Engineered GPCRs as Tools to Modulate Signal Transduction

Different families of G-protein-coupled receptors (GPCRs) have been engineered to provide exclusive control over the activation of these receptors and thus to understand better the consequences of their signaling in vitro and in vivo. These engineered receptors, named RASSLs (receptors activated solely by synthetic ligands) and DREADDs (designer receptors exclusively activated by designer drugs), are insensitive to their endogenous ligands but can be activated by synthetic drug-like compounds. Currently, the existing RASSLs and DREADDs cover the G\(_i\), G\(_q\), and G\(_\text{signaling pathways. These modified GPCRs can be utilized as ideal tools to study GPCR functions selectively in specific cellular populations.}

The role of GPCRs in signal transduction was first elucidated in the late 1960s (6). When triggered by extracellular stimuli such as photons, odorants, neurotransmitters, proteins, hormones, and other small molecules, GPCRs interact with a complex of heterotrimeric guanine nucleotide-binding proteins (G-proteins) and thus regulate a wide variety of intracellular signaling pathways including cyclic adenosine monophosphate (cAMP), phosphatidylinositol-4,5-bisphosphate (PIP2), Ca\(^{2+}\), small G proteins, arrestins, various types of protein kinases, ion channels, and transcription factors (26). To date, GPCRs have been recognized as the largest protein superfamily in the human genome (23). Currently, GPCRs represent the molecular target for ~50% of approved medications and remain the most popular target for therapeutic drug discovery (7, 14). However, the extreme diversity of GPCRs with respect to their tissue distribution (35), endogenous and exogenous ligands, and cellular functions as well as the complexity of the G-protein family makes it very difficult to manipulate the signaling pathways of specific GPCRs in vivo (17, 21, 40). In particular, there is no technology available that allows us to control the activation of a specific GPCR in a specific subpopulation of cells in vivo. Additionally, many GPCRs share common structures. Thus ligands acting on one GPCR may also have “off-target” effects on other molecular targets (see, for instance, Refs. 23, 36). Moreover, endogenous ligands modulate the receptor basal “tone,” making it difficult to distinguish the effects of some classes of ligands (e.g., allosteric modulators) in vivo.

Therefore, it would be valuable to generate an experimental system in which a GPCR could be exclusively linked to one specific ligand that has no effect on other molecular targets and then to place the system selectively in certain cell populations. RASSLs and DREADDs have been created based on this idea (5, 11, 12). These engineered receptors have proven to be enormously powerful tools for improving our understanding of GPCR signaling and have ushered in a novel synthetic biological approach for in vivo modulation of signaling.

A Brief History of RASSLs and DREADDS

The first attempt at an engineered GPCR was reported by Strader et al. in 1991 (47). By mutating the Asp113 residue, a genetically engineered \(\beta_2\)-adrenergic receptor was generated and utilized to explore ligand-receptor interactions. The mutant receptor lost the ability to respond to its endogenous ligands but could be fully activated by a group of designed compounds that have no affinity for the wild-type (WT) receptor (46, 47). Unfortunately, the potencies of these synthetic ligands were too low to be utilized in further in vivo studies (EC\(_{50}\) \(\geq\) 40 \(\mu\)M). Several years later, Coward et al. created a mutated \(\kappa\) opioid receptor (KOR) that was insensitive to its natural opioid peptide ligands but responded to nanomolar concentrations of synthetic KOR agonists (12). This first RASSL activated the G\(_i\) signaling pathway and was named Ro1. KOR was primarily chosen to be made into RASSL because its endogenous ligands are small peptides whose typical binding regions are in the extracellular loops of the receptor and are physically distinct from the binding sites of synthetic small molecules. Thus, hypothetically, one could easily mutate away the endogenous ligand site without affecting the affinity of synthetic ligands. As expected, when the second extracellular loop region of the \(\delta\) opioid receptors (DOR) was substituted for that of KOR, the mutated KOR greatly lost affinity for opioid peptides but retained high potency interactions with the KOR agonist spiradoline (EC\(_{50}\) = 5 nM) (FIGURE 1). Since then, a variety of GPCRs have been made into RASSLS by introducing well designed mutations into the natural ligands’ binding domains.

