Title
Controlling the Agonist-Mediated and Constitutive G-Protein Signaling of the Human 5-HT4 Receptor

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Publication Date
2007-11-07

Peer reviewed|Thesis/dissertation

Controlling the Agonist-Mediated and Constitutive G Protein Signaling of the Human 5-HT_4 Receptor

by

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Pharmaceutical Sciences and Pharmacogenomics

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

UMI Number: 3289299

ProQuest Information and Learning Company
300 North Zeeb Road
P.O. Box 1346
Ann Arbor, MI 48106-1346

"If I have seen further, it is by standing on the shoulders of giants."

Sir Isaac Newton

February 5, 1675

"I would thank you from the bottom of my heart,

but for you my heart has no bottom. "

~Author Unknown

http://www.quotegarden.com/thank-you.html

ACKNOWLEDGEMENT

This has been the most exciting journey in my life. As I begin the next chapter, I want to thank all the giants whose shoulders I stood on during this exciting journey. I owe them the deepest gratitude. They have not just allowed me to see further. They have brought out the best in me.

I would like to first thank my thesis advisor, Dr. Bruce R. Conklin. He has given me the key to explore the Garden of Eden in the past six years. In addition to serving as a phenomenal role model, his inspiring visions, scientific expertise, and innovation made this a voyage full of endless wonders, exhilarations, and possibilities. His munificent support, patience and mentoring have been the constant North Star guiding me through this adventure.

I would like to next thank Drs. Mark von Zastrow, Su Guo, Thiennu Vu, and Larry Tecott as my qualifying examination committee and Drs. Mark von Zastrow and Brian Black as my thesis committee members. Their scientific insights, patience, understanding, and generosity with both advice and time served as the compass for me to stay on the Golden Path through out this voyage.

I would like to thank members of Conklin lab and von Zastrow lab for all the generous technical support. In particular, I would like to thank Dr. Jennifer Ng, Ms. Trieu Nguyen, and Dr. Ed Hsiao for helping me stay focused on my

research. The expert computer and bioinformatics assistance from Dr. Alex Pico, Dr. Nathan Salomonis, and Ms. Kristina Hanspers were also invaluable.

I would like to thank all my collaborators: Dr. Sylvie Claeysen for generously sharing her expertise on the human 5-HT_4 receptor, and Drs. Kimberly Scearce-Levie, Yuko Terasawa, and John Forsaeyth for all the insightful scientific discussions. I would also like to thank Dr. Gary Howard and Stephen Ordway for all the editorial assistance these years.

Finally, I would like to thank my parents (Chen Ming Chang and Ming Meai Lin) and my brothers (Tom Wei-Yi Chang and James Wei-Kuo Chang) for their unconditional support and for continuously sharing both the joys and frustrations in my life. Their absolute faith in me enables me to aspire and to continue, even in the most challenging times.

PUBLICATIONS

Publications arising from this thesis

Chang WC, Ng J, Nguyen T, Pellissier L, Claeysen S, Hsiao E, Conklin BR Controlling the G_s-, G_q- and Basal Signaling of the human 5HT4 receptor. *PLoS-ONE*. (Submitted)

Hsiao E, Boudignon B, C**hang WC**, Bencsik M, Peng J, Manalac C, Halloran B, Conklin BR, Nissenson RA A Stony Mouse: Osteoblast Expression of an Engineered Gs-coupled Receptor Causes a Massive Increase in Bone Mass. *Proc. Natl. Acad. Sci*. (Submitted)

Conklin BR, Hsiao E, Claeysen S, Srinivasan S, Forsayeth J, Guettier J, **Chang WC**, McCarthy K, Nissenson R, Wess J, Bockaert J , Roth BL. Evolving new hormonal pathways by engineering receptors activated solely by synthetic ligands (RASSLs). *Natural Methods*. (In preparation)

Pellissier L, Cochet M, Quefeullou E, Marion P, **Chang WC**, Bockaert J, Claeysen S and Dumuis A. Functional selectivity of 5-HT4 receptor ligands on Gs, Gq and ERK coupling. (In preparation)

Other publications

Uchida N, Sutton RE, Friera AM, He D, Reitsma MJ, **Chang WC**, Veres G, Scollary R, Weissman I (1998) HIV, but not murine leukemia virus vector mediate high efficiency gene transfer into freshly isolated Go/G1 human hematopoetic stem cells. *Proc. Natl. Acad. Sci*. *USA* 95:11939–11944.

Melkonyan HS, **Chang WC**, Shapiro JP, Mahadevappa M, Fitzpatrick PA, Kiefer MC, Tomei D, Umansky SR (1997) SARP's: A family of secreted apoptosis-related proteins. *Proc. Natl. Acad. Sci. USA* 94:13636–13641.

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ABBREIVATIONS

i1	1st intracellular loop
i2	2nd intracellular loop
i3	3rd intracellular loop
c-terminus	carboxyl terminus
ELISA	enzyme-linked immunosorbent assay
GPCR	G protein coupled receptors
HTRF	Homogenous Time Resolved Fluorescence Resonance Energy Transfer
IRES	Internal Ribosomal Entry Site
RASSL	Receptor Activated Solely by Synthetic Ligands
Rs1	RASSL Serotonin 1
Ro1	RASSL Opioid 1
TET	tetracycline

ABSTRACT

G-protein-coupled receptors (GPCRs) signal through a limited number of G-protein pathways and are crucial in many biological processes. The molecular and functional diversity of GPCRs and the lack of ligands with specific signaling effects have complicated studies of their *in vivo* functions. To better compare the effects of activating different G-protein-signaling pathways through agonist-mediated or constitutive signaling, we developed a new series of RASSLs (receptors activated solely by synthetic ligands) activating through different G-protein-signaling pathways. These RASSLs are based on the human 5-HT_4 receptor, a GPCR with a high level of constitutive G_s signaling and strong agonist-mediated G-protein activation of G_s and $G_{s/q}$ pathways. The first receptor in this series, 5-HT_4-$D^{100}A$ or Rs1 (RASSL serotonin 1), is not activated by its endogenous agonist, serotonin, but is selectively activated by the small synthetic molecules GR113808, GR125487, and RO110-0235 (antagonists and inverse agonists for the 5-HT_4 receptor). All agonists potently induced G_s signaling, but only a few (e.g., Zacopride) also induced signaling via the G_q pathway. Zacopride-induced G_q signaling was enhanced by replacing the C-terminus of Rs1 with the C-terminus of the human 5-HT_{2C} receptor. Additional point mutations ($D^{66}A$ and $D^{66}N$) blocked constitutive G_s signaling and lowered agonist-mediated G_q signaling. Finally, replacing the third intracellular loop of Rs1 with that of human 5-HT_{1A} conferred ligand-mediated G_i signaling. This G_i-coupled RASSL, Rs1.3, exhibited no measurable signaling to the G_s or G_q

pathway. These findings show that the signaling repertoire of Rs1 can be expanded and controlled by receptor engineering and drug selection. Here, we describe a new series of RASSLs developed to modify the agonist-mediated and constitutive signaling of the human 5-HT_4 receptor. Since none of the inverse agonists for the 5-HT_4 receptor works in the presence of the $D^{100}A$ mutation, I also engineered a series of synthetic internal ribosomal entry sites and a single plasmid tetracycline-inducible (tet) system to better control GPCR expression. Combined with a tissue-specific promoter, these RASSLs can be expressed in a tissue-specific manner and at specific levels. With these additional tools, RASSLs will help us better study the effect of constitutive G_s signaling and agonist-mediated G_s, G_s/G_q, and G_i signaling *in vivo*.

CHAPTER 1: INTRODUCTION

1.1 Physiological Importance of G-Protein-Coupled Receptors

The heptahelical G-protein-coupled receptors (GPCRs) are integral membrane proteins with seven hydrophobic transmembrane domains. Sensing and transducing extracellular signals into intracellular signaling pathways, they represent the largest family of human cell-surface receptors, encompassing more than 340 hormone receptors and 350–460 olfactory receptors (Karchin *et al.* 2002; Fredriksson *et al.* 2003). They are activated by peptide hormones, odorants, photons, biogenic amines, phospholipids, purines, nucleotides, protein antigens, and many other extracellular signals. Upon activation, these receptors undergo conformational changes that allow active and reversible signaling through a limited number of G-protein pathways (G_s, G_i, G_q, $G_{12/13}$). These signals mediate a wide variety of physiological responses, including cellular differentiation (Luttrell 2002), immune response (Houshmand *et al.* 2003), smell (Gaillard *et al.* 2004), taste (Max *et al.* 2001), vision (Filipek *et al.* 2003), heart rate regulation (Myslivecek *et al.* 2003), learning and memory (Moldrich *et al.* 2003), and energy homeostasis (Goodfellow *et al.* 2003; Srinivasan *et al.* 2003).

Consequently, GPCR are important for normal physiological functions. The role of GPCR signaling in development was first established in tissue-culture models, in which cAMP and/or PKC activation induced dramatic phenotype changes, including adipogenesis (Otto *et al.* 2005), neutrophil formation (Taimi *et al.* 2001), and programmed cell death (S49 cells) (Zambon *et al.* 2005). In

humans, mutations in the G_s-signaling pathway cause multiple endocrine disorders and severe bone deformities (Spiegel 1996). In mice, disrupting genes in each of the major G protein pathways can lead to embryonic lethality (Wettschureck *et al.* 2005). In cardiac development, double knockout of the G_q pathway genes (G_q and G_{11}) leads to embryonic lethality and single-chamber hearts (Wettschureck *et al.* 2005). In addition, the G_q-coupled endothelin receptor appears to be essential for early cardiogenesis, and a downstream component of the G_q pathway (PKC) is required for cardiomyocyte formation in mouse ESCs (Wettschureck *et al.* 2005). Similarly, several G_q-coupled receptors have been implicated in the formation of vascular tumors, suggesting that this class of receptors is involved in vascular development (Seifert *et al.* 2002).

Not surprisingly, GPCR mutations cause a wide range of diseases. GPCR loss of function mutations result in diseases, such as Albright hereditary osteodystrophy (Simon *et al.* 2000), color blindness (Spiegel 1996), hypothyroidism (Ando *et al.* 2005), extreme obesity (Cody *et al.* 1999), and hormone deficiency. GPCR gain of function mutations, on the other hand, result in diseases, such as McCune-Albright syndrome (Sargin *et al.* 2006), night blindness (Kijas *et al.* 2002), male precocious puberty (Shenker 2002), hyperthyroidism (Di Cerbo *et al.* 1999), hypocalcemia (Felderbauer *et al.* 2005), and various forms of tumors (Spiegel *et al.* 2004).

Owing to their physiological importance, GPCRs are of great medical interest and importance. Only 4–10% of GPCRs are currently known targets

(Vassilatis *et al.* 2003; Tyndall *et al.* 2005), and they have already contributed to 30–50% of modern pharmaceuticals (Flower 1999; Robas *et al.* 2003; Brink *et al.* 2004). Many more drugs may target GPCRs since it has been estimated that 330 of the 367 non-olfactory GPCRs are potential drug targets (Landry *et al.* 2006).

1.2 Structure-Function Relationships in GPCRs

Since the elucidation of "side chain theory" and ""receptive substance" by Erhlich and Langley to describe how antigens bind to cells (Hinke 2002), we have learned much about GPCRs.

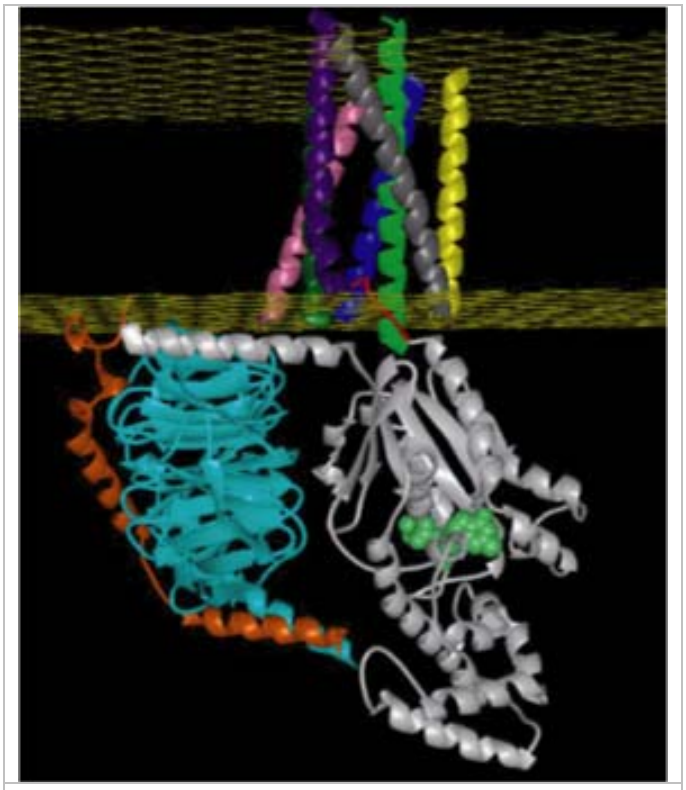


Figure 1. Heptahelical GPCRs.

Heptahelical GPCRs are represented by the seven α-helixes in the transmembrane. Bovine rhodopsin, (based on PDBID 1LN6) (Choi et al. 2002) is represented by seven helixes inside the membrane. G_{α}, G_{β} and G_{γ} subunits are represented by grey, cyanine and orange ribbons, respectively. This is a model by Dr. Taroh Iiri. Reprinted through the courtesy of Dr. Henry Bourne, UCSF.

1.2.1 GPCR Structure

A high-resolution three-dimensional structure of GPCR (Figure 1) is difficult to obtain due to complications for crystallization from the extensive attachment to the cell membrane. Until the high-resolution 2.8 Å crystallization of rhodopsin (Palczewski *et al.* 2000),

confirmation of predictions based on the primary sequence of GPCR have been modeled on bacteriorhodpsin (from *Halobacterium halobium* not coupled to G proteins) (Henderson *et al.* 1990; Larhammar *et al.* 1993) and low-resolution crystallization of rhodopsin (Unger *et al.* 1997). GPCRs have seven transmembrane helixes arranged in a counterclockwise barrel perpendicular to the membrane (Larhammar *et al.* 1993) and connected by three intracellular and three extracellular loops beyond the lipid bilyaer (Figure 1). Almost all GPCRs, except rhodopsin, have extracellular N terminus and intracellular C terminus.

The three-dimensional structure, and hence the function, of GPCRs involves multiple interactions, such as hydrogen bonding, ionic interaction, hydrophobic interactions, and cysteine bonds. For instance, the two cysteine residues in the 2^{nd} and 3^{rd} extracellular loops are predicted to form a disulfide bond crucial for the receptor stability. The cysteine residues in the C terminus are also important: palmityolation of these residues leads to formation of membrane anchors that allow a fourth intracellular loop (Bouvier *et al.* 1995). While the effects of mutations on these C-terminal cysteines are not universal, the C terminus of GPCRs has been hypothesized to serve as an anchorage to interact with many "functional protein networks" for many GPCRs (Bockaert *et al.* 2003; Bockaert *et al.* 2004). While the effects are not universal, mutations of these C-terminal cysteines led to functional uncoupling for β_2-adrenergic receptors (O'Dowd *et al.* 1989) and enhanced constitutive signaling for 5-HT_4 receptor (Bockaert *et al.* 2004), but not others (Ulloa-Aguirre *et al.* 1999).

1.2.2 Agonist-mediated activation of GPCR

According to the classical model, GPCRs isomerize between inactive (R) and active states (R*) in a dynamic equilibrium. Agonists bind preferentially to and stabilize R*. The activated receptor then functions as a guanine nucleotide exchange factor to catalyze the substitution of GTP with GDP on the guanidine nucleotide–binding protein (G protein) α subunit, leading to conformation changes in the Gα subunit that promote dissociation of the heterotrimer into Gα subunit from βγ complex. The activated Gα subunit then interacts with downstream effectors, such as phosphodiesterases, adenylyl cyclases, phospholipases, and ion channels, to release second messenger molecules, such as cyclic AMP (cAMP), cyclic GMP (cGMP), and inositol triphosphate (IP3). Secondary messengers are small, chemically stable molecules. Depending on the G_{α} protein activated, they regulate the activity of many effectors, such as protein kinase A (to phosphorylate ion channels, enzymes and transcription factors), cyclic nucleotide gated channels, and transcription factors. The signal transduction cascade is then rapidly turned off. The activated GPCR becomes desensitized and internalized. The intrinsic GTPase activity of Gα subunit converts GTP to GDP, thus inactivating the Gα subunit and allowing the heterotrimeric complex to reform.

1.2.3 Constitutive signaling versus agonist mediated signaling

In contrast to agonist-mediated signaling, constitutive (basal, ligand-independent) signaling occurs in the absence of ligands (Figure 2). The constitutive signaling also increased affinity for agonist and increased maximal response, leading to subsequent downregulation. Constitutive signaling depends on the receptor level of GPCR. An unequivocal identification of the constitutive signaling in the "native system" has often been obscured by the possible activation by endogenous agonist(s). Hence, constitutive signaling is often identified via recombinant system in cell lines (Milligan 2003).

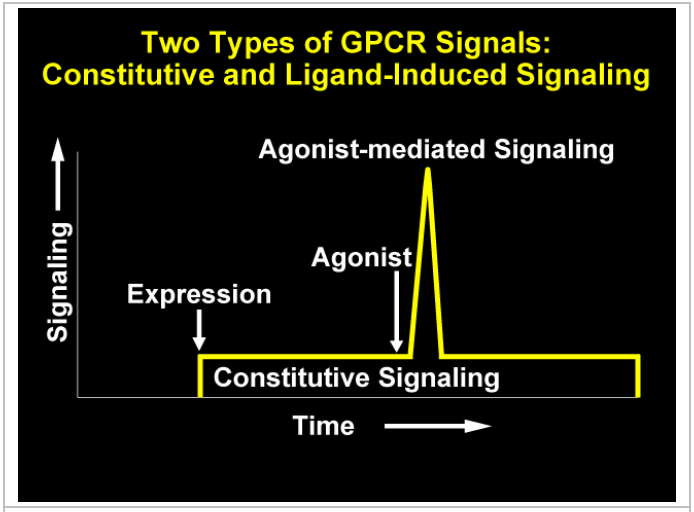


Figure 2: Constitutive versus agonist-mediated response.

In contrast to the two-state model (Samama et al. 1993), most GPCRs demonstrate both constitutive and ligand-induced signaling. While constitutive signaling is of long duration and highly dependent on receptor expression level, the agonist mediated signaling is short-lived.

1.2.4 Classification of GPCR ligands: agonist, antagonist, inverse agonist

A ligand (latin ligare = to bind) is a molecule that interacts with a receptor (via hydrogen, ionic, or covalent bonding). An agonist (Greek ago= lead, exert) binds and activate a receptor to generate a response. A partial agonist binds and activates a receptor to create a partial response. An antagonist blocks the effect of an agonist (Simmons 2005).

1.2.5 GPCR Signaling Transduction Cascade

Amplification is a very important part of the GPCR signaling transduction cascade. It occurs at almost every level of the signal transduction cascade (Lamb *et al.* 1992; Lagnado 2002). Composed of an opsin linked to 11-*cis*-retinal (Ovchinnikov Yu 1982), rhodopsin changes the retinal Schiff base cofactor in rhodopsin from *cis* to *trans* when it absorbs a photon, initiating receptor conformational changes that activated the rhodpsin (seven helixes inside the membrane in Figure 1). The activated rhodopsin activates about 10 Gα proteins. Each Gα protein then activates 200–1000 effector molecules (transducin) per second (Baylor 1996) (Vuong *et al.* 1984). Each transducin activates phosphodiesterase, and each phosphodiesterase hydrolyzes ~1000 cGMP molecules to GMP, closing the sodium channels and thus hyperpolarizing the cells (Baylor 1996).

This amplification in the signaling cascade allows GPCRs to efficiently detect and transducer signals from low concentrations of agonist. This amplification, combined with the low constitutive activity of 11-*cis*-retinal bound rhodopsin, allows human eyes to detect as little as five photons (Hecht *et al.* 1942).

1.3 Challenges to study GPCRs

In spite of our interest in GPCR signaling, there is still much we do not understand, due largely to significant challenges. Although many drugs target GPCRs, studies of GPCR signaling *in vivo* are complicated by the lack of specific agonists and antagonists for many of the receptors. According to the International Union of Pharmacology, more than 130 orphan GPCRs lack a known agonist as of August 2007 (IUPHAR 2007). Furthermore, GPCRs are often expressed in multiple organs, making activation of a GPCR in a specific tissue of an organ difficult.

The biggest challenge is that GPCRs display molecular and functional diversity, such as the type of G-protein-signaling pathway, different levels of constitutive activity, and functional selectivity (different responses of a receptor due to different ligand-selective conformations) (Kenakin 2003; Maudsley *et al.* 2005). For instance, more than 40% of wildtype GPCRs have constitutive signaling (Seifert *et al.* 2002). In one survey examining 173 GPCRs, 11% of

GPCRs were found to simultaneously couple to multiple G proteins (Hermans 2003; Wong 2003). In addition, members of GPCR subfamilies (such as serotonin) are activated by the same agonist but have different G-protein-signaling pathways. This diversity enables the receptors to transmit unique extracellular signals and function appropriately in multiple tissues but hampers efforts to sort out the relative contributions of each signaling pathway or the roles of constitutive signaling for each receptor.

1.4 Receptors Activated Solely by Synthetic Ligands (RASSL) Evolution

1.4.1 RASSL Concept

To better study the significance and molecular diversity of GPCR signaling, we developed receptors activated solely by synthetic ligands (RASSLs). RASSLs are genetically engineered receptors that are insensitive to their natural, endogenous ligand(s), but can still be fully activated by synthetic, small-molecule drugs. Based on the simple concept that GPCRs can have multiple ligand-binding domains, we modify the binding pockets for the endogenous ligands so that the receptor is no longer activated by endogenous hormones (Figure 3). Instead, they are activated by small-molecule drugs (Scearce-Levie *et al.* 2001), allowing them to be used to activate specific G-protein pathways rapidly and reversibly and to mimic the speed, localization, regulation, and amplification of endogenous GPCR signals (Srinivasan *et al.* 2007).

Since GPCRs are the major targets of commercially available drugs, they are attractive starting points for devising physiological controls for tissue engineering. In contrast to the bacterially derived tetracycline transactivator, the human GPCRs provide a scaffold for building drug-controlled switches that are less likely to induce an immune response. Since almost half of drugs currently in the market target GPCR signaling (Flower 1999; Robas *et al.* 2003; Brink *et al.* 2004), FDA-approved drugs are often available. For instance, erythromycin activates the putative RASSL based on the human motilin receptor to induce G_q

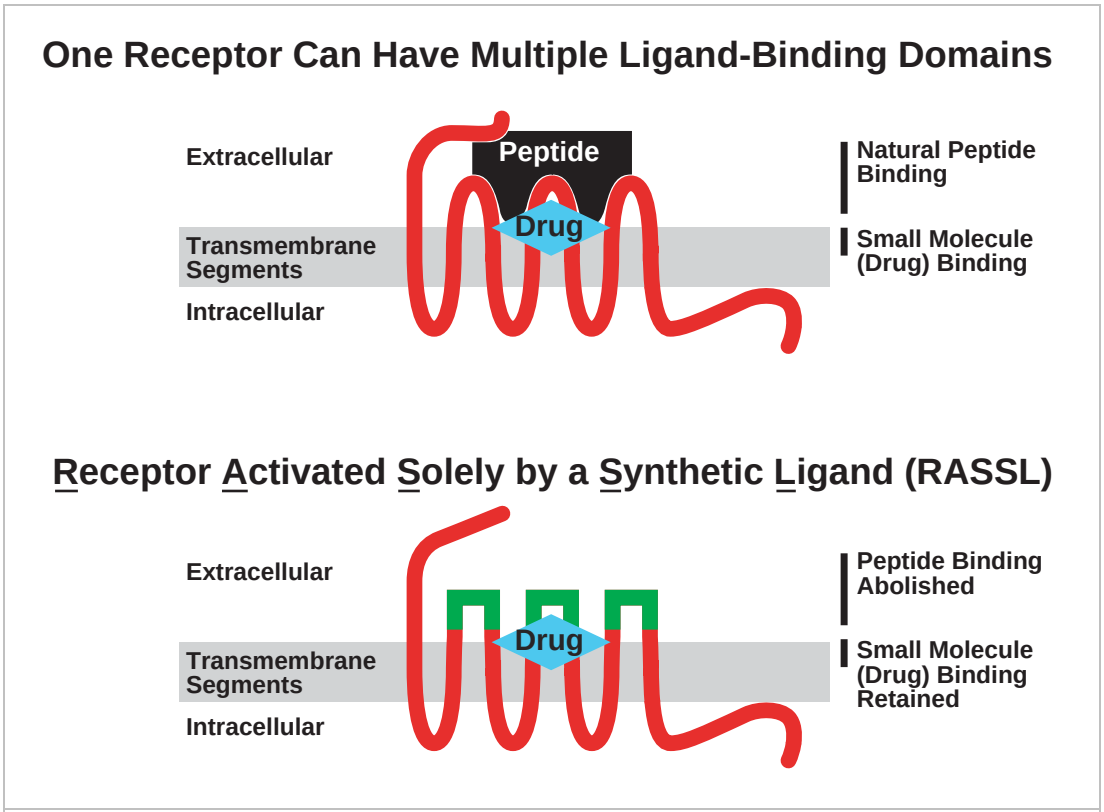


Figure 3. Design of a RASSL. *Peptide receptors are generally activated by the binding of endogenous hormones to the extracellular surface of the receptor, whereas small molecules typically activate the receptors by binding in the transmembrane segments. Although these binding domains can overlap, some residues bind only the peptide, allowing mutations to be made that selectively inhibit the binding of the natural peptide. The resulting RASSL is only activated by the synthetic drug.*

signaling and calcium mobilization. Erythromycin is safe and well tolerated by millions of people each year. Similar drug-GPCR combinations form the basis for designing new pharmacologically activated switches to modulate all the major GPCR signaling pathways for tissue engineering studies.

Since it is impractical to convert all GPCRs into RASSLs, we and others have focused on representative G_s-, G_i-, and G_q-coupled GPCRs, which stimulate adenylyl cyclase, inhibit adenylyl cyclase, and stimulate phospholipase C, respectively.

In early elegant attempts to make a designer GPCR, Strader and colleagues combined a custom ligand specifically designed to complement a mutant adrenergic receptor that had impaired binding to epinephrine (Strader *et al.* 1991). Unfortunately, this custom ligand was impractical for *in vivo* studies: the potency is too low by several orders of magnitude (EC_{50} = 40 μM). Other studies have designed new metal ion binding sites into GPCRs, yet these compounds may be toxic and require mM concentrations (Elling *et al.* 1999) that would be difficult to achieve *in vivo*. By comparison, the EC_{50} for spiradoline-induced responses is 5 nM (800-fold more potent than designer adrenergic agonists), allowing *in vivo* studies with drug doses that can be easily achieved. Since the RASSL concept is based on a pre-existing high-affinity agonist-binding site, we did not have to face the daunting task of building a new agonist-binding site that other systems face.

1.4.2 The first G_i coupled RASSL as proof of concept

Ro1 (RASSL opioid 1), the prototype RASSL, is based on a G_i-coupled κ-opioid receptor (Coward *et al.* 1998). In a proof of concept of this strategy, its G_i response to natural ligands is 0.001% of that of the wildtype receptor, but it is potently activated by the synthetic agonist spiradoline (Coward *et al.* 1998; Zhao *et al.* 2003; Sweger *et al.* 2007). Ro1 decreases the heart rate in mice (Figure 4A) (Redfern *et al.* 1999) and affects taste sensation in the tongue (Figure 4C) (Zhao *et al.* 2003). In addition to serving as powerful tools to dissect the G-protein signaling *in vivo*, RASSLs can yield insights into fundamental aspects of receptor diversity (Pauwels 2003). For instance, constitutive signaling of Ro1 led to cardiomyopathy (Figure 4B) (Redfern *et al.* 1999), diminished bone formation (Nissenson RA, personal communication), and hydrocephalus (Sweger *et al.* 2007) (Figure 4C). These constitutive signaling phenotypes would have been difficult or impossible to identify by studying endogenous receptors.

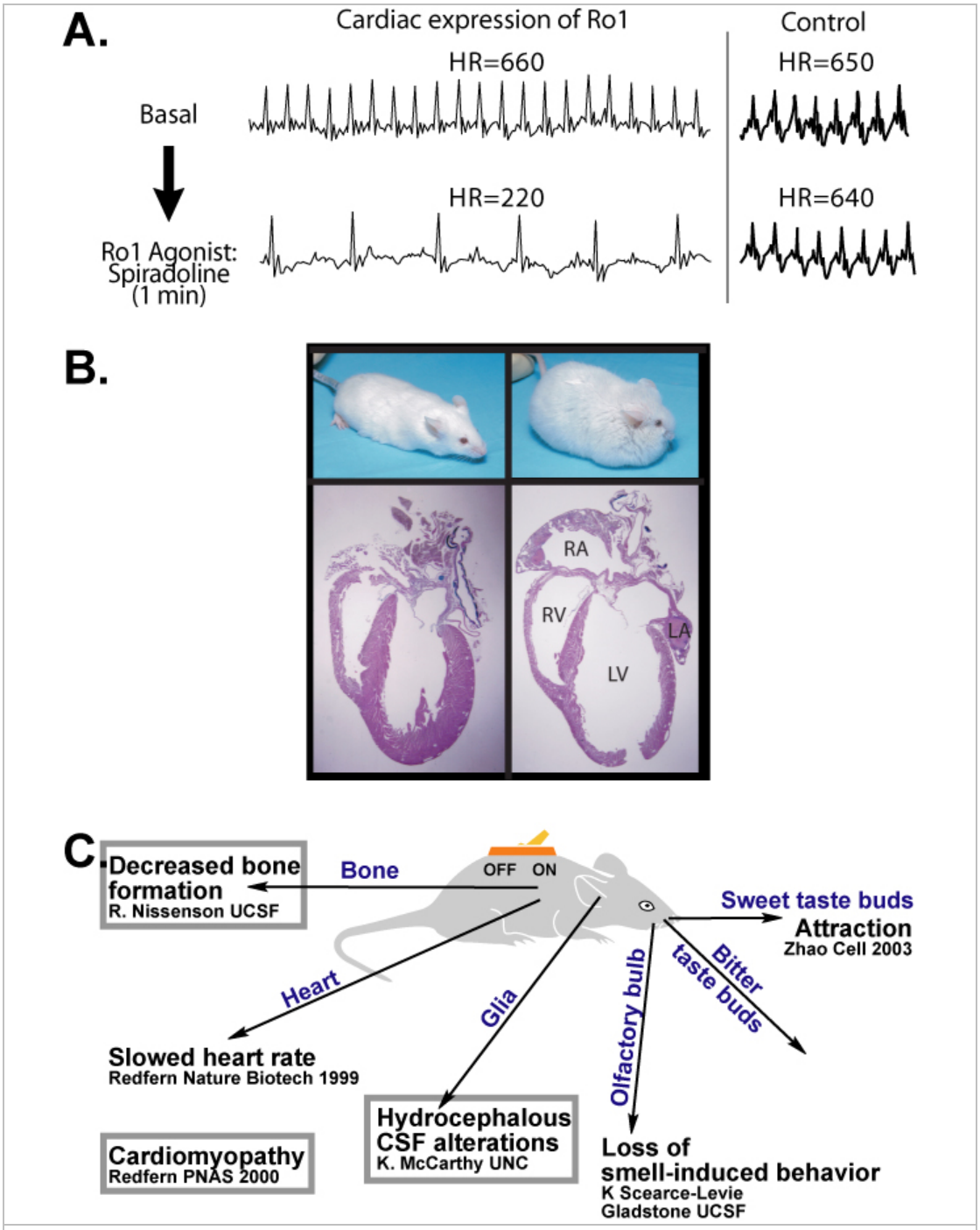


Figure 4. Ro1 has been used to study both agonist-mediated and constitutive signaling in vivo. (A) *Mice with Ro1 transgene driven by myosin-heavy chain promoter display decreased heart rate when stimulated with spiradoline.* ***(B)*** *Ro1 mice suffer peripheral edema, shortness of breath and lethargy in the absence of spiradoline. Hearts from Ro1 mice show reduced systolic function, fibrosis, and ventricular chamber dilation. The mice show normal phenotype when treated with NorBNI (the inverse agonist).* ***(C)*** *Summary of findings based on Ro1. Ro1 has already been tested in bone, heart, glia, olfactory bulb, and taste buds. Its agonist-mediated signaling decreases heart rate, loss of smell-induced behavior, and attraction. Its constitutive signaling led to RO1 expression results in embryonic/perinatal lethality. It decreased bone formation, cardiomyopathy and hydrocephalous CSF alterations. Grey box indicate constitutive signaling.*

1.4.3 Other approaches to control G protein signaling

Other approaches have been useful in the study of G protein signaling. One approach involved creating specifically designed protein kinase substrates to help identify the specific kinase targets. Another created protein kinases designed to identify specific kinase targets (Bishop *et al.* 2000). These chemical genetics approaches have a major impact on determining the specificity of protein kinases, building a growing toolbox for biomedical researchers (Knight *et al.* 2007). For example, chemical inducers of dimerization (CID) fuse a signaling domain with a drug-binding protein. The addition of a chemical dimerizer (i.e., FK1012 or rapamycin) that binds to the drug-binding domain can then cross-link the fusion proteins and activate signaling. These dimerization systems are powerful tools for molecules that are activated by proximity to other molecules (hetero- or homo-oligomerization) or by proximity of cellular compartments (plasma membrane, nucleus). They have been used to rescue protein functions (Pratt *et al.* 2007), cause selective proliferation (Siatskas *et al.* 2005), transformation and even apoptosis of select populations of cells (Siatskas *et al.* 2005). They have also been used in several animal models and have just begun human clinical trials.

