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Engineering GPCR signaling pathways with RASSLs

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Abstract

We are creating families of designer G-protein-coupled receptors (GPCRs) to allow for precise spatiotemporal control of GPCR signaling *in vivo*. These engineered GPCRs, called receptors activated solely by synthetic ligands (RASSLs), are unresponsive to endogenous ligands but can be activated by nanomolar concentrations of pharmacologically inert, drug-like small molecules. Currently, RASSLs exist for the three major GPCR signaling pathways (G_s, G_i, G_q). These new advances are reviewed here to help facilitate the use of these powerful and diverse tools.

G-protein-coupled receptors (GPCRs) are an ideal vehicle for engineering synthetic signaling systems. These receptors function as signaling switches throughout the body and regulate virtually every physiological response¹⁻³. GPCRs are also the largest gene family targeted for drug discovery⁴. GPCRs stimulate a variety of G protein pathways; for example, G_s stimulates

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Highlighted Papers (numbers refer to order in citations) 5. Coward et al., PNAS 1998: First use of the term RASSL and first description of a RASSL that responds to a drug with nanomolar potency allowing potential *in vivo* use.

8. Armbruster et al., PNAS, 2007. First application of directed evolution for engineering RASSLs/DREADDs that respond to an experimentally inert ligand (CNO).

10. Redfern et al., Nature Biotech 1999: First use of a RASSL in a transgenic animal. Expression of hRO-i (previously known as Ro1) in the heart allows control heart rate within seconds after administration of spiradoline.

11. Mueller et al., Nature 2005. First use of a RASSL to decode a sensory pathway *in vivo*. RASSL expression in the sweet or bitter taste buds of transgenic mice allows the RASSL ligand (spiradoline) to induce a sweet (attractive) or bitter (aversive) responses.

cAMP production, G_i inhibits cAMP production, and G_q stimulates phospholipase C and releases intracellular calcium stores. Since GPCRs have a relatively simple modular design and are encoded by small genes (usually <1.5 kb), engineered GPCRs can be easily transferred without loss of their functionality into different tissues and species. Finally, designer GPCRs could be useful for regulating physiologic processes and engineering tissues with stem cells and other technologies.

Attempts to engineer GPCRs that are activated solely by pharmacologically inert drug-like molecules have met with varying degrees of success (Table 1). Engineered receptors and engineered receptor/ligand pairs have been created by several approaches and bear different names—receptors activated solely by synthetic ligands (RASSLs)⁵, therapeutic receptor-effector complexes (TREC)s⁶, neoceptors⁷, and designer receptors exclusively activated by designer drugs (DREADDs)⁸. Here we will refer to them as RASSLs, and we propose a consensus nomenclature (Table 1) for those in widespread use or in development. This nomenclature links the name of the parent receptor to the major G-protein signaling pathway activated by the receptor.

Engineering RASSLs by directed mutagenesis

In the first attempt to make a designer GPCR, Strader and colleagues developed a series of compounds to selectively activate a mutant version of the α_2 -adrenergic receptor (α_2 -AR) that was unresponsive to its natural hormone⁹. This group focused on D113^{3,32}, which is conserved among all biogenic amine GPCRs and is critical for binding terminal amine groups (Fig. 1). Mutating D113^{3,32} to Ser greatly reduced activation of the α_2 -AR by biogenic amines. Remarkably, this mutation enabled the newly synthesized butanone derivative, 1-(3',4'-dihydroxyphenyl)-3-methyl-1-butanone (L-185,870), to activate the mutant receptor but not the wildtype (WT) receptor, albeit with relatively low potency ($EC_{50}=118 \mu\text{M}$)⁹. Although this original report was inspiring, the synthetic agonist had low affinities and unknown pharmacokinetics that rendered it impractical for *in vivo* use.

