors and Gαi1-91 subunit in order to ensure better cell survival. The assay works upon the principle that increase cAMP leads to increased protein kinase A activity, which results in a lower ATP concentration. The ATP is used to drive a coupled luciferase reaction so that an increase in cAMP will result in a lower luciferase reading. The results are presented Figure 9. cAMP levels were significantly decreased with 5-HT treatment in

untagged 5-HT_{1A} receptors. Decreased cAMP levels were also present in the GFP2 and S-tagged 5-HT_{1A} receptors; however although they approached significance, especially for the GFP2-tagged receptor ($p=0.053$), they did not reach it due to the variability in the cell-based assay; however the magnitude of cAMP inhibition was similar for tagged and wild-type 5-HT1A receptors.

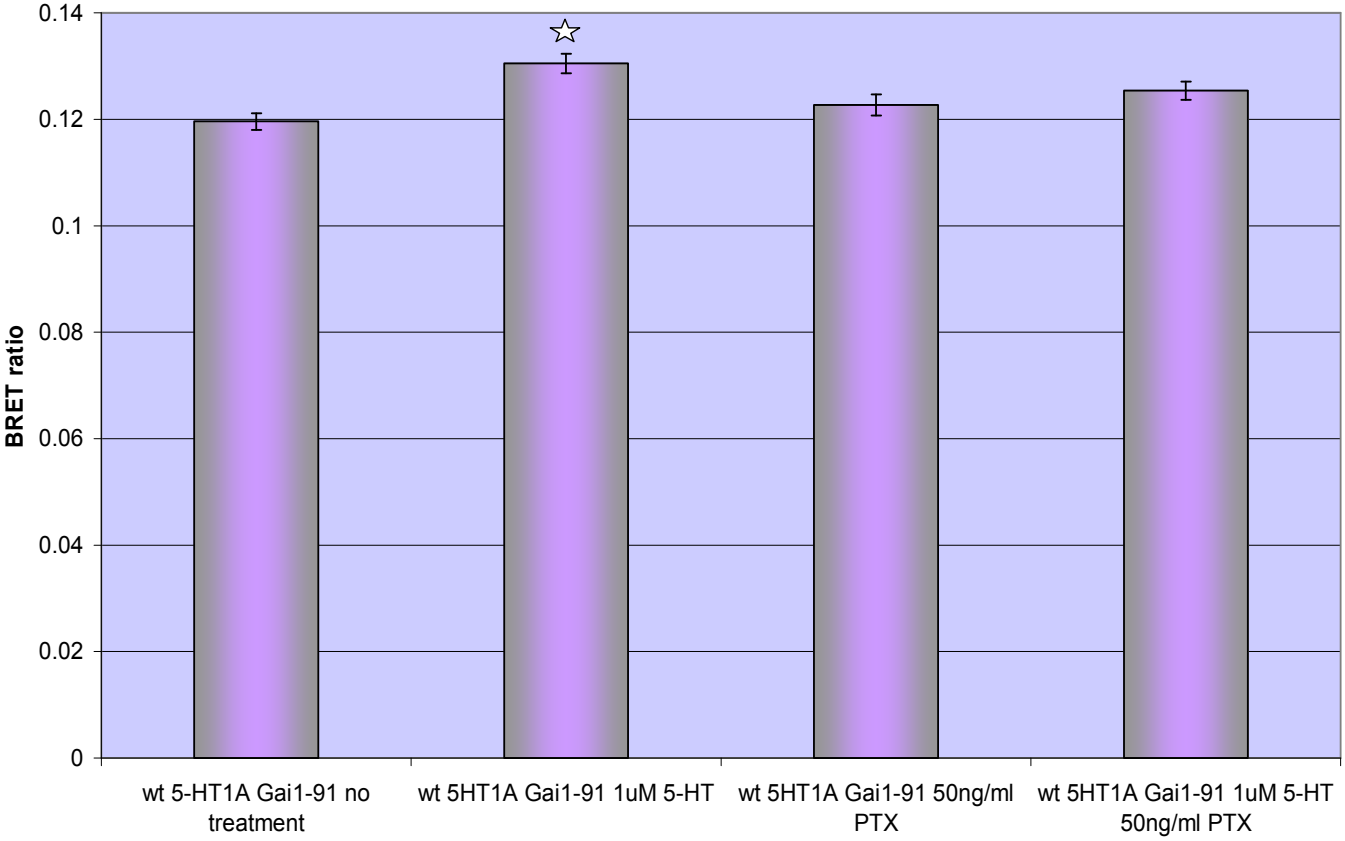

Figure 8. **BRET ratio of wild type 5-HT$_{1A}$-GFP2 and RLuc-Gai1-91 in the presence of 5-HT and PTX.** BRET ratios of the interaction between 5-HT$_{1A}$ and Gai1-91 were recorded in the presence of no treatment, treatment with 1 μM 5-HT, 50 ng/ml PTX, or 1 μM 5-HT and 50ng/ml PTX. All PTX treatment cell populations received the PTX treatment 16 hours prior to the BRET assay. Stars indicate significant ($p<0.01$) compared to the untreated controls (n=24-30).