In 2000, a modified 5-HT\(_{2A}\) receptor was constructed, which was activated by the inert ligand gramine but not by 5-HT (22). In 2001, Small et al. (43) developed a therapeutic receptor-effector complex (TREC) by modifying the ligand binding sites in the third transmembrane helix of the
β2-adrenergic receptor and fusing the COOH terminus of the receptor to an αG protein subunit. In 2003, Srivinasa et al. (45) designed a series of Gs-coupled RASSLs from the melanocortin-4 receptor (MC4R) via rationally designed mutagenesis. In the same year, another serotonin receptor-related RASSL, Rs1, which couples to Gs, was generated by introducing a single mutation at Asp100 of the mouse 5-HT4A receptor (10). In addition, the generation of Gs-coupled α2A-adrenergic receptor RASSLs was achieved following mutations at conserved serine residues in the fifth transmembrane region (31). In 2005, Bruysters et al. (9) made a F435A mutation in H1-histamine to create a Gs-coupled RASSL (8). In 2007, a group of RASSLs inducing different G-protein signaling pathways were created by modifying the 5-HT4A RASSL Rs1.

These RASSLs have significantly reduced ability to interact with their endogenous ligands and can be utilized as new tools to simplify the complexity of GPCR signaling pathways. Although these RASSLs have proved useful, they have real and potential problems as follows. 1) Many of the synthetic compounds that activate RASSLs exhibit appreciable affinities and potencies for the native receptor. This nonselective activity limits RASSLs’ utilization in vivo because they need to be expressed in knockout animals to eliminate the activation of endogenous receptors. 2) Some RASSLs lack ligands that have high enough potency to be utilized in vivo. 3) It is not clear whether these synthetic ligands have affinities for other protein targets. 4) Many RASSLs have high basal signaling in vivo that might obscure any ligand-induced phenotypes (49). To address these problems, Jacobson and colleagues (20) have attempted to improve on RASSLs by modifying adenosine receptors based on predictions from molecular modeling. They have christened the modified receptors “neoreceptors.” The synthetic ligands (“neoligands”), which activate them, have no effect on the native receptor; however, it is not known whether these neoligands have “off-target” actions.

We have developed another approach to overcome the limitations of RASSLs. We have recently created, via directed molecular evolution, a new generation of RASSLs, which we have dubbed DREADDs (5). After repeated cycles of random mutagenesis and selection of human muscarinic acetylcholine receptor (mAChR) subtype 3 (hM3) mutants in yeast (see Refs. 5, 11), we identified a double mutant (Y3.33C/A5.46G) that has no constitutive activity and no longer interacts with its natural ligand [acetylcholine (Ach)] but can be potently activated by a small molecule clozapine-N-oxide (CNO). CNO has drug-like properties but is pharmacologically inert and lacks appreciable off-target actions (3, 4, 37, 39). Constructing these two cognate mutations into all other human mAChRs resulted in a family of DREADDs, hM3L5 D, which is exclusively activated by CNO. Because of the novel biochemical and pharmacological properties of these engineered receptors, we believe that DREADDs will provide us with an ideal system to probe various signaling transduction pathways of different GPCRs in different tissues.

**Tissue-Specific Expression of Engineered GPCRs**

There are several conditional signaling systems that have been utilized to control the transcription of target proteins with administered drugs (2, 6, 13, 19, 23). Currently, in vivo studies of RASSLs and DREADDs use the tetracycline-controlled transcription system (Tet-system) and constitute transgenic approaches for the generation of RASSL and DREADD-expressing
mice. In this system, tissue-specific expression of the target protein is achieved by crossing transgenic mice carrying the transgene of interest downstream of a tetracycline-responsive promoter element (TRE or tetO) and a mouse line expressing the tetracycline transactivator (tTA or rtTA), for Tet-Off or Tet-On, respectively, gene under the control of a tissue-specific promoter (2, 6). In double transgenic mice, the tissue-specifically expressed tTA proteins bind to and activate tetO and start the transcription of its downstream protein expression. There are two different Tet systems: Tet-On and Tet-Off. In the Tet-On system, target gene expression is activated in the presence of doxycycline (DOX), whereas in the Tet-Off system the transgene will be expressed in the absence of DOX or tetracycline (TC). Thus gene expression can be easily and strictly regulated in a tissue-specific and timing-specific manner by adding or removing TC or DOX. For example, transgenic mice with Ro1 specifically targeted to the heart region were created by breeding the Tet-O-Ro1 mice with a myosin heavy chain (MHC)-tTA mouse strain containing the tTA driving from a heart-specific MHC promoter, and Ro1 gene expression was induced by the removal of doxycycline from the mice’s diet (15) (FIGURE 2).