The first engineered receptor or RASSL activated by an agonist with nanomolar affinity suitable for *in vivo* use was reported by Coward et al.⁵, who took advantage of potent synthetic drugs originally developed as potential analgesics, such as μ -opioid receptor (KOR) agonists (e.g., spiradoline). The first RASSL was created by introducing mutations in the KOR that abrogated signaling via the natural peptide ligands, yet preserved stimulation by spiradoline⁵. This engineered human RASSL (hRO-i; h=human, R=RASSL, O=Opioid, i= G_i ; referred to as Ro1⁵, a G_i -coupled receptor, has been expressed in at least six tissues (Fig. 2) in transgenic animals. Diverse phenotypes were induced, including ligand-dependent heart-rate modulation¹⁰, bitter and sweet taste sensations^{11,12}, and ligand-independent cardiomyopathy¹³, hydrocephalus¹⁴, and osteopenia¹⁵. These exciting results fueled efforts to develop RASSLs with improved ligand pharmacology and a greater range of signaling responses.

Once scientists understood that RASSLs could be designed to work with existing drugs, new RASSLs soon emerged from studies of a wide variety of receptors, including the 5-HT₄ serotonin¹⁶, α_2 -adrenergic⁶, H₁-histamine¹⁷, A₃ adenosine⁷, 5-HT_{2A}-serotonin¹⁸, and MC4-melanocortin¹⁹ receptors. In most, key residues essential for binding the native ligand were targeted by site-directed mutagenesis. For example, introduction of the D100^{3,32}A mutation (analogous to the D113^{3,32}A mutation in the α_2 -AR⁹) into the G_s -coupled human 5-HT₄ serotonin receptor abolished the ability of 5-HT to activate the receptor but did not affect the activity of many synthetic agonists, including carbazimidamides, benzamides, benzimidazolones, and aryl ketones; this RASSL, previously known as Rs1, will be referred to as hRS-s¹⁶. Many of these synthetic agonists have drug-like properties, nanomolar affinities,

and readily penetrate the central nervous system and can therefore be used effectively *in vivo*^{16,20}. In addition, hRS-s is activated by antagonists of the 5-HT₄ receptor, which have fewer *in vivo* side effects than 5-HT₄ receptor agonists. Intriguingly, when expressed in osteoblasts of young mice, hRS-s dramatically alters bone growth *in vivo*²¹, presumably due to constitutive activation of the G_s pathway. These experiments provide valuable insights into the specific cellular and temporal factors that allow G_s signaling to induce bone growth.

This same mutagenesis approach of replacing conserved residues of the binding pocket with alanines has been applied to other biogenic amine receptors. Thus, mutation of a conserved serine residue in the fifth transmembrane region (S204^{5.46}A) led to a sizeable loss of affinity and efficacy of (-)-adrenaline at α_2 -adrenoceptors (G_{i/o}-coupled receptors), while the mutated receptor could still be activated by synthetic agonists (UK14304, clonidine) or even by antagonists of the WT receptor (atipamezole, SKF86466)²². Similarly, a G_{q/11}-coupled RASSL was obtained by introducing the F435^{6.55}A mutation into the histamine H₁ receptor¹⁷. This RASSL could be activated by high concentrations of endogenous histamine and had improved affinity and potency for 2-phenylhistamines, a class of synthetic H₁R agonists (2-[3-chlorophenyl]histamine). Interestingly, alternative substitutions at this position (F435^{6.55}) resulted in RASSLs with different levels of constitutive activity; F435^{6.55}A (hRH-s) had the lowest level of constitutive signaling.

Creating RASSLs by directed molecular evolution

Despite these noteworthy advances, first-generation RASSLs were not ideal for experimentation. The ligands of first-generation RASSLs activated endogenous receptors (e.g., μ -opioid, H₁-histamine, 5-HT₄-serotonin, MC4-melanocortin), and had low affinities for the mutated receptor (A3-adenosine neoreceptor; α -AR TREC; 5-HT_{2A} serotonin RASSL). Moreover, profound phenotypes induced by constitutive activity were observed upon RASSL overexpression *in vivo* (RO-i and RS-s). Finally, development of new RASSLs by repeated cycles of directed mutagenesis was labor intensive and did not consistently yield receptors with ideal agonist affinities or controlled levels of constitutive signaling. To overcome these inherent difficulties, we developed a generic approach to create a new class of RASSLs that have low constitutive activity and respond specifically to drug-like, pharmacologically inert small molecules⁸.