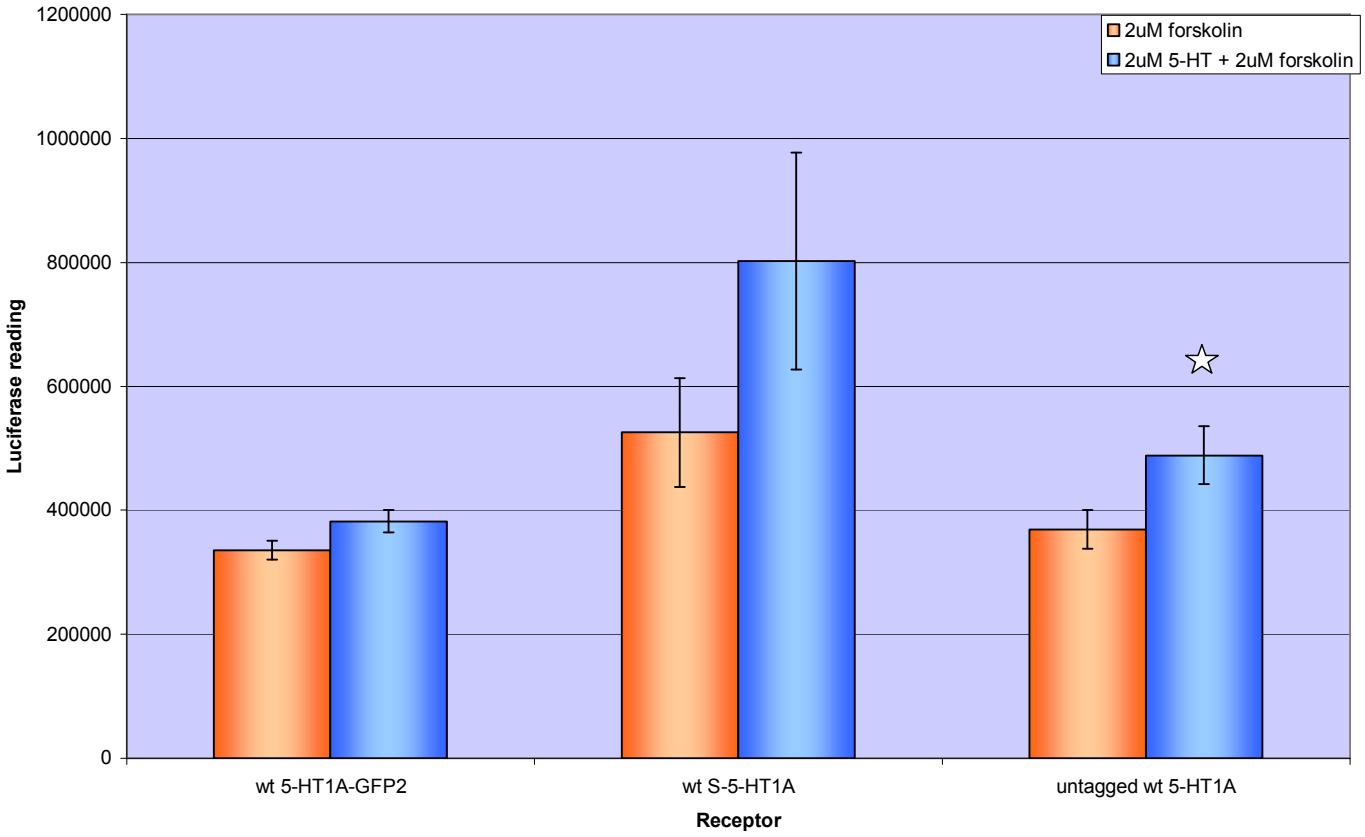

Figure 9. The ability to decrease cAMP of GFP2 and S-tagged 5-HT$_{1A}$ receptors compared to wild type. HEK293 cells were transfected with either 5-HT$_{1A}$-GFP2, S-5-HT$_{1A}$, or untagged 5-HT$_{1A}$ receptor and RLuc-Gαi1-91. Cells were treated with either buffer or 2 μM 5-HT followed by 2 μM forskolin. cAMP was measured by the cAMP Glo™ assay (Promega). cAMP is determined by a competition system for ATP availability. A decrease in cAMP results in a higher luciferase activation reading. Stars indicate significance ($p<0.05$) (n=10-16).

Verifying transfection efficiency and its impact on the BRET signal

Referring back to the titration studies, the ratio of the GFP2 and RLuc plasmids expressed in the HEK293 can change the strength of the BRET ratio. From the titration studies, however, it was clear that a ratio of as low as a 1:10 RLuc to GFP2 plasmid transfection was enough to obtain optimal BRET signal in the wild type 5-HT_{1A} receptor. It is currently unknown whether the mutant 5-HT_{1A} receptors are expressed at the same level as the wild type receptor. Thus correlation studies were used to see if there was any relationship between the BRET signal obtained and the level of GFP2 or RLuc signal. Because it was not possible to quantify the GFP2 or RLuc proteins directly, their expression was measured indirectly through their luminescence levels compared with background. Whereas the RLuc did not demonstrate a correlation with regards to the BRET signal levels (R2 = 0.004), there appeared to be a weak non-significant correlation when the expression of GFP2 was assessed (R2 = 0.2345) (Figure 10, Figure 11). Because the BRET ratio is calculated using the ratio between the GFP2 fluorescence and the RLuc luminescence, a similar formula was used on the fold increase over background levels of GFP2 and RLuc. This value, since it takes into account both GFP2 and RLuc expression, was correlated with the BRET ratio, and the results indicated again a weak correlation (R2 = 0.15) (Figure 12). Thus the data demonstrated that the differences in BRET signal levels between the wild type and mutant 5-HT_{1A} receptors were largely independent of expression levels of the two proteins.

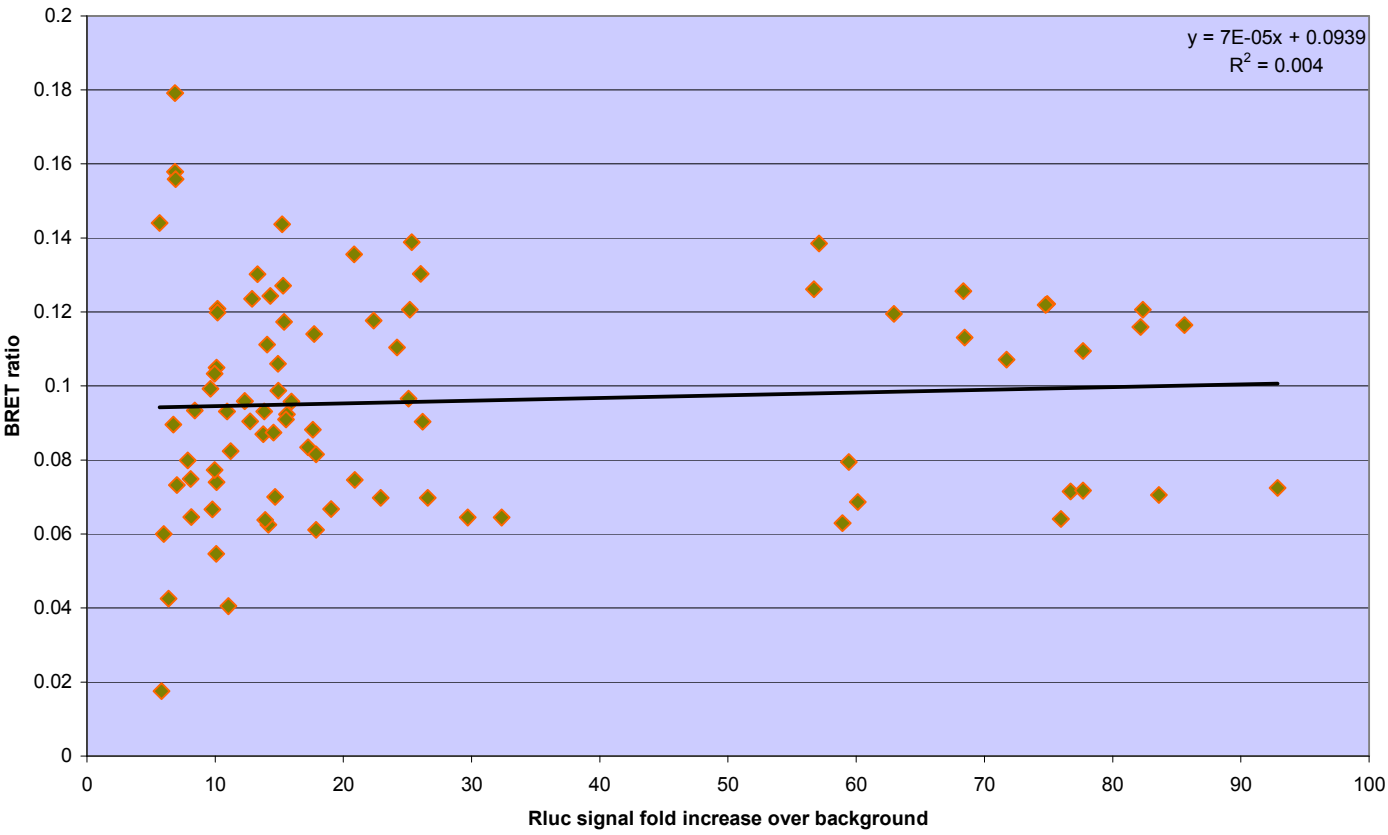

Figure 10. **Correlation between the expression of RLuc tagged proteins within HEK293 cells and their subsequent effect on the BRET signal strength.** The RLuc signals, after activation by coelenterazine 400a, of the untreated control group from select experiments were taken and normalized against the non-transfected control readings, described as fold increase compared with the background. Correlation between this RLuc signal and the BRET ratio was determined by a scatter plot. The regression equation and the R^2 value are noted on the graph (n = 84)