Signal Transduction of Engineered GPCRs In Vitro and In Vivo

The wide variety of physiological effects of a GPCR mainly rely on the heterotrimeric (α, β, and γ) G-proteins and accessory proteins to which it couples (1, 21, 22). To date, at least 23 α subunits have been identified and grouped into four families (Gαq/11, Gαs, Gαi/0, and Gα12) (7). The primary effect of Gαi0 activation is to suppress intracellular cAMP levels by inhibiting adenyl cyclase (AC). In addition, the activation of Gαi0-signaling pathway also has effects on ion channels and cyclic GMP phosphodiesterase. The Gαi-signaling pathway has the opposite effect on cAMP compared with Gαi (i.e., activating AC and stimulating cAMP levels), whereas Gαq/11, stimulates membrane-bound phospholipase C, which metabolizes phosphatidylinositol bisphosphate (PIP2) into two second messengers, inositol triphosphate and diacylglycerol. Specific physiological responses of each signaling pathway depend on the cell type in which the GPCR is activated. Currently, different GPCRs that activate Gαi, Gαs, and Gα12-mediated pathways have been modified into RASSLs and DREADDs (Tables 1–3).

Gαi-coupled RASSLs and DREADDs

The engineered GPCRs, which activate Gαi-signaling pathways, are summarized in Table 1. Currently, the first RASSL, Ro1, has been the most studied in vitro and in vivo. This Gαi-coupled RASSL has 200-fold decreased affinity for the KOR endogenous agonist dynorphin than the WT receptor but maintains high affinity and potency for the synthetic agonist spiradoline. Activation of Ro1 causes an agonist-dependent cAMP decrease in CHO-K1 cells (4). Several modified RASSLs derived from Ro1 have been generated, including Ro2, Rog, Rog-A, Rog-m, and Rog-μA (4, 17). Ro2 has one more mutation at the extracellular loop 3 (E297Q) than Ro1 and this mutation further reduces the binding ability of dynorphin (2000 fold less than WT). In Rat-1a cells, Ro2 induced cell proliferation via Gαi-signaling
pathway (4). Rog, Rog-A, Rog-µ, and Rog-µA performed similarly to each other in cell signaling assays but differently in measures of desensitization and internalization in response to spiradoline. Rog-A was generated by mutating phosphorylation sites in the COOH-terminal region of Rog, an NH2-terminal GFP-tagged version of Ro1. The resistance of Rog-A to internalization, to spiradoline-stimulated desensitization and internalization but could not induce any AC superactivation. Rog-µA is Rog-µ with phosphorylation sites removed from the rat MOR COOH terminus. Rog-µA signals and desensitizes normally but undergoes constitutive internalization. These Ro1 derivatives prove that internalization, desensitization, and AC superactivation are independent processes. These modified receptors are useful tools for studying the differential physiological regulations of GPCRs in vitro and in vivo (17).

As described previously, the ultimate goal of generating engineered GPCRs is to use them as tools to better understand the signaling pathways of each GPCR in specific tissues in vivo. To date, Ro1 has been expressed in at least six tissues in transgenic mice including heart, liver, salivary gland, taste buds, brain, and bones (10, 11, 14–16, 20, 24). Due to the limitation of Ro1 that spiradoline activates both

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**Table 1. Engineered GPCRs that activate G_s-signaling pathways**