We used a well-established yeast mutagenesis system to produce hundreds of thousands of mutant hM₃ muscarinic receptors and screened them for signaling characteristics of an “ideal” RASSL⁸. After multiple rounds of mutagenesis and iterative screening, we isolated mutants that had lost the ability to respond to the natural ligand (acetylcholine) but gained the ability to respond with nanomolar potency to clozapine-*N*-oxide (CNO), a pharmacologically inert, bioavailable²³ synthetic compound (Fig. 3). This new class of RASSLs was designated DREADDs (ref. 6). We will refer to this first DREADD as “hRMD-q” (RASSL M₃ DREADD, G_q-coupled; referred to as hM3-D in ref. 6). The RMD-q receptor is insensitive to acetylcholine but activates the G_q pathway to induce calcium mobilization upon binding of CNO. Analogous mutations in the closely related M₄ muscarinic receptor, which is G_i-coupled and inhibits cAMP accumulation, led to another RASSL/DREADD that we call hRMD-i (referred to as hM4D⁸). Intriguingly, when activated by CNO, hRMD-i silenced hippocampal neurons via G α -mediated activation of G-protein inwardly rectifying K⁺ (GIRK) channels⁸. It is likely that hRMD-i will be widely used to induce neuronal silencing *in vivo* via indirect activation of GIRKs. More recently, (Guettier, J.M., et al abstract, International Group on Insulin Secretion, St. Jean Cap-Ferrat, France; 2007), made chimeras of the rat equivalent of the hRMD-q that incorporates the second and third intracellular loops of the G_s-coupled α_1 adrenergic receptor to create a G_s-coupled RASSL (rRMD-s, see table 1). Thus, CNO can be used to activate the G_s, G_i, or G_q signaling pathways, depending on which of the new RASSLs is utilized.

With the current DREADD-type RASSLs, only two point mutations were required to create hRMD-I and hRMD-q. By contrast, the rRMD-s required two point mutations and swapping of two intracellular loops. Creating other DREADD-type RASSLs by directed molecular evolution will likely require at least multiple point mutations based on our own unpublished experience (Pei et al, in preparation). Thus far, all of the point mutations have been found in or near predicted binding sites for orthosteric ligands.

The general method we devised evolves GPCR ligand specificity toward pharmacologically "inert" ligands (e.g., drug-like compounds without known molecular targets). This technique is likely to be widely used to create designer GPCRs, owing to the availability of strains of yeast (*Saccharomyces cerevisiae*) engineered to express and respond to human GPCRs^{24,25}. When these GPCR-expressing yeast are activated by an agonist, the signal induces the expression of a variety of selectable markers under control of a Fus-1 promoter. This system allows for the facile screening and optimization of millions of mutant GPCRs in a relatively short time²⁶. Dozens of human GPCRs have been expressed in yeast²⁷, thereby opening up the potential to create families of designer GPCRs activated by specific small molecules.

RASSLs as molecular switches for tissue engineering

RASSLs may be valuable in controlling growth and ensuring appropriate control of function for experimental or therapeutic tissue engineering. GPCR signaling is essential for the growth and differentiation of many tissues². For example, the 5-HT_{2B} serotonin receptor is required for cardiac development and cell-cycle progression²⁸⁻³⁰. Ectopic signaling via GPCRs can promote abnormal growth³, leading to human disease. For instance, drug-induced valvular heart disease may be caused by excessive stimulation of cardiac 5-HT_{2B} receptors³¹. One can envision the use of RASSLs to activate discrete signaling pathways to promote the proper growth and differentiation of engineered tissues.