Another innovative tool uses engineered zinc finger proteins that work at the transcriptional level to control stem cell fate (Bartsevich *et al.* 2003), control gene expression (Liu *et al.* 2005), and target gene correction (Urnov *et al.* 2005; Miller *et al.* 2007).

Both approaches complement the RASSLs since each system delivers a fundamentally different type of biochemical signal and controls different physiologic responses. Because none of the other systems has the unique GPCR seven-transmembrane structure, none can mimic the speed (nanoseconds), amplification, and membrane-localized signal that can be achieved through a RASSL.

1.4.4 RASSL Evolution

RASSLs were first developed 16 years ago and are now being used by researchers in a wide variety of areas that are of great interest to tissue engineering (see letters of support). The first attempt at making a designer GPCR was lead by Catherine Strader, who developed a series of compounds to selectively activate a mutant version of the β-adrenergic receptor (Strader *et al.* 1991). Unfortunately, this elegant work only yielded compounds with millimolar affinities and unknown pharmacokinetics, making *in vivo* work impractical.

Our laboratory later devised a series of RASSLs with nanomolar agonist affinities, making *in vivo* use possible for the first time (Coward *et al.* 1998). The key to making these first RASSLs was to take advantage of potent pre-existing synthetic drugs, such as kappa opioid agonists (e.g., spiradoline) that had been developed by the pharmaceutical industry as potential analgesics. A RASSL could be made by introducing mutations that abrogated signaling via the natural

peptide ligands, yet preserved stimulation via the drug, spiradoline. The first in this series, RO1 (RASSL Opioid #1, Gi-coupled), has been expressed in at least six different tissues in transgenic animals to induce diverse phenotypes, such as heart-rate control (Redfern *et al.* 1999), cardiomyopathy (Redfern *et al.* 1999), and bitter and sweet taste sensations (Muller *et al.* 2000; Zhao *et al.* 2003).

Multiple RASSLs have since been made, including a G_s-coupled RASSL based on the melanocortin-4 receptor (Srinivasan *et al.* 2003), a G_q-coupled RASSL based on the histamine 1 receptor (Bruysters *et al.* 2005), and a series based on muscarinic receptors (Armbruster *et al.* 2007). These RASSLs are useful tools. However, it is still advantageous to derive a series of RASSLs with distinct G-protein signaling from the same parental GPCR. It can be difficult to compare the effects of RASSLs based on different parental GPCRs since these RASSLs could have different pharmacokinetics, constitutive activity, desensitization kinetics, and cellular localization.

1.5 Human 5-HT_4 Receptor

We based the series of GPCRs with different G protein signaling and constitutive signaling on the human 5-HT_4 receptor (Figure 1) for the following reasons. First, its pharmacological properties are well established (Claeysen *et al.* 2000), and non-antipsychotic agonists are available (Meltzer *et al.* 1991). Its agonists have

milder effects (increased gastrokinesis (Crowell *et al.* 2005), augmented memory acquisition and retention (Orsetti *et al.* 2003), increased chronotropic and ionotropic cardiostimulation (Kaumann *et al.* 2006), and enhanced cortisol release (Lefebvre *et al.* 2002), compared to other serotonergic drugs. In addition, the large number of ligands would allow us to identify differences in their effects on that receptor.

Second, the 5-HT_4 receptor has remarkable functional diversity. Eight isoforms of 5-HT_4 receptor due to mRNA splicing of the carboxyl tail (form a-g, n) and one based on a 14–amino acid insertion in the second intracellular loop have been identified (Bockaert *et al.* 2004). While they have similar pharmacological responses (Blondel *et al.* 1998), the isoforms show different signaling responses, such as different G protein signaling by the 5-HT_{4A}, enhanced desensitization by 5-HT_{4D} (Mialet *et al.* 2003), and enhanced constitutive signaling by 5-HT_{4E} and 5-HT_{4F} (Claeysen *et al.* 1999). With minor modifications, RASSLs based on the 5-HT_4 receptor would be the ideal tool for examining the significance of functional diversity *in vivo*.

Third, 5-HT_4 couples to other signaling pathways, such as activating L-type calcium channels (via increased cAMP and activated protein kinase C), activating sodium currents (via cAMP independent mechanism), activating potassium current (not via cAMP), and decreasing calcium-activated potassium currents (via increased cAMP and protein kinase), and voltage activated potassium (via increased cAMP and protein kinase C) (Greengard 2001).

Fourth, a single mutation ($D^{100}A$) in the mouse 5-HT_4 receptor dramatically reduced its affinity for serotonin, its endogenous ligand. This mutation also allows synthetic agonists and antagonists for the wildtype receptor to activate the mutant, turning 5-HT_4-$D^{100}A$ into a RASSL (Claeysen *et al.* 2003).

Finally, we reasoned that novel receptors coupling to other signaling pathways could be created by making chimeras of the 5-HT_4 receptor with the others (Felderbauer *et al.* 2005) because it is based on receptor conformation determined by multiple regions of the receptor (Wong 2003). Changing multiple regions involves large internal mutations that often lead to receptor instability. A

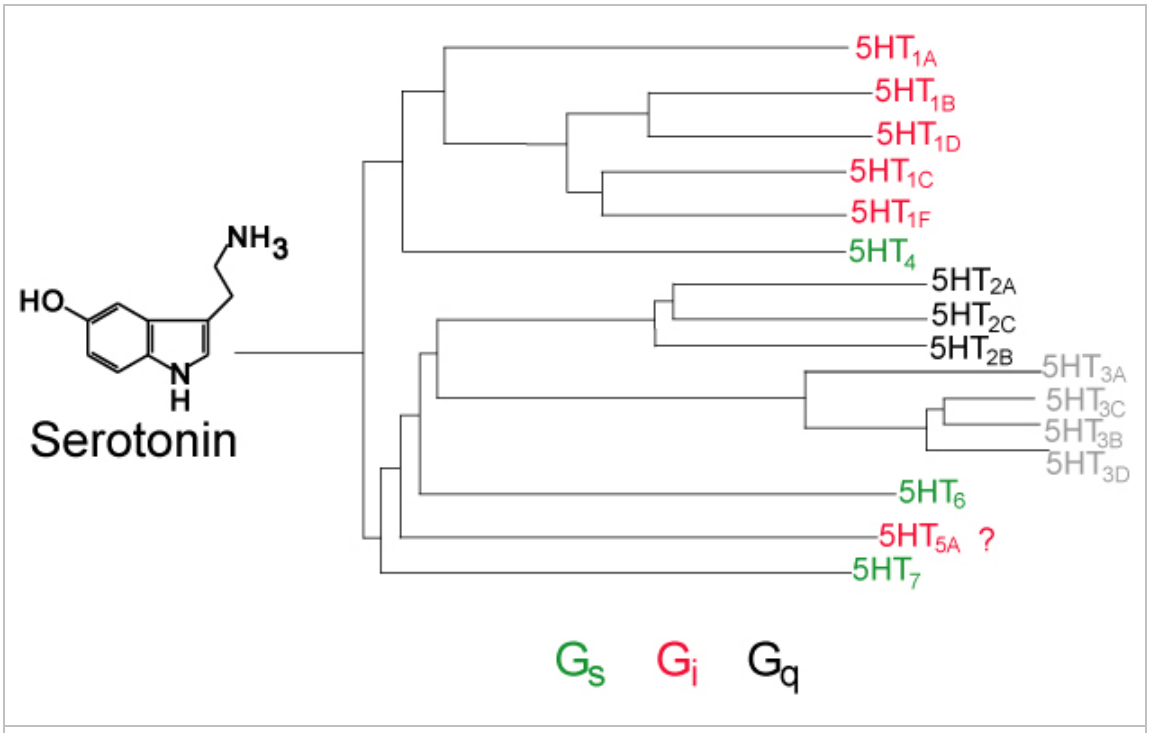


Figure 5. Human Serotonin Receptor Family.
Serotonin activates up to 16 members of serotonin receptors to activate the G_s, G_i and G_q signaling pathways. 5-HT_4, 5-HT_6, and 5-HT_7 are Gs coupled. In contrast, 5-HT_1 subfamily is G_i coupled, and 5-HT_2 subfamily is G_q coupled. The ionotropic 5-HT_3 subfamily members are ligand-gated ion channels. The dendrogram is based via Align X (Invitrogen) on amino acids with a gap-opening penalty of 10 and a gap-penalty of 0.1.

better strategy for altering G-protein signaling characteristics is to swap domains between structurally similar receptors within the same family. The 5-HT_4 receptor belongs to a family of at least 16 receptors, each with different subfamilies that engage different G-protein signaling pathways (Figure 5). The 5-HT_4, 5-HT_6, and 5-HT_7 subfamilies are G_s coupled, the 5-HT_1 subfamily is G_i coupled, and the 5-HT_2 subfamily is G_q coupled (Hoyer *et al.* 2002). These characteristics could expedite our efforts to make purely G_s-, G_i-, and G_q-coupled RASSLs.

CHAPTER 2: G PROTEIN SIGNALING PROPERTIES OF THE HUMAN 5-HT_4-BASED RASSL

2.1 Summary

After adding a flag epitope and signal peptide and making a point mutagenesis, we tested the ligand induced and constitutive signaling of the human 5-HT_4-$D^{100\ (3.32)}$A mutant. We found that it was not activated by serotonin, its endogenous agonists. Expressed at similar level as the 5-HT_4 receptor on the cell surface, it was instead potently activated by antagonists and inverse agonists against the 5-HT_4 receptor. In addition, we found that the mutant had a high level of constitutive G_s signaling. Finally, we discovered that different agonists caused differential effect on the mutant. It showed high levels of G_s/G_q signaling when treated with benzamide-based compounds (RS23597, RS39604, RS67333) and only Gs signaling when treated with indoleamine-based compounds (GR113808, GR125487, RO110-0235). Interestingly, the indoleamine-based compounds also selectively activated 5-HT_4-D^{100}A without affecting the 5-HT_4 receptor.

2.2 Introduction

2.2.1 Ligand binding domain and RASSL Engineering

The ligand binding domain is essential for proper GPCR function and crucial for engineering RASSLs. While the interaction of the ligand with TM V and TM VI is

important for receptor activation (Strader *et al.* 1987; Ulloa-Aguirre *et al.* 1999), the exact location of the ligand-binding domain depends on the receptors and the ligand size/structure. This is further complicated by different bidning domains for agonists and antagonists or endogenous agonists versus synthetic agonists. This, in turn, affects how RASSLs are engineered from their parental GPCRs.

For instance, the N terminus of κ-opioid receptor (a peptidogenic GPCR) serves as a ligand binding domain for antagonists, while the second and/or third extracellular loop(s) interact with the endogenous agonists (Kong *et al.* 1994). Thus, the first RASSL (Ro1) was made by replacing the second extracellular loop of the κ-opioid receptor with that of δ-opioid receptor so that endogenous agonists no longer activate the RASSL (Coward *et al.* 1998).

In contrast, the ligand binding domain for biogenic amines GPCRs, such as the β-adrenergic receptors and the 5-HT_4 receptor, involve interactions between the ligand and highly conserved residues in TM III, TM V, and TM VI (Dixon *et al.* 1987; Strader *et al.* 1987; Claeysen *et al.* 2000; Bockaert *et al.* 2004). More specifically, the ionic interaction between the amine in the ligand and a highly conserved aspartic acid (D^{100} for 5-HT_4 receptor) is essential for ligand binding (Strader *et al.* 1987; Claeysen *et al.* 2003).

This then led to Claeysen's finding that a base-pair mutation on a highly conserved residue in the ligand-binding pocket of the mouse serotonin 5-HT_4 receptor ($D^{100}A$) makes the receptor insensitive to its endogenous agonists (Claeysen *et al.* 2003). Aspartic acid $D^{100(3.32)}$ is a highly conserved residue in the

ligand-binding pocket of the 5-HT_4 receptor. Earlier studies suggest that the ionic interaction between the positively charged amine in serotonin and the negatively charged carboxylate of aspartic acid in $D^{100(3.32)}$ is crucial for functional activation of the 5-HT_{1A} (Ho *et al.* 1992), 5-HT_2 (Wang *et al.* 1993; Manivet *et al.* 2002), 5-HT_4 (Mialet *et al.* 2000; Joubert *et al.* 2002), 5-HT_6 (Boess *et al.* 1998), M_1 (Lu *et al.* 1999), and other biogenic GPCRs. Mutations, such as $D^{100}A$, disable the ionic interaction between the negative charge of the aspartic acid and the primary amine of serotonin, thus making the receptor insensitive to serotonin. More unexpectedly, the mouse 5-HT_4-$D^{100(3.32)}A$ mutant was activated by antagonists and inverse agonists for wildtype the 5-HT_4 receptor (Claeysen *et al.* 2003). This result could not be extended to other biogenic GPCRs (Claeysen, personal communication). However, its effects on the human 5-HT4 receptor have not been tested. We now extend these findings to the human 5-HT4 receptor

2.2.2 Physiological importance of constitutive signaling

The physiological significance of constitutive signaling is reflected by the diseases caused by GPCR gain-of-function mutations that were discussed earlier. However, constitutive signaling is probably also crucial for normal health. There are many examples. For instance, “gene dosage effect” of human melanocortin 4 receptor is inversely proportional to food intake and body weight (Huszar *et al.* 1997). The importance of constitutive signaling in normal physiological responses is also suggested by the multiple natural inverse

agonists, such as retinal for rhodopsin (Han *et al.* 1998), agouti for melanocortin MC1 receptor (Eberle *et al.* 2001), AgRP for melanocortin MC3 and MC4 receptors (Nijenhuis *et al.* 2001), and Exendin-(9-39) for Glucagon-like peptide 1 receptor (Serre *et al.* 1998). It is thus little surprise that 85% of the 380 antagonists targeting 73 GPCR targets in a survey are inverse agonists (Kenakin 2004).

Interestingly, the prevalence of constitutive signaling seems to be different for different G protein signaling pathways. Constitutive G_s signaling is much more common than the constitutive G_q or G_i signaling. It is estimated that 46%, 32%, and 22% of G_i-, G_q-, and G_s-coupled wildtype GPCRs, respectively, show constitutive signaling. On the other hand, about 50%, 25%, and 25% of constitutively active mutants (CAM) are $G_{i/o}$, G_q, and G_s coupled, respectively (Seifert *et al.* 2002).

The physiological effect of constitutives signaling in different G protein signaling pathways may also be different. There have been many instances of diseases caused by consitutively active G_s or G_q signaling (Seifert *et al.* 2002). On the other hand, except for a few exceptions (rhodopsins and leutenizing horomone receptor), there has not yet been any report of constitutive $G_{i/o}$ signaling as causing disease (Seifert *et al.* 2002).

2.3 Results

2.3.1 Human 5-HT$_4$ D^{100}A is a G$_s$-coupled RASSL (Rs1)

To determine if antagonists for the 5-HT$_4$ receptor also activate the human 5HT$_4$-D^{100}A mutant, we tested a variety of compounds. The mutant receptor was not activated by serotonin (serotonin, 15.8±1.3 nM; phosphate-buffered saline (PBS), 13.5 ± 1.8 nM; p = 0.672) (Figure 7, 8A), but it was activated by agonists

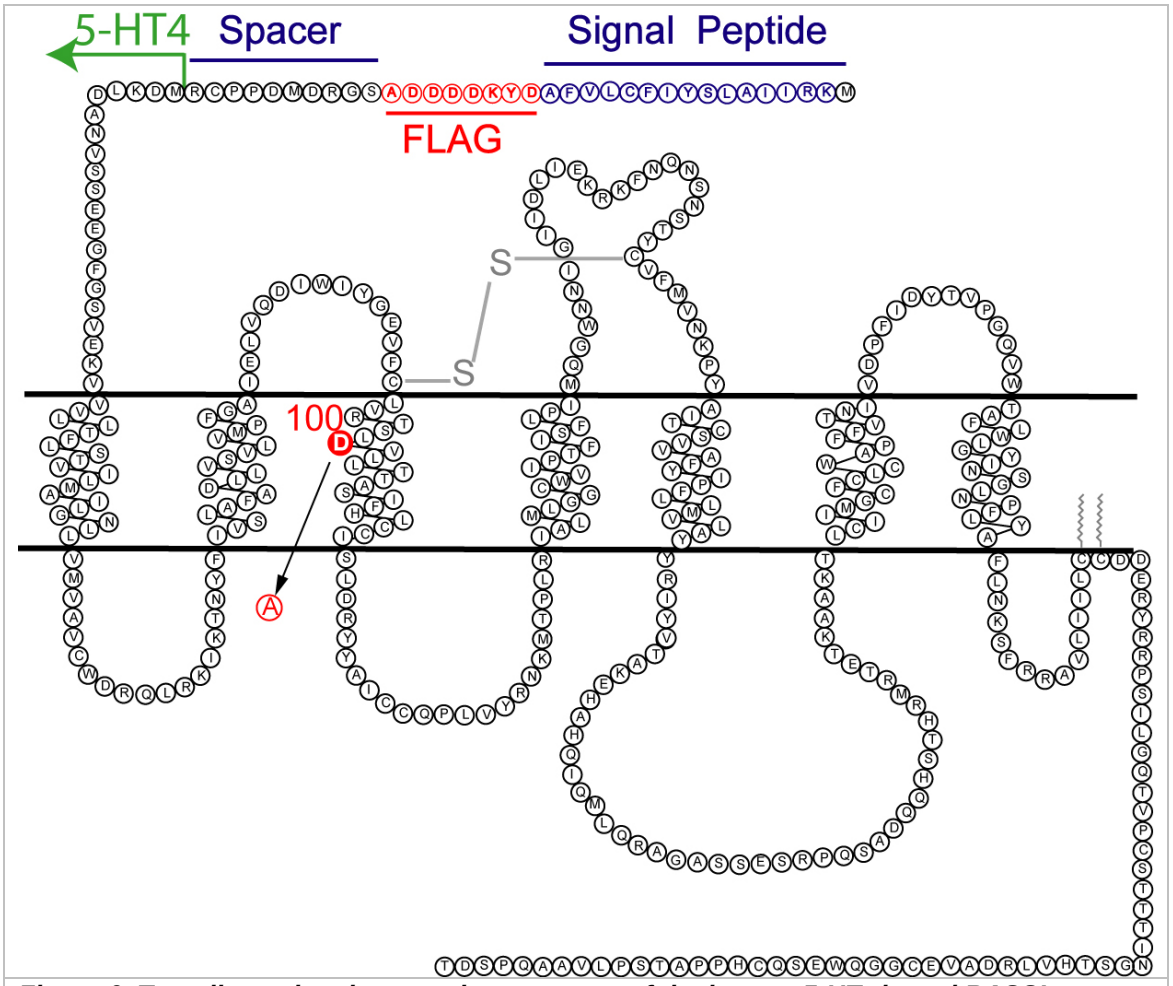


Figure 6. Two-dimensional serpentine structure of the human 5-HT$_4$-based RASSLs.
A signal peptide and the FLAG epitope were added to the N-terminus of the human 5-HT$_4$ receptor. The D^{100}A mutation was introduced by site-directed mutagenesis to create the G$_s$-coupled RASSL (Rs1).

(Cisapride, Zacopride), partial agonists (RS23597, RS39604, RS67333), antagonists (GR113808, RO110-0235), and an inverse agonist (GR125487) for the wildtype 5-HT_4 receptor (Figure 7). Rs1 retained agonist-dependent increases in cAMP production in response to several synthetic 5HT4 receptor agonists, including zacopride (Figure 7), demonstrating that the receptor is folded properly, inserted into the plasma membrane, and capable of signaling through the native G_s pathway. Since the mutant receptor is selectively activated by multiple synthetic ligands (GR113808, GR125487, and RO110-0235) but not serotonin, we named it Rs1 (RASSL serotonin 1) (Figure 1).

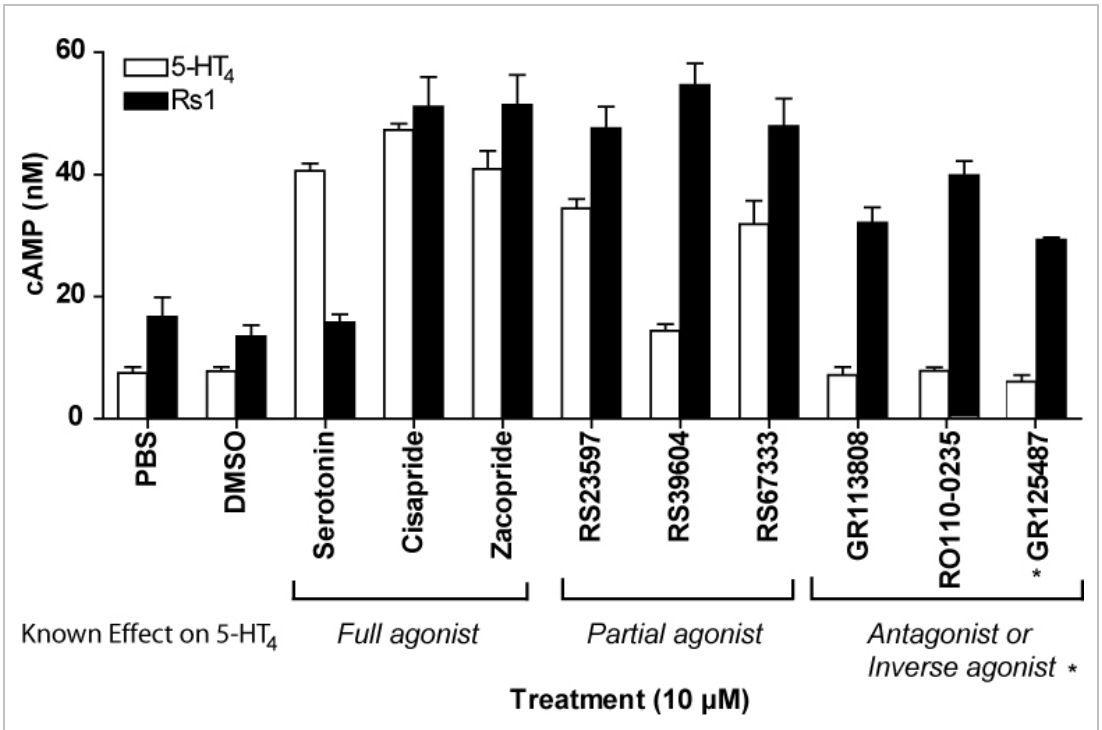
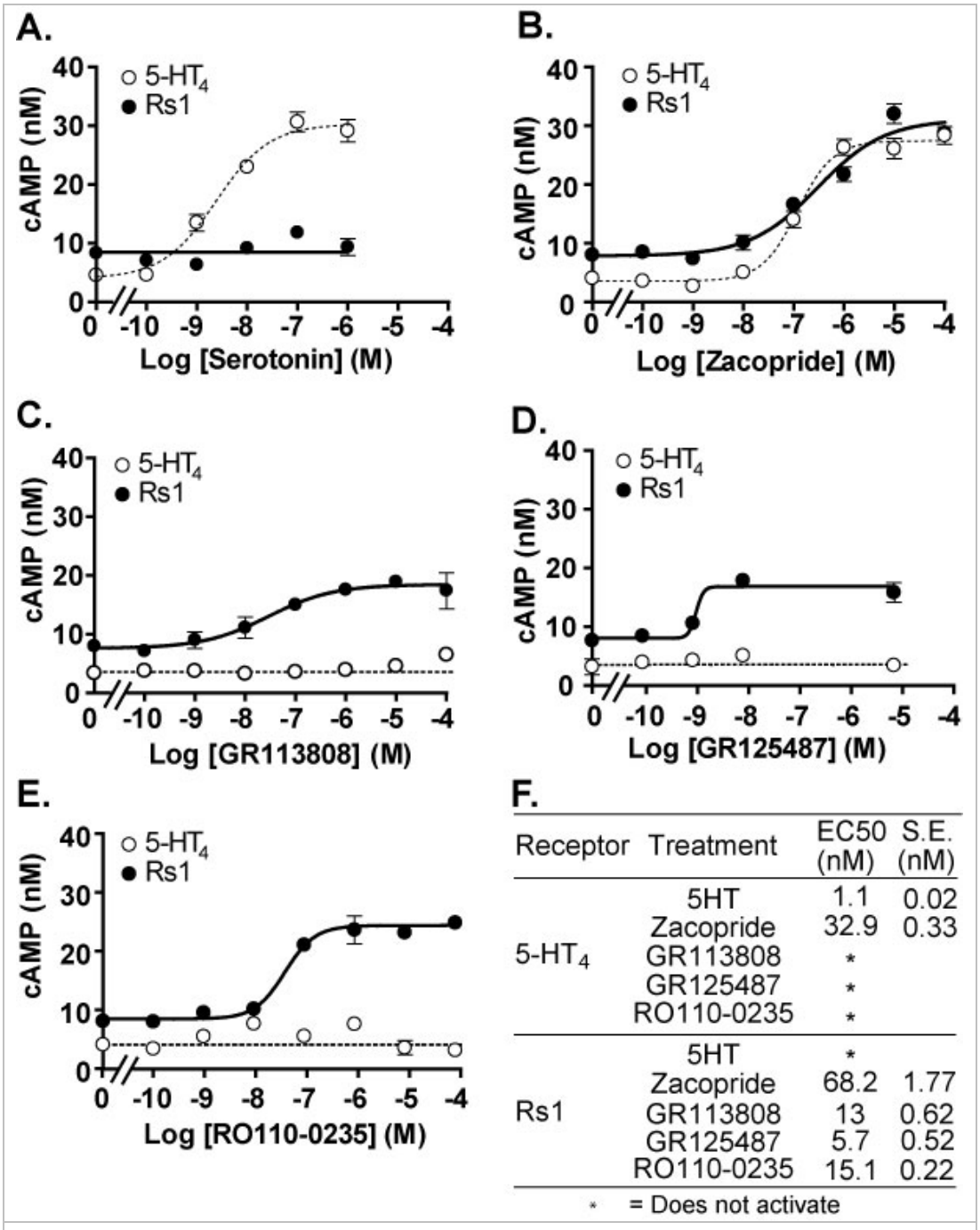


Figure 7. Rs1 is a G_s- coupled RASSL.
Rs1 was efficiently activated by small compounds known to be full agonists (Cisapride, Zacopride), partial agonists (RS39604, RS67333, and RS23597), antagonists (GR113808, RO110-0235), or inverse agonists (GR125487) for the wildtype 5-HT_4 receptor. It was not activated by its endogenous agonist (serotonin). Values are mean ± standard deviation of three independent experiments in which 25 ng of 5-HT_4 or Rs1 receptor cDNA was electroporated into 5x10^6 cells. DMSO, dimethyl sulfoxide.

2.3.2 Rs1 is activated by multiple agonists in the nanomolar range

We next explored the potency of Rs1 agonists. We found that antagonists and synthetic agonists for the 5-HT4 receptor activate Rs1. Without stimulating the wildtype receptor, antagonists and inverse agonists such as GR113808, GR125487, and RO110-0235 activated G_s signaling of 5-HT_4-$D^{100}A$ (EC_{50} of 13, 5.7, and 15.1 nM cAMP, respectively) more portently than Zacopride (EC_{50} of 68.2 nM) (Figure 8 B–E).



F.

Receptor	Treatment	EC50 (nM)	S.E. (nM)
5-HT4	5HT	1.1	0.02
	Zacopride	32.9	0.33
	GR113808	*	
	GR125487	*	
	RO110-0235	*	
Rs1	5HT	*	
	Zacopride	68.2	1.77
	GR113808	13	0.62
	GR125487	5.7	0.52
	RO110-0235	15.1	0.22

* = Does not activate

Figure 8. Rs1 activation is in the nanomolar range.

(A–E) *Rs1 transfectants were stimulated with increasing amounts of drugs. The D100A mutation in Rs1 makes the receptor insensitive to serotonin. It was efficiently activated by GR113808, GR125487, and RO110-0235, which do not activate the wildtype 5-HT4 receptor. Values are mean ± standard deviation of three independent experiments in which 25 ng of 5-HT4 or Rs1 receptor cDNA was electroporated into 5x106 HEK293 cells.* ***(F)*** *Best-fit estimate of the half-maximal effective concentration (EC50). Values are mean ± SEM of three independent experiments.*

2.3.3 Rs1 has a high level of constitutive signaling

We next examined the constitutive signaling of Rs1 in more detail. Rs1 showed greater constitutive signaling than the wildtype receptor at all levels of transfection (Figure 9A). The highest level of constitutive activity, achieved with 5.4 μg of receptor cDNA per 5 x 10^6 HEK293 cells, was 1.5 times greater than that of the wildtype 5-HT_4 receptor (49.6±1.25 nM vs. 32.5± 4.04 nM, $p < 0.005$) and >10-fold higher than that of the control receptors (the β_2-adrenergic and parathyroid hormone receptors), which have low levels of constitutive signaling (Figure 9A). Despite the high level of constitutive signaling, both the 5-HT_4 receptor and Rs1 could still be further activated by Zacopride (Figure 9B).

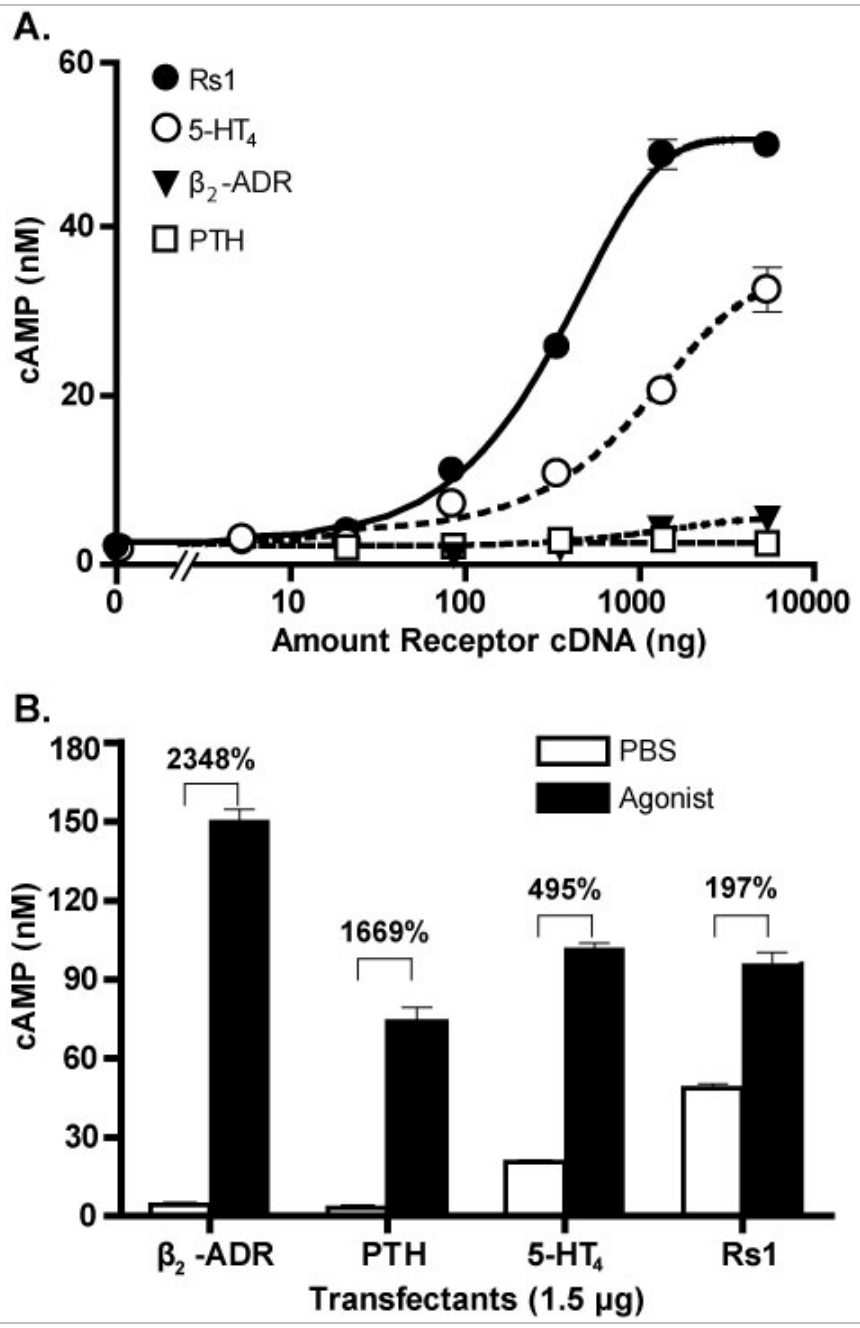


Figure 9. Rs1 has significant constitutive activity. (A) *Rs1 and the wildtype 5-HT$_4$ receptor both had much higher constitutive activity than the β$_2$-adrenergic receptor (β$_2$-ADR) and parathyroid hormone receptor (PTH).* ***(B)*** *Rs1 was still activated when 1.5 µg of receptor cDNA was electroporated into 5x10^6 HEK293 cells. Rs1 only showed 197% increase over constitutive signaling. 5-HT$_4$, and Rs1 were stimulated with 1 µM of Zacopride. β$_2$-ADR and PTH were stimulated with 1 µM of isoproterenol and human PTH (1-34) peptide, respectively. All experiments were repeated three times. Values are mean ± standard deviation.*

2.4 Discussion

We report here that the human 5-HT_4-D^{100}A mutant is a G_s-coupled RASSL (Rs1). We found that the mutant could be activated by a series of agonists and antagonists for the 5-HT4 receptor. In addition, we found that the mutant has high levels of constitutive signaling.