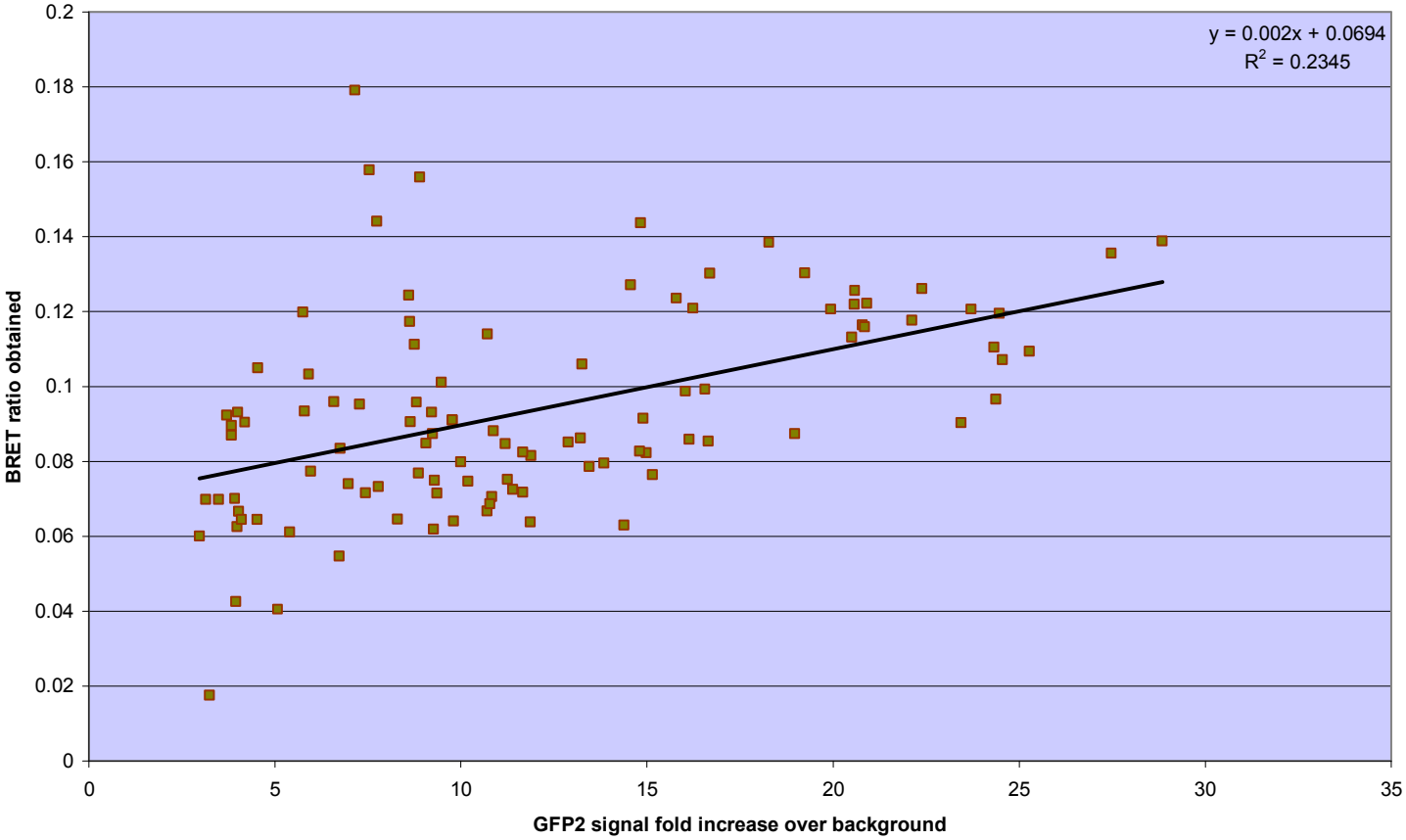

Figure 11. **Correlation between the expression of GFP2 tagged proteins within HEK293 cells and their subsequent effect on the BRET signal strength.** The GFP2 readings of untreated controls from select experiments were taken and normalized against the non-transfected control readings, described as fold increase compared with the background. Correlation between this GFP2 signal and the BRET ratio was determined by a scatter plot. The regression equation and the R^2 value are noted on the graph (n = 84).