Another potential use of RASSLs is to gain precise control of signaling in neurons and other tissues. Currently, this control in defined neuronal populations can be facilitated by expressing RASSLs in a neuron-specific manner. We reported that CNO-mediated activation of the G_i-coupled hRMD-i induces neuronal silencing when expressed in hippocampal neurons⁸. When expressed in hippocampal neurons, hRMD-q induces neuronal excitation and intracellular Ca⁺⁺ release in CNO-dependent fashion (Rogan and Roth, manuscript in preparation). Using these two engineered muscarinic receptors, one could gain precise bi-directional control of neuronal firing *in vitro* and *in vivo*. These modified receptors could also be used in other excitable tissues, such as cardiac pacemaker cells, where G_s stimulation speeds diastolic depolarization and accelerates heart rate, and G_i stimulation slows heart rate. Expression of different G_s (e.g., hRS-s or rRMD-s) and G_i (e.g., RO-i or hRMD-i) RASSLs in pacemaker cells could allow for the precise regulation of heart rate without affecting cardiac muscle function.

The importance of constitutive signaling by RASSLs

In studies of first-generation RASSLs expressed *in vivo*, constitutive signaling (constitutive activity) has often produced the most profound effects. Overexpression of a G_i-coupled RASSL (hRO-i) in cardiomyocytes led to cardiomyopathy¹³, while overexpression in osteoblasts led to osteoporosis¹⁵. Recently a G_s RASSL (hRS-s) expressed in osteoblasts induced marked bone growth¹⁵. Constitutive activity is a common property of native GPCRs^{32,33} and is essential for the normal function of certain GPCRs³⁴. Therefore, RASSLs with different levels of constitutive activity (high and low) will be needed to recapitulate normal GPCR functions. Because of the potential ligand-independent effects, RASSL expression ideally should be controlled through conditional expression systems (e.g., Tet or Cre). With these systems, a single RASSL transgenic line can be used to drive expression in diverse tissues with tighter

temporal control. The second-generation RASSLs (hRMD-q, hRMD-i, rRMD-s) were created to lack constitutive activity⁸, and thus far, their overexpression in mice has not elicited baseline phenotypes (Rogan, Roth, Guettier and Wess, unpublished observations). These second-generation RASSLs will be most useful for studies in which ligand-dependent effects (rather than baseline phenotypes) are sought.

Future directions

The RASSL field has undergone dramatic growth in the past decade, but many more challenges lie ahead. For instance, the ideal family of RASSLs would respond to a clinically approved, biologically inert drug (e.g., antibiotic or antiviral) that has no intrinsic effect on human cells, allowing tissue engineering without the drug safety studies needed for relatively new compounds such as CNO. The optimal series of RASSLs would also selectively couple to each of the GPCR pathways, including noncanonical pathways, such as those involving arrestins, GRKs, and intracellular kinases²⁹. Furthermore, each RASSL would have different constitutive responses, desensitization properties, and subcellular targeting that could be fine-tuned with simple mutations. For many of these goals, we will need spatiotemporal control of RASSL expression to allow for direct comparisons of RASSL actions that could be applicable to virtually any tissue. Several groups are now testing a variety of approaches (BAC transgenics, knock-ins, and inducible systems) to meet these new challenges and provide new tools to RASSL researchers. With these highly refined tools, biologists will have a better understanding of how to use RASSLs and GPCR signaling pathways for tissue engineering.

The cross-disciplinary nature of RASSL-related research fosters a highly collaborative community that makes protocols, reagents, and transgenic animals publicly available whenever possible. Even though individual members of the RASSL community initially created tools specifically for their own research, the potential uses of these tools go well beyond any individual project. Indeed, precisely because GPCR signaling is important to such a wide swath of biology, it is impossible for us to accurately predict how and where RASSLs will be ultimately used.