The discovery of a large set of agonists with different activities for the 5-HT_4 RASSLs could be useful in future studies. Many of the Rs1 agonists used in this study activated Rs1 with an EC_{50} in the nM range, allowing to us efficaciously activate Rs1. Having an abundant selection of ligands for Rs1 helped us find strong evidence of agonist-mediated functional selectivity on Rs1, and having a greater variety of drugs to choose from could be valuable for *in vivo* studies. For instance, we could use GR125487 to activate Rs1 while suppressing the basal activity of wildtype 5-HT_4. We could potentially study Rs1 constitutive signaling by using drugs such as RO116-0086 or RO116-1148 (Joubert *et al.* 2002) to decrease constitutive signaling of the wildtype 5-HT_4 receptor but not of Rs1.

In addition, we found that Rs1 has very high levels of constitutive signaling. Even though there are currently no inverse agonist for Rs1, Rs1 could still be a powerful tool to study constitutive signaling *in vivo* when combined with tetracycline inducible system. We are now ready to investingate the physiological significance of the constitutive G_s signaling in Rs1 *in vivo*.

CHAPTER 3: MODIFYING THE CONSTITUTIVE G_s SIGNALING OF Rs1

3.1 Summary

To investigate the physiological significance of Rs1 constitutive signaling, Rs1 mice were made. *In vivo,* Rs1 expression induces a dramatic anabolic skeletal response with mid-femur girth increasing 1200% and femur mass increasing 380% in 9-week-old mice. Bone volume, cellularity, mineral density, and serum markers of bone turnover were also elevated. No unusual phenotype developed when Rs1 was expressed after the first 4 weeks of postnatal life, indicating an exquisite temporal sensitivity of osteoblasts to G_s signaling. Since Rs1 does not respond to serotonin and we did not detect significant G_q signaling, the magnitude of G_s-mediated signaling by Rs1 should depend solely on the level of Rs1 expression and not on endogenous serotonin levels, which we cannot control.

Since the $D^{100}A$ mutation in Rs1 turned known inverse agonists (Claeysen *et al.* 2000) into either inverse agonists (GR125487, ML10375) or antagonists (RO116-0086, RO116-1148, SB207266) (Joubert *et al.* 2002; Claeysen *et al.* 2003) for Rs1, we examined mutations ($D^{66}A$, $D^{66}N$, $W^{272}A$) that decrease constitutive signaling of 5-HT_4 receptor. We found that these mutations decrease both the agonist dependent and constitutive signaling of Rs1 even though the mutants have similar expression level as Rs1.

3.2 Introduction

3.2.1 Physiological significance of constitutive signaling

Since first reported by Costa and Herz in δ-opioid receptor (Costa *et al.* 1989), the role of constitutive signaling is now recognized as an essential component for many GPCRs (Spiegel *et al.* 2004; Srinivasan *et al.* 2004). Many GPCRs have some basal activity and may be activated without ligands (Chen *et al.* 2000). This constitutive signaling may be physiologically significant. For example, KSHV-GPCR is a naturally occurring G_q- and G_i-coupled GPCR homologous to human CXCR2. The constitutive activity of KSHV enhances the activity of various downstream effectors, such as phospholipase C and MAPK, in fibroblasts and lymphatic cells, and leads to excessive angioproliferation (Yang *et al.* 2000). More recently, the G_q-coupled sphingosine 1-phosphate receptor ($S1P_5$) was shown to constitutively inhibit adenylate cyclase and extracellular signal regulated kinase, independent of its natural ligands (Niedernberg *et al.* 2003). It contributes significantly to its activation of downstream effectors, such as MAPK (Lee *et al.* 1996) and affects angiogenesis. Despite the well-established roles of the S1P receptor in endothelial proliferation, and apoptosis, the physiological significance of the basal activity *in vivo* is not well understood. If the basal activity is critical, then treatment of these constitutively active GPCRs with inverse ligands may affect human disease.

An early lesson in the use of RASSLs was that constitutive signaling often produced the most profound effects. Constitutive signaling occurs in over 40% of

GPCRs that have been tested (Seifert *et al.* 2002). We need to engineer RASSLs with varying levels of constitutive activity to truly recapitulate a native receptor (Figure 5). Even receptors with no measurable constitutive biochemical response in tissue culture can exhibit constitutive physiological responses when expressed at high levels in animals (Redfern *et al.* 1999; Scearce-Levie *et al.* 2001).

Because RASSLs have such potent physiological effects, they are best studied using conditional expression systems, such as the tetracycline transactivator system (Gossen *et al.* 1992; Gossen *et al.* 1995). With the Tet system, a single RASSL transgenic line can be used to drive expression in multiple tissues with tight temporal control. Using these tools, we can direct the expression of RASSLs to dissect complex physiological processes, such as the growth and development of cardiac function.

3.3.2 Rs1 constitutive signaling causes increased bone phenotype

Osteoblasts are essential for maintaining bone mass, avoiding osteoporosis, and repairing injured bone. Activation of osteoblast GPCRs, such as the parathyroid hormone receptor, can increase bone mass; however, the anabolic mechanisms are poorly understood.

Osteoblast dysfunction leading to bone loss is thought to be a major mechanism in osteoporosis, which affects over 10 million people in the United States and contributes to 1.5 million fractures each year (Foundation 2002). In

addition, bone fractures constitute over 3 million emergency department visits a year in the United States (Surgons 2007). Cells from the osteoblast lineage play key roles in regulating bone development, acquisition of peak bone mass, maintenance and repair of the adult skeleton, and calcium homeostasis (Aubin *et al.* 2006). Activation of the parathyroid hormone receptor (PTHR1) by recombinant PTH(1-34) (teriparatide) can increase bone mass (Lanske *et al.* 1996), but the exact *in vivo* roles of the different G protein signaling pathways and how they interact with other aspects of skeletal biology have not been clearly elucidated.

Human genetic diseases involving the Gsα subunit (GNAS) suggest that Gs signaling can influence bone growth (Weinstein *et al.* 2004). Inactivation of GNAS in humans leads to delayed bone growth and multiple endocrinopathies, as seen in Albright's hereditary osteodystrophy (AHO; OMIM #103580). Mouse models of AHO with chondrocyte- or osteoblast-specific inactivation of GNAS show severe alterations in chondrocyte maturation (Sakamoto *et al.* 2005) or cortical bone formation (Sakamoto *et al.* 2005), respectively. In contrast, abnormal genetic activation of GNAS in humans leads to McCune-Albright syndrome (MAS; OMIM #174800), which is characterized by alterations in bone and cartilage formation as well as multiple types of endocrine tumors (Weinstein 2006). Mice expressing a constitutively active PTHR1 in osteoblasts show increased trabecular bone volume and decreased cortical bone thickness at 12 weeks of age, with grossly normal femur shape and size (Calvi *et al.* 2000).

Since many GPCRs, including PTHR1, can signal through multiple G-protein signaling pathways, and a mouse model with constitutively active GNAS in osteoblasts has not been developed, the direct role of activating G_s signaling in osteoblasts has not been clearly tested.

3.3 Results

3.3.1 Rs1 constitutive signaling shows increased bone formation

We used the tetracycline transactivator system ("TetOff") to provide temporal control of Rs1 expression (Figure S1B) since G_s is crucial in a variety of tissues and likely causes embryonic lethality (Furth *et al.* 1994; Kistner *et al.* 1996). To obtain spatial control, the TetO-Rs1 transgenic mice were mated with transgenic mice expressing the tetracycline transactivator (tTA) under the control of the osteoblast-specific Col1α-1 2.3-kb promoter fragment (R. Nissenson, manuscript in preparation). In the absence of doxycycline, double transgenic progeny (designated ColI(2.3)+/Rs1+) showed high levels of Rs1 expression in whole femurs but not in non-skeletal tissues as assayed by quantitative real-time PCR (Figure 2A). These results are consistent with published descriptions of the Col1α-1 2.3-kb promoter fragment being active in maturing osteoblasts (Dacic *et al.* 2001; Dacquin *et al.* 2002).

Because of the high level of Rs1 transgene expression in ColI(2.3)+/Rs1+ mice, we hypothesized that the basal Rs1 signaling activity might be sufficient to alter bone mass *in vivo*. ColI(2.3)+/Rs1+ mice that were maintained off of

doxycycline from conception were phenotypically indistinguishable from littermate controls at birth, but displayed notable asymmetric enlargement of the skeleton starting at 3 weeks of age. As determined by bone densitometry (DEXA) scanning, the ColI(2.3)+/Rs1+ mice displayed an osteosclerotic phenotype with increased bone mineral (Figure 10B). At 9 weeks of age, both female (Figure S2E) and male (Figure S2F) mice showed dramatic increases (380%) in whole-body areal bone mineral density (BMD) (Figure 10C). No significant differences were observed in BMD, weights, or lengths within the littermate control genotypes (ColI(2.3)+ or Rs1+ single transgenics and wildtypes) or between males and females. In addition, three distinct TetO-Rs1 responder transgenic mouse lines gave similar results confirming that the bony changes were not due to transgene integration effects (data not shown). ColI(2.3)+/Rs1+ mice maintained off of doxycycline continued to show progression of the bone phenotype, requiring euthanasia by 30 weeks of age from complications of spinal stenosis, infection, or failure to thrive. Mice maintained on doxycycline from conception did not develop the bone phenotype.

A more detailed characterization of the bony lesions was obtained by microCT analysis (Figure 11A). The bones of ColI(2.3)+/Rs1+ newborn mice were normal in structure, location, and size, indicating grossly normal

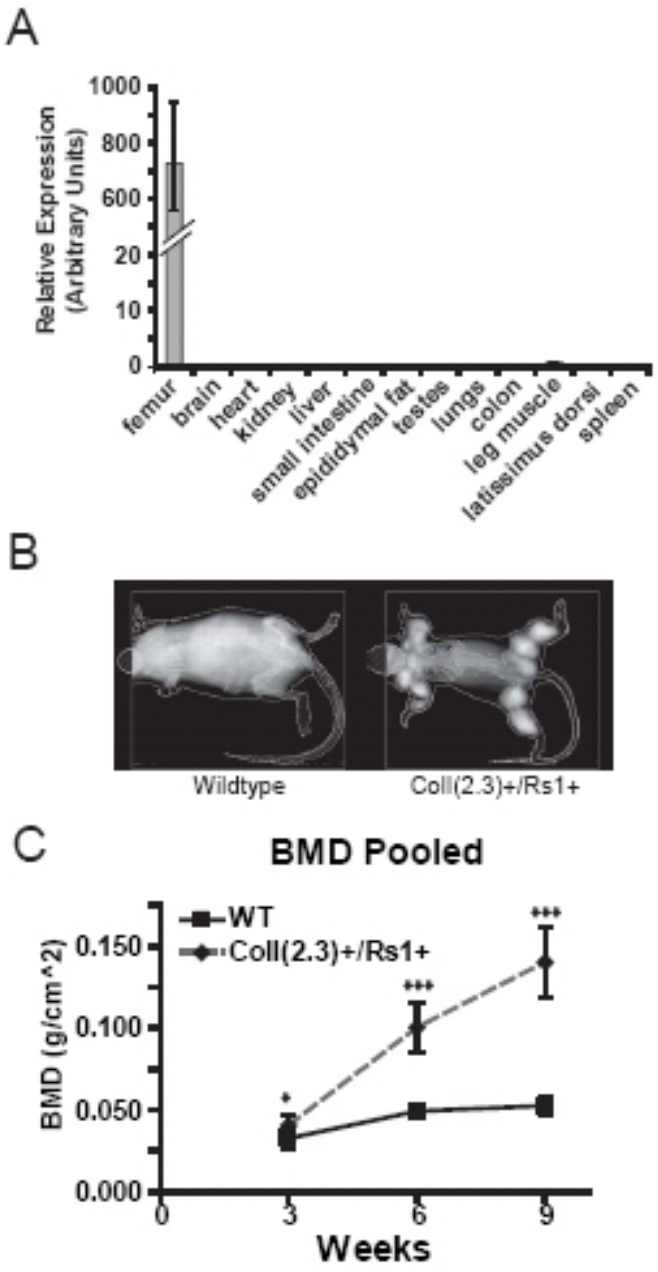


Figure 10. Effects of osteoblast expression of Rs1. (A) *Coll(2.3)+/Rs1+mice with the tetracycline-transactivator ("TetOff") system were conceived and maintained off doxycycline. Bone-specific expression of Rs1 was confirmed by qPCR. A representative adult mouse is shown, with similar expression profiles seen in three independent animals. Error bars represent +/- 1 SD for technical triplicates per tissue.* ***(B****) DEXA images show enhanced mineral accrual in the bones of 9- week-old double transgenic mice compared to littermate controls.* ***(C)*** *BMD measured in age matched littermates at 3 (n=10 WT, 14 mutant), 6 (n=10 WT, 10 mutant), and 9 (n=8 WT, 14 mutant) weeks showed continued progression of the phenotype. Error bars represent +/- 1 SD. * p<0.05, *** p<0.0005 by t-test of Rs1-expressing mice vs control.*

developmental patterning. Whole skeleton alizarin red staining confirmed normal development of the craniofacial structures with normal tooth eruption (data not shown). At 3 weeks of age, a moderate increase in femur size and an increase in bone accrual within the skull were evident in the ColI(2.3)+/Rs1+ mice. High-resolution CT scans of femurs from 3-week-old mice showed a significant replacement of the normal long-bone structures within the diaphysis by disorganized trabecular bone (Figure 11B, C). Morphometric measurements of femurs from 3-week-old mice showed large increases in femur weight and mid-diaphyseal diameter, but not femur length (Figure 11D–F).

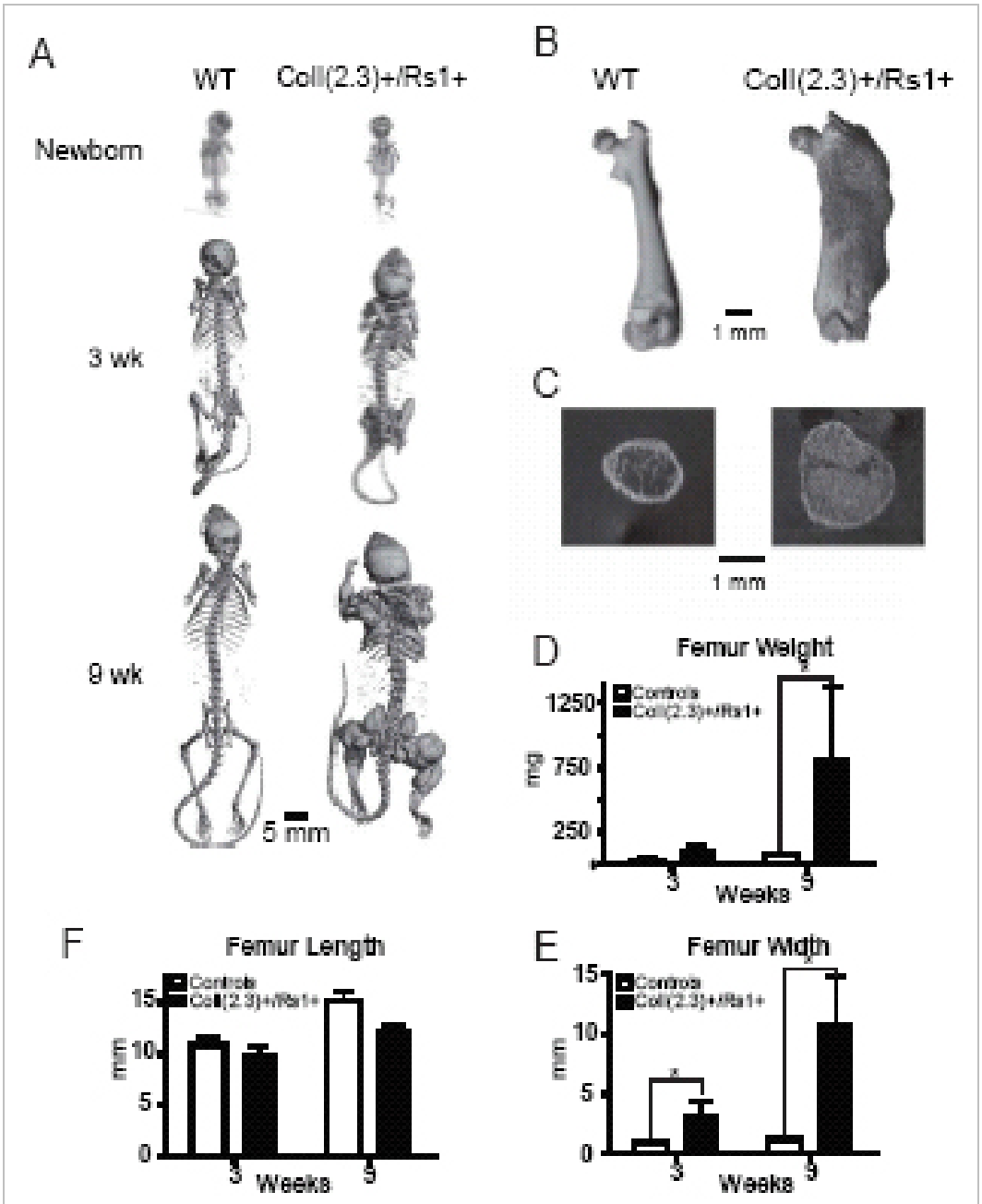


Figure 11. Skeletal effects of osteoblast expression of Rs1 by microCT. *(**A**) Whole-body CT analysis of Coll(2.3)+/Rs1+ mice and wildtype (WT) littermate controls (50-µm resolution) shows dramatically enhanced bone accumulation in double transgenic mice that progresses with age. (**B**) Femurs from WT and double transgenic mice illustrating the increase in bone width and effacement of cortical bone produced by Rs1 expression. Note that the articular surfaces of the bones appear minimally affected. (**C**) Cross-sectional CT images (10-µm resolution) of femurs from WT (left image) and Coll(2.3)+/Rs1+ (right image) mice show a predominance of trabecular bone with effacement of the cortical shell in double transgenic mice. (**D–F**) Double transgenic mice display increased femur width and weight, but not length. n=4 WT and 4 mutants at 3 weeks; 2 WT and 5 mutants at 9 weeks. Error bars represent +/- 1 SD. * p<0.05 by t-test of Rs1- expressing mice vs. wildtype controls.*

3.3.2 Rs1 shows little constitutive G_q signaling in HEK293 cells

Before making Rs1-5HT_{2C} chimeras to make a G_q signaling RASSL, we assayed for inositol phosphate 1 (IP1) accumulation. Constitutive G_q signaling was not easily observed, even in cells transfected with large amounts of cDNA (Figure 5). Upon activation by Cisapride, Zacopride, RS23597, RS39604, and RS67333, Rs1 showed much higher G_q signaling than the wildtype 5-HT_4 receptor (Figure 5).

3.3.3 A purely G_s signaling RASSL with low levels of constitutive signaling

Since the high constitutive activity of the 5-HT_4-D^{100}A mutant results in significant phenotypes and could not be controlled by inverse agonists, such as ML10375, Ro116-0086, and RO116-1148 (Joubert *et al.* 2002; Claeysen *et al.* 2003), we attempted to lower the Rs1 constitutive activity via additional point mutations. To do this, we focused on the D^{66}N and W^{272}A mutations that reduce constitutive signaling of the mouse 5-HT_4 receptor (Joubert *et al.* 2002; Rivail *et al.* 2004). Rs1-D^{66}A, Rs1-D^{66}N, and Rs1-W^{272}A (Figure 1) significantly reduced constitutive signaling (Figure 6A). The cell-surface expression of Rs1-D^{66}A and Rs1-D^{66}N was similar to that of Rs1 (Figure 6B), so the reduction in constitutive signaling was probably not linked to lower cell-surface expression. Surprisingly, the D^{66}A and D^{66}N mutations also abolished Zacopride-induced G_q signaling (Figure 6C). Thus, we created two RASSLs exhibiting pure G_s signaling and low constitutive

signaling. Unfortunately, the efficacy of the agonist-mediated G_s response was significantly compromised.

3.4 Discussion

These new RASSLs could help us better dissect the physiological significance of constitutive signaling *in vivo*. Constitutive signaling is crucial for many physiological processes and diseases. Up to 40% of all GPCRs (Seifert *et al.* 2002), including the 5-HT_4 receptor (Claeysen *et al.* 2001), show significant constitutive activity, and Rs1 could be a good model for these receptors. As reported in the mouse 5-HT_4-D^{100}A receptor (Claeysen *et al.* 2003), Rs1 had a higher level of constitutive signaling than the wildtype 5-HT_4 receptor. An Rs1 transgenic mouse has already been made using the tetracycline transactivator system, and Rs1 expression driven by the osteoblast-specific $Col1_{\alpha}$-1 2.3-kb promoter fragment dramatically increased bone formation. Bone volume, cellularity, mineral density, and serum markers of bone turnover were also elevated. No unusual phenotype developed when Rs1 was expressed after the first 4 weeks of postnatal life, indicating an exquisite temporal sensitivity of osteoblasts to Gs signaling. This pathway likely represents an important determinant of peak bone mass and may be a potential therapeutic target for enhancing bone repair and treating metabolic bone diseases.

Our data show that osteoblast-lineage cells in pre-pubertal mice have an enormous bone-forming capacity that, if harnessed, could be exploited to improve bone healing after fractures or surgical repair. Many clinical conditions affecting bone show age dependence. Several types of primary skeletal tumors, including osteosarcoma and Ewings sarcoma, have a higher prevalence in children and adolescents than in adults (23, 24). In addition, older patients (25-28) and animals (29-31) recover more slowly after certain types of fracture. Recent animal studies in young rats (32) and clinical studies in young children (33) also suggest that exercise during childhood can lead to an increase in bone mass that persists with age.

Our finding that Rs1-mediated bone accrual requires Rs1 expression during gestation and early post-natal growth suggests that osteoblasts during this time are particularly susceptible to Gs activation and may provide a cellular explanation for these age related differences in bone growth and healing. We are actively investigating whether early, limited expression of Rs1 in growing animals will also lead to a lifelong increase in bone mass. We are developing stem cell and osteoblast model systems to further investigate the roles of specific signaling pathways as downstream mediators of the anabolic actions of Rs1 in bone. This promises to reveal new physiological mechanisms for the control of bone formation as well as molecular mechanisms and potential treatments for skeletal diseases such as fibrous dysplasia (9), osteoblastomas, osteoid sarcomas (34, 35), and osteoblastic bone metastases (36). Since Rs1-expressing mice show a striking sparing of the joints, this model will also be useful for elucidating the

mechanisms regulating bone shape and patterning and may help develop targeted treatments for diseases where regulated bone sculpting or reconstruction is needed. Our findings have exciting implications for understanding the causes of and developing novel treatment strategies for fractures, abnormal bone formation, and diseases of bone loss.

These and other findings strongly suggest that constitutive signaling can drive potent phenotypic changes *in vivo*. In our studies, we found only constitutive G_s signaling in Rs1 but did not observe any constitutive G_q signaling. It will be interesting to express RASSLs with increased G_i or G_q constitutive signaling in the future. To answer if the bone phenotype observed is due to constitutive G_s signaling and to differentiate the roles of G_s and G_q signaling, we need to control the constitutive and agonist-mediated signaling. Unfortunately, even though a majority of antagonists are inverse agonists and can be used to study the effect of constitutive signaling (Rossier *et al.* 1999; Bakker *et al.* 2001; Weiner *et al.* 2001; Greasley *et al.* 2006), the $D^{100}A$ mutation in Rs1 turned inverse agonists into either agonists (GR125487) or antagonists (RO116-0086, RO116-1148) (Joubert *et al.* 2002) for Rs1. Since signaling would only be dependent on expression rather than on circulating hormones, these RASSLs have an intrinsic advantage for studying constitutive signaling when combined with a Tet system.

CHAPTER 4: A SERIES OF RASSLs ACTIVATED BY THE SAME AGONIST

4.1 Summary

To better compare results among RASSLs activating different G-protein-signaling pathways, I built a set of RASSLs based on chimeras of 5-HT_{1A} (G_i-coupled), 5-HT_{2C} (G_q-coupled), and 5-HT_4 (G_s-coupled) receptors. Since G protein coupling is dependent on multiple regions and the G-protein-coupling domains are variable among receptors, I made a series of chimeras of the 5-HT_4 $D^{100}A$ mutant with intracellular loops from 5-HT_{1A} and 5-HT_{2C} to create G_i- and G_q-coupled RASSLs.

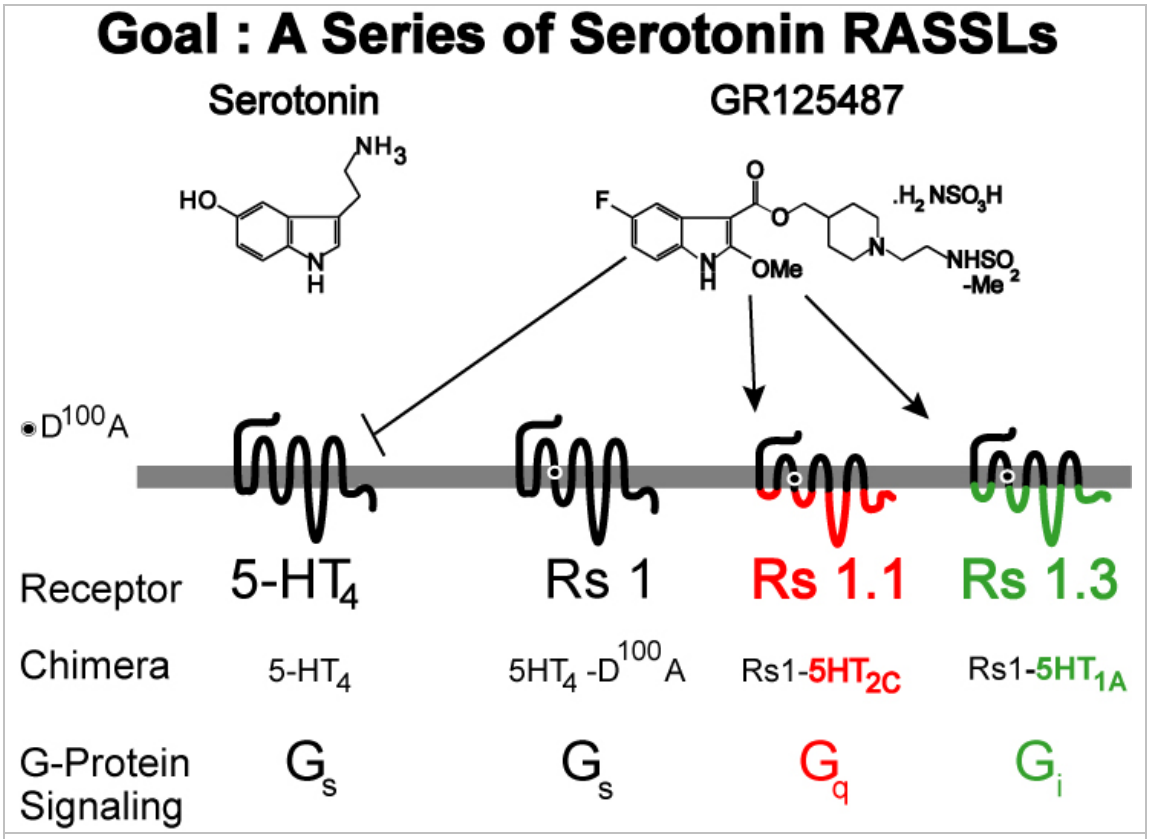


Figure 12. A series of RASSLs under the control of a single ligand.
A single point mutation at arginine-100 prevents activation by serotonin, thereby converting mouse 5HT4 receptor into a RASSL. We hypothesize that making chimeras of human 5HT4 D100A mutant with second or third intracellular loop or the C-terminus from G_i-coupled $5HT_{1a}$ receptor or G_q-coupled $5HT_{2c}$ receptor would convert it into a G_i or G_q RASSL, respectively.

4.2 Introduction

To create the series of RASSLs with different G protein signaling properties (Figure 12), I had to switch the G_s coupling domains of Rs1 with G_q and G_i coupling domain(s) of 5-HT_{2C} and 5-HT_{1A}, respectively.

In contrast to the ligand binding domains, the location(s) of G-protein-coupling domain(s) is/are much more illusive. G-protein-coupling domains often involve the second (i2) or third (i3) intracellular loops or sometimes the carboxyl terminus, but more often a combination of different intracellular loops. Wong recently conducted a comprehensive review of all the reported chimeric GPCRs (Wong 2003). Based on the successful examples, such as α_2-adrenergic receptor and β-adrenergic receptor (Kobilka *et al.* 1988) and the muscarinic M_2 and M_3 receptors (Wess *et al.* 1990) where the G protein signaling has been exchanged, he hypothesized that substitution of i3 in "closely structurally related" GPCRs from similar receptor subfamilies is sufficient to change the G protein coupling while the i2 serves as "G protein selectivity domain" (Wong 2003). However, this observation only holds for "structurally related" GPCRs. In addition, since G protein coupling is determined "conformation and charge" of multiple regions of intracellular loops instead of sequence homology (Wong 2003) and we still do not have high resolution crystallography of most GPCRs, the exact location of the G-protein-coupling domains must determined on a case-by-case basis by mutations, deletions and/or domain swapping.

4.3 Results

4.3.1 Replacing the C-terminus of Rs1 with 5-HT_{2C} increases G_q signaling

To make a purely G_q signaling RASSL from Rs1, we exchanged the intracellular loops of Rs1 with those of the G_q-coupled human 5-HT_{2C} receptor. By swapping domains at different junctions of intracellular loops, we made 12 different Rs1-5-HT_{2C} chimeras (Figure 13). To characterize them, we used RS23597 because it activated G_q signaling of Rs1 but not the wildtype 5-HT_4 receptor, as measured by calcium mobilization (Figure 13C).

4.3.2 The second and third intracellular loops are both necessary for G_s-coupling of the 5-HT_4 receptor

Replacing the second intracellular loop (i2) or third intracellular loop (i3) of Rs1 eliminated both G_s and G_q signaling (Figure 13). Only the carboxyl chimera (Rs1-C-5-HT_{2C}) showed enhanced G_q signaling in response to Cisapride, Zacopride, RS23597, RS39604, and RS67333 (Figure 13C), showing that the agonist-mediated specificity of signaling was preserved. Constitutive and agonist-mediated G_s signaling were also largely preserved (Figure 13).

These data indicated that i2 and i3 are both necessary for G_s-coupling of the 5-HT_4 receptor. They also suggested that the C terminus of 5-HT_{2C} contains a G-protein coupling domain for G_q signaling or the C terminus of 5-HT_4 receptor

promotes G_q signaling. However, we were unable to completely alter the G-protein preference of this receptor for G_q signaling. We amplified the G_q signaling of Rs1 by domain swapping the C terminus with 5-HT_{2C}. However, none of the other 12 chimeras showed enhanced G_q signaling, even when multiple internal segments were combined. We did not proceed further with these experiments, since substitutions of multiple internal domains also decreased the cell-surface expression of the receptors (data not shown). Since G_q signaling of Rs1-C-5-HT_{2C} was activated by Cisapride, Zacopride, and RS23597, but not serotonin, we named it Rs1.1 (Table 1).