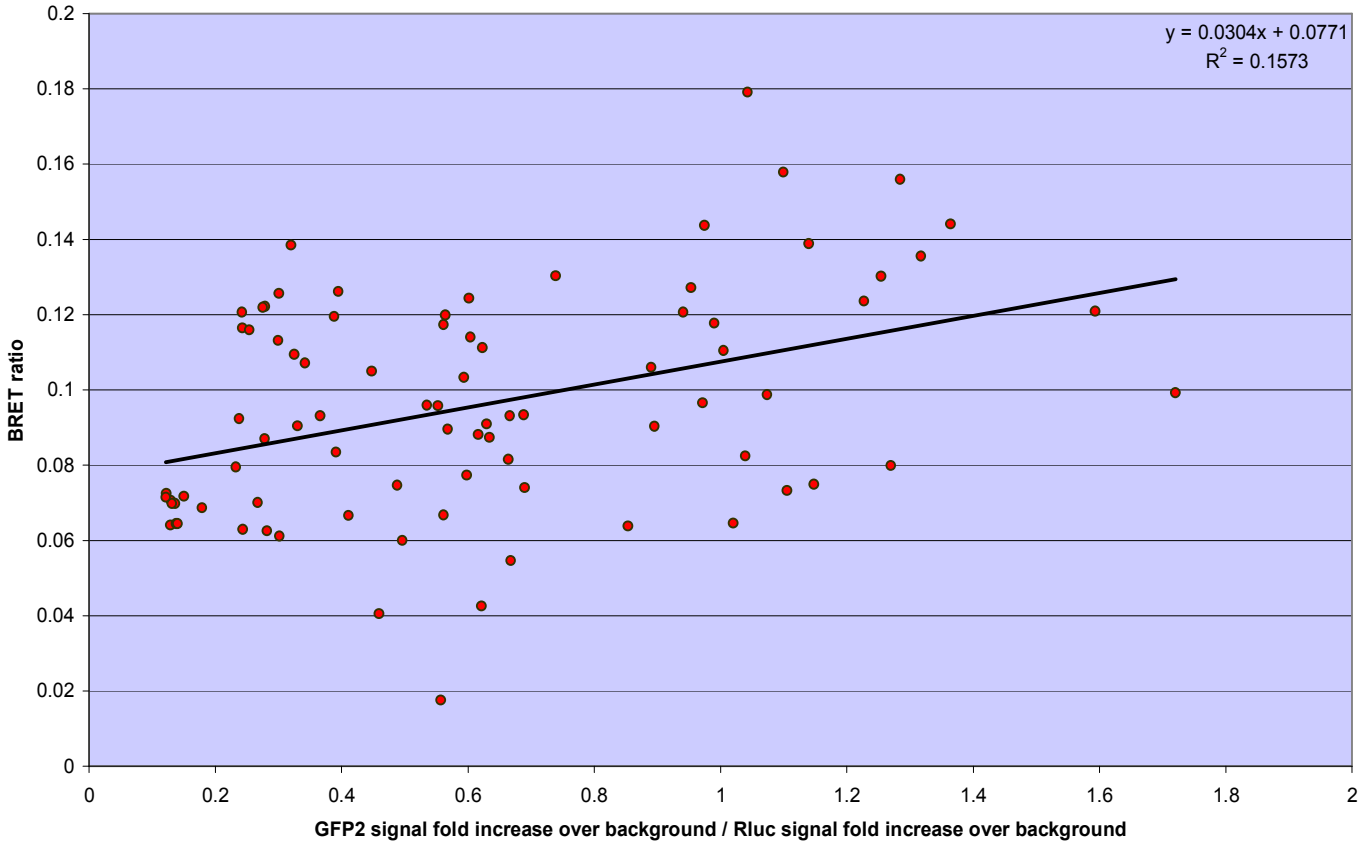

Figure 12. **Correlation between the expression of GFP2 and RLuc tagged proteins within HEK293 cells and their subsequent effect on the BRET signal strength.** The GFP2 and RLuc readings of untreated controls from select experiments were taken and normalized against the non-transfected control readings, described as fold increase compared with the background. The GFP2 values were divided by the RLuc much like how the BRET ratio is calculated. Correlation between this GFP2/RLuc signal and the BRET ratio was determined by a scatter plot. The regression equation and the R^2 value are noted on the graph (n = 84).

BRET interaction between wild type and mutant 5-HT_{1A} receptor and Gαi1-91

Next the BRET interaction between the 5-HT_{1A} receptor and the Gαi1-91 subunit was studied. HEK293 cells were transfected with 2.5 μg of either wild type or mutant 5-HT_{1A} receptor as per protocol described in the methods. BRET profiles of interactions were constructed under conditions of no treatment, WAY100635, 5-HT, and 5-HT with WAY100635 conditions and are presented in Figure 13. First the BRET profile for the wild type 5-HT_{1A} receptor is similar to that seen previously during the characterization of the wild type 5-HT_{1A}-GFP2/RLuc-Gαi1-91 BRET interaction. That is to say, there is a basal associated BRET signal normally or when WAY100635 was present. When the agonist 5-HT was present, however, the BRET signal increased significantly. The agonist-mediated increase in BRET signal was abolished in the presence of WAY100635. The various mutants exhibited different BRET profiles but can be grouped into three general categories. As indicated in the study by Dr. Kushwaha (Kushwaha et al, 2006) the T149E and T149V mutants were previously found to be Gαi weakly coupled but Gβγ uncoupled. The data obtained from the BRET study were in agreement with this finding as they were both agonist and antagonist sensitive, much like the wild type 5-HT_{1A} receptors. The Y144A, R152D, E340G, and E340K 5-HT_{1A} receptor mutants were all Gαi uncoupled, as well as selectively Gβγ uncoupled. Thus, not surprisingly, all of these mutants failed to show any responsiveness to 5-HT treatment. The last category was the inverse agonist-like effect, seen with the R148K mutant. The BRET profile for this mutant demonstrated no response towards 5-HT, however, the presence of WAY100635 appeared to decrease the BRET signal relative to the basal untreated condition. It is important to note that the basal and stimulated BRET signal levels were unequal. With the exception of the R148K mutant, all of the mutants had lower BRET ratios compared to the wild type 5-HT_{1A} receptor. The difference may be accounted in

part by the level of expression of the receptors themselves within the cell as some receptors are not as well expressed (Kushwaha et al, 2006). In addition, the altered BRET signal levels also indicate that the mutants not only have different responses to agonist mediated Gαi1 interaction, but also that they have a different spatial configuration between them and the Gαi1 subunit compared to the wild type.