We anticipate that our RASSL delivery systems will be deployed for a wide range of tissue engineering applications in neurological disease, pain perception, immunology, bone metabolism, and diabetes. In each case, our efforts will provide enabling technologies to rapidly advance those fields. For instance, in many neurological diseases (e.g., Parkinson's disease), RASSLs may be useful for correcting the imbalance of neural pathways, in a manner that could complement the surgical/electrical approaches in current clinical practice. Similarly, many groups envision using tissue-engineering approaches to study pain perception pathways. RASSLs, which selectively modulate neuronal firing, should be ideal for this application. Although GPCRs are clearly important in bone metabolism, many key receptors signal via multiple pathways and exhibit constitutive signaling. RASSLs allow researchers to stimulate discrete signaling pathways in bone metabolism. Finally, in diabetes, GPCRs play a role in the growth, development, and function of insulin-secreting pancreatic β -cells³⁵. Dissecting the precise roles of different G-protein signaling pathways in β -cell function should be of considerable therapeutic interest.

Perhaps most importantly, the use of RASSL technology may shed light on relatively unknown aspects of GPCR signaling. For instance, many researchers are investigating nonclassical signaling responses of GPCRs, such as signaling by $G_{12/13}$, arrestins, receptor kinases, regulators of G protein signaling, Wnt receptor signals, and scaffolding proteins²⁹. It should be of interest to create two RASSLs that only differ in their ability to activate the arrestin pathways. Expression of these two RASSLs in the same spatial and temporal pattern would then allow determination of the true physiological roles of arrestin signaling.

Some of these non-G-protein signaling pathways could prove essential for robust tissue engineering and for uncovering the pathways responsible for stem cell differentiation. One can envision scenarios in which RASSLs are selectively expressed in different stem cell lineages and then activated (with an exogenous ligand or by overexpression) to determine which pathways are responsible for lineage choices and tissue differentiation.