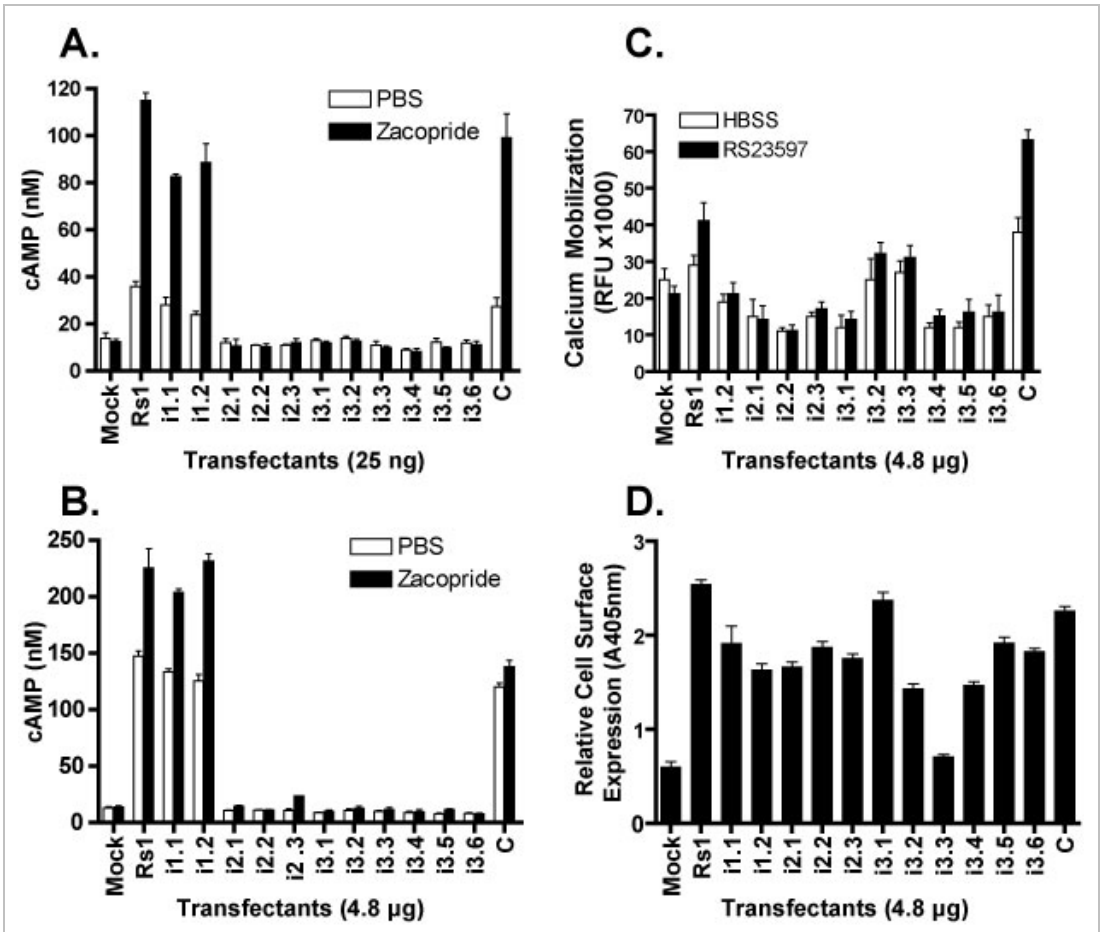


Figure 13. The second and third intracellular loops (i2 and i3) of Rs1 are crucial for G_s and G_q signaling

(A, B) *Rs1-5-HT_{2C} chimeras with swaps of the second and third intracellular loops could no longer process G_s signals, at either 25 ng or 4.8 µg of receptor cDNA per 5 x 10^6 HEK293 cells.*

(C) *The G_q signaling of Rs1 was abolished when the second and third intracellular loops of Rs1 were replaced with those of 5-HT_{2C}. The G_q signaling was measured by calcium mobilization assay.*

(D) *Only chimeras with a single domain swap were expressed on the cell surface. The results represent three independent experiments. All figures were representative of three independent experiments.*

4.3.3 The third intracellular loop of 5-HT_{1A} is sufficient for G_i signaling

We next attempted to make a G_i-signaling RASSL based on Rs1. To engineer G_i signaling into Rs1, we replaced its intracellular loops with those of 5-HT_{1A}, a G_i-signaling receptor (Liu *et al.* 1999). Of four Rs1-5HT_{1A} chimeras (Figure 14), only the two containing i2 and i3 from Rs1 were expressed at a level similar to Rs1 (Figure 14D). Replacing those loops abolished constitutive and agonist-mediated G_s signaling at both low and high levels of receptor cDNA (25 ng and 4.8 µg per $5x10^6$ cells) (Figure 14A, 14B).

Interestingly, no evidence of constitutive signaling via the G_i or G_s pathway was seen in this new RASSL. These findings strongly imply that both i2 and i3 are required for G_s signaling of Rs1. In addition, activation of the Rs1-i3-5-HT_{1A} chimera with Zacopride significantly inhibited cAMP accumulation induced by 10 µM Apomorphine (agonist for dopamine 1 receptors) in HEK293 cells co-transfected with 1.5 µg of Rs1 receptor and 0.5 µg of dopamine 1 receptor (per $5x10^6$ cells; Figures 14C and 15A). This inhibition was smaller than that of µ-opioid receptor stimulated by [D-Ala2, D-Leu5]-enkephalin (DADLE). Both inhibitions were abolished by 50 nM pertussis toxin, suggesting the involvement of G_i signaling (Figure 15B). Unfortunately, the potency (amount of drug needed to reach an effect) of agonist-mediated G_i signaling was significantly reduced (Figure 15C). Since Rs1-i3-5HT_{1A} exhibited G_i but not G_s signaling, we named it Rs1.3 (Figure 16, Table I).

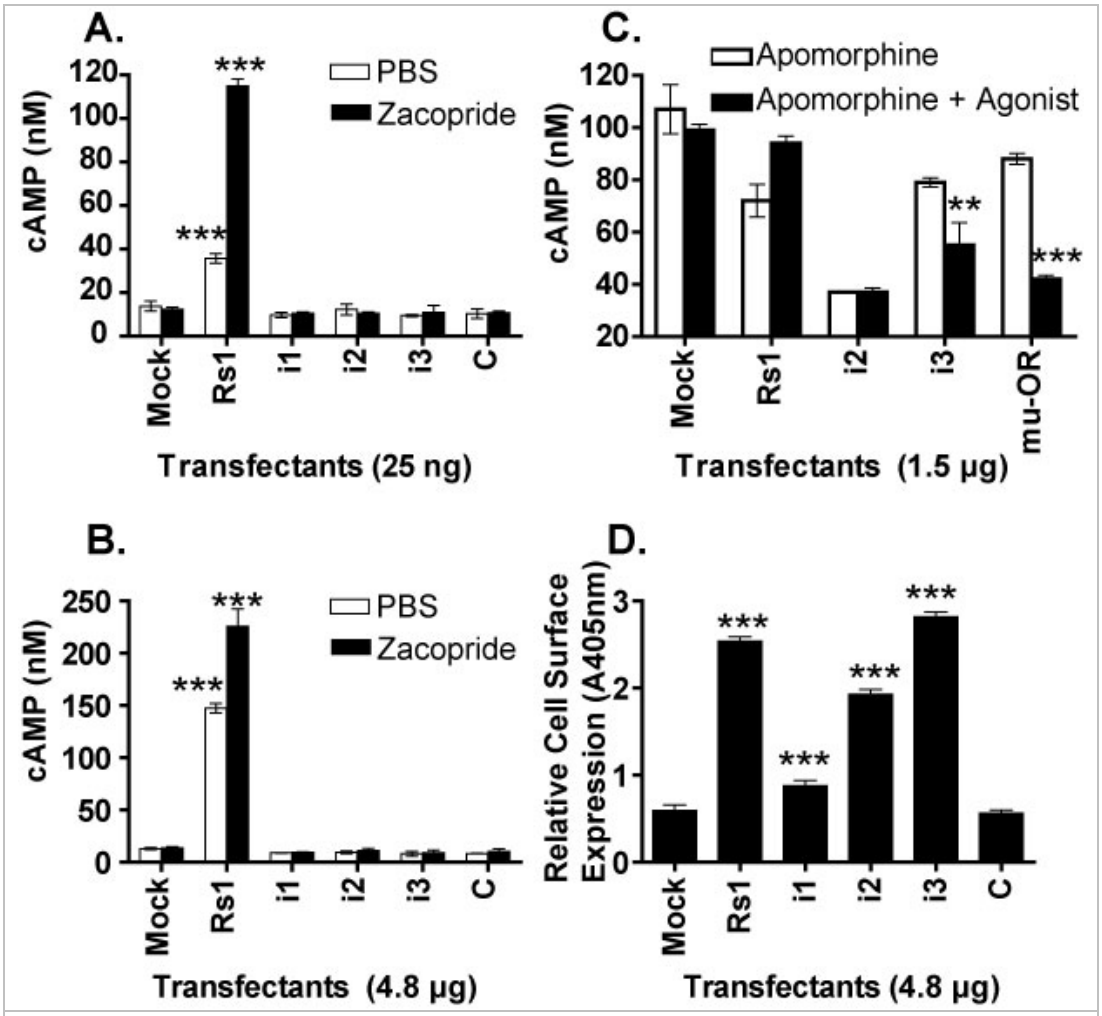


Figure 14. Replacing the second or third intracellular loop (i2 or i3) of Rs1 with 5-HT$_{1A}$ alters G protein signaling.

(A, B) *Rs1-5-HT$_{1A}$ chimeras with swaps of the second (i2) and third (i3) intracellular loops no longer signal via the G$_s$ pathway, regardless of whether cells were transfected with 25 ng or 4.8 µg of receptor cDNA per 5x10^6 HEK293 cells. This suggests that the second and third intracellular loops are crucial for acute G$_s$ signaling of Rs1.*

(C) *Replacing the third intracellular loop of Rs1 resulted in G$_i$ signaling receptor. All HEK293 transfectants were electroporated with 0.6 µg of the human dopamine 1 receptor and 1.5 µg of Rs1, Rs1-5HT$_{1A}$ chimeras, or the mu-opioid receptor. Rs1 and Rs1-5HT1a chimeras were treated with 10 µM Apomorphine (an agonist for dopamine 1 receptor) or with10 µM Apomorphine and 10 µM Zacopride. Transfectants with 0.6 µg of the human dopamine 1 receptor and 1.5 µg of the mu-opioid receptor served as positive controls. 10 µM DADLE was used in place of Zacopride to stimulate mu-opioid receptors. ** p<0.05*

(D) *Rs1, i1, i2 and i3 were expressed on the cell surface. ***p<0.001 vs. Rs1 (Student's t tests). All the cell-surface expression and calcium mobilization of the chimeras were examined at 4.8 µg per 5x10^6 HEK293. The results are representative of three independent experiments. Values are mean ± standard deviation.*

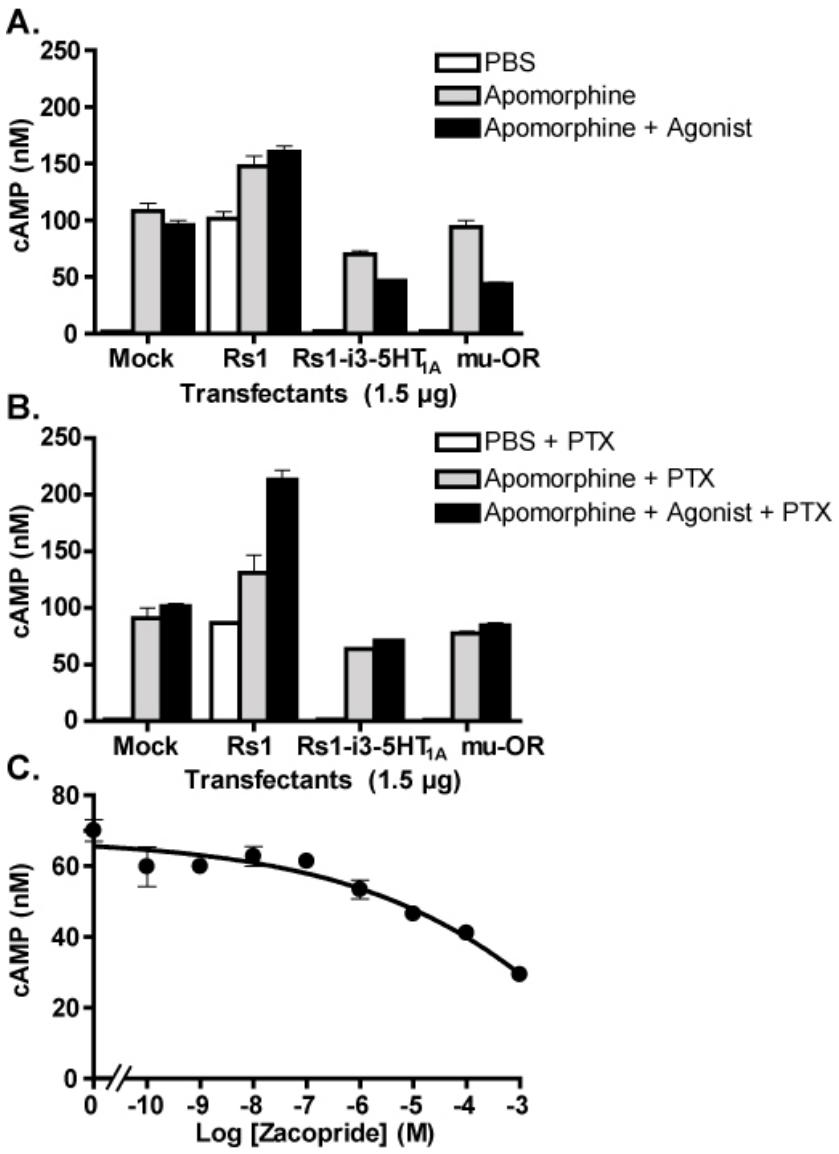


Figure 15. Replacing i3 of Rs1 with that of 5-HT$_{1A}$ results in a receptor, Rs1-i3-5-HT$_{1A}$, with weak G$_i$ signaling.

(A) *Rs1-i3-5-HT$_{1A}$ chimera decreased cAMP accumulation and also showed little constitutive G$_s$ signaling, in contrast to Rs1. All HEK293 transfectants were electroporated with 0.6 µg of human dopamine 1 receptor and 1.5 µg of Rs1, Rs1-i3-5-HT$_{1A}$ chimera, or human mu-opioid receptor. The transfectants were stimulated with 10 µM Apomorphine (agonist for the dopamine 1 receptor) to increase basal cAMP level and to observe G$_i$ signaling. Rs1 and Rs1-i3-5-HT$_{1A}$ were then stimulated with 10 µM Zacopride. The mu-opioid receptor was stimulated with 10 µM DADLE.*

(B) *Treatment with pertussis toxin (PTX) abolished the decreased cAMP accumulation of Rs1-i3-5HT$_{1A}$ and mu-opioid receptor, indicating that the decreased cAMP accumulation seen in panel (A) was due to G$_i$ signaling. The results are representative of three independent experiments. Values are mean ± standard deviation.*

(C) *Rs1-i3-5HT$_{1A}$ required a large amount of Zacopride for maximal G$_i$ response. The data are representative of two independent experiments.*

4.4 Discussion

We report here a new series of RASSLs (Figure 16) to study multiple G-protein-signaling pathways. Many GPCRs activate multiple G-protein-signaling pathways and exhibit a wide range of constitutive signaling activities. Our new RASSLs could help us better study the effect of stimulating canonical signaling pathways (G_s/G_q, G_s, G_i), with different constitutive signaling using a single receptor

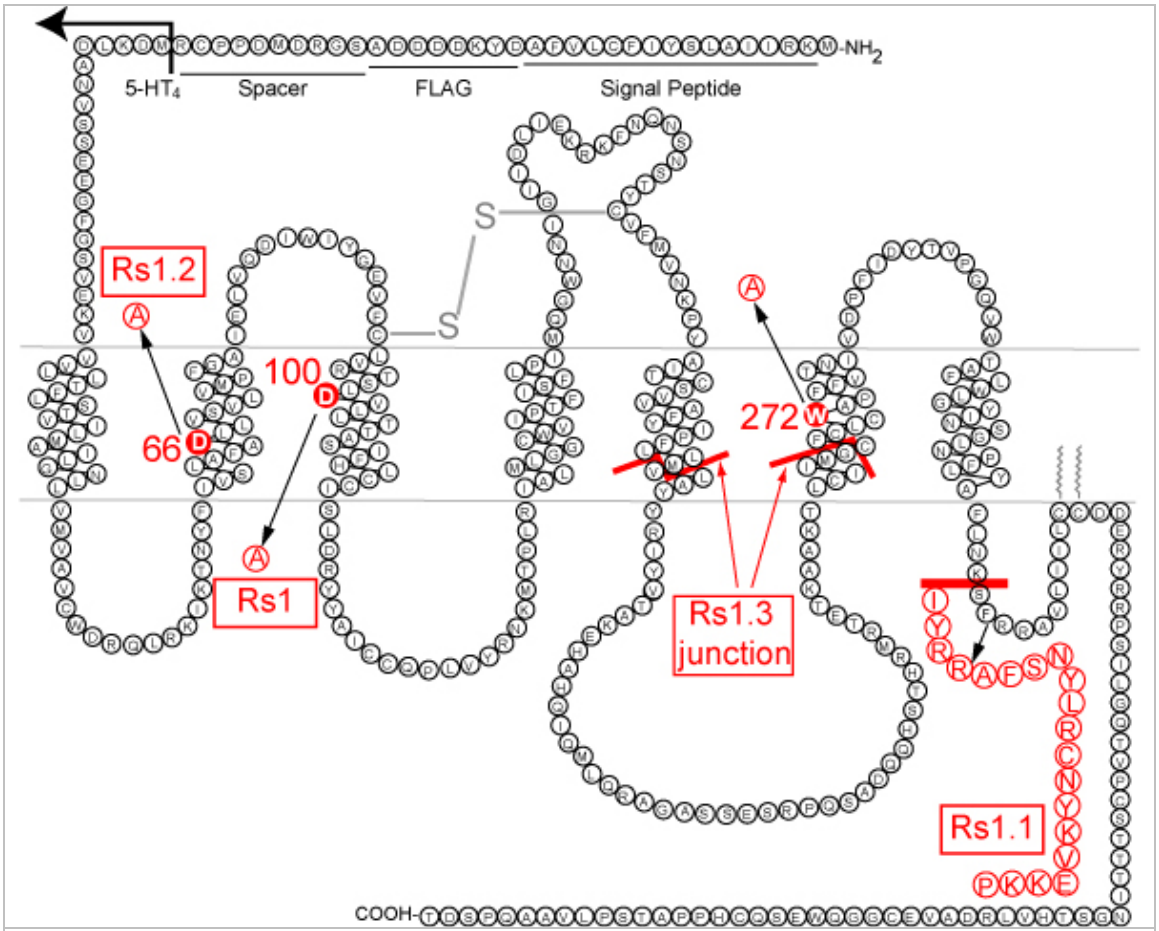


Figure 16. Two-dimensional serpentine structure of the human 5-HT$_4$-based RASSLs. *A signal peptide and the FLAG epitope were added to the N-terminus of the human 5-HT$_4$ receptor. The D^{100}A mutation was introduced by site-directed mutagenesis to create the G$_s$-coupled RASSL (Rs1). An additional point mutation (D^{66}A, D^{66}N, or W^{272}A) was added to Rs1 to modulate constitutive signaling. Rs1.1 is the Rs1-C-5-HT$_{2C}$ chimera with enhanced G$_q$ signaling. Rs1.2 is Rs1 with an extra D^{66}A mutation that decreased constitutive G$_s$ signaling. Rs1.3 is the Rs1-i3-5-HT$_{1A}$ chimera with G$_i$ signaling.*

backbone and a single collection of synthetic agonists.

Of the 12 Rs1-5-HT_{2C} chimeras that are expressed on the cell surface, none of the i2 or i3 chimeras showed any G_s or G_q signaling. Evidently, these intracellular loops of Rs1 are crucial for signaling via those pathways. The importance of i2 and i3 for G_s signaling of Rs1 is further supported by the lack of G_s signaling in the Rs1-i2-5-HT_{1A} and Rs1-i3-5-HT_{1A} chimeras. This is the first study showing the importance of i2 and i3 in both G_s and G_q signaling of the human 5-HT_4-D^{100}A receptor.

We also found that i3 domain swapping abolished all G_s signaling and enabled Rs1 to stimulate G_i signaling of 5-HT_{1A}. The role of i2 and i3 in the G_i

RASSL	Description	Constitutive signaling G_s	G- protein signaling G_i	G_s	G_q
Rs1	**5-HT_4-D^{100}A**	**+++**	**–**	**+++**	**+**
Rs1.1	**Rs1-C-5-HT_{2C}**	**+++**	**N/A**	**+++**	**++**
Rs1.2	**Rs1-D^{66}A**	**+**	**N/A**	**+**	**–**
Rs1.3	**Rs1-i3-5-HT_{1A}**	**–**	**+**	**–**	**N/A**

Table 1: A series of RASSLs based on the human 5-HT_4 receptor.
Constitutive signaling and ligand-induced signaling of Rs1 were successfully controlled by point mutations, drug choice, and domain swapping. G_q signaling of Rs1 could be activated by Zacopride or RS23597 but not GR113808, GR125487 or RO110-0235. The signaling was significantly increased by switching the carboxyl tail (Rs1.1). Attempts to decrease constitutive activity also decreased ligand-induced G_s signaling and abolished G_q signaling (Rs1.2). Replacing the third intracellular loop of Rs1 with that of 5-HT_{1A} resulted in a G_i-coupled RASSL with no $G_{s/q}$ signaling and G_i signaling (Rs1.3).

signaling of 5-HT_{1A} receptor has been extensively reported. The entire N terminus (IALDRYWAITD) (Thiagaraj *et al.* 2007) and C terminus of i2 (DYVNKRTPRR) (Kushwaha *et al.* 2006) of 5-HT_{1A} are thought to be sufficient to support G-protein coupling, but not signaling. On the other hand, the N terminus (TC Ortiz *et al.* 2000) and C terminus of i3 (IFRAARFRIRKTVKK) of 5-HT_{1A} (Hayataka *et al.* 1998; Malmberg *et al.* 2000) seem to be essential for the G_i signaling of 5-HT_{1A}. In fact, replacing the N terminus of the i3 of the α_2-adrenergic receptor with that of 5-HT_{1A} resulted in a chimera that signals like a 5-HT_{1A} receptor when stimulated by a α_2-adrenergic receptor agonist (Eason *et al.* 1995). Since Rs1-5HT_{1A} chimeras with multiple internal domains replaced are not significantly expressed on the cell surface (data not shown), it may be difficult to further improve of the potency of the Rs1.3 using our current approach. We hypothesize that replacing the N- and C-terminal portions of i2 and i3 instead of the whole i2 and i3 loops may increase the potency of Rs1.

This new series of RASSLs based on 5-HT_4 receptor might make it possible to perform *in vivo* studies in which Rs1 is activated with minimal side effects. Since knockout of the 5-HT_4 receptor showed only “decreased reactivity to novelty” and “increased sensitivity to PTZ induced seizure” without causing overt side effects (Compan *et al.* 2004), treatment with GR113808, GR125487, and R0110-0235 (antagonists and inverse agonists for the wildtype receptor) may have minimal side effects as well. This eliminates the need to laboriously knock out the endogenous 5-HT_4 receptors for most *in vivo* experiments (Sweger *et al.* 2007). Since the same agonists can be used to activate all of the RASSLs

within this series and thereby engage different G-protein-signaling pathways (Table 1), we can more easily compare the effect of activating the G_s, $G_{s/q}$, or G_i pathway.

Recently, Brian Roth and colleagues made a series of RASSLs based on the G_q-coupled muscarinic M_3 receptor with low constitutive activity. They used a well-established yeast mutagenesis system to produce thousands of mutant hM_3 muscarinic receptors and screened them for signaling characteristics of an ideal RASSL. After multiple rounds of mutagenesis and repeated screening, they isolated mutants that had lost the ability to respond to the natural ligand (acetylcholine) but gained the ability to respond to the inert compound, clozapine-N-oxide (CNO), an inert ligand with high bioavailability (Armbruster *et al.* 2007). By making analogous mutations in the M_2, they developed a G_i-coupled RASSL that can be activated by CNO (Armbruster *et al.* 2007).

More recently, Jurgen Wess and colleagues followed an earlier report (Wong *et al.* 1990) and swapped the second and third intracellular loops of the hM_3-based RASSL with those of rat G_s-coupled β_2-adrenergic receptor (personal communication). This new RASSL no longer couples to G_q, but it strongly activates G_s signaling in response to CNO. Thus, CNO can activate the G_s-, G_i-, or G_q-signaling pathways, depending on which RASSL is expressed.

These RASSLs nicely complement our Rs1 RASSLs with varying constitutive activity. We predict that some RASSLs with the same canonical G-protein signaling (G_s, G_i, or G_q) will have different *in vivo* phenotypes due to non-

canonical signaling. This growing collection of RASSLs will greatly facilitate our efforts to understand the physiological significance of the inherent signaling diversity of GPCRs.

CHAPTER 5: FUNCTIONAL SELECTIVITY

5.1. Summary

We found that all all indoleamine-based ligands activate G_s- but not G_q-signaling of Rs1 (Figure 18). In contrast, all benzamide-based ligands activate both G_s- and G_q-signaling of Rs1 (Figure 19). The activation of G_q-signaling activity by benzamide-based ligands is further enhanced when the C terminus of Rs1 is replaced with that of 5-HT_{2C}.

5.2. Introduction

Since its first observation by Portoghese in opiates (Portoghese 1965), functional selectivity is activation of a receptor to different responses by different drugs. The effect has since been known by many different terms, including agonist-directed signaling, relative activity, biased agonism, stimulus-trafficking, conformational selection, and differential selection, to explain all the different transduction events differentially activated by different agonists. (Simmons 2005).

Based on the selective activation of the multiple activation states of GPCRs by ligands (Azzi *et al.* 2003; Gbahou *et al.* 2003), functional selectivity affects various aspects of signaling transduction in many receptors. It led to pharmacological effects, such as divergent fates of internalization for the dopamine D_1 receptor (Ryman-Rasmussen *et al.* 2007) and enhanced internalization of μ-opioid receptor by DAMGO, more effectively than morphine

(Whistler *et al.* 1999). It led to differential binding and signaling, such as various binding specificity for gonadotropin-releasing hormone receptors, and, mu-opioid (Saidak *et al.* 2006), dopamine D_2 (Gay *et al.* 2004), and human 5-HT_{2A} (Urban *et al.* 2007) receptors. It led to biochemical effects, such as different receptor phosphorylation of different G-proteins for the β_2-adrenergic (Ghanouni *et al.* 2001; Trester-Zedlitz *et al.* 2005). There is even genetic evidence in the constitutive active mutants of complement factor 5a receptor supporting functional selectivity (Whistler *et al.* 2002).

5.3. Results

5.3.1 Rs1 demonstrates ligand-specific G_s- and $G_{s/q}$-signaling

We showed that various drugs could differentially activate G-protein signaling of Rs1. We found that GR113808, GR125487, and RO110-0235 (all indoleamine derivatives) did not fully activate G_q-signaling of Rs1, Rs1.1, or the 5-HT_4 receptor. On the other hand, the benzamide derivatives (Cisapride, Zacopride, RS23597, RS39604, and RS67333) activated the G_q signaling of Rs1. These findings may reflect distinct conformational changes caused by indoleamine and benzamide derivatives.

5.3.2 Rs1-C-5HT$_{2C}$ demonstrate a more potent ligand-specific G$_q$-signaling

Surprisingly, GR113808, GR125487, and RO110-0235 activated only G$_s$- (Figure 2) and not G$_q$-signaling (Figure 17) by Rs1. These are the same ligands used to selectively activate the G$_s$-signaling of Rs1 without activating the G$_s$-signaling of the wildtype 5-HT$_4$ receptor (Figure 7). We therefore could use specific drugs to activate either the G$_s$- or G$_s$/G$_q$-signaling of Rs1. The once controversial use of conformation-specific ligands to alter G-protein coupling and other receptor functions has now been demonstrated in several other GPCRs (Ghanouni *et al.* 2001; Gay *et al.* 2004; Saidak *et al.* 2006; Ryman-Rasmussen *et al.* 2007; Urban *et al.* 2007). This is the first time that agonist-dependent functional selectivity has been shown in a RASSL.

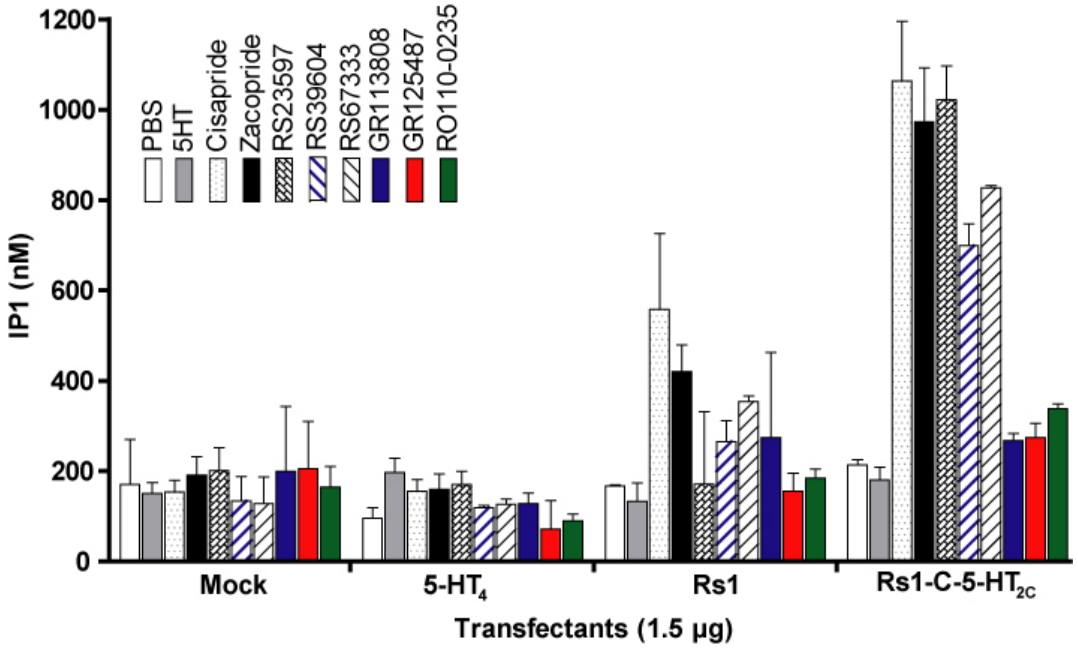


Figure 17. The wildtype 5-HT$_4$ receptor, Rs1, and the Rs1-C-5-HT$_{2C}$ chimera exhibit different G$_q$-signaling properties.

G$_q$-signaling was analyzed by measuring the accumulation of inositol 1 phosphate (IP1). 5-HT$_4$ receptor showed increased G$_q$-signaling when activated by serotonin, Cisapride, and Zacopride but not by RS39604 or RS67333. Rs1 showed significantly higher G$_q$-signaling than the wildtype 5-HT$_4$ receptor when activated by Cisapride, Zacopride, RS39604, and RS67333 but not serotonin. G$_q$ signaling of Rs1-C-5HT$_{2C}$ chimera was activated by Cisapride, Zacopride, RS23597, RS39604, and RS67333 and was minimally activated by RO110-0235. GR113808 and GR125487 did not activate G$_q$-signaling by any of the three receptors. Values are mean ± standard deviation of three experiments.

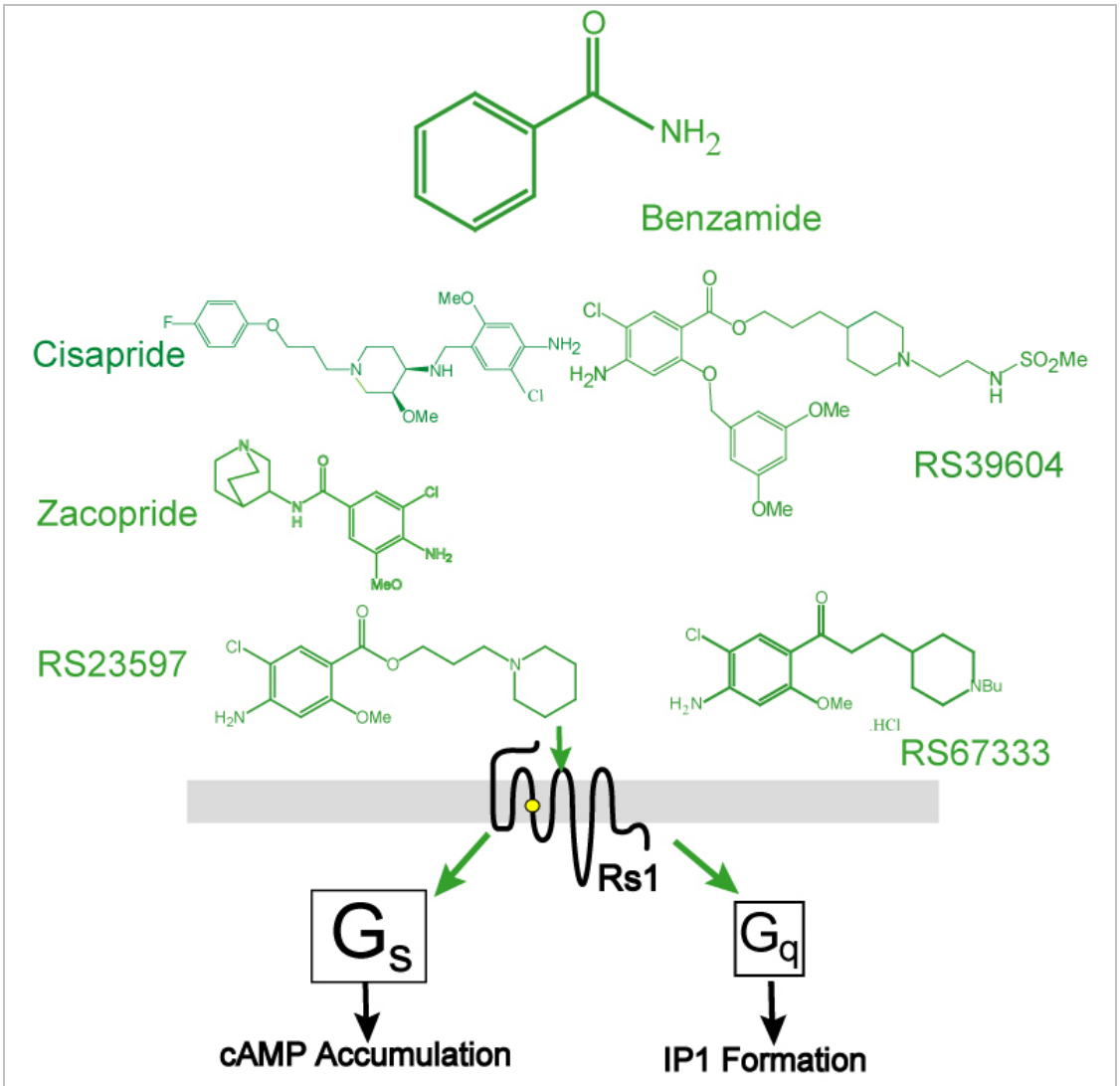


Figure 18. Benzamide analogous compounds activate both G_s- and G_i-signaling of Rs1 and Rs1-C-5HT$_{2C}$.
Benzamide analogous compounds activate both the 5-HT4 receptor and Rs1 and also both G_s- and G_q-signaling of Rs1.