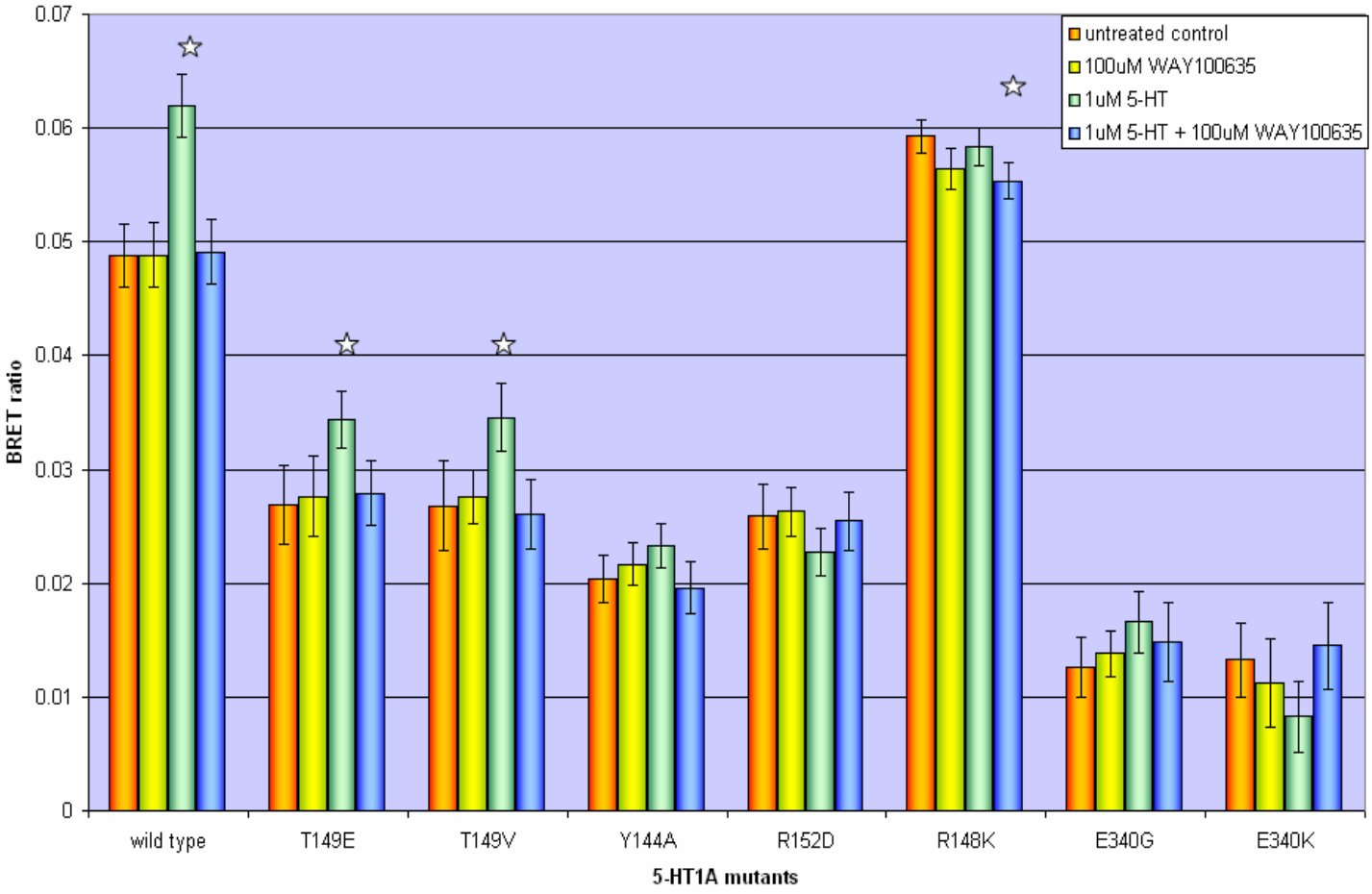

Figure 13. Combined BRET interaction between 5-HT$_{1A}$-GFP2 (wild type and mutants) and HCN4-GFP2 with RLuc-Gαi1-91 under both agonist and antagonist treatment. HEK293 cells were transfected with GFP2-tagged wild type or 5-HT$_{1A}$ receptor and RLuc-tagged Gαi1-91. BRET readings were taken with non-treated controls, 100 μM WAY100635, 1 μM 5-HT, or 1 μM 5-HT and 100 μM WAY100635. Mutants are designated by their specific amino acid mutations. All BRET ratio values were normalized against the non-specific BRET signal recorded from the interaction between GFP2-tagged HCN4 and RLuc-Gαi1-91. Stars indicate significance compared to the untreated controls ($p > 0.05$) (n=12-28)

Pull downs

The interaction between the 5-HT_{1A} receptor and Gαi was further investigated using the pull down method. In the pull down method, HEK293 cells were transfected with both the FLAG-tagged Gαi3 and the N terminal S-tagged 5-HT_{1A} receptors were used, since the 5-HT_{1A} receptor has been shown to preferentially couple to Gαi3 in raphe tissue. Previous studies into the functional ability of the wild type S-tag receptor have shown its retaining some of the ability to activate AC and reduce cAMP (Figure 9). The S-tag is an epitope tag, derived from the pancreatic ribonuclease A, with the sequence KETAAAKFERQHMDS. It can be detected using the S-tag HRP conjugate, which is a highly specific one step antibody directed towards the S-tag. The FLAG tag, also found at the N terminus, is a commonly used epitope with the sequence amino acid sequence of DYKDDDDK that can be detected with anti-FLAG antibody and subsequently anti-rabbit HRP conjugate. Due to construct availability, only the wild type and the T149E mutant 5-HT_{1A} receptor were used in conjunction with FLAG-tagged Gαi3-Q204L, a constitutively active form of the Gαi3 subunit, in the pull down study. The interaction between the receptor and Gαi3 subunit was preserved using the cross-linker DTBP. DTBP is a homobifunctional and cleavable cross-linker that is both water soluble and membrane permeable. The maximum cross-linking distance is 11.9 angstroms, which is considerably shorter than the maximum BRET distance of 10 nm, thus only proteins within very close proximity would be cross-linked. Pull-downs were achieved using S protein agarose beads (Novagen) that binds to the S-tag on the 5-HT_{1A} receptors. The de-cross-linked pull-down samples each lysate were separately probed for the presence of the S-tagged 5-HT_{1A} receptor and the FLAG tagged Gαi3-Q204L (Figure 14, Figure 15). Strong signals were detected for both the 5-HT_{1A} receptor (~45 kDa) and the Gαi3 (~40 kDa) in the pull-down samples for both the wild type 5-HT_{1A} receptor

and the T149E mutant across all treatment conditions. Strangely, however, the input samples did not show consistently strong bands, compared to the pull-down, which is likely caused by both the lower quantity of protein concentration relative to the pull-down samples and the degradation of the protein following freeze thaw cycles. A further observation is that treatment with the 5-HT caused a slight increase in the amount of Gαi3-Q204L being pulled down with the 5-HT_{1A} receptor as indicated by a darker band (Figure 14b, Figure 15b). This effect was abolished following treatment with the antagonist, WAY100635 in the wild type 5-HT_{1A} receptor (Figure 15b).