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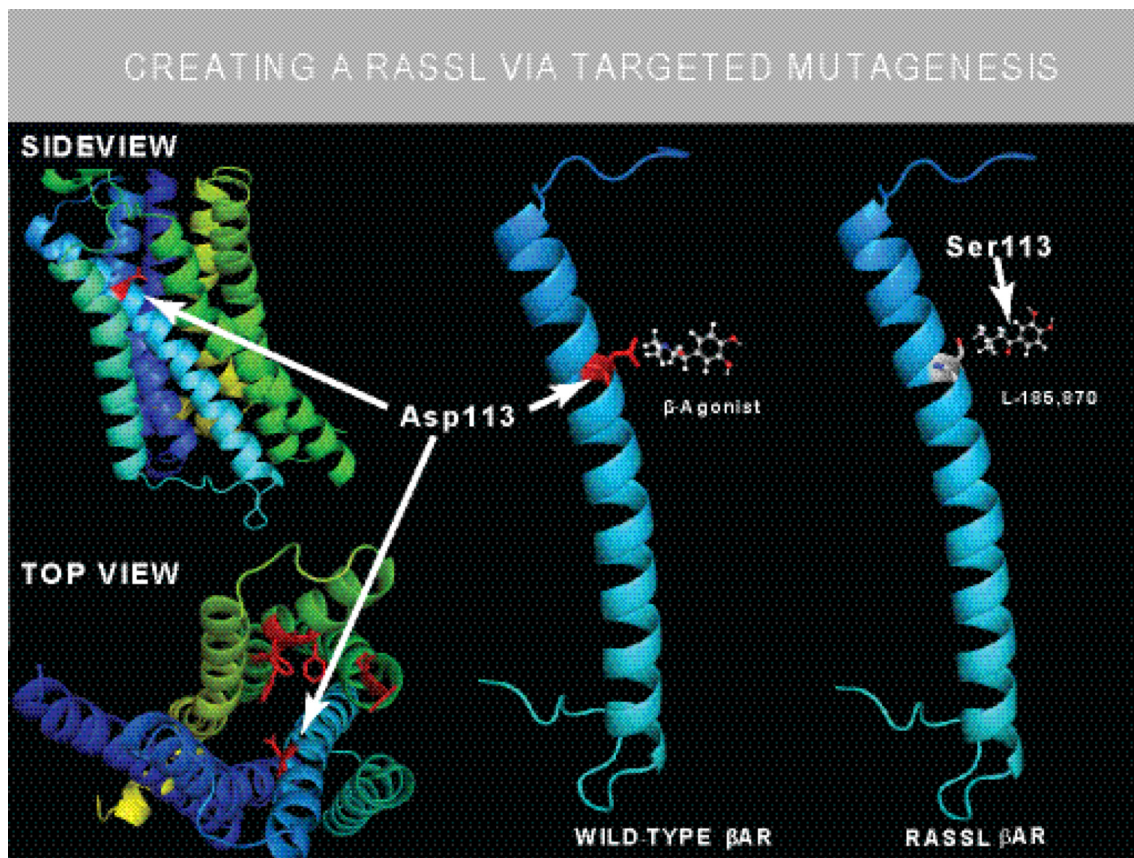
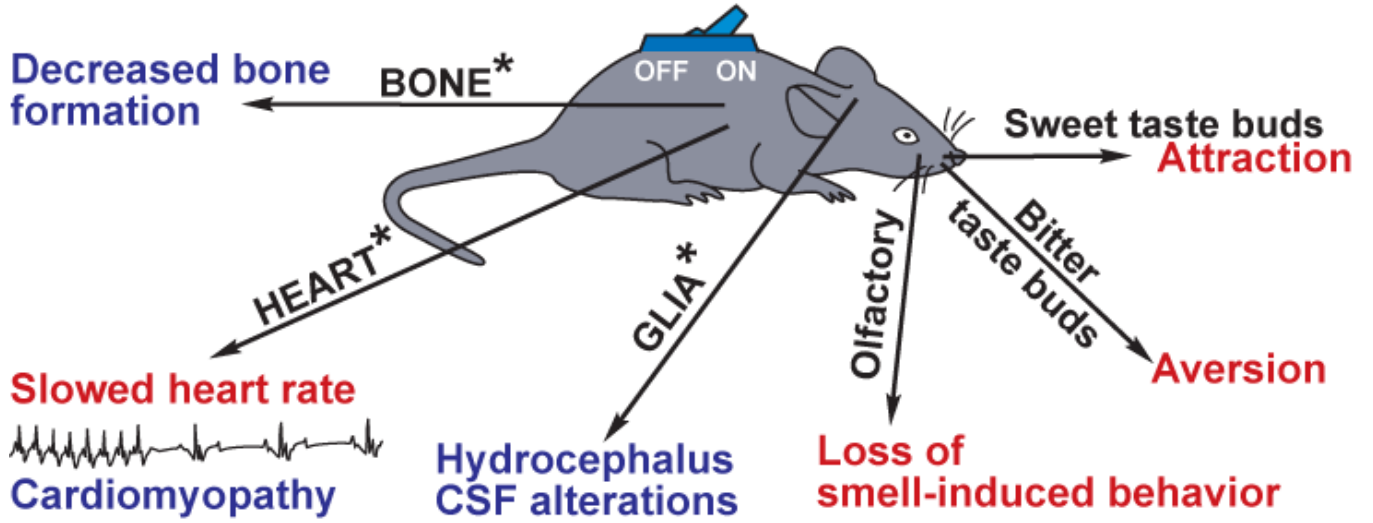


Figure 1. Creating RASSLs by targeted mutagenesis. A conserved residue(s) in the canonical binding pocket of biogenic amine receptors (e.g., adrenergic, serotonin, histamine) is mutated to eliminate the binding and activation of the receptor for the native ligand. A model using the coordinates of the β -adrenergic receptor structure³⁰ is used to illustrate this. In all biogenic amine GPCRs, the binding pocket is composed of a conserved aspartic acid (D113^{3,32} in the β -2-AR model; SIDE VIEW) and conserved aromatic and polar residues (TOP VIEW). Mutation of the highly conserved Asp to Ser (D113S) renders the β -2-AR insensitive to β -AR exogenous and endogenous agonists, such as isoproterenol, epinephrine, and norepinephrine. However, the D113S mutant receptor could be activated by the synthetic ligand L-185, 870. Such a targeted mutagenesis approach was used to create both peptidergic RASSLs (e.g., μ -opioid, MC4-melanocortin) and nonpeptidergic RASSLs (H1-histamine, 5-HT2A serotonin, 5-HT4 serotonin, β -2-adrenergic) (see text for details).