<table>
<thead>
<tr>
<th>Endogenous Receptor</th>
<th>Name</th>
<th>Mutation Site(s)</th>
<th>In Vitro Binding and Signaling</th>
<th>In Vivo Expression</th>
<th>Physiological Effects in Transgenic Mice</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>KOR</td>
<td>Ro1</td>
<td>KOR-EL2-DOR</td>
<td>Showed 200-fold decreased affinity for dynorphin</td>
<td>Heart</td>
<td>Caused dysfunctions in calcium transients and myofila-ment, which were followed by heart rate decrease and dilated cardiomyopathy</td>
<td>12, 25, 33, 34</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Remained high affinity and potency for spiradoline</td>
<td></td>
<td>Taste cells</td>
<td>27, 56</td>
</tr>
<tr>
<td>Ro2</td>
<td>Ro1</td>
<td>E297Q,</td>
<td>Showed 2,000-fold reduced signaling ability with dynorphin</td>
<td>N/A</td>
<td>Transgenic mice expressing Ro1 in the &quot;sweet&quot;/&quot;bitter&quot; cells exhibited strong attraction/aversion to spiradoline</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Induced cell proliferation</td>
<td></td>
<td>Astrocytes</td>
<td>32</td>
</tr>
<tr>
<td>R أ ج</td>
<td>GFP-Ro1</td>
<td></td>
<td>Performed similar to Ro1</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>R أ ج A</td>
<td>Rog-5356A/T357A/5358A/T363A</td>
<td></td>
<td>Resistant to internalization and desensitization</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>R أ ج µ</td>
<td>Rog-C-MOR</td>
<td></td>
<td>Could not induce any AC superactivation</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>R أ ج µ A</td>
<td>Rog-µµ-T383A/E391Q/E397Q/T394A</td>
<td></td>
<td>Had constitutive internalization.</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>α_{2a}-AR</td>
<td>α_{2a}-AR-S200A/S204A</td>
<td></td>
<td>Showed abolished potency to epinephrine but could be activated by several synthetic ligands</td>
<td>N/A</td>
<td>N/A</td>
<td>31</td>
</tr>
<tr>
<td>RASSL</td>
<td>Rs1.3</td>
<td>Rs1-13-5-HT_{1a}</td>
<td>Inhibited cAMP accumulation with low potency</td>
<td>N/A</td>
<td>N/A</td>
<td>9</td>
</tr>
<tr>
<td>R أ ج _4</td>
<td>Hm4</td>
<td>Hm4- Y3.33C/A5.46G</td>
<td>Insensitive to ACh Inhibited cAMP production potently by CNO Caused neuron silencing when activated by CNO</td>
<td>5-HT neurons</td>
<td>Currently under investigation</td>
<td>5</td>
</tr>
</tbody>
</table>
Ro1 and the natural receptor, this RASSL needs to be expressed in knockout animals or in tissues that express few endogenous KORs to eliminate nonspecific receptor activations. Therefore, under regulated control using the Tet-off system, Ro1 was first inducibly expressed in the heart, a tissue that lacks KOR (15). Spiradoline exhibited no effects on control animals. However, <1 min after intraperitoneal drug administration, the double transgenic mice exhibited a 50% decrease in heart rate. This result is consistent with previous observations of Gi coupling in the heart (5, 8). Moreover, symptoms consistent with dilated cardiomyopathy (DCM) were observed in these transgenic mice. However, the effect of spiradoline on the double transgenic mice can be entirely removed by suppressing the Ro1 expression with DOX. Although Ro1 did not show appreciable constitutive activity in transfected CHO-K1 cells, HEK 293 cells, or bone marrow stromal cells (4, 14), double transgenic mice developed DCM in the absence of spiradoline, indicating high basal Gi signaling of Ro1 in the heart. These observations, for the first time, demonstrated the tight relationship between Gi signaling and DCM (16). Recently, the mechanisms of the Gi-induced DCM were further investigated (10). After induction of Ro1 expression but before heart failure occurred, reduction of sarcoplasmic reticulum Ca2+-ATPase (SERCA), subsequent impairment in calcium transients, and abnormal myofilament function were observed. Since Ca2+ handling and myofilament function are important for heart contractile function, their dysfunctions during Ro1 activation could contribute to the G_i-induced DCM.

In addition to the heart, Ro1 has also been expressed in the sweet and bitter taste buds of the tongue to help to understand the basis of taste recognition (11, 24). The taste receptor TR1 mediates sweet and umami tastes in mammals, whereas TR2s are responsible for bitter tastes. Interestingly, engineering TR1-expressing cells to express RO1 drove transgenic mice to seek the tasteless spiradoline rather than sweet compounds (24). Consistently, transgenic mice expressing Ro1 in the bitter cells with TR2 exhibited strong aversion to spiradoline (11). These results suggested that the sense of the taste is determined by the activation of specific cells that express taste receptors instead of by the activation of taste receptors themselves.