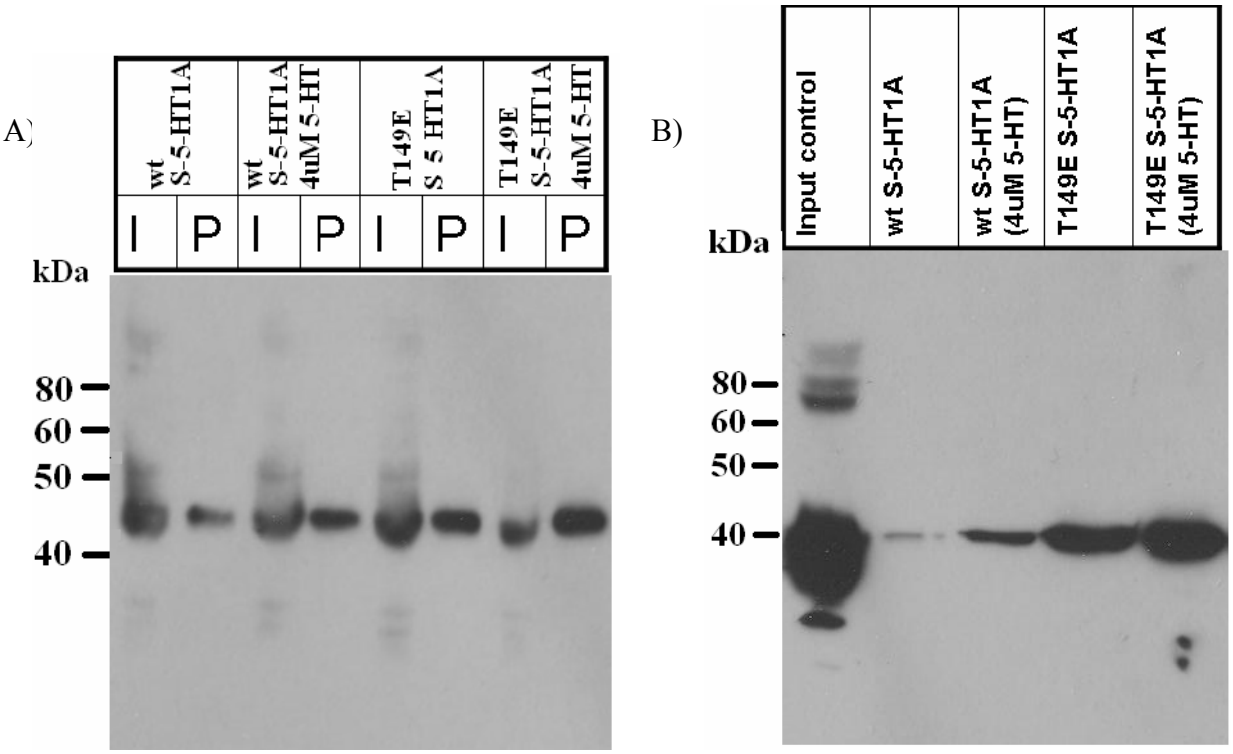

Figure 14. **Pull-down results of S-tagged 5-HT$_{1A}$ and Flag-Gαi3-Q204L interaction.** Two simultaneous western blots using the same cell lysate sample are shown in this figure. The different treatment conditions and S-tagged 5-HT$_{1A}$ receptors are indicated above each lane. **A**. Membrane probed for the S-5-HT$_{1A}$ (~45 kDa) showing both the input (I) and pull-down (P) samples. **B**. Membrane shows input and pull-down samples that were probed for Gαi3-Q204L (~40 kDa) using Flag antibody. Pull-down was performed using S-agarose beads for the S-tagged-5HT$_{1A}$, which was previously cross-linked with Flag-Gαi3-Q204L. The loading of the wells are indicated above each column. Input from wt S-5-HT$_{1A}$ used as a positive control in the first column while the rest are pull-downs containing only 5-HT$_{1A}$ and Gαi3-Q204L. Pull-down samples probed for Gαi3-Q204L used the same lysate as the pull-downs probed for S-5HT$_{1A}$ controls shown in Figure 14A. Films were exposed for 10 minutes.

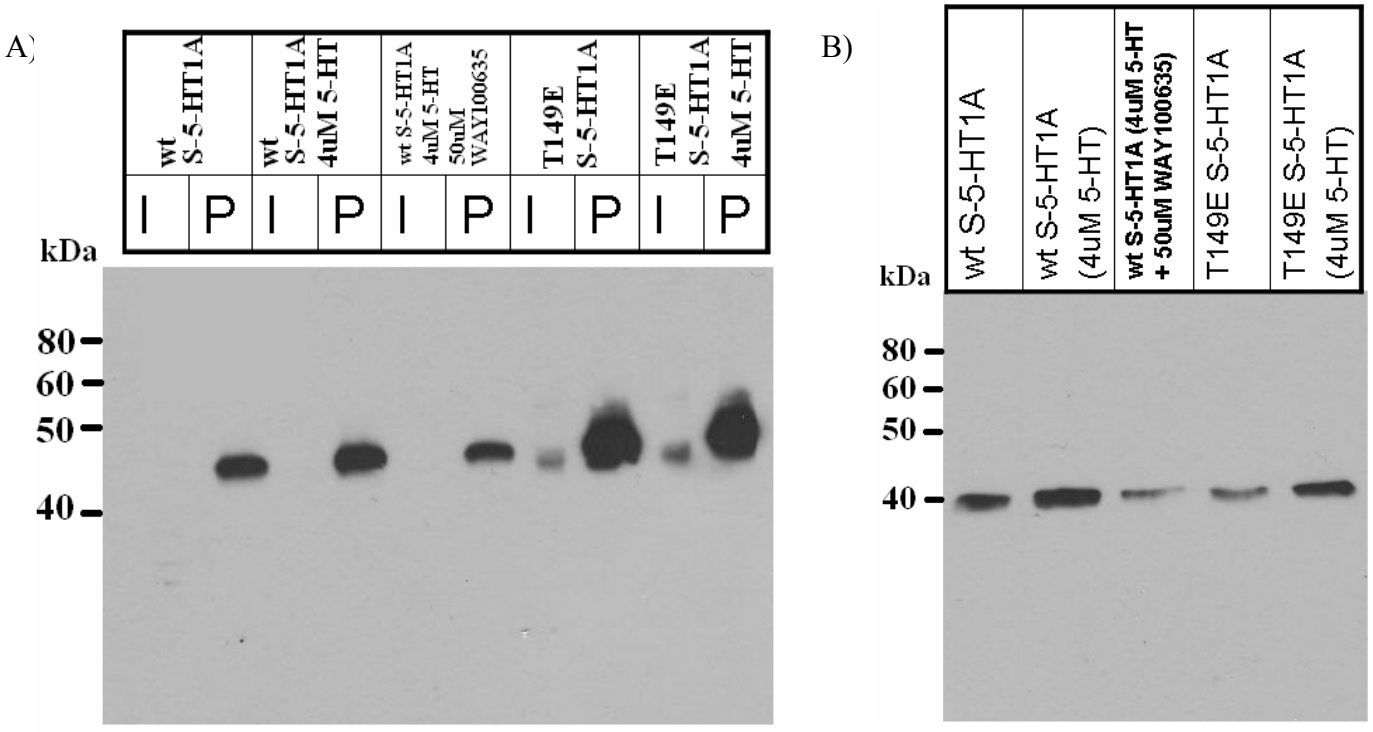

Figure 15. **Pull-down results of S-tagged 5-HT$_{1A}$ and Flag-Gαi3Q-204L interaction.** Two simultaneous western blots using the same cell lysate sample are shown in this figure. The different treatment conditions and S-tagged 5-HT$_{1A}$ receptors are indicated above each lane. **A**. Membrane probed for the S-5-HT$_{1A}$ (~45 kDa) showing both the input (i) and pull-down (p) samples. **B**. Membrane shows pull-downs that were probed for Gαi3Q-204L (~40 kDa) using flag antibody. Pull-down was performed using S-agarose beads for the S-tagged-5HT$_{1A}$, which was previously cross-linked with Flag-Gαi3-Q204L. The loading of the wells are indicated above each column. Pull-down samples probed for Gαi3-Q204L used the same lysate as the pull-downs probed for S-5HT$_{1A}$ controls shown in Figure 15A. Films were exposed for 5 minutes.

Discussion

Verification of Gαi interaction with wild type and mutant 5-HT_{1A} receptors

In this study, we have set out to verify the changes in G-protein, specifically the Gαi, coupling to the mutant 5-HT_{1A} receptors in light of previous studies that have shown mutations' causing signalling pathway uncoupling. To that end, the BRET assay was used to assess the interaction and spatial orientation between the receptor and the Gαi subunit.

Resonance energy transfer (RET) assays, such as BRET and FRET, are gaining widespread use in studying interaction between proteins. These assays have a few advantages compared to more traditional methods such as yeast-two-hybrid and co-IP or pull down assays. First the assay is performed in live cells under a much more natural cellular environment. In addition both the kinetics of protein interaction and spatial proximity of the two interacting pairs can be monitored in real time (Hamdan et al, 2006). There are, however, drawbacks compared to the other assays, the main one being the introduction of altered proteins into cells, which causes two potential areas of concern. The position where the fluorescent proteins are added to the protein of interest must be taken into consideration so that there is as little interference on the function of the protein as possible. As a general rule, the fluorescent proteins are attached to the N or C terminus, but there are exceptions to this case such as the in the Gαi1 where viable fusion proteins are made when inserted internally between protein domains. Sometimes due to physical constraints, it is impossible to attach the protein at the site of least hindrance. This is the case with the GPCR such as the 5-HT_{1A} receptor. The C terminus of the 5-HT_{1A} receptor has been implicated in coupling to Gi/o G-proteins and receptor trafficking on account of the presence of two palmitoylation sites at residues C417 and 420 (Papoucheva et al, 2004, Kobe et al, 2008).