Tissue-specific Effects of hRO-i, a G_i-RASSL



Blue = Constitutive phenotype Red = Ligand induced phenotype

Figure 2. Myriad ligand-dependent and -independent phenotypes are induced by tissue-specific expression of a single RASSL. Ligand-induced phenotypes are noted in red; constitutive signaling-induced phenotypes are in blue. Asterisks indicate tissues in which RO1 expression results in embryonic or perinatal lethality. Conditional expression allows RASSL researchers to avoid embryonic lethality and analyze adult phenotypes.

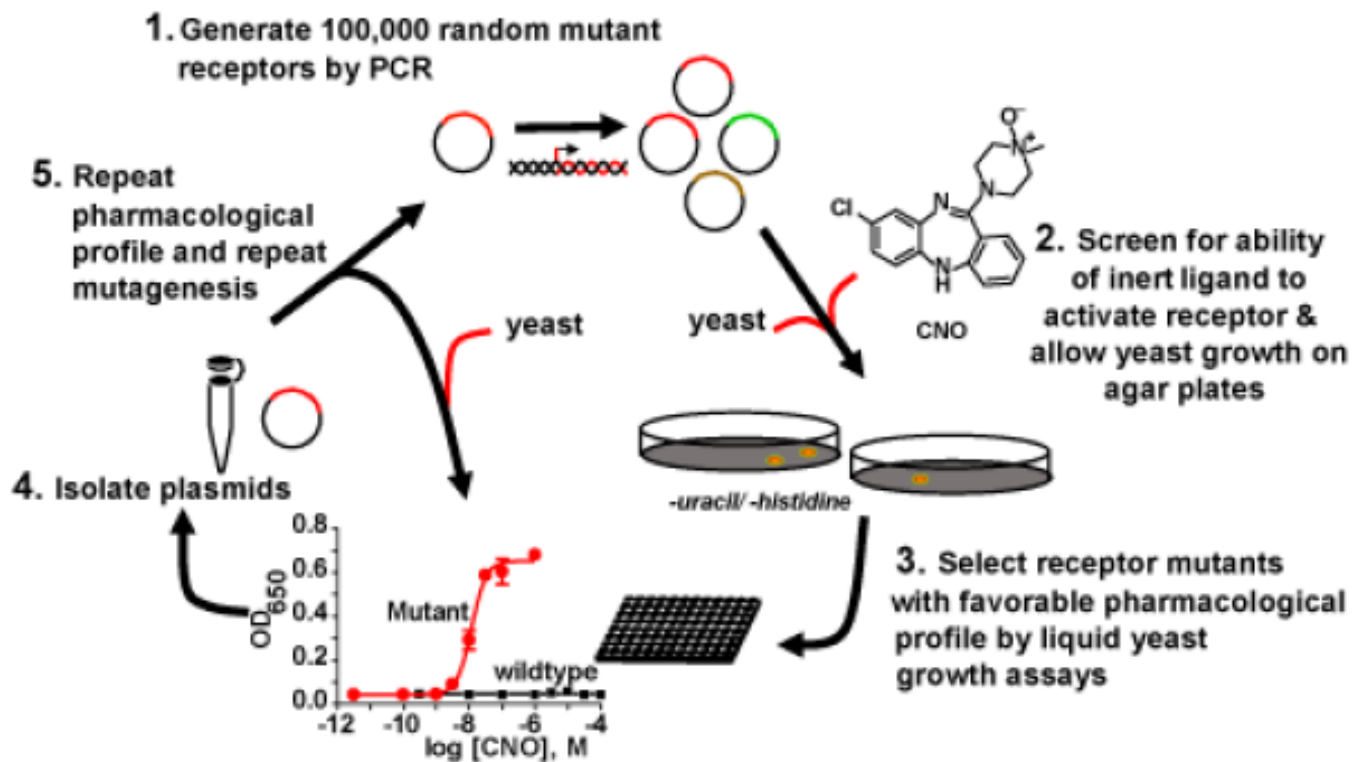


Figure 3.