Since KORS are widely distributed in the brain, studying Ro1 in KOR knockout mice could provide us a clean background with no endogenous receptor activation. Sweger et al. (49) expressed Ro1 in astrocytes of KOR knockout mice by the DOX-controlled Tet-Off system under the human glial fibrillary acidic protein (hGFAP) promoter to study whether and how G_i signaling in this region affects brain neurophysiology (20). Control animals appeared normal at all ages. Unfortunately, all the Ro1-expressing mice developed hydrocephalus when off DOX. By week 12 of life, half the population of Ro1 mice had died from severe hydrocephalus; the remaining half eventually developed significant hydrocephalus. As was seen with the development of DCM following Ro1 expression in the heart, hydrocephalus occurred in the absence of any applied agonists, suggesting that, as in the heart, Ro1 also has high constitutive activity in astrocytes. These data indicated that the development of hydrocephalus resulted from Ro1 expression; however, the mechanism by which increased Gi signaling in astrocytes causes hydrocephalus is still unclear.

Recently, the role of Gi-signaling in bone formation has been investigated by conditionally expressing Ro1 in osteoblasts using the Tet-Off system under the control of mouse Col I promoter (14). Again, as in the heart and brain, Ro1 showed basal activity in trabecular bone cells. The volume of trabecular bone, but not cortical

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Table 2. Engineered GPCRs that activate Gq-signaling pathways

<table>
<thead>
<tr>
<th>Endogenous Receptor</th>
<th>Name</th>
<th>Mutation Site(s)</th>
<th>In Vitro Binding and Signaling</th>
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<th>Physiological Effects in Transgenic Mice</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>hH1R</td>
<td>hH1R-RASSL</td>
<td>hM1R-F435A</td>
<td>Resulted in 200-fold decreases in potency for histamine and 2,600-fold improved potencies for the synthetic drug 2-phenylhistamines</td>
<td>N/A</td>
<td>N/A</td>
<td>8</td>
</tr>
<tr>
<td>hM3</td>
<td>hM3D</td>
<td>hM3-Y3.33C/AS.46G</td>
<td>Did not respond to ACh Potently elicited PI hydrolysis</td>
<td>Pancreatic beta-cells Cortex and hippocampus 5-HT neurons</td>
<td>Caused CNO-dependent decrease in blood glucose and improvement in glucose tolerance and insulin release Caused strong seizure with CNO treatment Currently under investigation</td>
<td>5, 16</td>
</tr>
</tbody>
</table>

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bone, is considerably decreased after expression of Ro1, suggesting that the activation of this G_i-coupled RASSL in osteoblasts can cause trabecular osteopenia, probably by decreasing bone formation. On the other hand, expression of the G_s-coupled RASSL Rs1 in the same cells induces the opposite phenotype: significant bone mass increases, as will be described later.

Compared with RASSLs, DREADDs that we have developed using high-throughput yeast screening, the G_s-coupled human M_4 DREADD (hM4D) and G_i-coupled hM3D could be activated by CNO and functions of a series of ligands including agonists, antagonists, and a potentiator were characterized. Except for clozapine analogs, which have increased affinities for and potencies at hM4D, the other orthosteric agonists cannot activate the hM4D receptor, even though some of these ligands maintain their ability to bind to the receptor. Interestingly, the allosteric modulator LY2033298 can significantly rescue the affinity and potency of the endogenous compound ACh at hM4D but has no effect on clozapine-like compounds, indicating the existence of a functional allosteric site on the hM4D, which may explain the functional selectivity of the DREADD receptor. Moreover, the results suggested that the two mutation sites in hM4D (Y3.33C/A5.46G) are important in the binding and activation motifs of both orthosteric and allosteric ligands (12).

To further characterize hM4D in vivo, we are currently using mice with hM4Ds targeted to serotonin neuron populations using an ePet BAC, which is able to direct 5-HT neuron-specific transgene expression (18) (Rogan S.C., Roth B.L., unpublished observations). The unique ability of CNO to induce

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### Table 3. Engineered GPCRs that activate G_s-signaling pathways