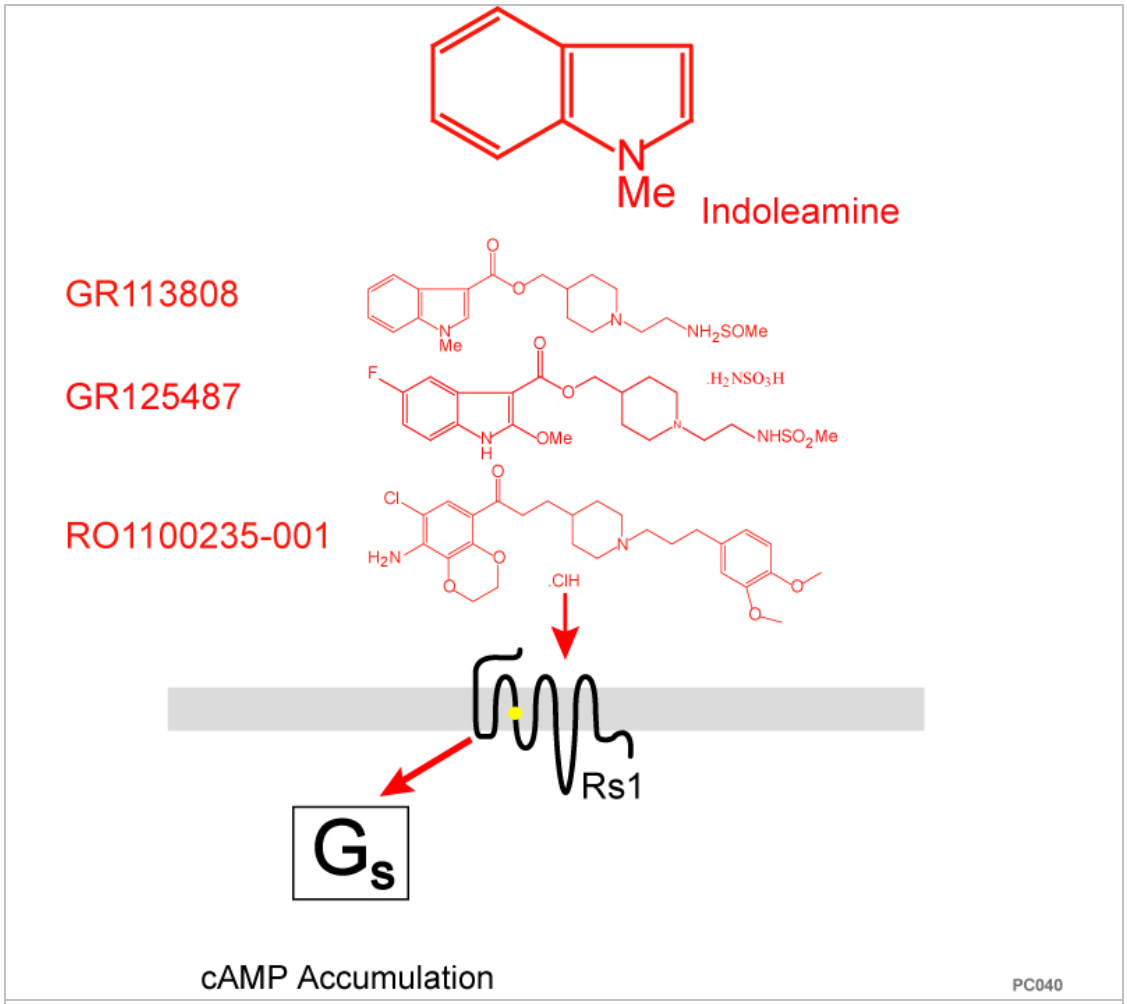


Figure 19. Indoleamine analogous compounds activate only the G_s signaling of Rs1. *Benzamide analogous compounds activate both the 5-HT4 receptor and Rs1. In addition, they activate both G_s and G_q signaling of Rs1.*

5.4 Discussion

In addition to the creation of a new set of RASSLs that can be activated by common agonists, our study uncovered insights into the G-protein selectivity and functional selectivity (differential effects of ligands on the same receptor) of Rs1.

The possibility of functional selectivity is further supported by the results obtained with Rs1-C-5HT$_{2C}$ and Rs1 point mutants (Rs1-D^{66}A and Rs1-D^{66}N). The D^{100}A mutation and replacement of the C terminus amplified the G_q signaling of the 5-HT$_4$ receptor. The addition of D^{66}A and D^{66}N abolished G_q signaling. Since D^{100}A is located in the binding pocket of the 5-HT$_4$ receptor, this mutation in Rs1 may have changed the configuration of the binding pocket, making the receptor more susceptible to G_q activation by Zacopride and RS23597. This response was even more pronounced when the D^{100}A mutation was combined with domain swapping of the C terminus with that of 5-HT$_{2C}$. Thus, it is reasonable to hypothesize that these changes modified the ligand-selective receptor conformation (Kenakin 2003), changing the receptor susceptibility to functional selectivity.

While we still need to examine G_q signaling in other cell lines and tissues, our finding has interesting implications. While the clinical implication of functional selectivity is still unknown, it may be possible to make functionally selective agonists and antagonists (Roth *et al.* 1987). A closer examination of the chemical structures of these and other ligands targeting 5-HT$_4$ receptor may lead to safer and more efficacious agonists. A better understanding of the 5-HT$_4$ receptor resulting from this study such as the functional specificity due to different

functional groups may be able to develop or find better drugs. Many drugs targeting 5-HT_4 receptor are clinically available or in clinical trials, such as Mosapride, Tegaserod, and TD-5108 for treatment of functional gastrointestinal disorders (functional dyspepsia and irritable bowel syndrome) (Mizuta *et al.* 2006; Baun *et al.* 2007) or PRX-03140 for treatment of Alzheimer's disease.

CHAPTER 6: MODULATING RASSL EXPRESSION LEVEL BY INTERNAL RIBOSOMAL ENTRY SITES (IRES)

6.1 SUMMARY

To have a series of IRESes with different translational efficiency so that basal activity of RASSLs may be better controlled, a collection of IRES based on three 9-bp IRES modules (GTX1 (Chappell *et al.* 2000), ICS1, and ICS2 (Owens *et al.* 2001)) were made. The translational initiation of 2nd cistron was compared in cell lines from different origins. All three IRES modules show a more efficient internal translational efficiency. In most cell lines, GTX1 modules give the best expression of second cistron and ICS2 modules give the worst. In addition, we observed that the more efficacious IRES modules lowered the expression of the first cistron.

6.2 INTRODUCTION

Expressing multiple genes in a plasmid is often crucial when expressing recombinant proteins. Using multiple promoters or making fusion proteins is a common method to express multiple genes in a plasmid. However, the use of multiple promoters to drive different genes sometimes leads to promoter attenuation, and fusion proteins are sometimes misfolded or mis-targeted (Wong *et al.* 2002). IRES allow multicistronic expression from the same promoter without these problems.

First discovered in poliovirus RNA (Pelletier *et al.* 1988) and encephalomyocarditis virus (EMCV) RNA (Jang *et al.* 1988), IRES is an unique sequence of mRNA that attracts the 40S ribosomal subunit. This allows cap-independent, internal initiation of translation of gene downstream, and thus bicistronic expression from the same promoter.

Even though the first discoveries are viral in origin, IRESs have also been discovered in eukaryotic mRNA. While not all reported IRESs necessarily enable internal translational initiations (Kozak 2005), the list of viral and eukaryotic IRESs has increased exponentially. As of June 2007, there are at least 62 viral and 90 eukaryotic mRNAs with IRES (Mokrejs *et al.* 2006).

Even though the IRES-dependent translation of the second gene is 20–50% that of the first gene in a bicistronic plasmid, it is a remarkable improvement from the 0.1–0.5% without IRES (Mizuguchi *et al.* 2000). Thus, multiple IRESes have since been used for bicistronic expression of transgene in plasmids. Of all the IRESes discovered so far, EMCV IRES is one of the best studied and most popular of the IRES. It is frequently used for bicistronic expression or even tri-cistronic expression (Zhu *et al.* 1999) from the same promoter in a plasmid. In addition, EMCV IRES sequences in gene traps have been used to drive a variety of marker genes, such as GFP and placental alkaline phosphatase, resulting in expression patterns that are identical to those of the endogenous genes (Mitchell *et al.* 2001).

The optimal internal initiation of translation is limited by several constraints (Borman *et al.* 1997; Michael *et al.* 1999). EMCV IRES requires the transgene to be driven from the 11th ATG in the IRES. In addition, it has multiple cryptic splice donor and acceptor sites, which could interfere with the trapped genes. Removal of these splices sites, however, diminishes IRES efficiency because the natural sequences around 11th ATG have been altered and the transgene can no longer be transcribed from the 11th ATG. Thus, a large section of the ECMV IRES (from 273 to 845) is required for optimal translational initiation (Bochkov *et al.* 2006), greatly limiting the cloning capacity of vectors, such as rAAV and retrovirus vectors (de Felipe *et al.* 1999), and their viral titer (Klump *et al.* 2001). In addition, ECMV IRES has different translational efficiencies in cultured cells of different origin (Borman *et al.* 1997). This could complicate the experiment when the RASSL is expressed in different tissues *in vivo*. Third, to study basal signaling, we would need to make a series of IRES with different translational efficiencies to express different level of receptors (Figure 20A). IRES without these limitations would be useful for the proposed project.

There are other limitations with other IRES. For instance, the cellular IRES are more “complex” (Martinez-Salas 1999; Beales *et al.* 2003), show a more limited tissue tropism (Shaw-Jackson *et al.* 1999; Hennecke *et al.* 2001), and are usually larger in size (Houdebine *et al.* 1999), thus limiting their usefulness for multicistronic expression (Fux *et al.* 2004).

To expand the range of translational efficiency of IRES, I made a series of IRES sequences with graded levels of efficiency based on newly described IRES modules, GTX (CCGGCGGGT) (Chappell *et al.* 2000; Owens *et al.* 2001), ICS1 (CAGCGGAAACGAGCG) (Owens *et al.* 2001), and ICS2 (TCCGGTCGT) (Owens *et al.* 2001) IRES modules are 9-bp oligonucleotides (Figure 5a), complementary to 18S ribosomal RNA. GTX is 100% complementary to 18S rRNA at 1132–1124. ICS1 and ICS-2 are complementary to 18S rRNA at 1311–1324 and 68–76, respectively.

Even though the mechanism is still unclear, it is thought that GTX base pairs to 18S ribosomal RNA to increase the concentration of ribosomal subunits around the mRNA (Chappell *et al.* 2004). This increase in turn enhances the translational efficiency. This enhanced translational activity was reported to be increased with increasing number of tandem repeats (Chappell *et al.* 2000). In contrast to picornaviral IRES, the IRES modules lack cryptic splice sites and are mammalian in origin and, thus, less likely to cause immunological responses. Furthermore, they require only that the second transgene be within 75 bp of the 3' end of IRES for optimal activity (Mauro VP, personal communication). I then examined the translational efficiency of the IRES modules in cell lines of different origin.

6.3 RESULTS

Reasoning that more efficacious IRES would yield more GFP, I compared the ratios of GFP to DsRed2N1 of GFP and DsRed2N1 double positive transfectants. I examined HEK293 (human embryonic kidney), HeLa (human epithelioid carcinoma), N2a (mouse neuronal and amoeboid stem cells), 3T3L1 (mouse fibroblast), C6 (rat glial tumor cell line), and NRK (rat epithelial kidney) cell lines.

6.3.1 Comparing the translational efficency and tissue tropism of three IRES modules in different cell lines

As expected, increasing the number of copies of IRES modules resulted in higher ratios of fluorescent means (Figure 20C). Even though IRES modules have not been tested in EBs or endothelial cells, its translational efficiency was consistently higher than that of EMCV in all the cell lines tested (Figure 20D), an important proof of concept.

The three different IRES modules (GTX, ICS1 and ICS2) also showed tissue tropism. GTX always showed a greater GFP:DsRed ratio than ICS2. GTX showed a greater response in ICS1 in cell lines of human origin (HEK293, HeLa). The translational efficacy of GTX IRES modules is more variable in cell lines of mouse and rat origin. It showed the same response in N2a (mouse) and C6 (rat) but a weaker response in NRK (rat) and 3T3L1 (mouse). The comparative response of other IRES modules (ICS1, ICS2) is even more varied in the cell

lines of rodent origin; it showed a similar response in rodent neuronal cell lines (C6, N2a) but a weaker response in rodent epithelial/ fibroblast cell lines (NRK, 3T3L1). While the number of cell lines examined was limited and more cell lines from different origins should compared, the choice of IRES modules depends on

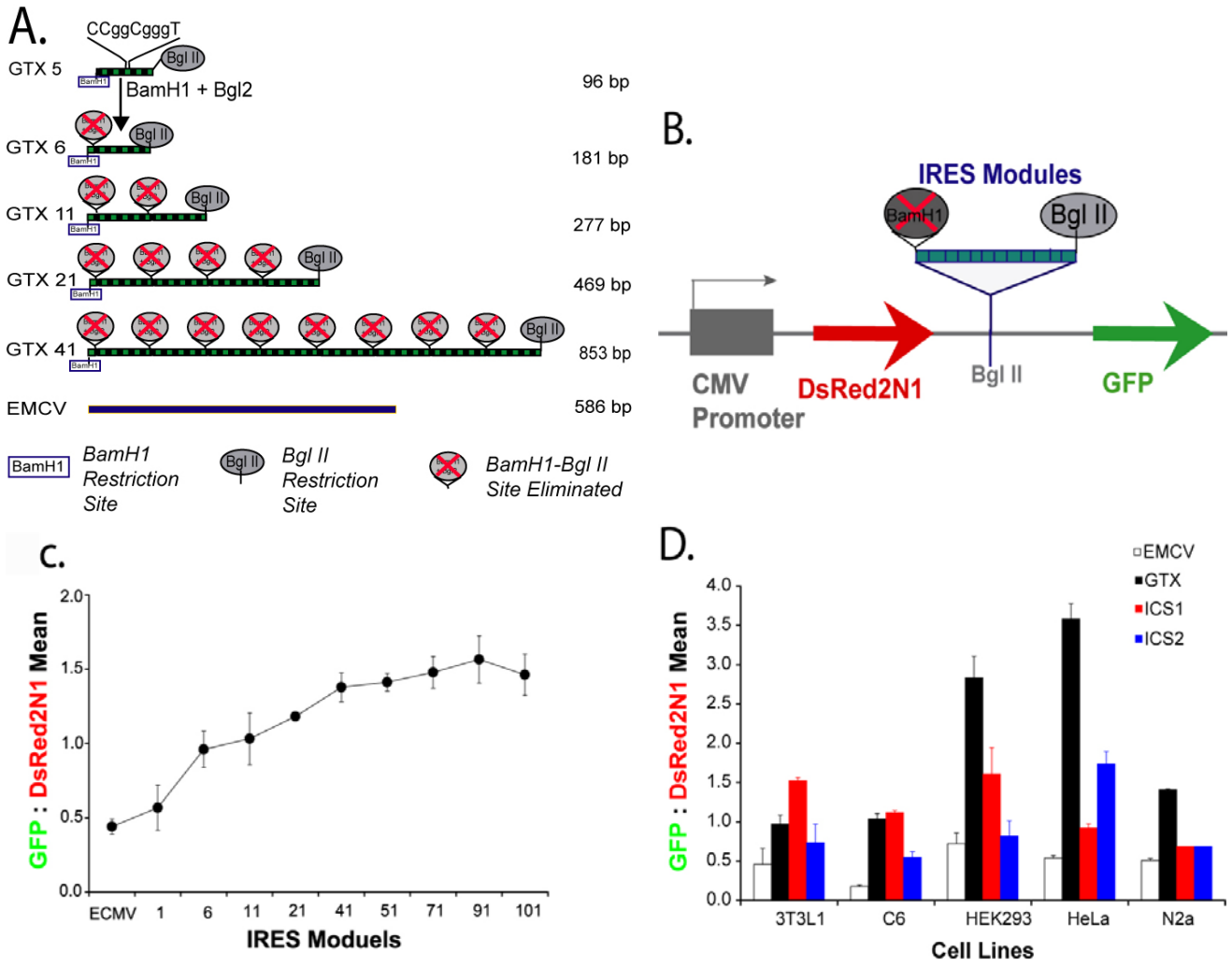


Figure 20. Enhanced IRES activity with increased number of tandem copies. (a) *Comparison of GTX IRES modules to EMCV. Black boxes represent spacers (ttctgacat) and green boxes, IRES modules. GTX IRES = CCGGCGGGT. Even though EMCV is about the same size as GTX 26, it is not modular, and there is no evidence that multiple copies of EMCV enhanced translational efficiency.* ***(b)*** *The completed GTX IRES modules are cloned into a reporter plasmid between Ds Red2 N1 fluorescent protein and GFP. IRES activity is analyzed as a ratio of the median fluorescence of GFP to that of DsRed2 N1.* ***(c)*** *Translational efficiency of GTX increase with the number of tandem repeats. Similar results were seen for ICS1 (CAGCGGAAACGAGCG) and ICS2 (TCCGGTCGT), two known IRES modules.* ***(d)*** *Translational efficiency of 41-repeat IRES modules. Even though translational efficiency varied across different cell lines, the IRES modules were consistently more efficient. Similar results were seen with different tandem copies of IRES.*

the organism, and the tissue. This differential translational efficiency has been reported in other IRES such as hepatitis C IRES (Laporte *et al.* 2003).

6.3.2 Increasing the number of IRES modules enhances the translation of the second cistron at the expense of the first cistron

We notice that increasing the number of GTX IRES modules increases the relative translational efficiency of the second cistron to the first cistron by enhancing the translation of the second cistron (GFP). This increased ratio, however, is due to decreased translational efficiency of the first cistron increased translational efficiency of the second cistron. This is affected by tissue tropism. While this trend is repeatedly observed in HEK293, 3T3L1, and C6 cell lines, the magnitude of the decreased translation of DsRed is affected by tissue tropism. There is no any additional increased translational efficiency of the second cistron beyond 51, 11, and 11 copies of GTX IRES modules in HEK293, 3T3L1, and C6. Similarily, the diminished translation plateaus around 41 copies in HEK293 and 71 copies in 3T3L1, while there is no plateau in C6 (Figure 21B, D, F).

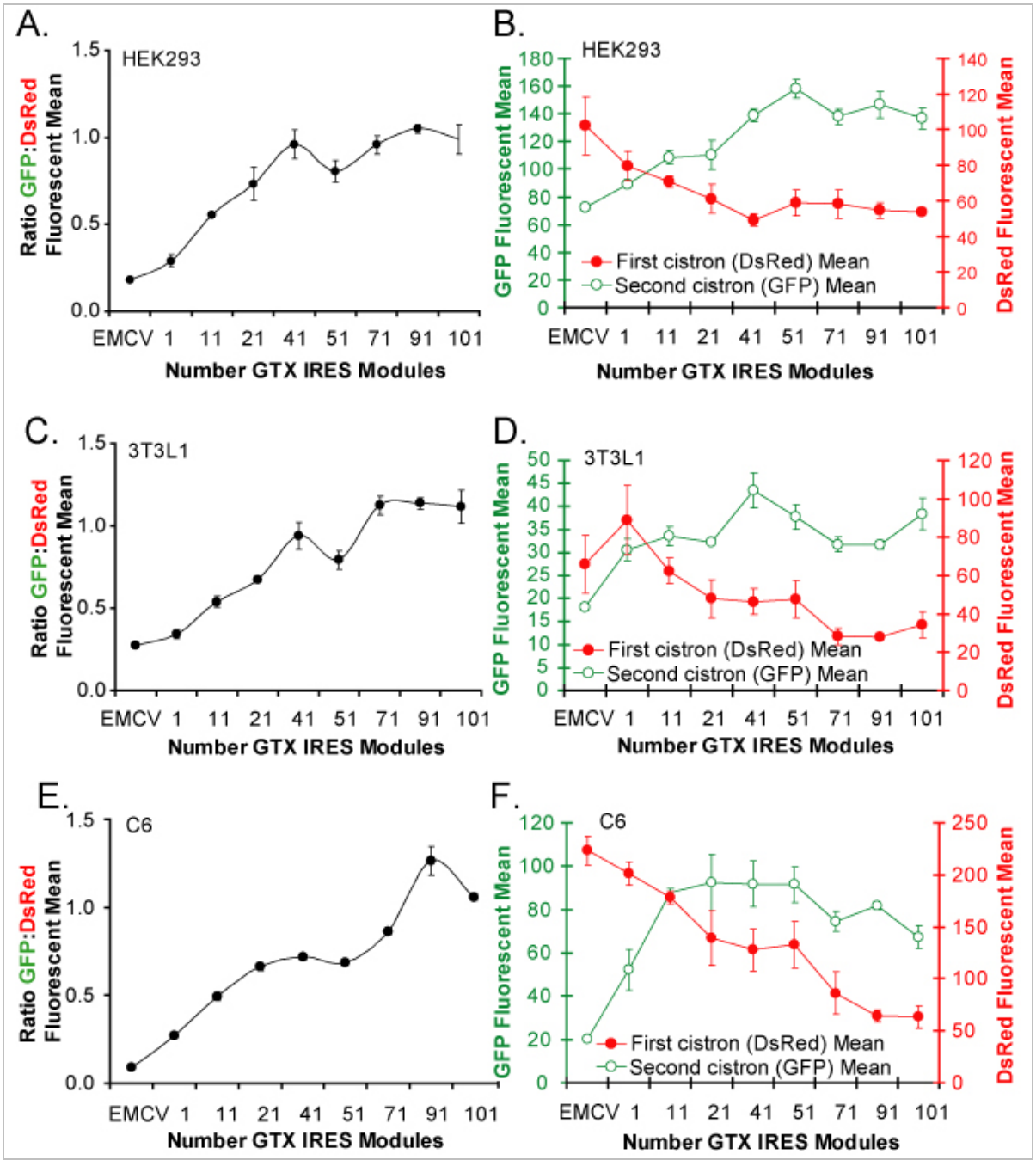


Figure 21. Increasing GTX IRES modules increases the fluorescent mean of the second cistron (GFP) but decreases the fluorescent mean of first cistron (DsRed). *Increasing the number of GTX IRES modules enhances the relative translational efficiency of the second cistron to the first cistron in HEK293* ***(A)****, 3T3L1* ***(C)****, and C6* ***(E)****. This increased ratio is due to increased translation of the second cistron (GFP) and decreased translation of the first cistron (DsRed). The decreased translation of the first cistron has been observed in HEK293* ***(B)****, 3T3L1* ***(D)****, and C6* ***(F)****.*

6.4 Discussion

In these experiments, we successfully constructed a series of IRES with different activities (Figure 4) to express different levels of RASSLs and thus different levels of basal signaling. This series of IRES modules greatly extends the range and possibilities of IRES to use.

We observed that the synthetic IRES modules are much more efficient than the EMCV-based IRES. This observation confirms multiple reports (Chappell *et al.* 2000; Owens *et al.* 2001; Wang *et al.* 2005). In addition, we observed that the synthetic IRES modules showed differential internal translational initiation, increasing tandem repeats results in IRES with greater translational efficiency. This could allow different level of RASSL expression from an endogenous promoter. In contrast to viral IRES, the use of the synthetic IRES modules based on mammalian IRES may minimize the possibility of an immune response due to the viral sequences of viral IRS (Wong *et al.* 2002). This could have important implications. Combining some IRESes (foot-and-mouth disease virus IRES and cellular immunoglobulin heavy chain binding protein IRES) in a plasmid leads to IRES interference and thus a decreased translational efficiency (Reigadas *et al.* 2005). Expressing multiple genes with IRES from the origin may be less likely to cause IRES translational interference.

While we still need to repeat our observations in mouse embryonic stem cells and with other reporters (luciferase) and test the other synthetic IRES based on ICS1 and ICS2 IRES modules, our results suggest that these synthetic IRES

modules are still affected by tissue tropism. More importantly, the greater number of IRES modules decreases the translation of the first cistron. Tissue tropism might make it difficult to use these synthetic IRES modules to translate the second cistron without affecting the first cistron.

For a most stochiometric expression, we would need a different method. Recently, a new method involving picornaviral “self-cleaving” 2A peptides was described that allows for “stoichiometric coexpression of at least four genes” (Szymczak *et al.* 2004). Together with this new technology, we now have the ability to more fully control the RASSL expression levels.

CHAPTER 7: CONTROLLING RASSL EXPRESSION LEVEL BY SINGLE PLASMID TETRACYCLINE INDUCIBLE SYSTEM

7.1 SUMMARY

We report here a single plasmid tetracycline-inducible system for better control of RASSLs expression. To ease cloning, tetO, tTA/ rtTA, insulators, and transgenes, such as GFP and RASSLs, are cloned into two entry plasmids. Multisite Gateway is then used to combine it with any destination plasmid. We observed robust induction of transgene (~fivefold) in HEK293 treated with 10 μg/m of doxycycline.

7.2 INTRODUCTION

Despite its usefulness, the RASSL technology is limited by the relatively inefficient and inconsistent expression patterns that can be achieved by traditional transgenic technologies. Expressing a RASSL under the control of a tissue-specific promoter in transgenic mice can take months or years of work. Even then, each transgenic line has a slightly different expression pattern due to different genomic integration sites. This makes direct comparisons between different RASSL mutants nearly impossible. To solve this technical challenge, we combined RASSLs with the tetracycline system.

7.2.1 Tetracylcine inducible system

First developed by Hermann Bujard and Manfred Gossen (Gossen *et al.* 1992), the tetracycline-controlled transcriptional activation is ideal for controlling transcriptional activity of transgene *in vivo*. It is highly inducible: induction up to 10,000-fold may be possible. More importantly, it offers a tight and readily reversible method to induce transgene expression in a tissue-, time-, and level-specific manner.

The tetracycline-controlled transcriptional activation system has three components. A tet-responsive element (TRE) made of seven optimized and multimierized copies of tetR-binding sequence (tetO) is coupled to a minimal promoter upstream of a minimal element with a TATA box and a transcriptional initiation site. The second component is the transcriptional transactivator (tTA) made of tetracycline repressor from *Escherichia coli* and VP16 from herpes simplex virus. The third is an antibiotic from the tetracycline family to turn on or turn off the system.

The use of tetracycline and doxycycline (a tetracycline derivative) to control induction is ideal for *in vivo* studies. The high lipophilicity of the tetracycline derivatives greatly facilitate crossing cell membranes (Barza *et al.* 1975; Argast *et al.* 1984). Both doxycycline and tetracycline efficiently bind all commonly used tetR variants (Gossen *et al.* 1995; Blau *et al.* 1999). In particular, doxycycline has minimal side effects in human and other organisms (Michel *et al.* 1979; Malmborg 1984; Meijer *et al.* 1993; Santos *et al.* 1996; Abu-Basha *et al.* 2006)

and has long half-life (18–22 hours) and 100% oral bioavailability, greatly facilitating the drug administration into animal models (Barza *et al.* 1975; Mansuy *et al.* 1998). It also efficiently crosses placental barrier and the blood-brain barrier (2–14%), enabling transgene induction in neurons and infants (Andersson *et al.* 1976).

7.2.2 Tet-ON versus Tet-OFF

In contrast to the doxycline, different complementary versions of the transactivator have been developed to meet different experimental needs. In the original setup, tTA (often driven by a tissue-specific promoter) is a fusion protein of TetR repressor (1–207) and the C-terminal 127 amino acids of the herpes simplex VP16 transactivation domain (Triezenberg *et al.* 1988). This fusion converts the tetR from a transcriptional repressor to a transcriptional activator. tTA binds to tetO to activate the promoter coupled to TRE. Upon binding by tetracycline or other tetracycline derivatives such as doxycycline, however, tTA can no longer bind to the promoter. Since transcription is off in the presence of tetracycline, this is commonly known as “tet-off.”

The reverse tetracycline-controlled transactivator (rtTA) contains additional four amino acids in the tetR DNA binding moiety. This alters the binding and dimerization so that the resulting protein can only bind to the tetO sequence in TRE the presence of doxycycline (Hillen *et al.* 1994; Hinrichs *et al.* 1994; Gossen

et al. 1995; Orth *et al.* 1998). Since transcriptional is off in the presence of tetracycline, this is commonly known as “tet-on.” However, rtTA can only be activated by doxycycline (Gossen *et al.* 1995).

Both systems are complementary to each other. Even though Tet-on is the most commonly used system (Berens *et al.* 2003), Tet-off is often more effective (Mizuguchi *et al.* 2002; Xu *et al.* 2003). For kinetic reasons, it more rapidly adds the activators to induce rather than to remove the inhibitors (Kistner *et al.* 1996), and rtTA has several disadvantages. It requires 1–2 μg/mL doxycyclines. This high concentration may be difficult to achieve in certain organs. Furthermore, rtTA demonstrates residual binding to tetO without doxycycline, shows lower stability, and is harder to express in certain tissues (Urlinger *et al.* 2000). Finally, rtTA is more sensitive to site of integration and mouse strain (Robertson *et al.* 2002).

In addition, the tetracycline-inducible system has certain level of leaky expression thatis further exacerbated by the site of integration. There have been many breakthrough in tetracycline-inducible delivery (Sun *et al.* 2007 Apr) and many attempts have been made to reduce the transgene leaky expression and maximize inducibility of the tetracycline inducible system (Forster *et al.* 1999), including a transcriptional silencer (Zhu *et al.* 2001; Bockamp *et al.* 2007) or using rtTA-M2 (a rtTA with tighter expression) (Baron *et al.* 1997). The four amino acid changes in rtTA-M2 decrease the residual binding to tetO and increase the sensitivity so that only 0.1 μg/mL of doxycycline is needed for full

activation (Urlinger *et al.* 2000). rtTA-M2 has since been used in multiple instances (Lamartina *et al.* 2002; Koponen *et al.* 2003; Lamartina *et al.* 2003; Welman *et al.* 2006).

7.2.3 Single Plasmid Tetracycline inducible system

Despite the improvements on the tetracycline-inducible system, it still suffers multiple limitations. The efficiency of the tet system is especially sensitive to the strain of mice and the site of integration of the transgene (Robertson *et al.* 2002). In addition, the system requires multiple crossing of different transgenic lines in mice, increasing both the cost and time. In the case of the embronyic stem cells, multiple electroporations and selections are needed, complicating the need to minimize the passaging.

To solve these challenges, many different versions of single plasmid tetracycline-inducible systems (or single autoregulatory cassettes) built in plasmid DNA, adeno-associated vectors (McGee Sanftner *et al.* 2001; Mizuguchi *et al.* 2002) retroviruses (Hofmann *et al.* 1996), lentiviruses (Vogel *et al.* 2004), and adenoviruses (Corti *et al.* 1999)

These systems have many clear advantages over the traditional tetracycline-inducible systems, but they have not been widely utilized due to the following limitations. First, the inclusion of both tetO and rtTA in the same plasmid forms an autoregulatory loop that increases the leakiness (Hofmann *et al.* 1996).

Furthermore, replacing the tissue-specific promoter, tTA/rtTA, or transgene of interest is difficult due to the size of the plasmid. Finally, inclusion of the complex tetracycline-indicuble units into a single viral vector (Hwang *et al.* 1996; Lindemann *et al.* 1997) often led to few clones due to low viral titer (Blau *et al.* 1999).