Directed molecular evolution to create novel RASSLs. Shown is a generic scheme for obtaining RASSLs via directed molecular evolution. In brief, a large library of randomly mutated GPCRs is obtained by error-prone PCR and used to efficiently transform the appropriate yeast strain. Yeast with functional GPCRs is grown in histidine-deficient medium in the presence of the inert ligand (in this case clozapine-N-oxide, CNO), and surviving colonies are expanded and characterized pharmacologically. GPCRs with the appropriate pharmacological profiles are subjected to iterative rounds of additional mutagenesis and selection until the ideal RASSL is obtained. Candidate RASSLs are subjected to growth assays in the presence and absence of candidate ligands to screen out those with elevated levels of constitutive activity. Typically several candidate inert ligands are used in the initial screens to determine which are most suitable for directed molecular evolution and then one or more chosen for further testing. The final choice of the candidate ligand is based on its potency and drug-like properties.

Table 1

Commonly used RASSLs

Name	Alternative names: major versions	Signaling notes	Agonist and antagonists (relative affinities in nM)	In vivo phenotypes
hRO-i	Ro1 ^{5,10} : human kappa opioid receptor with delta opioid extracellular loop, and N-terminal FLAG epitope tag hRO3-i (RO3 or Rog) ³⁶ : same as Ro1, but with point mutation and N-terminal GFP tag	G _i signaling, with constitutive signaling in heart, but not no apparent constitutive signaling in cultured cells	Agonists Spiradoline (low nM) Salvinorin A (low nM) Antagonist: Nor-binaltorphimine-dihydrochloride (Nor-BNI) (low nM)	Cardiac, neurological, sweet taste, bitter taste, bone. See Figure 1.
hRS-s	Rs1 ^{16,20,21} : human serotonin 4 receptor with D100A mutation with N-terminal FLAG epitope tag hRS2-s (Rs2) ²⁰ : has N-terminal GFP tag	G _s -signaling, with high level of constitutive activity	Agonist RS67333, RS39604, Cisapride, ML10375, GR113808 (all nM) Antagonist None known	Massive increase in bone formation
hRMC-s	RM1 ¹⁹ : human melanocortin 4 receptor with point mutations	G _s -signaling constitutive signaling essential for native receptor function	Antagonist	None known
hRMD-q	DREADD hM3D ⁸ : human M ₃ muscarinic receptor with point mutations	G _q signaling	None known Agonist CNO (low nM) Antagonist Atropine (reduced affinity vs. WT; high nM)	None known
hRMD-i	DREADD hM4D ⁸ : human M ₄ muscarinic receptor with point mutations M ₂ receptor RASSL is also Gi coupled	Gi signaling	Agonist CNO (low nM) Antagonist Atropine (reduced affinity vs. WT; high nM)	Inhibits electrical signaling in brain slices
rRMD-s	DREADD RASSL2: rat M ₃ receptor with point mutations and intracellular loops from the turkey •1-AR	G _s signaling	Agonist CNO (low nM) Antagonist	None known

Name	Alternative names: major versions	Signaling notes	Agonist and antagonists (relative affinities in nM)	In vivo phenotypes
			Atropine (reduced affinity vs. WT; high nM)	

This table only includes RASSLs that are actively used in transgenic animals and have a commercially available ligand with low side effects. A uniform RASSL naming system is used. For instance hRMD-q stands for: h=human, R=RASSL, M=Muscarinic, D=DREADD, q=signaling via G_q. The shortest possible name is used. For the parent RASSL we do not add a number but numbers can be added, to indicate versions. For instance hRO3-i, is a GFP tagged version of hRO-i.