<table>
<thead>
<tr>
<th>Endogenous Receptor</th>
<th>Name</th>
<th>Mutation Site(s)</th>
<th>In Vitro Binding and Signaling</th>
<th>In Vivo Expression</th>
<th>Physiological Effects in Transgenic Mice</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>m5-HT_4a</td>
<td>S-HT_4 RASSL</td>
<td>S-HT4-R-D100A</td>
<td>Totally insensitive to serotonin</td>
<td>N/A</td>
<td>N/A</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Respond to synthetic ligand in nanomolar concentrations</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β2-AR</td>
<td>β2-AR-TREC</td>
<td>β2-AR (with 19 modification)-Gαs</td>
<td>No response to albuterol</td>
<td>N/A</td>
<td>N/A</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Stimulate cAMP accumulation when activated by L-158,870, which has no affinity to the WT β2-AR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>No agonist-induced desensitization or phosphorylation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>h5HT_4b</td>
<td>Rs1</td>
<td>5-HT_4bD100A</td>
<td>Preferably coupled to G_s when activated, but some ligands can induce Rs1 to activate both G_s and G_i pathways</td>
<td>N/A</td>
<td>Osteoblast</td>
<td>Significantly increased bone mass</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Increased G_s coupling, but still preferably coupled to G_i</td>
<td>N/A</td>
<td>N/A</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Rs1.1</td>
<td>Rs1-C-5-HT_4c</td>
<td>Exhibited pure G_s signaling and low basal signaling</td>
<td>N/A</td>
<td>N/A</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Rs1.2</td>
<td>Rs1-D66N</td>
<td></td>
<td>N/A</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MC4R</td>
<td>MC4R with point mutations</td>
<td>Had decreased affinity for α-MSH but can be activated by synthetic small molecules</td>
<td>N/A</td>
<td></td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>rM3</td>
<td>GsD</td>
<td>Insensitive to ACh Caused CNO-dependent cAMP accumulation</td>
<td>N/A</td>
<td>Pancreatic beta-cells</td>
<td>Caused CNO-dependent decrease in blood glucose and improvement in glucose tolerance and insulin release</td>
</tr>
</tbody>
</table>

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**Emerging Technology**

CNO triggers the hM_4D to activate G-protein inwardly rectifying potassium channels (GIRKs), leads to Ca^{2+}-mobilization in a pertussis toxin-sensitive fashion, and induces neuronal silencing (all without affecting endogenous M_4 receptors), proving that the DREADD and its natural receptor are independent systems with no effect on each other’s functions (3, 9). Recently, hM4D was utilized as a tool to investigate structure-activity relationships of different allosteric and orthosteric ligands with M4 WT and DREADD receptors (12). The binding and functions of a series of ligands including agonists, antagonists, and a potentiator were characterized.
neuronal silencing in neurons via hM₄D provides us a powerful tool to investigate the functions of Gₛ-coupled GPCRs in the serotonergic system.

**G₉-coupled RASSLs and DREADDs**

In 2005, Bruysters et al. (8) reported a new RASSL that activated Gₛ signaling. Using computational modeling to identify critical ligand binding residues, F435 of the H1-histamine receptor (H1R) was mutated to an alanine to break the aromatic interaction with the endogenous ligand histamine. As expected, this H1R mutation resulted in a 200-fold decrease in histamine’s potency and a 2,600-fold improved potency of the synthetic drug 2-phenylhistamine, as measured in an NF-κB-driven reporter gene assay (8) (Table 2). Currently, no studies have reported using this Gₛ RASSL in vivo.

The generation of hM₄D (Table 2), a Gₛ-coupled DREADD that can be potently and exclusively activated by the pharmacologically inert compound phenylalanine, is important for studying Gₛ-mediated signaling in vivo. Preliminary reports indicate that, when activated in pancreatic beta-cells, hM₄D showed dramatic effects on beta-cell functions (16). Additionally, we have produced mice that express hM₄D under control of the CAMKII-inducible Tet system, and preliminary findings indicate a strong phenotype when hM₄D is activated by CNO but, importantly, no basal phenotype (8). This Rₛ₁ exhibited a high constitutive activity and could be activated by nanomolar concentrations of the synthetic ligand zacopride. Previous studies have indicated that bone mass can be affected by the activation of various GPCRs (38). To investigate the role of Gₛ signaling in skeletal formation, Rs1 was targeted to osteoblasts in transgenic mice, regulated by the Tet-Off system under control of the mouse 2.3-α1 collagen promoter. When Rs1 was expressed during the first 4–6 weeks after removing DOX from the mouse diet, several significant abnormal skeletal phenotypes were observed in 9-week-old double transgenic mice: dramatically increased bone volume, bone mass, bone formation rate, and bone mineral density. No significant change was detected in those of the G₉-coupled Ro1 inhibiting bone formation, as described previously (32). Therefore, the cAMP pathway might play an essential role in bone development.

“Further studies on these transgenic mice should aid us in understanding the role of Gₛ and G₉-mediated signaling pathways in β-cell growth, survival, and functions.”