Regardless of this, the best feasible place to insert the GFP2 protein is at the end of the 5-HT_{1A} sequence because the N-terminus is extracellular. Thus it is important to test the GFP2-tagged receptor for its functional abilities. Although the 5-HT_{1A}-GFP2 showed retention of sensitivity to PTX treatment (Figure 8), it appeared to have a slight impairment in its ability to reduce forskolin-mediated cAMP increase (Figure 9). Despite the results' showing a decrease in receptor-mediated cAMP decrease, they fall just short of significance at $p = 0.053$. Studies have shown that the C terminus fusion of a fluorescent protein onto the β_2 and α_2 adrenergic receptors produced functional proteins, and 5-HT1A fused with fluorescent proteins retained proper cell targeting and dimerization (Galés et al, 2005, Galés et al, 2006, Renner et al, 2007).

The other major issue regarding the RET assays is the possibility of non-specific interaction signals, which results from over-expression of the fluorescent tagged proteins. In the BRET experiments using the negative control, HCN4-GFP2, increased expression of the HCN4-GFP2 did not show any appreciable increase in BRET signal (Figure 6, Figure 7). Therefore, the BRET assay is relatively specific for interacting proteins. The negative control, however, did show a background level of BRET signal (~0.06) that is solely caused by the presence of the GFP2 protein. This background BRET signal is consistent across all treatment conditions and GFP2-tagged or RLuc-tagged plasmid concentrations. The fact that expression of the fluorescent-tagged proteins have little non-specific BRET signals can be explained by organizational micro-domains within the plasma membrane that sequester and restrict the diffusion of the receptors and signalling effectors to specific regions (Papoucheva et al, 2004, Pontier et al, 2008, Ganguly et al, 2008).

One final point regarding the BRET assay that one needs to address is the possibility regarding RLuc or GFP2-tagged protein expression levels and their effects on total BRET signals. Results from the study revealed a very minor correlation between GFP2 expression and BRET signal strength (Figure 10, Figure 11, Figure 12). On the other hand, the BRET ratio, being a ratio between the GFP2 signal over the RLuc signal, accounts for variations in protein expression. In addition, the ability of attaining saturation of the BRET signal indicates specific interactions.

The C terminal i2 loop of the 5-HT_{1A} receptor has been previously found to be involved in mediating G-protein coupling, especially towards Gβγ (Kushwaha et al, 2005, Kushwaha et al, 2006). In contrast the i3 loop is implicated primarily in Gα coupling, but both regions play a role in stabilizing the interface between the 5-HT_{1A} receptor and the G-protein subunits. Therefore it is not surprising that mutations within i2 and i3 loops caused changes in the 5-HT_{1A} receptor's signalling pathway activation. Previously it has been observed that the T149 amino acid is important in activation of N-type calcium channels, a Gβγ mediated pathway (Wu et al, 2002). Thus mutations at T149 generally preserved Gαi signalling as demonstrated previously and by this study (Figure 13) (Kushwaha et al, 2006). Mutations at other positions in the i2 loop and i3 loop, with the exception of the R148, showed uncoupling from the Gαi subunit in addition to Gβγ uncoupling, suggesting that they play a role in stabilizing the Gαi subunit interaction. The R148 position is interesting in that mutation at this residue produced an inverse agonist-like effect for Gβγ mediated ACII activation, which resulted in negative cAMP accumulation (Kushwaha et al, 2006). Although in agreement with previous results, the R148K mutant did not show changes in interaction with Gαi1-91 upon agonist treatment; however, the BRET signal for the interaction between R148K and Gαi1-91 saw a significant decrease in the presence of the

antagonist, WAY100635 (Figure 13). This significant decrease was absent in the other mutant receptors and may play a role in explaining the inverse-agonist like effects that the R148K receptor possesses.

In summary the BRET coupling data obtained for the interaction between wild type and mutant 5-HT_{1A} receptors are in agreement with the hypothesis that changes in altered signalling pathway is the result of changes in G-protein coupling. The BRET assays have in addition provided insight into the dynamics and spatial orientation of the interaction between the 5-HT_{1A} receptor and Gαi subunit.

Elucidation of spatial interaction between the 5-HT1A receptor and the Gαi subunit

The nature of interaction between GPCR and its effectors were studied by Tolkovsky and Levitzki who evaluated two models of interaction: the pre-coupled and the collision-coupling models of G-protein activation (Tolkovsky and Levitzki, 1978). The two coupling models were defined by the group using the following two equations shown in Figure 16.

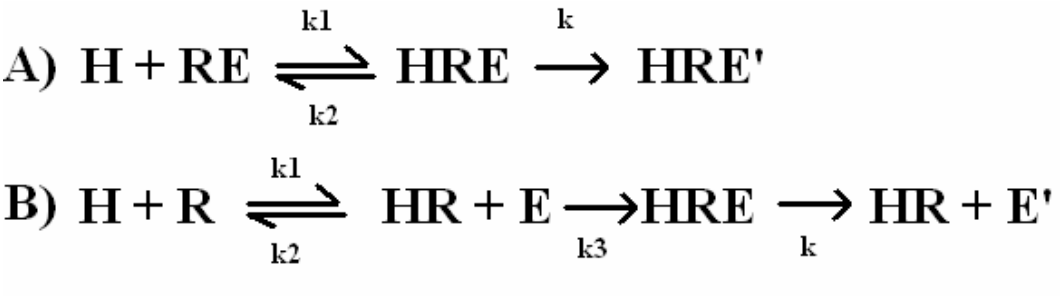

Figure 16. Receptor-ligand-and G-protein kinetic equations for pre-coupled and collision coupling models. H represents the ligand; R, the receptor; E, the G-protein effector; and E', the activated G-protein. k, k1, k2, k3 represents the individual kinetic constants at each step of the activation. A) Equation for the pre-coupled model. B) Equation for the collision-coupling model.

Whereas with the collision-coupling model, the activated receptor activates numerous downstream G-protein effectors and is subsequently regenerated following each cycle, the pre-coupled model is suggested to become permanently active following ligand association. Based on their observations the authors concluded that the coupling of the G-protein to GPCR most likely followed the kinetics presented in the collision coupling model. This, argument, however, is dependent upon the assumption that the pre-coupled model's end result, the activated HRE' complex, is a permanently active state and that the G-protein plays a kinetically significant role in receptor binding. It entirely possible that the G-protein is associated with the receptor prior to activation, and this association, along with other signalling and scaffolding proteins, may explain the rapid transduction of signal and cell specific signalling pathway activation.