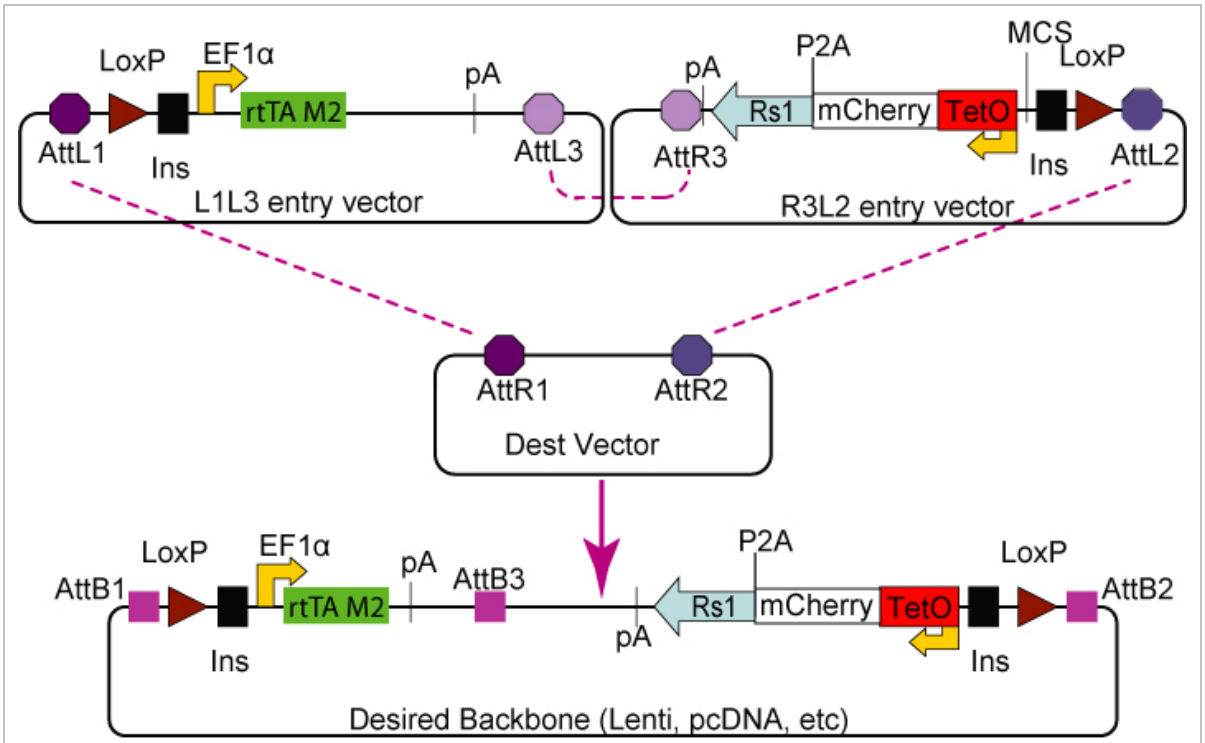


Figure 22. Schematic of the single plasmid tetracycline inducible system.
To facilitate the easy transfer of promoters, tTA or rtTA, and transgene, a system based on Gateway recombination (AttL/R 1, AttL/R 2, Att L/R 3) has been developed. Each entry vector has unique restriction sites to allow ease replacement of promoter, rTTA, and transgene. Insulators have been designed to minimize site-integration effects.

7.3 RESULTS

As proof of concept, we transfected a confluent six-well HEK293 (~1.5 x106 HEK293 cells) culture with 250 ng of our tetracycline-inducible plasmid. A fivefold induction was observed after 21 hours of 10 μg/mL doxycycline treatment by microscopy or flow cytometry. At the dosage tested, little leakage was observed.

7.4 DISCUSSION

We report here a single plasmid Tet-inducible system (Figure 22) to simplify the system so that fewer crossings need to be done in mice. The final plasmid utilizes a bidirectional TetO promoter, like the previous single-plasmid tetracycline-inducible systems (Baron *et al.* 1995). To minimize read-throughs from either the TetO or the elongation factor 1 alpha (EF1α) promoter as described in the figure, the cassettes are arranged in opposing orientations. Our system has several advantages over the existing single tetracycline-inducible systems.

First, we constructed the components in different entry plasmids to maximize the flexibility so that one can easily mix and match promoter-tTA or rtTA (eg MH-tTA, ColI) with different TetO-marker systems (mOrange, GFP, RASSLs). The modular set-up allows us to combine it with any destination vector (such as plasmid, retrovirus, lentivirus). The inclusion of the Gateway recombination system further facilitates the process to combine the two entry plasmids with any

destination vectors, such as plasmid (pDest26 or pDest27) and lentiviral pLenti6/V5-DEST, to create the final single-plasmid tetracycline-inducible system.

Furthermore, inclusion of Cre-technology adds tissue specificity for activation at specific times in development, when the tissue specificity is optimal (e.g., newborn mice). Then RASSL expression can be turn on later in the life of the animal (adults). This is important since cardiac expression of the RO1 RASSL is lethal in newborn animals (90% mortality, unpublished results). Therefore, the Cre-tet vector is ideally suited for expression of highly bioactive RASSLs. In addition to driving RASSL, it can be used to drive RNAi or transgenes in a adenoviral or single lentiviral vector (Mizuguchi *et al.* 2003; Vogel *et al.* 2004).

Finally, we included both rtTA-2S-M2 and an insulator (Burgess-Beusse *et al.* 2002; Recillas-Targa *et al.* 2002). The combination of rtTA-2S-M2 and an insulator increases long-term homogeneity and decreases leaky expression (Qu *et al.* 2004). This should decrease position effect variegation, regardless of the site of integration.

While the system still needs to be tested in embryonic stem cells and mice, we are realistically optimistc. The relatively low leakiness and threefold incution observed in human HEK293 transient transfectants demonstrated important proof of concept. The fold of induction and leakiness may be improved by making and selecting monoclonal colonies of stable transfectants. In the case of the experiment shown, after antibiotic selection, the pool may be selected by flow

cytometry of mCherry-expressing cells, and monoclonal colonies can be made. The colonies can then be selected for minimal leakiness and maximal induction. The combination of the single tetracycline-inducible system with RASSLs should allow us to better control RASSL expression and thus allow us to better study the role of constitutive signaling *in vivo*.

7.5 Materials and Methods

7.5.1 Making the single tetracycline-inducible system

Dr. Ed Hsiao and I collaborated on making of the two entry plasmids. I annealed two oligos (PC270F, TCGACgtttaaacataacttcgtatagcatacattatacgaagttatctcgagcc-tcaggcagcatatgttaattaagcccgggcgaattcgcggccgcagatctCTGCA and PC270R, GCAAATTTGTATTGAAGCATATCGTATGTAATATGCTTCAATAGAGCTCGGA GTCCGTCGTATACAATTAATTCGGGCCCGCTTAAGCGCCGGCGTCTAGAG) to make the L3 recombination site. This was then cloned into Sal1 and Pst1 sites of the pENTR2b entry plasmid. Another pair of oligonucleotides for LoxP site were then annealed and cloned into Bgl II and Pst I sites of the pENTR 2b (PC272F, GATCTcaactttattatacaaagttggcattataaaaaagcattgcttatcaatttgttgcaac-gaacaggtcactatcagtcaaaataaaatcattatttgatatcCTGCA and PC272R, AGTTG-AAATAATATGTTTCAACCGTAATATTTTTTCGTAACGAATAGTTAAACAACGT-TGCTTGTCCAGTGATAGTCAGTTTTATTTTAGTAATAAACTATAGG) to add LoxP sites adjacent to the L3 recombination site. The insulator sequences from

p5HT1a-insulator (Miles Berger, UCSF) was cloned into the Nde1 and Bsu36I restriction sites to make WC359-pENTR2bL3LoxPInsulator. rtTA-M2-polyA from polyA-BS/CMVrtTAM2-polyA was cloned into EcoR I and NotI sites. EF1a promoter from WC307-EF1a-GFP was excised (Spe1 and EcoR1, Klneowed) and cloned into the EcoRI site of pENTR2b-TetComponentL3-Insulator-EF1arttAM2.

Dr. Hsiao prepared the other entry plasmid pTETO-mcherry-P2A-Rs1-insulator). Two ligos for R3 recombinatino site were annealed, then digested with AflII and BglII, blunt ended with klenow fragment, and ligated into pEntR2B to create the plasmid pEntr2B/R3rev-MCS.

Sense AflII-R3-MCS-XhoI:

5’

CTTAAGCAACTATGTATAATAAAGTTGAACGAGAAACGTAAAATGATATAAAT
ATCAATATATTAAATTAGATTTTGCATAAAAAACAGACTACATAATACTGTAAA
ACACAACATATCCAGTCACTATGGAGATCTCTGAATGCGGCCGCTATCTTAA
TTAACCTCAGGACTTTCATATGACTCGAG 3’

Antisense AflII-R3-MCS-XhoI:

5’

CTCGAGTCATATGAAAGTCCTGAGGTTAATTAAGATAGCGGCCGCATTCAGA
GATCTCCATAGTGACTGGATATGTTGTGTTTTACAGTATTATGTAGTCTGTTT
TTTATGCAAAATCTAATTTAATATATTGATATTTATATCATTTTACGTTTCTCGT
TCAACTTTATTATACATAGTTGCTTAAG 3’

The LoxP site was then introduced by annealing ECH070 (TATGataacttcgtatagc-atacattatacgaagttatGTTTAAAC) and ECH071 (tcgagtttaaacataacttcgtataatgtatgc-tatacgaagttatca) into NdeI/XhoI restriction site of the plasmid aboveto create pEntr2B/R3rev-MCS/LoxP.

TetO with a beta globin intron was then PCR amplified with ECH069 (gcttaattaAGGCCCTTTCGTCTTCACTCGAG) and ECH062 (GAgcggccgcat-CCTGCAGGgcgtcgacgaattctttgcca)and cloned into Amplified by PCR from a TetO plasmid obtained from Mark Anderson and introducing NotI and PacI restriction sites of pEntr2B/R3rev-MCS/LoxP to creates pENTR2B/R3rev-MCS-LoxP-TetoBG.

Poly A tail was next amplified from pDEST27 with ECH060 (gacctgcaggCGGG-ACTCTGGGGTTCGAAA) and ECH061 (gagcggccgcTTCACACAAAAAACCAA-CACACAGATG) and cloned into Not1 and Sbf1 sites of pENTR2B/R3rev-MCS-LoxP-TetoBG to create pENTR2B/R3rev-MCS/LoxP/TetoBG/pA.

Insulator was then subcloned into Bsu36I and NdeI sites of pENTR2B/R3rev-MCS/LoxP/TetoBG/pA to create pEntR3L2 TetoBG. Judy Kim, a summer rotation student working with Ed, then created the derivative vector pENTR2B/R3 TetoBG-Ins mCherry MT2 which has just the mCherry driven by TetOBG (now renamed pENTR3L2 TetoBG-mCherry). She did this by PCR amplifying mCherry with JK001 (TCTACCTGCAGGATGGTGAGCAAGGGCGAGGA) and JK003 (GTTACCTGCAGGTTACTTGTACAGCTCGTCCATGCC)and subcloning into the Sbf1 site of pENTR3L2 TetoBG-mCherry. I then subcloned Rs1 into Sal1 and Sbf1 site of pENTR3L2 TetoBG-mCherry.

Invitrogen Multisite Gateway system was then used to combine the two entry vectors with destination vector pDEST-27 to create the single plasmid tetracycline-inducible system.

7.5.2 Measuring the induction in HEK293

A confluent plate of HEK293 (seeded at $1x10^6$ cells the previous day) were transfected with 250 ng of WC401-TETO-mcherry-P2A-Rs1, WC402-TETO-mcherry-P2A-Rm3, pDEST27 (empty plasmid), or pEF1α-mCherry (constitutively active promoter) pre-mixed with 1750 ng of pCDNA3 in 250 μl of Opti-MEM, and

5 μl of Lipofectamine 2000 (Invitrogen) was used for the transfection, following the manufacturer's protocol. The medium was replaced with either 0 or 10 μg/mL of doxycline the next day. After 21 hours of doxycyline treatment, the cells were then examined with microscopy and then analyzed via Becton Dickenson LSR II.

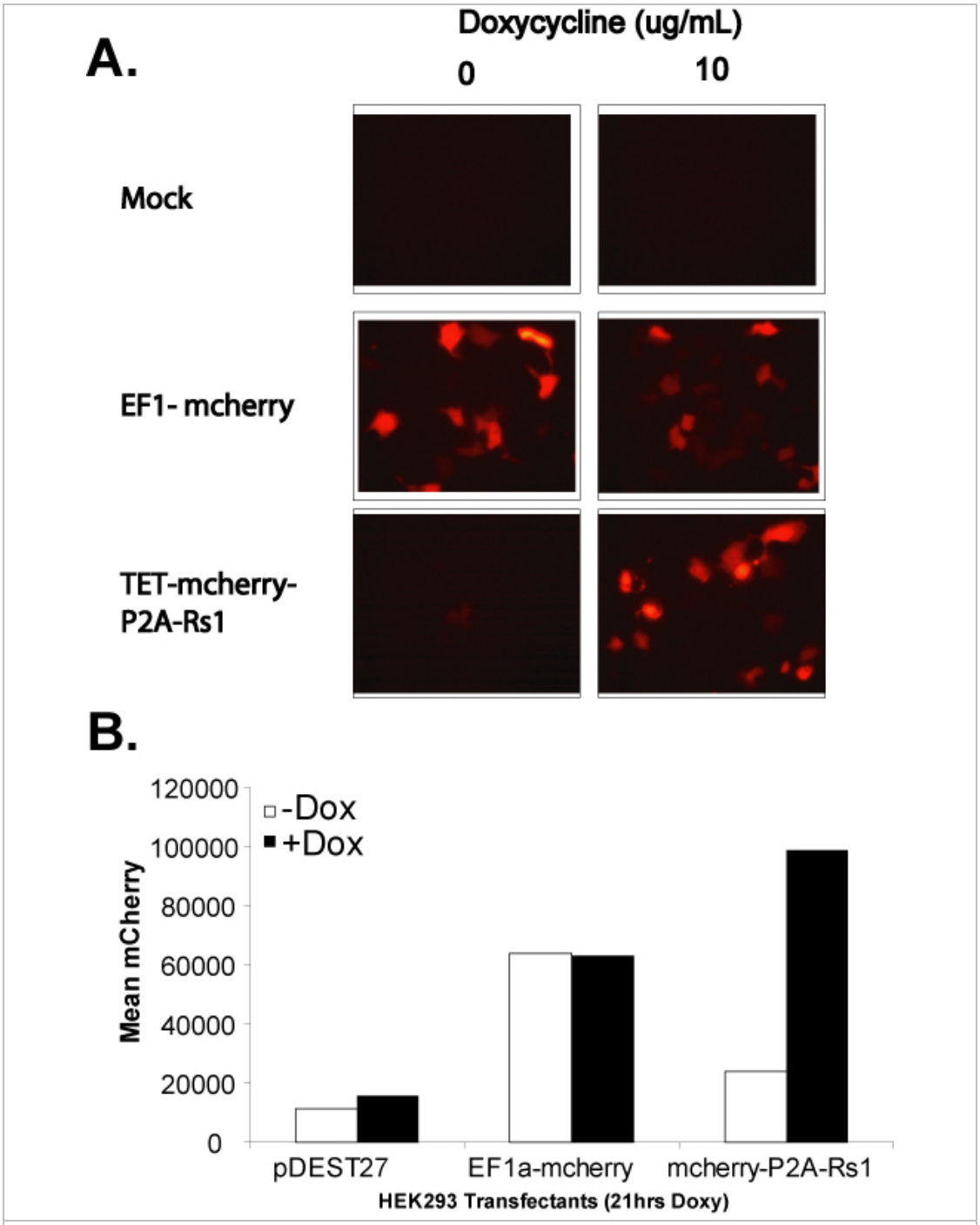


Figure 23. Single plasmid tetracycline-inducible system results in robust transcriptional activation with minimal leakage.

mcherry transcription is robustly transcribed when treated with 10 µg/mL of doxycycline for 21 hours. 1x10^6 HEK293 cells were transfected with 250 ng of tetracycline inducible plasmid with 1750 ng of empty plasmid and 5 μl of Lipofectamine 2000.

CHAPTER 8: SIGNIFICANCE AND FUTURE DIRECTION

8.1 Significance and Implications

In the process of engineering these new RASSLs, we learned much about the human 5-HT_4 receptor. In addition to facilitating a better understanding of the GPCRs that the RASSLs were based on, the RASSLs may also help us better understand the molecular diversity of GPCRs so that we can develop more efficacious and specific drugs and develop better tools for tissue engineering.

8.1.1 A better understanding of receptors may lead to better drugs

We hypothesize that improved GPCR drugs can be made if we understood how each G-protein pathway contributes to cardiac physiology via acute and constitutive signaling. RASSLs could prove to be an ideal tool to match discrete signal transduction event or molecular diversity of GPCRs to specific physiological responses.

8.1.2 Challenges of tissue engineering for growth and development

Despite recent promising breakthroughs in cell and gene therapy, there is still little control over stem cells *in vivo*. The need for external control of engineered tissues was highlighted in a trial of fetal cell transplants for Parkinson's disease (Freed *et al.* 2001; Lindvall *et al.* 2004). The trial was stopped prematurely

because the fetal cells grew aberrantly and caused permanent movement disorders from over-activity. Unfortunately, there were no means of selectively controlling the activity or proliferation of the transplanted cells.

The body's immune system poses another major challenge to tissue engineering, since any newly engineered protein could induce an immune response that would eliminate the therapeutic cells. Bacterially derived tetracycline transactivator was used in human gene therapy trials, but the immune response to this foreign intracellular protein eliminated the engineered cells. Similar problems will likely confront cell-surface receptors. Several groups have suggested that RASSLs should be made from GPCR-hormone pairs from distantly related organisms (Redfern *et al.* 1999), but these nonhuman receptors are likely to elicit a strong immune response in humans. By contrast, human GPCRs provide a scaffold for building drug-controlled switches that are less likely to induce an immune response.

The potential application of RASSLs to cell therapy is supported by the crucial roles of many GPCRs such as α- and β-adrenergic receptors (Niedernberg *et al.* 2003) and earlier work from Conklin lab (Redfern *et al.* 1999). RASSLs may be incorporated as a tool to control differentiation and proliferation. We hypothesize that RASSL-specific agonists and inverse angoinsts may be utilized to control differentiation.

RASSLs expressed in transplanted cells may provide a means of controlling transplanted electrical tissues. Similarly, RASSLs might be used to control

specific overactive neurons in Parkinson's disease and seizure disorders. Currently, neurosurgery is often used to ablate overactive neural circuits. However, RASSLs could provide a means of pharmacologically modulating brain activity with inert ligands, such as CNO.

We also hypothesize that engineered receptors can be used to modulate cardiac development and physiological responsiveness through each of the major G protein pathways (G_s, G_i, and G_q). RASSLs may find uses in other forms of therapeutic tissue engineering where it would be advantageous to control growth for development**.** GPCR signaling is essential for the growth and differentiation of many tissues (Malbon 2005; Wettschureck *et al.* 2005).

8.2 Future Direction

In conclusion, we made a series of RASSLs by modifying the agonist-mediated and constitutive signaling of the 5-HT4 receptor via both genetic and chemical means. We discovered possible functional selectivity in Rs1 and Rs1-C-5HT_{2C}. We also constructed a series of IRES with different activities and single plasmid tetracycline-inducible system to better study constitutive signaling.

We focused primarily on the effect of mutations, domains swappings and drug choice on the classical G-protein signaling (G_s, G_i, and G_q) of the 5-HT_4-based RASSLs. It will be interesting to examine how these changes affect other

5-HT_4 receptor-mediated signaling, such as coupling to tetrodotoxin-insensitive sodium channels (Cardenas *et al.* 1997) and other signaling proteins.

The pharmacological control and genetic manipulation for studying the significance of GPCR signaling and the molecular diversity of GPCR is a field ripe for discovery. These new tools in molecular pharmacology will greatly facilitate our understanding of GPCRs and may address fundamental questions of how specific G-protein-signaling pathways can used to control specific cells. This will have much impact on the medical utility of cell therapy.

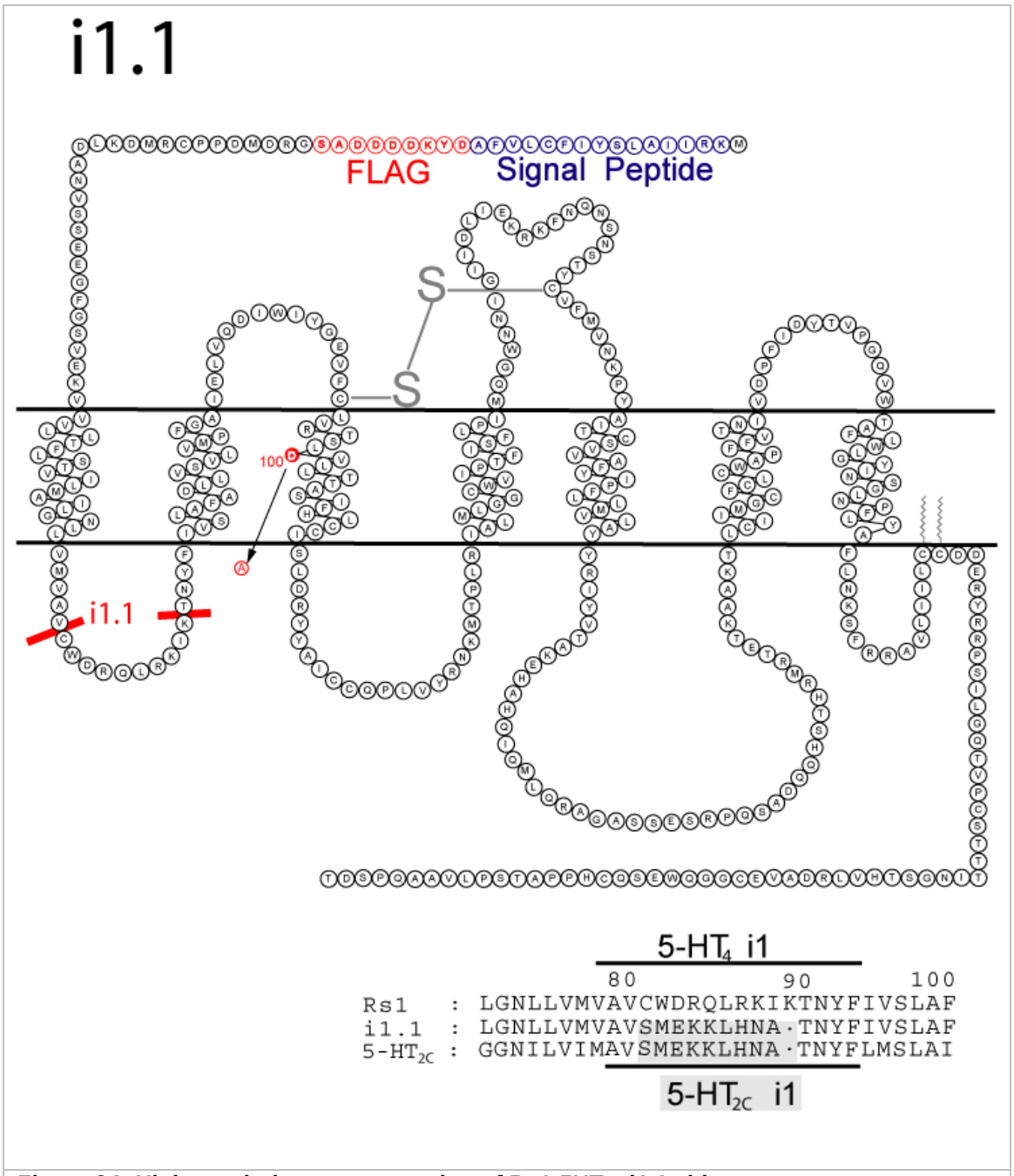


Figure 24: High-resolution representation of Rs1-5HT$_{2C}$ i1.1 chimera.
All modifications were made on Rs1 (Figure 1). Red lines indicate the junctions of chimeras. The exchanged amino acids are shown by the amino acid alignment.

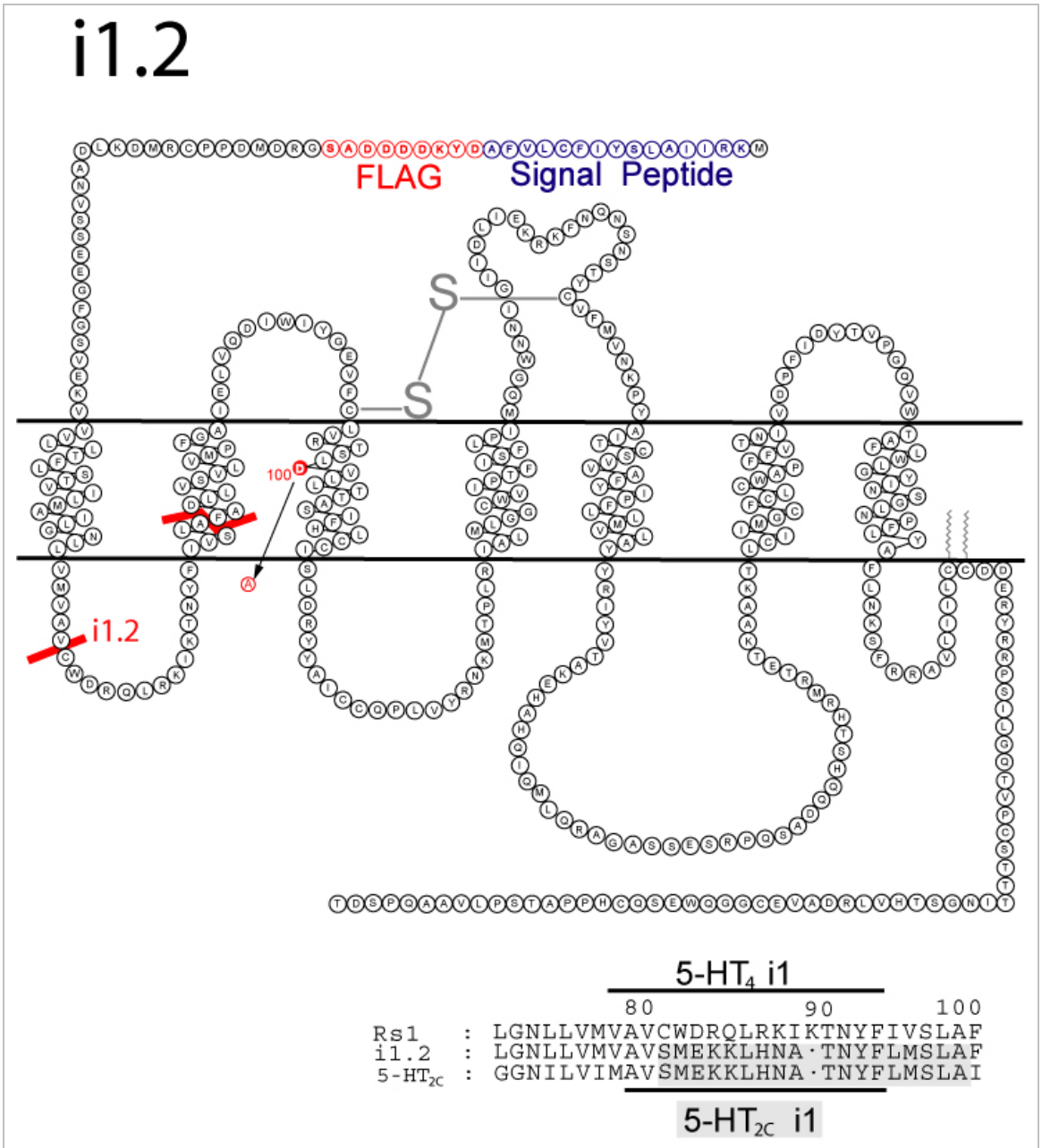


Figure 25: High-resolution representation of Rs1-5HT$_{2C}$ i1.2 chimera.
All modifications were made on Rs1 (Figure 1). Red lines indicate the junctions of chimeras. The exchanged amino acids are shown by the amino acid alignment.

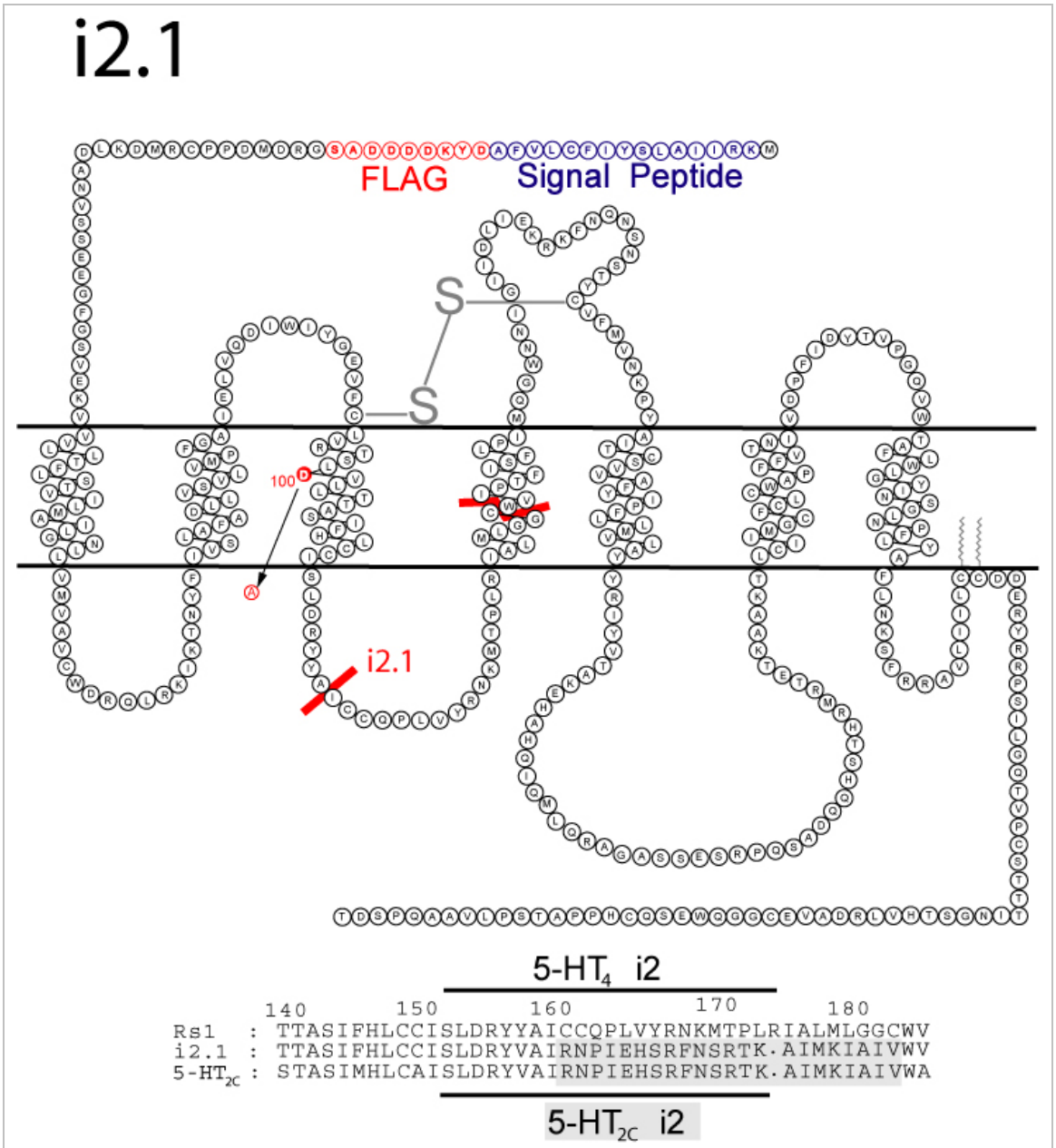


Figure 26: High-resolution representation of Rs1-5HT$_{2C}$ i2.1 chimera.
All modifications were made on Rs1 (Figure 1). Red lines indicate the junctions of chimeras. The exchanged amino acids are shown by the amino acid alignment.

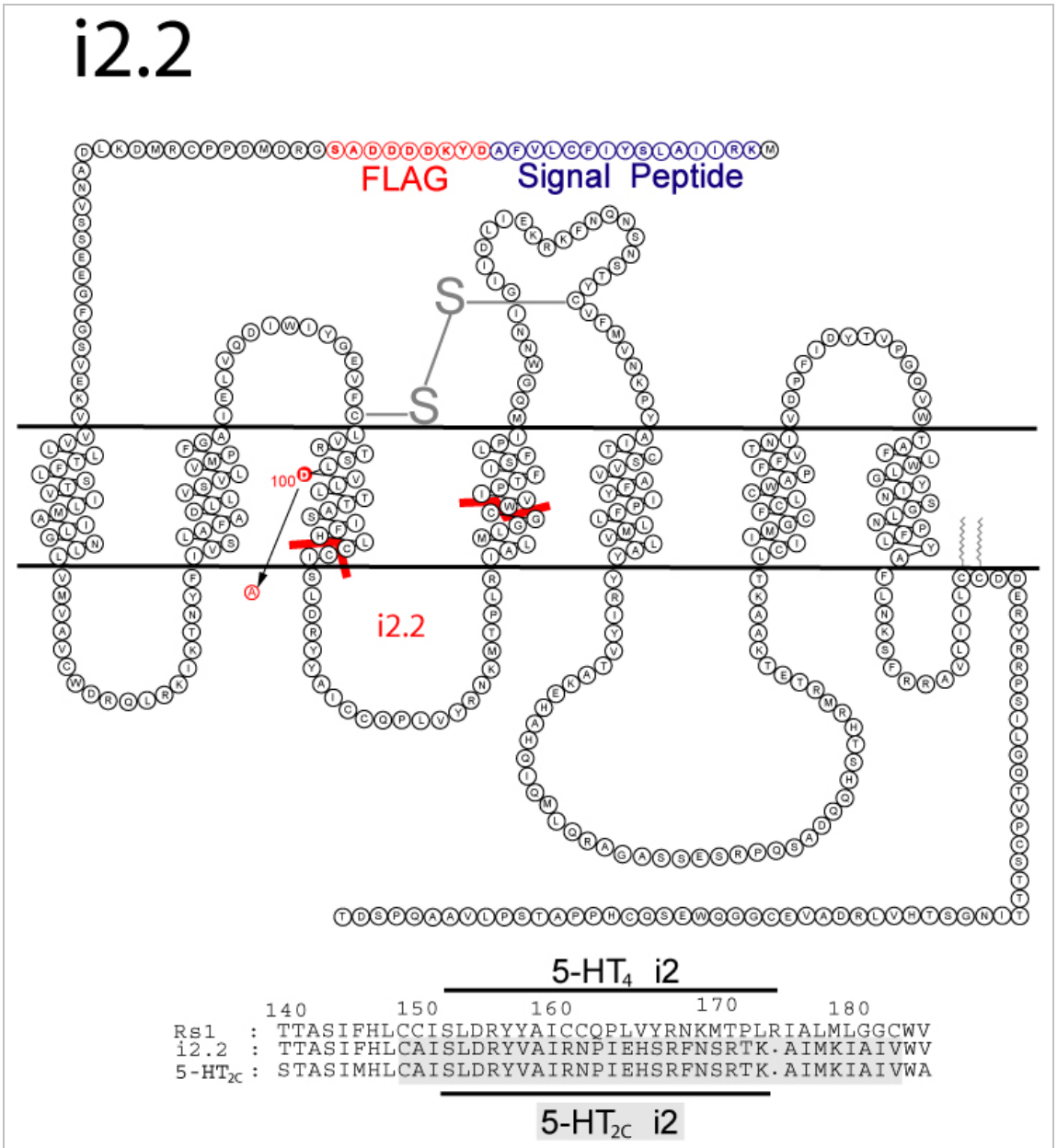


Figure 27: High-resolution representation of Rs1-5HT$_{2C}$ i2.2 chimera.
All modifications were made on Rs1 (Figure 1). Red lines indicate the junctions of chimeras. The exchanged amino acids are shown by the amino acid alignment.