Based on Rs1, a series of other 5-HT₄B RASSLs were generated by exchanging the extracellular loops of Rs1 with those of Gₛ- or G₉-coupled 5-HT receptors (9) (Table 3). As mentioned above, Rs1 exhibited a high level of basal signaling in vitro and in vivo. This Gₛ-coupled Rs1 might also exhibit some level of Gₛ signaling with specific ligands, although this signaling is not as predominant as its Gₛ signaling. By substituting the COOH terminus of Rs1 with the corresponding region of 5-HT₂C, the chimera Rs1.1 was generated. Rs1.1 has increased Gₛ signaling but maintains its coupling preference to Gₛ. On the other hand, introducing a point mutation at D66 of Rs1 (Rs1.2) completely diminished the Gₛ signaling and significantly decreased the constitutive activity of the receptor. In addition, Rs1 was also reengineered to a Gₛ-coupled RASSL by replacing the third intracellular loop with that of 5-HT₄A. Activated by zacopride, this chimera RASSL Rs1.3 inhibits cAMP accumulation in HEK293 cells, but with low potency (9).

The first Gₛ-DREADD (GₛD) was created by introducing homologus mutations from hM₄D into a mRₛ1 receptor with its second and third extracellular loops substituted with those of the Gₛ-coupled β₃ adrenergic receptor. GₛD can be potently activated by CNO (EC₅₀ = 1.76 nM) but not ACh to elevate intracellular cAMP accumulation (16) (Table 3). Two transgenic mouse lines expressing GₛD (Table 3) and the Gₛ-coupled DREADD
hM3D (Table 2) in pancreatic beta-cells were generated. When CNO was peripherally administered, the activation of G_D or hM3D exhibited significant effects on beta-cell functions such as glucose tolerance, insulin release improvements, and hypoglycemia (16). Further studies on these transgenic mice should aid us in understanding the role of G_, and G-mediated signaling pathways in beta-cell growth, survival, and functions. In addition, we are generating transgenic mice expressing the G_D in striatum to investigate whether or how activation of G pathway affects animal movement (Pei Y., Farrel M., Roth B.L., unpublished observations).

**Potential problems of in vivo studies of RASSLs and DREADDs**

Expressing engineered GPCRs in vivo might present several potential difficulties that need consideration. First, it is possible that tissue-specific promoters will not appropriately restrict receptor expression. Researchers should be sure to fully characterize receptor localization. Second, potential problems might also arise from receptor expression levels. The strength of tissue-specific promoters varies, and some promoters might not drive transgene expression sufficiently to provide detectable phenotypes. On the other hand, we should also be aware that overexpression of designed receptors in animals, especially those with constitutive signaling activity, might affect the signaling of native receptors; compensatory changes in endogenous receptor expression or activity could confound data collection. Even though our mACHR DREADDs do not show basal activity in vitro and in vivo, activation of these receptors might still interrupt the natural signaling network. It is important that we remain cognizant of these potential pitfalls and that we fully evaluate new expression systems so as to avoid misinterpreting data.

**Summary**

GPCRs are involved in many physiological events and currently hold the most potential as targets for drug discovery. However, the complexity and diversity of GPCRs have greatly challenged studies on these receptors. Over the last 10 years, many GPCRs coupling to different G-proteins have been engineered to regulate the receptors’ functions selectively and to modulate their signal transduction via drug-like compounds. The first generation of these modified receptors, which includes RASSLs, TREC, and neo-receptors, was created by designed mutagenesis. Recently, DREADDs, a new generation of engineered GPCRs that overcame most of the limitations of RASSLs, have been developed by directed molecular evolution. DREADDs have low basal activity and are no longer responsive to their endogenous ligands but can be exclusively activated by a synthetic, drug-like compound, which is pharmacologically inert at other molecular targets. Currently, RASSLs and DREADDs that activate G_, G-, and G-mediated signal transduction have been well characterized in vitro and are available for in vivo studies. In a tissue-specific and timing-specific manner, some of these RASSLs, such as the G-coupled Ro1 and G-coupled Rs1, have been expressed and activated in transgenic mice to validate their functions in various populations of cells including bone, heart, astrocytes, and taste cells. Moreover, the muscarinic G-, G-, and G-DREADDs we have created are currently widely utilized in different animal models. These engineered receptors provide us powerful tools to control GPCR-mediated signal transduction and to reveal mechanisms behind specific physiological events. 

**References**


