Engineering GPCR signaling pathways with RASSLs

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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 and G_q). We review these advances here to facilitate the use of these powerful and diverse tools.

GPCRs are an ideal vehicle for engineering synthetic signaling systems. These receptors function as signaling switches throughout the body and regulate virtually every physiological response[1–3]. GPCRs are also the largest gene family targeted for drug discovery[4]. GPCRs stimulate a variety of G-protein pathways; for example, G_s stimulates cyclic AMP production, G_i inhibits cyclic AMP production, and G_q stimulates phospholipase C and releases intracellular calcium stores. Because 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. Therefore, 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 have different names: RASSLs[5], therapeutic receptor-effector complexes (TRECs)[6], neoceptors[7] and designer receptors exclusively activated by designer drugs (DREADDs)[8]. Here we 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 compounds to selectively activate a mutant version of the β2-adrenergic receptor (β2-AR) that was unresponsive to its natural hormone[9]. They focused on Asp113$^{3.32}$ (named using the Ballesteros-Weinstein amino acid numbering system for GPCRs[10], where 3.32 refers to the third transmembrane segment of the GPCR and residue number 32 of that segment) which is conserved among all biogenic amine GPCRs and is critical for binding terminal amine groups (**Fig. 1**). Mutating Asp113$^{3.32}$ to serine greatly reduced activation of the β2-AR by biogenic amines. Notably, this mutation allowed the newly synthesized butanone derivative, 1-(3′,4′-dihydroxyphenyl)-3-methyl-1-butanone (L-185,870), to activate the mutant receptor but not the wild-type receptor, albeit with relatively low potency (half-maximum effective concentration,

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Table 1 | Commonly used RASSLs

Name	Alternative names: notable versions	Signaling notes	Agonists (relative affinity)	Antagonists (relative affinity)	*In vivo* phenotypes	References
hRO-i	Ro1: human κ-opioid receptor with δ-opioid extracellular loop and N-terminal Flag tag hRO3-i (RO3 or Rog): same as Ro1, but with point mutation and N-terminal GFP tag	G_i signaling, constitutive in heart but no apparent constitutive signaling in cultured cells	Spiradoline (low nanomolar) Salvinorin A (low nanomolar)	Nor-binaltorphimine-dilhydrochloride; Nor-BNI (low nanomolar)	Decreased heart rate, cardiomyopathy, decreased bone formation, induced sweet and bitter taste	5,11,37
hRS-s	Rs1: human serotonin 4 receptor with point mutation and N-terminal Flag tag hRS2-s (Rs2): same as Rs1 but has N-terminal GFP tag	G_s-signaling with high constitutive activity	RS67333, RS39604, cisapride, ML10375, GR113808 (nanomolar)	None known	Massive increase in bone formation	17,21,22
hRMC-s	RM1: human melanocortin 4 receptor with point mutations	G_s constitutive signaling essential for native receptor function	Tetrahydroisoquinoline (low nanomolar)	None known	None known	20
hRMD-q	DREADD hM3D: human M_3 muscarinic receptor with point mutations	G_q signaling	CNO (low nanomolar)	Atropine (reduced affinity versus wild type; high nanomolar)	None known	8
hRMD-i	DREADD hM4D: human M_4 muscarinic receptor with point mutations; M_2 receptor RASSL is also Gi coupled	G_i signaling	CNO (low nanomolar)	Atropine (reduced affinity versus wild type; high nanomolar)	Inhibits electrical signaling in brain slices	8
rRMD-s	DREADD RASSL2: rat M_3 receptor with point mutations and intracellular loops from the turkey β1-AR	G_s signaling	CNO (low nanomolar)	Atropine (reduced affinity versus wild type; high nanomolar)	None known	

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, in hRMD-q h stands for human; R, RASSL; M, muscarinic; D, DREADD; and 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.