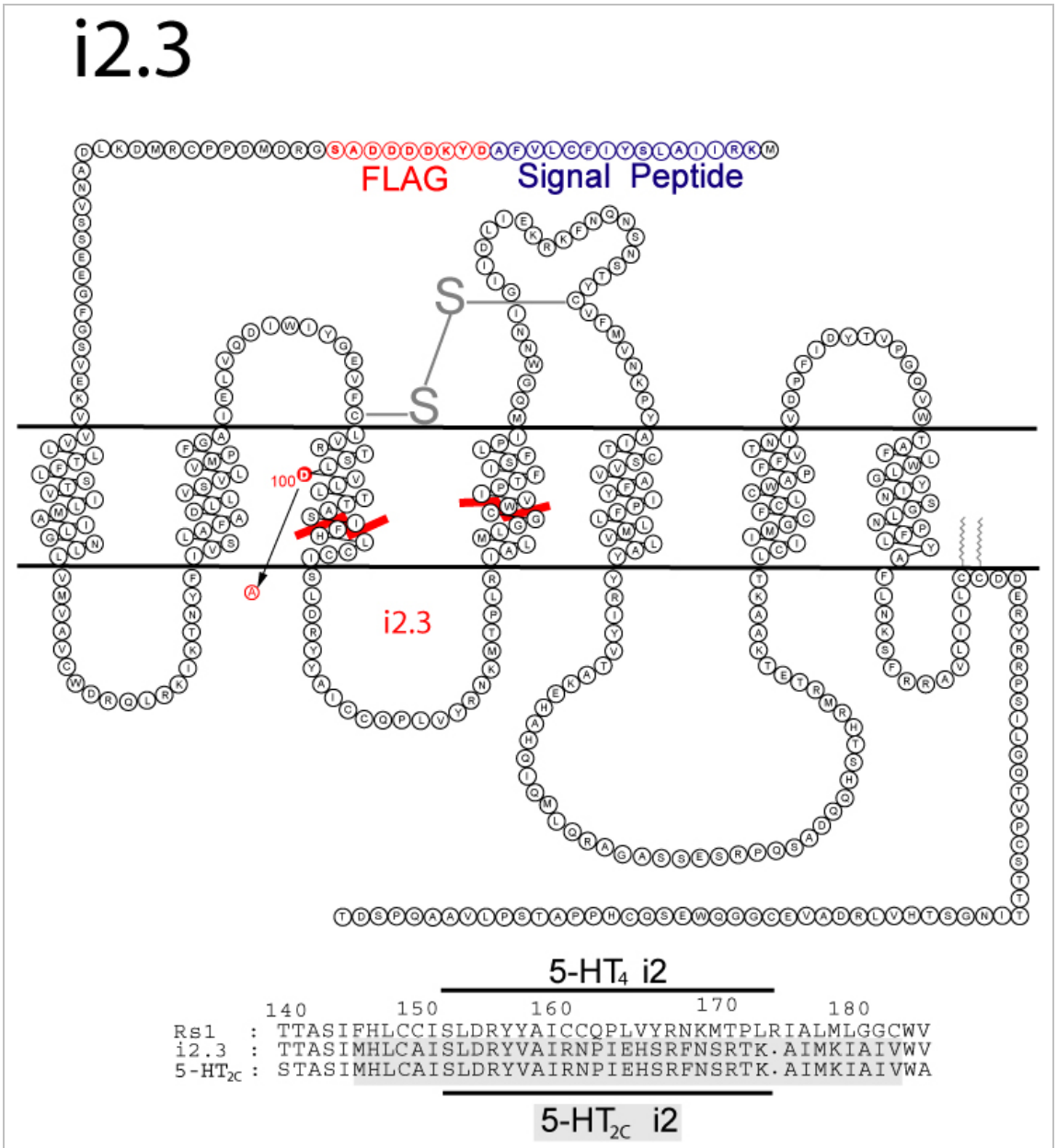


Figure 28: High-resolution representation of Rs1-5HT$_{2C}$ i2.3 chimera.
All modifications were made on Rs1 (Figure 1). Red lines indicate the junctions of chimeras. The exchanged amino acids are shown by the amino acid alignment.

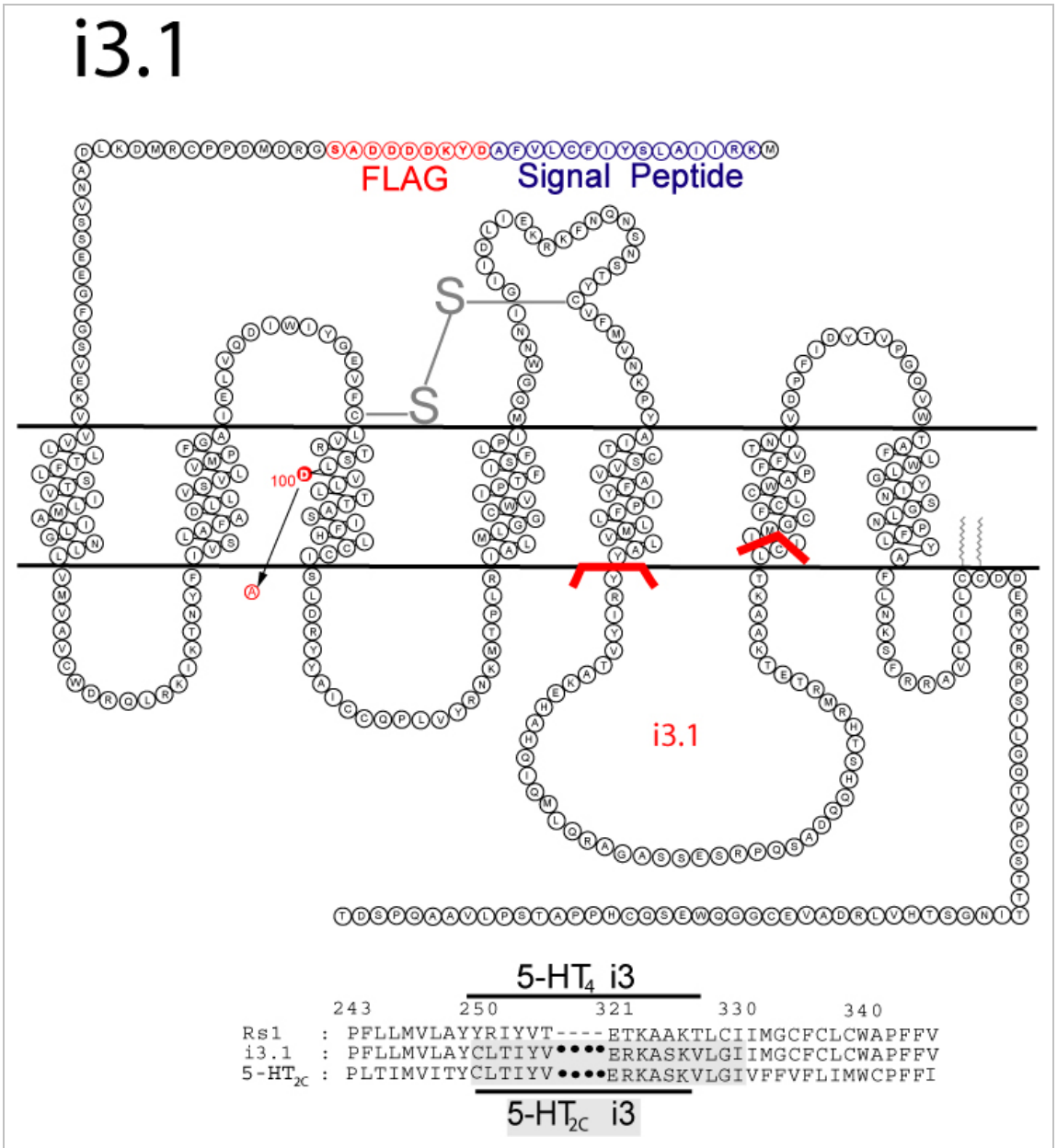


Figure 29: High-resolution representation of Rs1-5HT$_{2C}$ i3.1 chimera.
All modifications were made on Rs1 (Figure 1). Red lines indicate the junctions of chimeras. The exchanged amino acids are shown by the amino acid alignment.

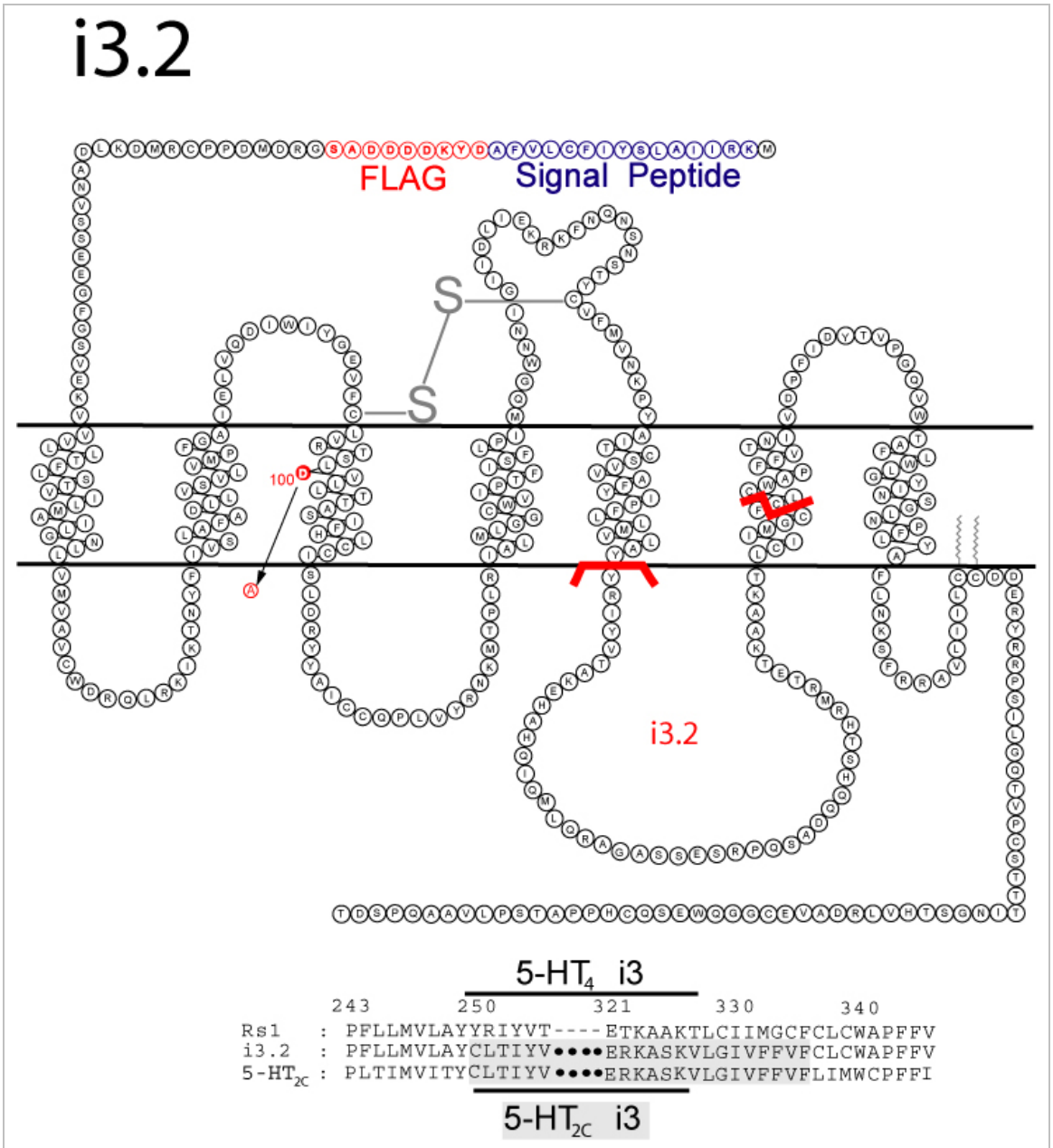


Figure 30: High-resolution representation of Rs1-5HT$_{2C}$ i3.2 chimera.
All modifications were made on Rs1 (Figure 1). Red lines indicate the junctions of chimeras. The exchanged amino acids are shown by the amino acid alignment.

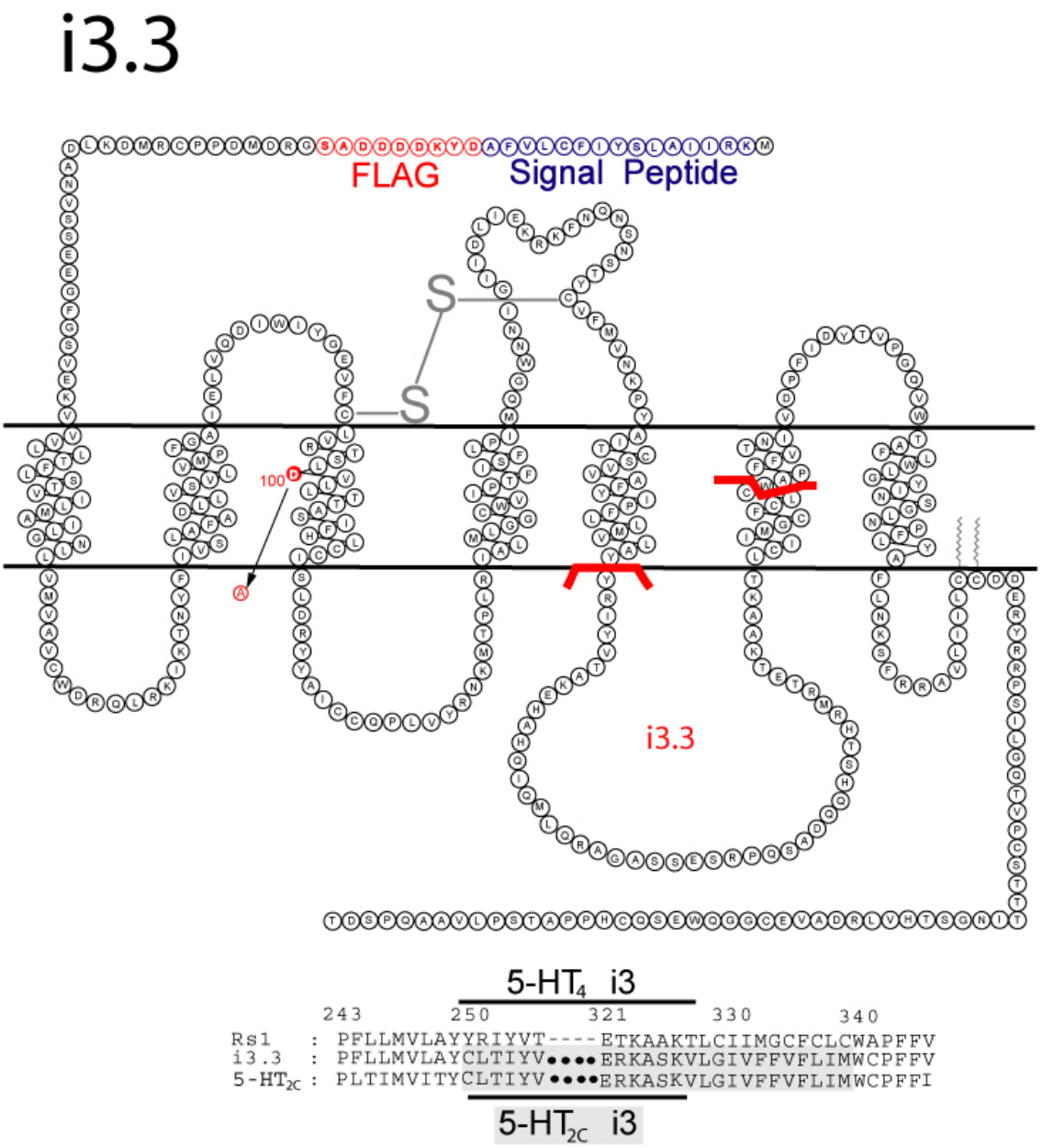


Figure 31: High-resolution representation of Rs1-5HT$_{2C}$ i3.3 chimera.
All modifications were made on Rs1 (Figure 1). Red lines indicate the junctions of chimeras. The exchanged amino acids are shown by the amino acid alignment.

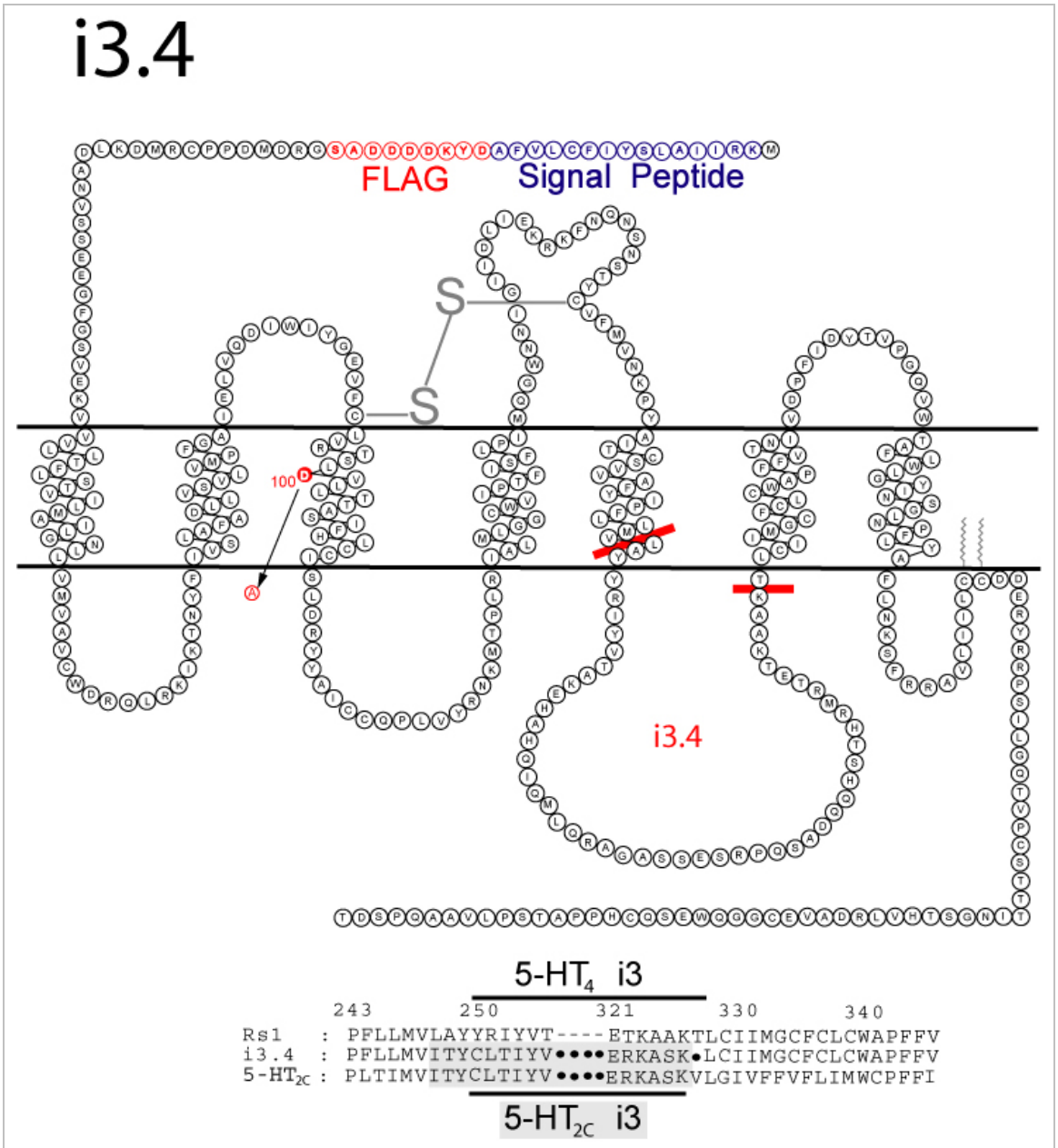


Figure 32: High-resolution representation of Rs1-5HT$_{2C}$ i3.4 chimera.
All modifications were made on Rs1 (Figure 1). Red lines indicate the junctions of chimeras. The exchanged amino acids are shown by the amino acid alignment.

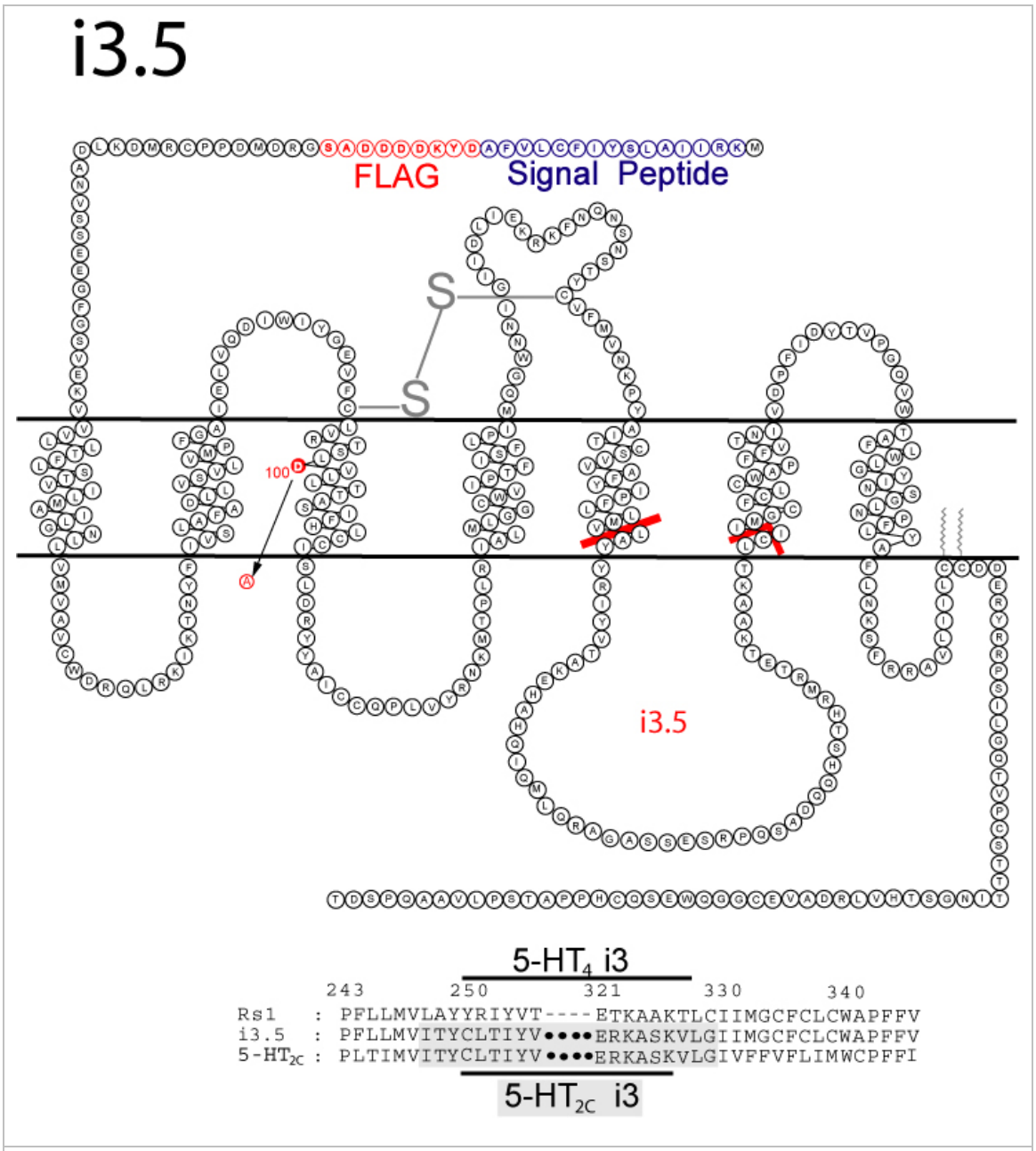


Figure 33: High-resolution representation of Rs1-5HT$_{2C}$ i3.5 chimera.
All modifications were made on Rs1 (Figure 1). Red lines indicate the junctions of chimeras. The amino acids exchanged are shown by the amino acid alignment.

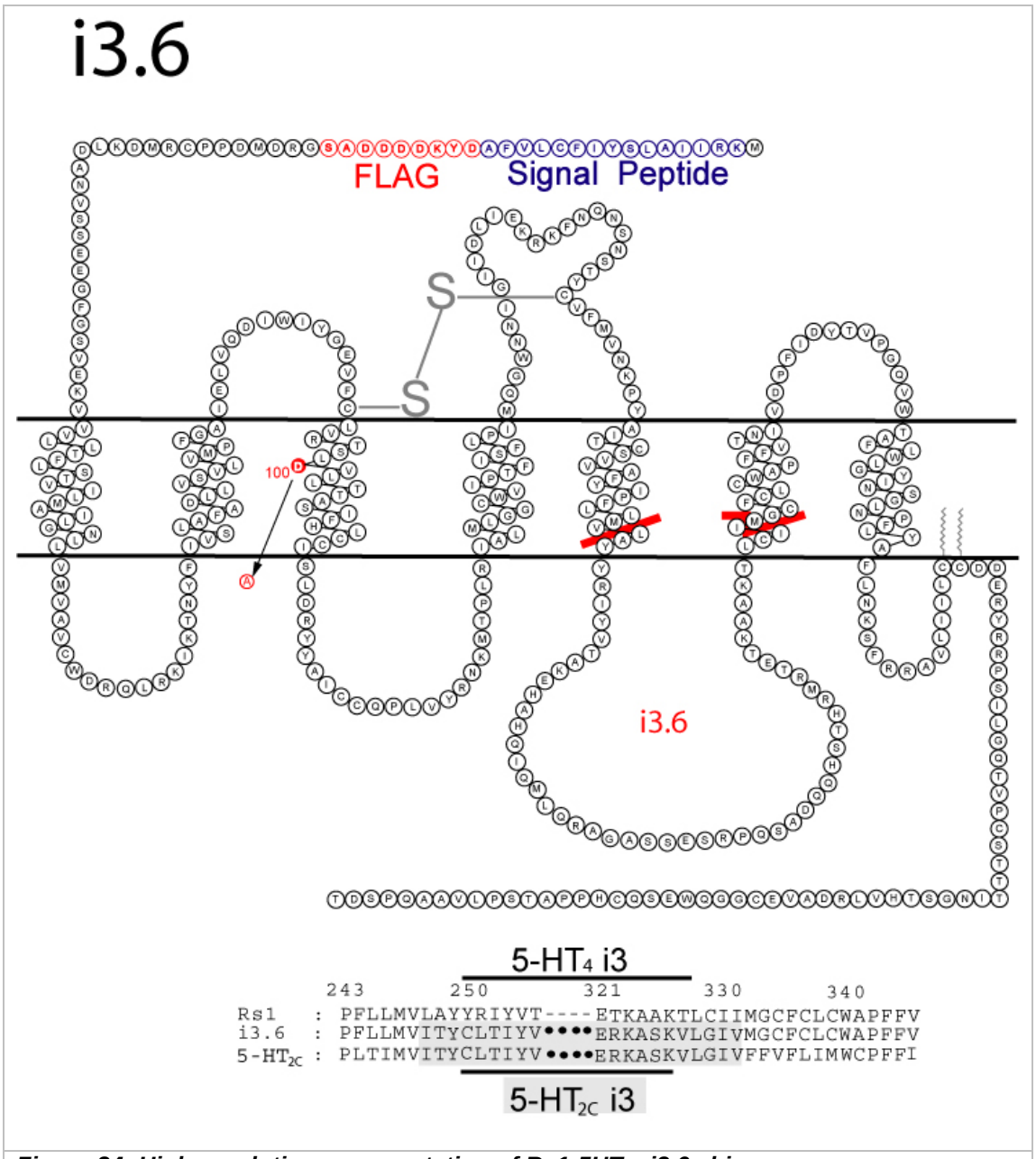


Figure 34: High-resolution representation of Rs1-5HT$_{2C}$ i3.6 chimera.
All modifications were made on Rs1 (Figure 1). Red lines indicate the junctions of chimeras. The amino acids exchanged are shown by the amino acid alignment.

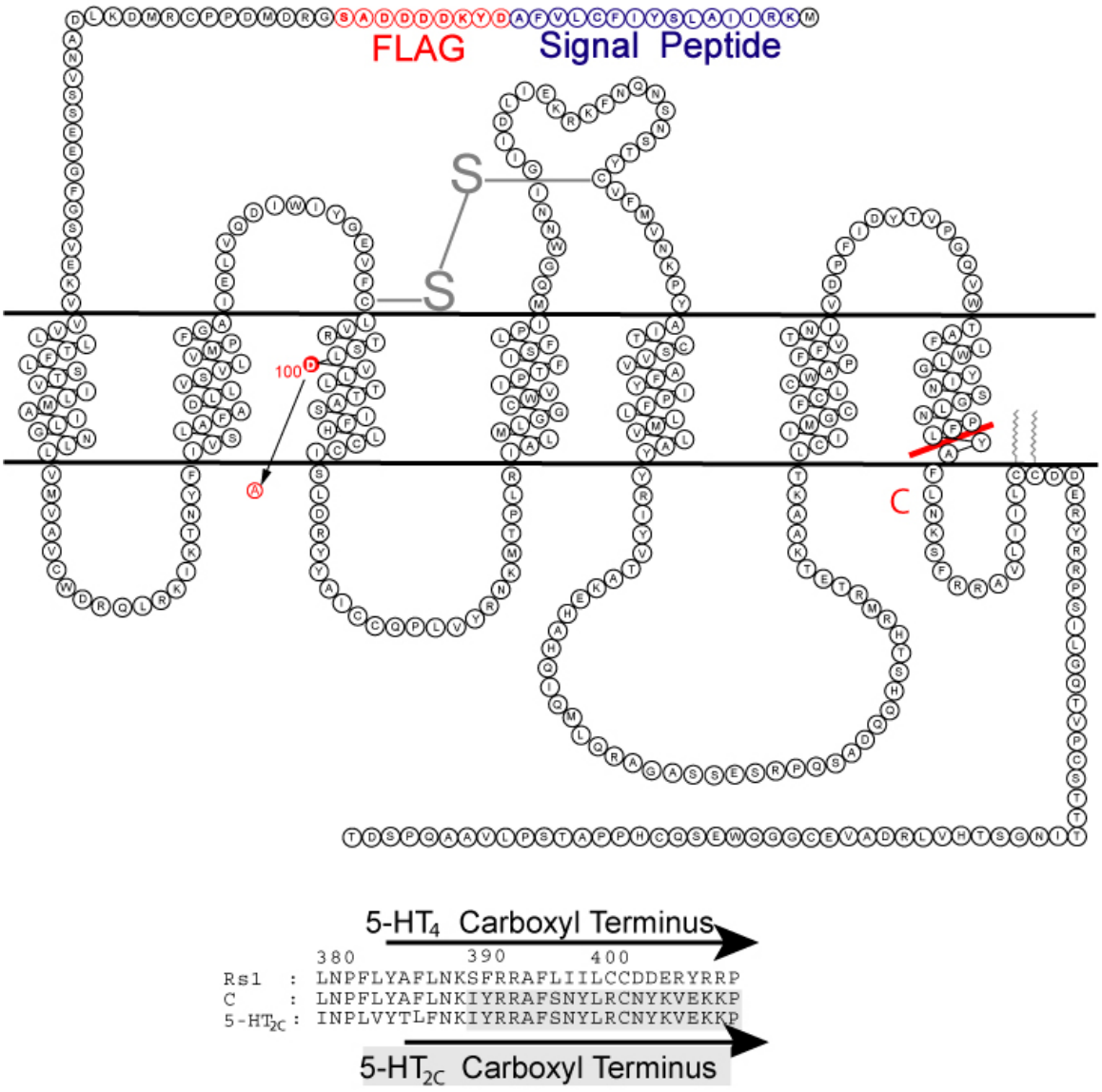


Figure 35: High-resolution representation of Rs1-C-5HT$_{2C}$ chimera.
All modifications were made on Rs1 (Figure 1). Red lines indicate the junctions of chimeras. The amino acids exchanged are shown by the amino acid alignment.