With the advent of newer assays, there have been studies that suggest a pre-coupled GPCR to G-protein model. Through the use of BRET assays, constitutive coupling between GPCR and G-proteins have been demonstrated with the β_2-adrenergic receptor, α2-adrenergic receptor, and the δ-opioid receptors (Audet et al, 2008, Galés et al, 2005, Galés et al, 2006). Spatial studies into GPCR-G-protein interaction demonstrated that following activation of the receptor by its ligand, the GPCR and its G-proteins do not completely dissociate as previously thought (Galés et al, 2006, Vilardaga et al, 2004). By inserting the RLuc protein at different positions within the Gαi1 subunit, both increased and decreased BRET signals were obtained following α_2 adrenergic receptor activation. This suggests that instead of dissociating, there is spatial or conformational rearrangement of the receptor and G-protein. Apart from members of the adrenergic family, this conformational change has been found also in the metabotropic glutamate receptor 1α (mGluR1α) (Tateyama et al, 2004). Furthermore, it has been shown that even the Gαi and Gβγ

subunits remain associated and instead change conformations following activation (Bünemann et al, 2003). In this study, the interaction between the 5-HT_{1A} receptor and the Gαi1 subunit were studied using the RLuc-Gαi1 construct received from Dr. Bouvier's lab. Compared to the α_2-adrenergic receptor, which showed BRET signal increase following agonist treatment for the Gαi1-60 and a signal decrease for the Gαi1-91, and Gαi-122, the 5-HT_{1A} receptor demonstrated slight to modest increases in BRET signal for all of the RLuc-Gαi1 constructs following agonist activation of the receptor (Figure 5, Figure 6, Figure 7, Figure 13) (Galés et la, 2006). In agreement with previous studies involving GPCR-G-protein interactions, there was a basal level of association between the 5-HT_{1A} receptor and the Gαi1 subunit as shown by the higher BRET signal compared to that of the HCN4 negative control (Figure 6, Figure 7).

A surprising effect of the neutral antagonist for 5-HT_{1A} receptors, WAY100635, was seen for the RLuc-Gαi1-122 subunit. Whereas the other two constructs demonstrated the ability to return the BRET signal to basal levels following agonist addition, for the RLuc-Gαi1-122, it worked synergistically with the agonist to further increase the BRET signal obtained. This suggests that the WAY100635's interaction with the 5-HT_{1A} receptor causes conformational changes between the receptor and the G-proteins where the 122 region of the Gαi1 subunit approaches the C-terminus of the 5-HT_{1A} receptor. WAY100635 does not appear to directly interfere with the ability of 5-HT to alter the spatial conformation of the 5-HT_{1A} receptor and the Gαi1 subunit, as shown by the retention of agonist responsiveness. A possible explanation for the mechanism of the WAY100635 can be its altering the normal conformational change caused by 5-HT, which results in a shift that is not pharmacologically active compared to normal response in the absence of WAY100635. It is interesting to note that WAY100635 has been found to be a partial agonist

towards 5-HT_{1B} and 5-HT_{1D} receptors (Davidson et al, 1997). Perhaps WAY100635 exerts its agonist-like effects in these receptors by causing a specific orientation between the receptors and the G-proteins that is pharmacologically active. The altered BRET profiles of the three Gαi1 constructs provide further evidence of conformational changes of the Gαi subunits following receptor activation. Furthermore, there is also support for the idea of multiple activation states of GPCR, which recruit different signalling effectors based on conformational changes to initiate various signalling pathways (Audet et al, 2008).

By and large, the current evidence for the pre-coupled model and the rearrangement of the GPCR signalling effectors have been primarily discovered using the BRET assay. Thus it is important to verify the potential for constitutive coupling and changes in conformation following receptor activation through another assay. This study employed the pull down technique, using a short cross-linker to stabilize protein interaction, in order to further provide evidence for constitutive coupling. Due to the availability of the constructs, only the wild type and T149E mutant 5-HT1A receptor were studied, and they were cross-linked with interacting Gαi3-Q204L. The Q204L mutant of Gαi3 has been previously described as a constitutively active mutant that lacks the ability to hydrolyze bound GTP into GDP. Thus if one were to consider the collision coupling model in explaining GPCR signalling, then it would be expected that would be no detection of Gαi in the pull down. This, however, was not the case as all of the pull down samples showed Gαi3-Q204L cross-linked and pulled down with the 5-HT_{1A} receptor (Figure 14, Figure 15). Association between the Gαi3-Q204L and the 5-HT_{1A} receptor was found not only in the wild type, but also the T149E and T149A mutants that have been previously found to couple weakly to Gαi mediated signalling pathway (Kushwaha et al, 2006). Although the Gαi3-

Q204L does not provide conclusive evidence of pre-assembly between the 5-HT_{1A} receptor and G-protein, it demonstrated that upon activation, the Gαi3 does not dissociate from the receptor, an observation that contradicts the collision-coupling model of GPCR activation. Despite the constitutive activity of the Gαi3-Q204L mutant, the pull down data demonstrated that the interaction is still sensitive to agonist and antagonist treatment. The fact that agonist appeared to promote greater pull-down of the Gαi3-Q204L subunit, which is abolished by the introduction of an antagonist hint at another population of Gαi3-Q204L that is recruited to the receptor following agonist treatment. This, however, seems inconsistent with the results so far, for it provides evidence for the collision-coupling model. The collision-coupling model may very well play a role in tandem with the pre-coupled model, but its mechanism may be altered.

Recently, there has been a focus on studying the organization of signalling molecules within the cell membrane. Whereas previously, it was thought that the cell membrane was homogenous, the rate and specificity of certain signalling pathways are too fast to be explained by random collisions of the proteins in order to initiate the signal. Instead studies have shown that receptors and their effectors are organized into specific domains with the help of cellular structures such as actin fences and lipid rafts (Ganguly et al, 2008, Pontier et al, 2008). For the β_2 adrenergic receptor, it has been shown that there is a significant population of the receptors found at the periphery of the lipid rafts but are spatially segregated from its signalling effectors such as G-proteins and AC (Pontier et al, 2008). It is believed that with an increased requirement for cell signalling, through receptor activation, more of the signalling effectors are released from the rafts so that they may interact with non-coupled receptors. This observation does have elements of the collision coupling model as receptor and G-proteins come together to transduce the signal

following agonist activation, and it may explain the increased pull-down of the Gαi3-Q204L mutant following 5-HT treatment.

Future directions:

So far we have confirmed the interaction between the mutant 5-HT_{1A} receptors and Gαi subunits and also received insight into the 5-HT_{1A} receptor and G-protein signalling complex and mechanism. The coupling towards Gβγ by the seven mutant receptors, however, still remains to be elucidated. Furthermore because of the various coupling characteristics of the 5-HT_{1A} receptor exhibits depending on the cell in which it is expressed, verification of the interaction should also be performed within other cell lines, preferably neuronal, so that the interaction would better mimic conditions underlying behavioural disorders.

Better understanding of the spatial orientation of the 5-HT_{1A} receptor and G-protein subunits will also help to better clarify the mechanism of interaction, and perhaps help explain the uncoupling effects of the 5-HT_{1A} mutations.

The ultimate goal following elucidation of 5-HT_{1A} receptor signalling is to produce therapeutically active pepducins towards depression. Pepducins are short peptides derived from GPCR that can trigger receptor signalling (Covic et al, 2002). Thus by developing pepducins to only active specific 5-HT_{1A} receptors we hope to generate better treatments for depression with minimal side effects.

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