EC_{50}, = 118 μM)[9]. Although this original report was inspiring, the synthetic agonist had low affinity 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 and colleagues[5], who took advantage of potent synthetic drugs originally developed as potential analgesics, such as κ-opioid receptor agonists (for example, spiradoline). The first RASSL was created by introducing mutations in the κ-opioid receptor that abrogated signaling via the natural peptide ligands yet preserved stimulation by spiradoline[5]. This engineered human RASSL, human RASSL opioid G_i-coupled receptor (hRO-i), referred to as Ro1 (ref. 5), has been expressed in at least six tissues in transgenic mice (**Fig. 2**). This expression resulted in the induction of various phenotypes including ligand-dependent heart-rate modulation[11], and bitter and sweet taste sensations[12,13] as well as ligand-independent cardiomyopathy[14], hydrocephalus[15] and osteopenia[16]. 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-HT_4 serotonin[17], β2-adrenergic[6], H_1-histamine[18], A3 adenosine[7], 5-HT_{2A}-serotonin[19] and MC4-melanocortin[20] receptors. In most of these receptors, scientists targeted key residues essential for binding the native ligand by site-directed mutagenesis. For example, introduction of the $D100^{3.32}A$ mutation (analogous to the $D113^{3.32}A$ mutation in the β2-AR[9]) into the G_s-coupled human 5-HT_4 serotonin receptor abolished the ability of serotonin (the endogenous ligand) 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-s[17]. 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*[17,21]. In addition, hRS-s is activated by antagonists of the 5-HT_4 receptor, which have fewer *in vivo* side effects than 5-HT_4 receptor agonists. Notably, when expressed in osteoblasts of young mice, hRS-s dramatically alters bone growth *in vivo*[22], presumably owing 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 substituting 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_{2A}-adrenoceptors ($G_{i/o}$-coupled receptors), and the mutated receptor could still be activated by synthetic agonists (UK14304 and clonidine) or even by antagonists of the wild-type receptor (atipamezole and SKF86466)[23]. Similarly, a $G_{q/11}$-coupled RASSL was developed by introducing the $F435^{6.55}A$ mutation into the histamine H_1 receptor[18]. This RASSL could be activated by high concentrations of endogenous histamine and had improved affinity and potency for 2-phenylhistamines, a

class of synthetic H_1R agonists (2-[3-chlorophenyl]histamine). Notably, alternative substitutions at this position (Phe435$^{6.55}$) 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 (for example, κ-opioid, H1-histamine, 5-HT_4-serotonin and MC4-melanocortin receptors), or had low affinities for the mutated receptor (A3-adenosine neoceptor, β2-AR TREC and 5-HT_{2A} serotonin RASSL). Moreover, profound phenotypes induced by constitutive activity were observed upon RASSL overexpression *in vivo* (RO-i and RS-s). Lastly, 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 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 molecules[8].

We used a well-established yeast mutagenesis system to produce hundreds of thousands of mutant hM_3 muscarinic receptors and screened them for signaling characteristics of an 'ideal' RASSL[8]. 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, bioavailable[24] synthetic compound (**Fig. 3**). We designated this new class of RASSLs as DREADDs (ref. 6). We refer to this first DREADD as hRMD-q (RASSL M_3 muscarinic receptor DREADD, G_q-coupled; referred to as hM3-D in ref. 6). The hRMD-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 M_4 muscarinic receptor, which is G_i-coupled and inhibits cyclic AMP accumulation, led to another RASSL or DREADD that we call hRMD-i (referred to as hM_4D in ref. 8). When activated by CNO, hRMD-i silenced hippocampal neurons via Gβγ-mediated activation of G-protein inwardly rectifying K^+ (GIRK) channels[8]. It is likely that hRMD-i will be widely used to induce neuronal silencing *in vivo* via indirect activation of GIRKs. More recently, researchers made chimeras of the rat equivalent of the hRMD-q that incorporates the second and third intracellular loops of the G_s-coupled β1-adrenergic receptor to create a G_s-coupled RASSL (rRMD-s; **Table 1**) (Guettier, J.-M., *et al.* abstract, International Group on Insulin Secretion, St. Jean Cap-Ferrat, France; 2007). 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 used.