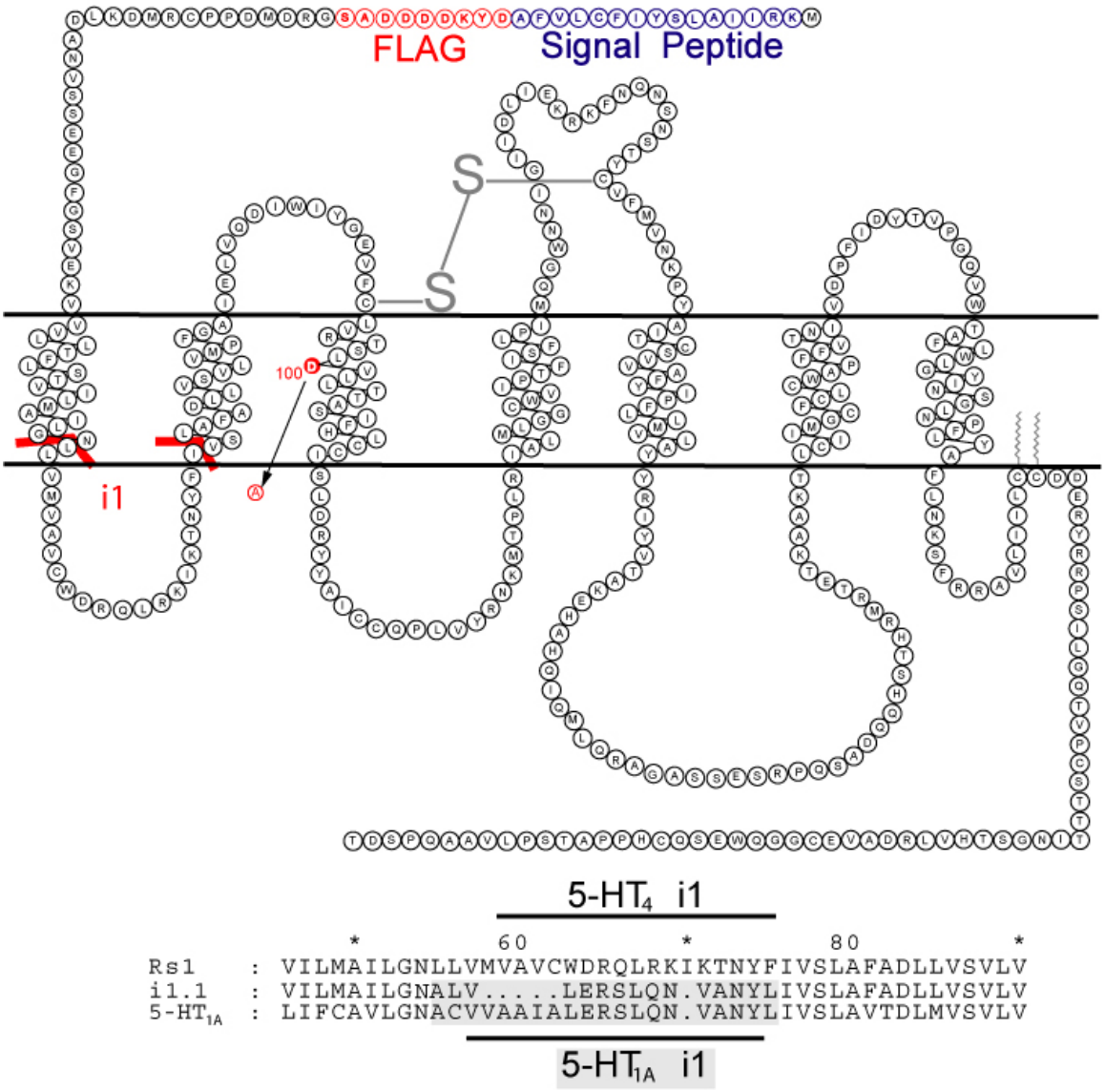


Figure 36: High-resolution representation of Rs1-i1-5HT$_{1A}$ chimera.
All modifications were made on Rs1 (Figure 1). Red lines indicate the junctions of chimeras. The amino acids exchanged are shown by the amino acid alignment.

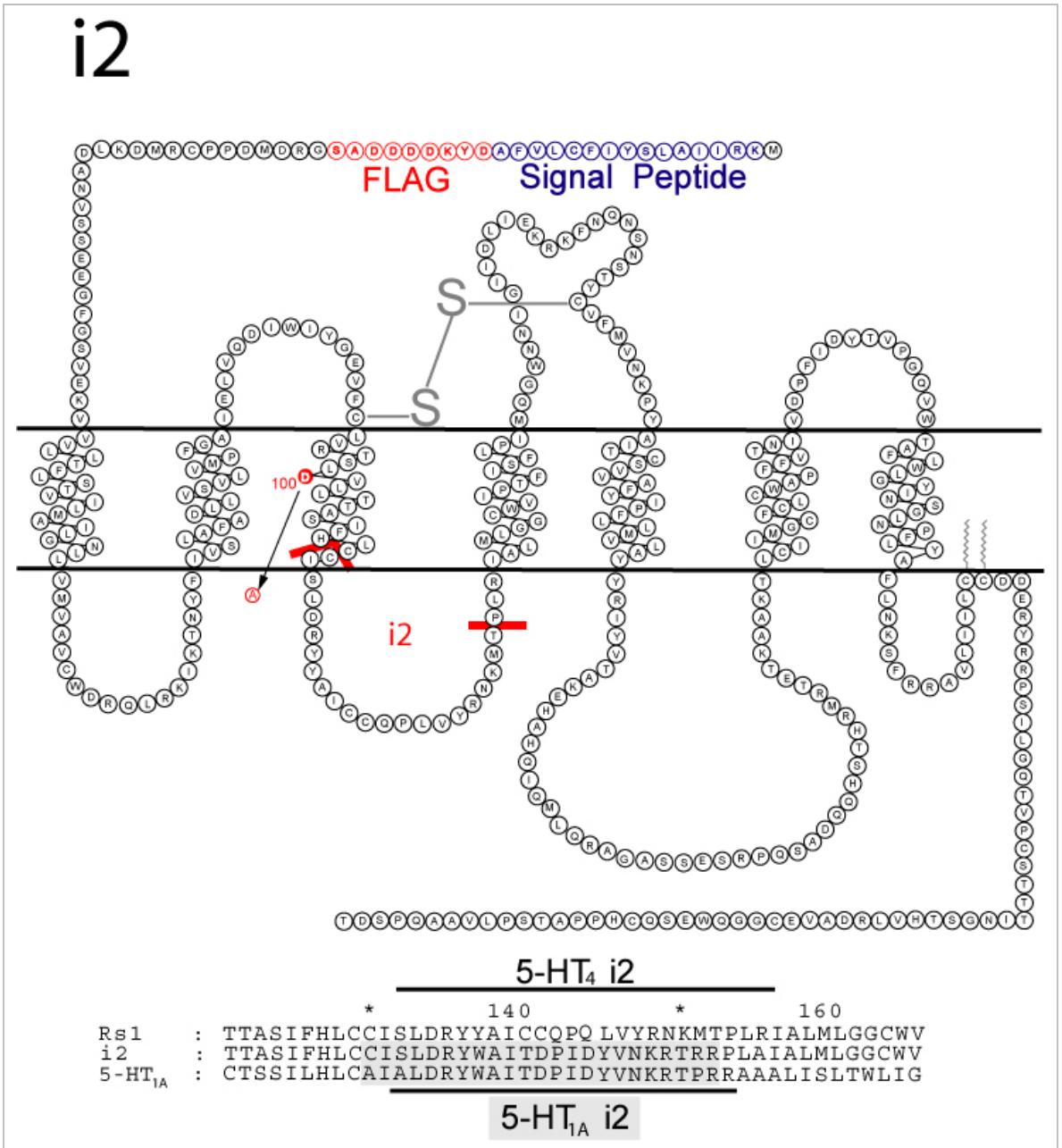


Figure 37: High-resolution representation of Rs1-i2-5HT$_{1A}$ chimera.
All modifications were made on Rs1 (Figure 1). Red lines indicate the junctions of chimeras. The amino acids exchanged are shown by the amino acid alignment.

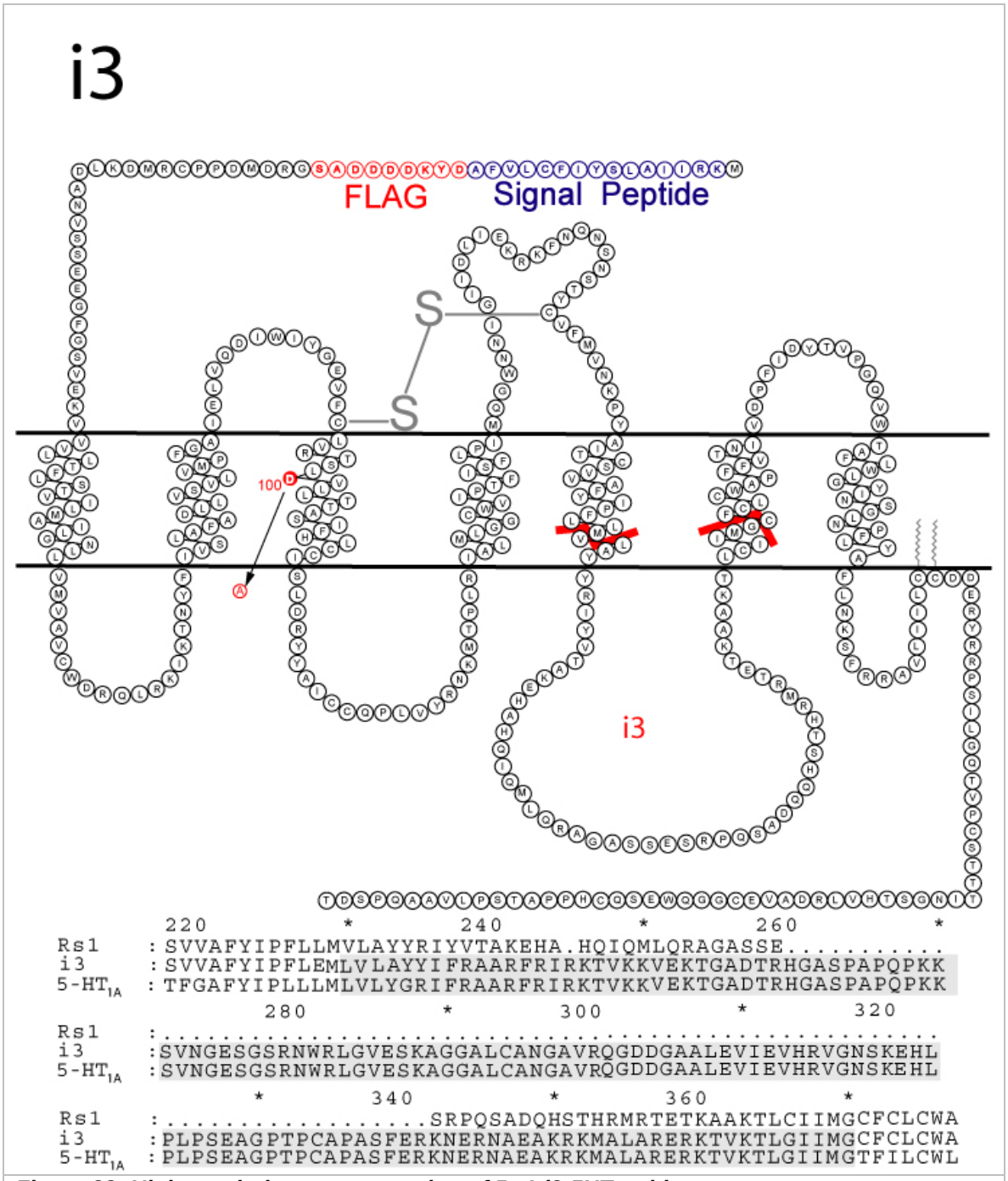


Figure 38: High-resolution representation of Rs1-i3-5HT$_{1A}$ chimera.
All modifications were made on Rs1 (Figure 1). Red lines indicate the junctions of chimeras. The amino acids exchanged are shown by the amino acid alignment.

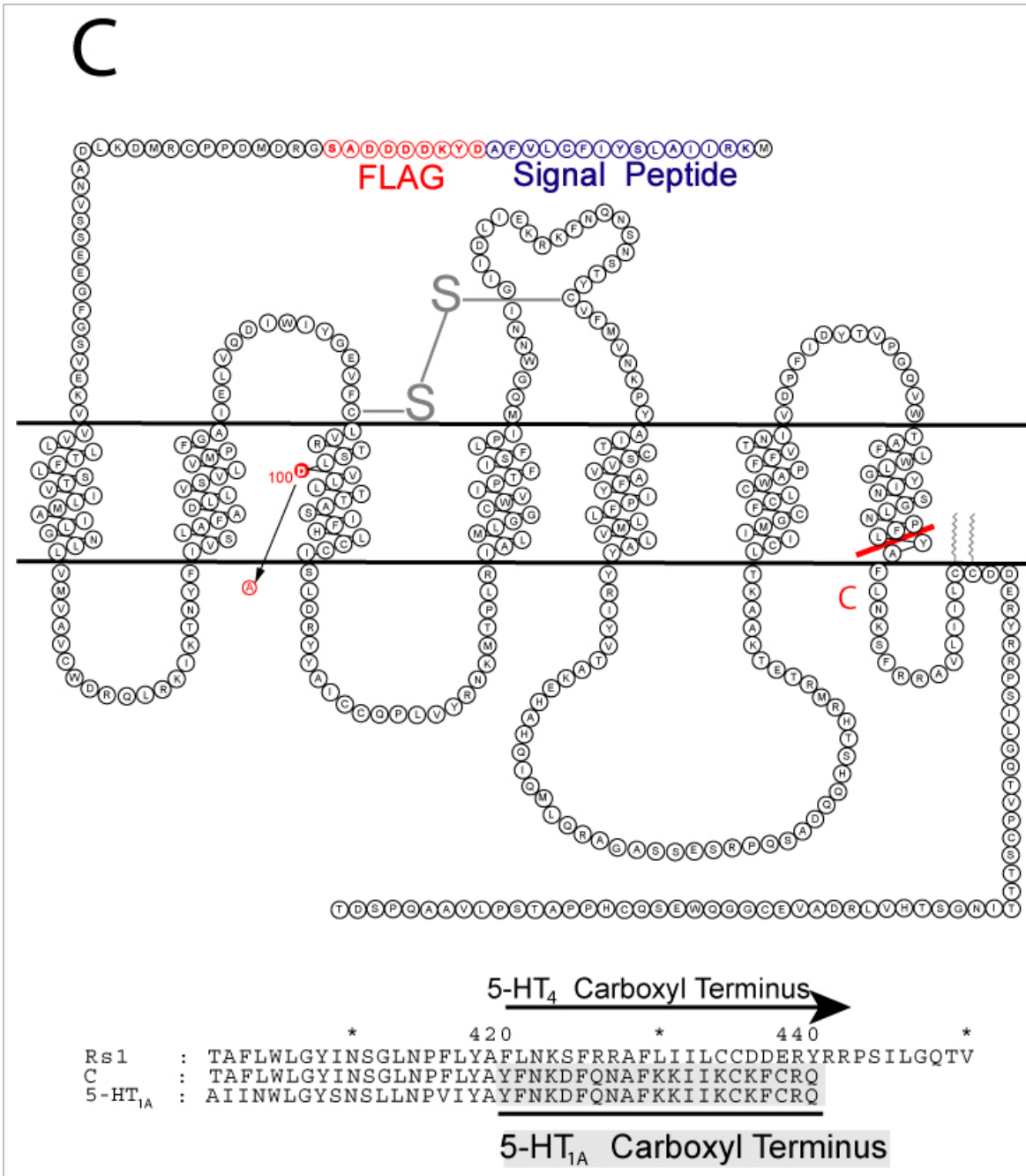


Figure 39: High-resolution representation of Rs1-C-5HT$_{1A}$ chimera.
All modifications were made on Rs1 (Figure 1). Red lines indicat the junctions of chimeras. The amino acids exchanged are shown by the amino acid alignment.

CHAPTER 9: MATERIALS AND METHODS

9.1 Plasmid Construction

9.1.1 Constructing human 5-HT_4 mutant cDNA and Rs1-5-HT_{1A}, and Rs1-5-HT_{2C} chimeras

The human 5-HT_4 receptor cDNA (a gift from Dr. Bryan Roth, University of North Carolina) was used in all experiments. To improve expression and allow detection of the receptor, we added a signal peptide from influenza hemagglutinin (Guan *et al.* 1992) and a FLAG epitope (DYKDDDDA) at the N-terminus. 5-HT_4 was then PCR-subcloned with the primers ATCGATCGgcggccgcGTGAGCAAG-GGCGAGGAGCTGTTC and ATCGATCGgcggccgcCTAAGTGTCACTGGGCTG-AGCAGCC into the Not1 restriction site (gcggccgc) of pUNIV-5-HT_{2C}-INI plasmid to replace the 5-HT_{2C}-INI (a gift from Dr. Bryan Roth) in frame of the signal peptide and the FLAG epitope. The receptor was then mutated ($D^{100}A$) with a Quick-Change site-directed mutagenesis kit (Stratagene, La Jolla, CA) with primer GTCTTGTTCGGACATCTCTG**gcc**GTCCTGCTCACAACGGCATCG (Figure 6). The following mutant sense primers were used: 5-HT_4-$D^{66}A$, TTCATTGTATCTCTTGCTTTTGCG**gca**CTGCTGGTTTCG-GTGCTGGTGATG; 5-HT_4-$D^{66}N$, TTCATTGTATCTCTTGCTTTTGCG**aac**CTGC-TGGTTTCGGTGCTGGTGATG, and 5-HT_4-$W^{272}A$, GTTGCTTCTGCCTCTGCT-GGGCGCCA**gcc**TTTGTCACCAATATTGTGG. The sense primer used to replace

the carboxyl chimera for Rs1.1 (Table 1) was AGTTACTCTTCC**gcg**GCCGCGA-ATTCAGTGGATCCACTAGTAAC. The Rs1-5-HT_{1A} and Rs1-5-HT_{2C} chimeras were made by PCR fusion. The mutations are indicated by boldface, lower case letters.

9.1.2 Constructing Internal Ribosomal Entry Sites

To better control the level of RASSL expression in a locus, I made tandem repeats of a series of IRES modules characterized earlier: GTX, ICS1, and ICS2 (Chappell *et al.* 2000; Owens *et al.* 2001). Oligonucleotides of five-copy IRES modules separated by spacers and flanked by 5' BamH1 and 3' Bgl II sites were cloned into the Bgl II site of pcDNA3, and tandem repeats of IRES modules were made in the plasmid (Figure 5A). Similarly, one repeat IRES module was cloned into the Bgl II site between DsRed2N1 and GFP in pDsRed2N1-GFP. BamH1-Bgl II fragments from pCDNA3 IRES modules were sub-cloned into the 3' Bgl II site 3' of the pDsRed2N1-IRES module-GFP, thus allowing for directional addition of IRES modules (Figure 5b). The sub-cloning was repeated to reach 101 copies.

9.2 Cell line maintenance and electroporation

Early-passage (≤20) HEK293 cells were maintained in high-glucose DMEM (Invitrogen, Carlsbad, CA) supplemented with sodium pyruvate (Invitrogen) and 10% Fetalplex (Gemini Bio-Products, West Sacramento, CA). Receptors were electroporated into HEK293 cells as described (Dumuis *et al.* 1988). The electroporated cells were reconstituted into a suspension using DMEM with 10% heat-inactivated, dialyzed fetal bovine serum (Thermo-Fisher Scientific, Logan, UT). The transfection efficiency was monitored by flow cytometry, and the cell-surface expression of the receptor was determined by FLAG ELISA the next day.

9.3 Drugs

5-HT, isoproterenol, and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma-Aldrich (St. Louis, MO). Cisapride (cis-N-[1-[3-(4-fluorophenoxy), Zacopride ((S)-N-(1-azabicyclo[2.2.2]oct-3-yl)-4-amino-5-chloro-2-methoxybenzamide monohydrochloride), GR113808 ((1-[2-(methylsulphonylamino) ethyl]-4-piperidinyl)methyl 1-methyl-1H-indole-3-carboxylate maleate), GR1254875 (Fluoro-2-methoxy-[1-[2-(methylsulfonyl)amino)ethyl]-4-piperidinyl]- 1H-indole-3-methylcarboxylate sulfate), RS23597-190 HCl (3 (piperidine 1 yl)propyl 4 amino 5 chloro 2 methoxybenzoate hydrochloride), RS39604 HCl (1 [4 amino 5 chloro 2 (3,5 dimethoxybenzyloxy)phenyl] 3[1 [2 [(methylsulfonyl)amino]ethyl] 4 piperidinyl] 1

propanone), and RS67333 HCl (1-(4-amino-5-chloro-2-methoxy-phenyl)-3-(1-butyl-4-piperidinyl)-1-propanone) were from Tocris (Bristol, UK). Human parathyroid hormone peptide (amino acids 1–34) was from Bachem Biosciences (King of Prussia, PA). RO110-0235 was generously donated by Renee Martin (Roche, Palo Alto, CA).

9.4 Measuring cell-surface expression by FLAG ELISA

Cell-surface receptor expression was measured with a FLAG ELISA method as described (Scearce-Levie *et al.* 2005). Cells seeded in poly-D-lysine-coated 96-well plates were fixed with 100 µl of 4% paraformaldehyde for 10 min at room temperature, washed, and stained with 100 µl of staining buffer (DMEM, 10% FBS, and 1 mM $CaCl_2$) containing anti-FLAG M1 antibody (1:1000; Sigma-Aldrich) for 1 h at 25^{o}C. The samples were washed three times with wash buffer (PBS and 1 mM $CaCl_2$) and stained with 100 µl of staining buffer with rat anti-mouse Ig G antibody conjugated with horseradish peroxidase (1:1000; Bio-Rad Laboratories, Hercules, CA). After 30 min, the samples were washed with wash buffer, placed on a rocker for 10 min, and washed again. This process was repeated two more times. 200 µl of 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) liquid substrate (Sigma-Aldrich) was added to the samples. After rocking for 30 min, 200 µl of the substrate was transferred to new 96-well plates, and optical density was measured with HTRF capable Victor 3 (PerkinElmer, Waltham, MA)

at 405 nm. All samples contain three technical replicates, and all experiments were repeated at least three times.

9.5 Determination of the cAMP production in intact cells

To improve assay consistency and minimize pipetting error in the 384-well plates, we modified the protocol for the High-Range HTRF assay (CisBio International, Bagnols-sur-Cèze, France) so that the cells were seeded, stimulated, and lysed in 96-well plates. The lysate was then used in place of the live cells for the determination of the cAMP production. Briefly, the cells were electroporated as above. After the cell density was confirmed by Beckman Z2 Coulter Particle Counter and Size Analyzer, 10^5 cells were seeded onto 96-well plates pre-coated with poly-d-lysine. The next day, cells in backup 96-well plates were counted to determine the amount of lysate to use for the cAMP assay. The cells were stimulated with 50 µl of 0.75 mM IBMX in KRBG buffer (Sigma-Aldrich) for 10 min at 25^{o}C. They were then treated with 25 µl of agonists at 37^{o}C for 10 min and lysed with 25 µl of lysis-conjugate buffer (CisBio International). Isoproterenol (1 nM) was used to stimulate β_2-adrenergic receptor, as an internal control for each cAMP assay point. In place of the live cells, 10 µl of lysate (10^5 cells) was transferred to white opaque, narrow-volume, 384-well microplates (Greiner bio-one). The recommended protocol for the High-Range HTRF assay was then followed to set up the HTRF assay. Briefly, 5 µl of cAMP-d2 and anti-cAMP-

Cryptate solution were then added to each well. After incubation at room temperature for 1 h, the reaction was detected with an HTRF-capable Victor 3.

9.6 Determination of IP1 production in intact cells

A modified version of the IP1 protocol was used (CisBio International). HEK293 cells were washed once with calcium-free PBS, dissociated from flasks with cell dissociation buffer (Invitrogen). HEK293 ($5x10^6$), and electroporated as described above. Then, 10^5 cells were placed in DMEM supplemented with 10% decomplemented, dialyzed FBS, and seeded onto 96-well plates coated with poly-D-lysine. The next day, the cells were stimulated with agonists in 50 µl of 1x stimulation buffer for 30 min at 37^oC and lysed for 10 min with 9 µl of lysis/detection buffer. Then, 14 µl of lysate was added to 384-well plates and subjected to High-Range HTRF assay as described above, except that 3 µl of cAMP-d2 and anti-cAMP-Cryptate solution were added to each well.

9.7 Fluorometric imaging plate reader assay to measure calcium mobilization

To measure calcium mobilization, 4.8 µg of receptors cDNA, 0.6 µg of DsRed plasmid, and 0.6 µg of human bombesin receptor cDNA were electroporated into $5x10^6$ HEK293 cells as above (Dumuis *et al.* 1988). Hank's balanced salt solution

(10 ml) with 20 mM HEPES, 0.25 mM probenecid acid (Sigma-Aldrich), and 2% pluronic acid (Sigma-Aldrich) was added to each bottle of Calcium 4 (Molecular Devices, Sunnyvale, CA), and 100 µl of the resulting solution was added to each well for 1 h at 37^{o}C before measurement. Assays were performed with a FLEX Station (Molecular Devices), with excitation of 485 nm, emission of 525 nm, and cut-off of 515 nm, as recommended by the manufacturer.

9.8 G_i Assay

G_i signaling was examined in cells transfected with 1.5 µg of receptors cDNA, 0.6 µg of human dopamine 1 receptor cDNA, and pcDNA3 (up to 6 µg). The co-transfectants were stimulated first with 100 µl of KRBG buffer containing IBMX for 10 min at room temperature and then with 50 µl of PBS containing 10 µM Apomorphine (agonist for the dopamine 1 receptor) and 10 µM Zacopride for 10 min at 37^{o}C. The cells were lysed in 50 µl of lysis buffer, and 5 µl of lysate was used in the HiRange HTRF assay.

9.9 Testing efficacy of modular synthetic ribosomal entry sites and single plasmid tetracycline inducible system by flow cytometry

To analyze the translational efficiencies of the IRES modules, multiple cells lines are transiently transfected, and analyzed by flow cytometry. The IRES sequences were tested in a reporter plasmid in tissue culture cell lines by flow cytometry. The ratio of the reporter gene downstream of IRES (GFP) to another reporter gene upstream of IRES (red fluorescent protein DsRed2N1) is used to estimate efficiency (Chappell *et al.* 2000; Owens *et al.* 2001). Thus, more efficacious IRES would increase the ratios of eGFP to DsRed2N1 (Figure 4a).

9.10 Data analysis

cAMP and IP1 values were analyzed with GraphPad Prism 4 (GraphPad Software, San Diego, CA). Calcium mobilization results were analyzed with SoftMax Pro v5 (Molecular Devices). Statistical significance was determined with paired Student's *t* tests.

CURRICULUM VITAE

(PETER) WEI CHUN CHANG

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EDUCATION

PhD, Pharmaceutical Sciences and Pharmacogenomics, 2001–2007
University of California, San Francisco
Thesis Advisor: Bruce R. Conklin

MS, Biochemistry 1992–1994
University of California, Riverside

BS, Biochemistry 1988–1992
University of California, Riverside

RESEARCH AND PROFESSIONAL EXPERIENCE

DOCTORAL RESEARCH Sept. 2002–

- Conducted high throughput assays to identify agonists to activate G_s- and G_q-signaling of a series of designer receptors based on the human 5-HT_4 receptor.
- Modified the G protein signaling of designer receptor via point mutagenesis and domain swapping with 5-HT_{2C} and 5-HT_{1A} to make a series of G_s- and G_q-coupled RASSLS (receptors activated solely by synthetic agonists) that can be activated for tissue engineering and gene therapy.
- Engineered synthetic internal ribosomal entry sites and a single-plasmid tetracycline inducible system with insulator sequences and ribosomal skip motifs to better control the expression of the designer receptor in embryonic stem cells.
- Explored the role of G protein signaling in the cardiac differentiation of mouse embryonic stem cells

INFECTIOUS DISEASES
Protein Design Labs
Scientist IV July 2000–Sept. 2001

- Routinely performed knockouts of bacterial candidate genes and conducted differential fluorescence induction in *Stalphylcoccus aureus* and *Streptococcus aureus* to identify possible therapeutic genes.

HIV GENE THERAPY
Systemix-Novartis, Inc.
Scientist III Oct. 1996–July 2000

- Engineered retroviral and lentiviral vectors to optimize gene delivery into human T-cell lines, human stem cells, and peripheral blood lymphocytes.
- Engineered and evaluated cell-surface biomarkers based on muscle, skeletal, receptor tyrosine kinase and epidermal growth factor receptor to avoid bioactivity *in vivo*.
- Studied the effectiveness of pre-integration (chemokines and novel pol anti-sense chimeras from HIV-1 strains Eli and HXB3 on multiple strains of HIV) and post-integration (Rev M10) strategies against HIV infection

- Conducted pre-clinical safety studies of retroviral HIV gene therapies with tumorgenicity assay, clonogenicity assay, northern analysis, flow cytometry and PCR.

GENE DISCOVERY

L.X.R. Biotechnology, Inc.

Research Associate July 1994–Oct. 1996

- Investigated anion-channel inhibitors as potential anti-apoptotic compounds by performing bioassays, ELISA, and antigen capture on mammalian cell lines, isolated mouse thymocytes, and splenocytes.
- Made recombinant constructs of novel genes isolated from a mammalian fibroblast cell line and its human homologues. Generated MBP and GST fusion proteins for novel genes.
- Examined potential function of novel genes in mammalian cell lines via Northern hybridization and other standard cell biology techniques and instruments.

TECHNICAL SKILLS

Tissue Culture: Extensive experience with mammalian tissue culture cell lines, primary human lymphocytes and macrophages. Routine transfection, transduction, bead selection and cloning. Working experience with infectious HIV-1 in BL3 environment and microscopy

Cellular Techniques: Expertise on measuring cAMP accumulation (HTRF) and calcium mobilization (Calcium 4). Extensive experience with immunological procedures such as immunocytochemistry, immunoassays, Western blotting, and intracellular assays for protein expression or DNA content, ELISA, FACS, Calibur, and Sorter. Working experience with high definition fluorescence microscopy.

Molecular Techniques: Expertise in subcloning, PCR cloning, RT-PCR, high-output PCR, RNA isolation, and Northern hybridization. Working experience with genomic DNA isolation, Southern, and Western hybridization, and optimizing protein expression.

Computer Programs and Databases: Expertise of CLONE-MANAGER, GENEDOC, DNA Strider, Vector-NTI, and GENEWORK for sequence analysis. Extensive use of EXCEL, Soft-Max, and Prism4 for data analysis. Experience with OpenLab and Metamorph for acquiring and analyzing data.

PUBLICATIONS

RESEARCH ARTICLES

Chang WC, Ng J, Nguyen T, Pellissier L, Claeysen S, Conklin BR. Controlling the G_s-, G_q- and Basal Signaling of the human 5-HT_4 receptor. (Submitted)

Hsiao E, Boudignon B, C**hang WC**, Bencsik M, Peng J, Manalac C, Halloran B, Conklin BR, Nissenson RA. A stony mouse: Osteoblast expression of an engineered Gs-coupled receptor causes a massive increase in bone mass. (Submitted)

Conklin BR, Hsiao E, Claeysen S, Srinivasan S, Forsayeth J, Guettier J, **Chang WC**, McCarthy K, Nissenson R, Wess J, Bockaert J , Roth BL. Evolving new hormonal pathways by engineering receptors activated solely by synthetic ligands (RASSLs). In preparation

Pellissier L, Cochet M, Quefeullou E, Marion P, **Chang WC**, Bockaert J, Claeysen S and Dumuis A. Functional selectivity of 5-HT4 receptor ligands on Gs, Gq and ERK coupling. In preparation

Uchida N, Sutton RE, Friera AM, He D, Reitsma MJ, **Chang WC**, Veres G, Scollary R, Weissman I (1998) HIV, but not murine leukemia virus vector mediate high efficiency gene transfer into freshly isolated Go/G1 human hematopoetic stem cells. *Proc. Natl. Acad. Sci. USA* 95:11939–11944.

Melkonyan HS, **Chang WC**, Shapiro JP, Mahadevappa M, Fitzpatrick PA, Kiefer MC, Tomei D, Umansky SR (1997) SARP's: A family of secreted apoptosis-related proteins. *Proc. Natl. Acad. Sci. USA* 94:13636–13641.

ABSTRACTS

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HONORS AND AWARDS

- American Heart Association Pre-doctoral Fellowship (2004–2006)
- Merck Scholar (2004–2006)

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