With the current DREADD-type RASSLs, only two point mutations were required to create hRMD-i and hRMD-q. By contrast, creation of 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 experience (Y.P. *et al.*, manuscript 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 (for example, 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[25,26]. 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 time[27]. Dozens of human GPCRs have been expressed in yeast[28], thereby opening up the potential to create families of designer GPCRs activated by specific small molecules.

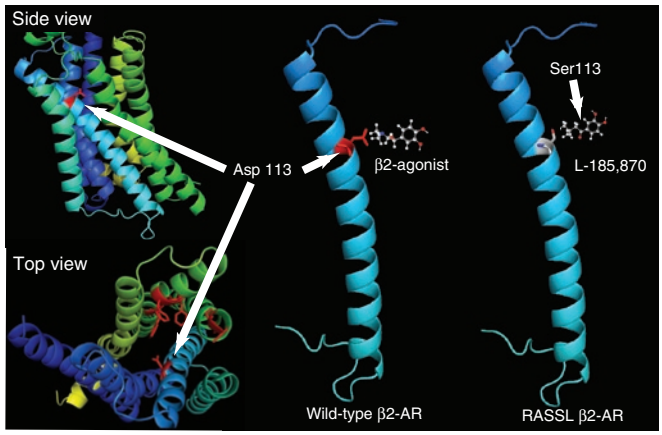

Figure 1 | Creating RASSLs by targeted mutagenesis. A conserved residue(s) in the canonical binding pocket of biogenic amine receptors (for example, adrenergic, serotonin and histamine receptors) is mutated to eliminate the binding and activation of the receptor for the native ligand. A model using the coordinates of the β2-adrenergic receptor structure[31] is used to illustrate this. In all biogenic amine GPCRs, the binding pocket is composed of a conserved aspartic acid (Aps113$^{3.32}$ in the β2-AR model; side view) and conserved aromatic and polar residues (top view). Mutation of the highly conserved aspartic acid to serine (D113S) renders the β2-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 (for example, κ-opioid, MC4-melanocortin) and nonpeptidergic RASSLs (H1-histamine, 5-HT_{2A} serotonin, 5-HT_4 serotonin, α_2-adrenergic).

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 tissues[2]. For example, the 5-HT_{2B} serotonin receptor is required for cardiac development and cell-cycle progression[29–31]. Ectopic signaling via GPCRs can promote abnormal growth[3], leading to human disease. For instance, drug-induced valvular heart disease may be caused by excessive stimulation of cardiac 5-HT_{2B} receptors[32]. 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 neu-

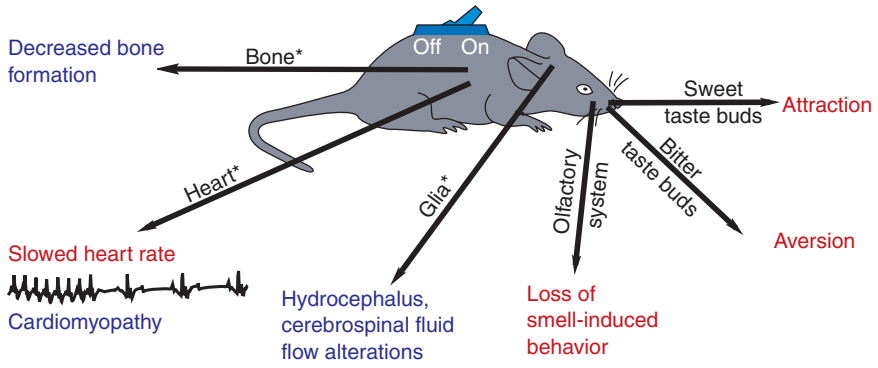

Figure 2 | Ligand-dependent and -independent phenotypes are induced by tissue-specific expression of a hRO-i, a G_i RASSL. Ligand-induced phenotypes are noted in red; constitutive signaling-induced phenotypes are in blue. Asterisks indicate tissues in which Ro1 expression results in embryonic or perinatal lethality. Conditional expression allows RASSL researchers to avoid embryonic lethality and analyze adult phenotypes.

ronal silencing when expressed in hippocampal neurons[8]. When expressed in hippocampal neurons, hRMD-q induces neuronal excitation and intracellular Ca^{2+} release in CNO-dependent fashion (S. Rogan and B.L.R., manuscript in preparation). Using these two engineered muscarinic receptors, one could gain precise bidirectional 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 (for example, hRS-s or rRMD-s) and G_i (for example, 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 cardiomyopathy[14], whereas overexpression in osteoblasts led to osteoporosis[16]. Recently a G_s RASSL (hRS-s) expressed in osteoblasts induced marked bone growth[16]. Constitutive activity is a common property of native GPCRs[33,34] and is essential for the normal function of certain GPCRs[35]. 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 (for example, tetracycline-inducible or Cre-*loxP*). 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 activity[8], and thus far, their overexpression in mice has not elicited baseline phenotypes (S. Rogan, B.L.R., J.-M.G. and J.W., 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, physiologically inert drug (for example, 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 RASSLs would also selectively couple to each of the GPCR pathways, including noncanonical pathways, such as those involving arrestins, GRKs and other intracellular kinases[30]. Furthermore, each RASSL would have different constitutive responses, desensitization properties, and subcellular targeting that could be fine-tuned by introducing 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 (bacterial artificial chromo-

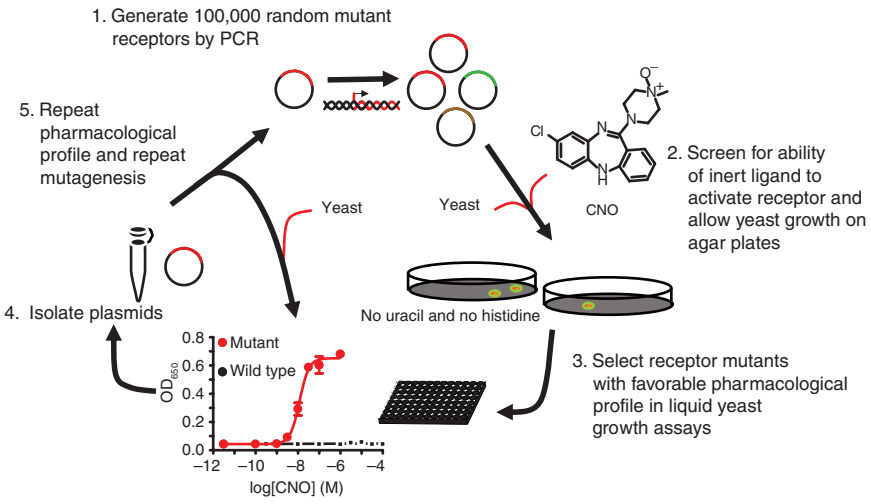

Figure 3 | Directed molecular evolution to create new RASSLs. Shown is a generic scheme for obtaining RASSLs via directed molecular evolution. In brief, a large library of randomly mutated GPCRs is obtained by error-prone PCR and used to efficiently transform the appropriate yeast strain. Yeast with functional GPCRs is grown in uracil- and histidine-deficient medium in the presence of the inert ligand (in this case, 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 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 are chosen for testing. The final choice of the candidate ligand is based on its potency and drug-like properties.

some 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 (for example, Parkinson's disease), RASSLs may be useful for correcting the imbalance of neural pathways, in a manner that could complement the surgical or 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, could 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 will also allow researchers to stimulate discrete signaling pathways in bone metabolism. Finally, in diabetes, GPCRs have a role in the growth, development and function of insulin-secreting pancreatic β-cells[36]. 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 proteins[30]. 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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