

# Tools for Dissecting Signaling Pathways in Vivo: Receptors Activated Solely by Synthetic Ligands (RASSLs)

Kimberly Scearce-Levie<sup>\*</sup>, Peter Coward<sup>\*||</sup>, Charles H. Redfern<sup>\*‡</sup>,  
and Bruce R. Conklin<sup>\*‡§†</sup>

<sup>\*</sup>Gladstone Institute of Cardiovascular Disease, Gladstone Institute of Neurological Disease, and Departments of <sup>‡</sup>Medicine and <sup>§</sup>Pharmacology, University of California, San Francisco, CA 94141

<sup>||</sup>Present address: Tularik Inc., Two Corporate Drive, South San Francisco, CA 94080

<sup>†</sup>Address correspondence to: Bruce R. Conklin, Gladstone Institutes, P.O. Box 419100, San Francisco, CA 94141-9100, USA

Tel: 415-826-7500; Fax: 415-285-5632; E-mail: [bconklin@gladstone.ucsf.edu](mailto:bconklin@gladstone.ucsf.edu);

Web address: <http://gladstone.ucsf.edu/labs/conklin/> or [www.GenMAPP.org](http://www.GenMAPP.org)

**Running Head:** RASSLs: Hormone Signaling by Design

## Introduction

The diversity of G protein-coupled receptors (GPCRs) presents a challenge to understanding the connection between a single receptor signaling pathway and a specific physiological or pathological response. Receptors activated solely by synthetic ligands (RASSLs) offer control over the location, timing, and specificity of a G protein signal *in vivo*. These novel, reversible switches for G protein signaling have clarified the role of G<sub>i</sub> signaling in cardiac physiology and are now being used to probe sensory transduction and complex neurobehavioral responses. This review summarizes the design of RASSLs and their first use *in vivo*. We supplement a concurrent review<sup>1</sup> on the subject by providing methods for expressing, detecting and activating RASSLs in a wide variety of tissues.

GPCRs are the largest known family of cell-surface receptors, encompassing over 1000 distinct receptors.<sup>2</sup> These receptors can be activated by a variety of natural ligands, including peptide hormones, odorants, photons, biogenic amines, and lipids. Activation of these receptors results in many different physiological responses, including heart rate changes, chemotaxis, cell proliferation, neurotransmission, and hormonal responses.<sup>3</sup> Prolonged stimulation of GPCRs can alter gene transcription and therefore may mediate long-term changes in the biochemistry, physiology, and behavior of an organism.

The same diversity of receptors, ligands, and responses that makes GPCRs biologically important has also complicated the study of their functions *in vivo*. The ability to stimulate a specific GPCR in a particular tissue *in vivo* would be a valuable aid to understanding the resultant changes in signaling and physiology. However, in a whole animal, numerous factors confound this type of study. Although it is feasible to inject a specific GPCR ligand directly into the tissue of interest, there is no way to restrict receptor activation to a specific subpopulation of cells. Furthermore, many GPCRs belong to large families of closely related receptor subtypes and may be activated by similar ligands. For example, the specific agonists and antagonists for many of the serotonin receptor subtypes remain unknown.<sup>4</sup> Finally, the actions of endogenous ligands

can complicate the interpretation of experimental results. In recent years, scientists have developed several “designer” signaling systems that use artificial ligands.<sup>5</sup> These systems, however, have not been able to mimic the complex conformational changes undergone by GPCRs when activated.

One approach to these difficulties has been to develop RASSLs.<sup>6</sup> These genetically engineered receptors are insensitive to their natural, endogenous ligand(s), but can still be fully activated by synthetic, small-molecule drugs. Expression of RASSLs in transgenic mice allows us to study GPCR signaling *in vivo*. Using tetracycline-regulated gene expression technology, we can control where and when these RASSLs are expressed. By administering the synthetic drug, we can stimulate a single G protein pathway in a specific tissue quickly and reversibly. This system has already yielded important insights into cardiac function.<sup>6,7</sup> By expressing RASSLs in other tissues, researchers can explore the role of G protein signaling in many physiological and pathophysiological responses.

#### Construction of a RASSL

To construct our first RASSL, we modified the kappa opioid receptor (KOR). Opioid receptors respond to endogenous peptides and signal via the G<sub>i</sub> pathway. Because of the importance of this receptor family for pain modulation, the pharmaceutical industry has developed many high-affinity opioid receptor agonists. Small-molecule ligands of the KOR are structurally distinct from the endogenous peptide ligands, including dynorphin.<sup>9, 10</sup> Unlike mu or delta opioid receptor agonists, kappa receptor agonists are nonaddictive.<sup>11</sup>

Structure/function studies of the KOR have revealed that the second extracellular loop is critical for the binding of dynorphin and other neuropeptides.<sup>12-14</sup> However, small-molecule agonists have a different binding pocket close to the transmembrane

region. This raised the possibility that mutating the extracellular regions of the receptor could interfere with the binding of endogenous, but not synthetic, ligands. To construct our first RASSL Ro1 (RASSL opioid 1), we substituted the second extracellular loop of the delta opioid receptor for the corresponding portion of the KOR (Fig. 1).<sup>6</sup> This substitution results in low affinity for both dynorphin and delta opioid receptor ligands.<sup>5,13,14</sup> Our second RASSL (Ro2) contains all the mutations in Ro1, as well as a substitution of glutamine for glutamic acid 297, located at the junction of transmembrane domain 6 and extracellular loop 3. This residue is thought to contribute to specific opioid peptide binding.<sup>16</sup> Both RASSLs showed reduced affinity for dynorphin, without a significant reduction in the response to spiradoline or other small-molecule ligands. The binding affinity of Ro1 for dynorphin was reduced to < 0.5%, while Ro2 showed dynorphin binding that is < 0.05% of the native KOR.<sup>6</sup>

Despite the changes in the peptide binding characteristics of Ro1 and Ro2, signaling in response to spiradoline remained intact.<sup>6</sup> This was demonstrated *in vivo* in transfected cells with calcium mobilization and cell proliferation assays. In both cases, spiradoline but not dynorphin induced the expected G<sub>i</sub>-mediated response.

#### Controlling RASSL Expression *in Vivo*

To take full advantage of the signaling control offered by a RASSL, it was necessary to control the timing and location of RASSL expression *in vivo*. To achieve this, we used the tetracycline (tet) transactivator system developed by Bujard and Gossen.<sup>17</sup> In the tet system, transgene expression is driven by a minimal promoter fused downstream of the tetracycline response element (tetO) from the bacterial *tet* operon (Fig. 3). Expression of the transgene requires the presence of tTA, a transcriptional transactivator protein that binds to tetO. Therefore, the mice must also be transgenic for a second transgene expressing tTA. Tissue specificity comes from the promoter element used to express tTA. Typically, two lines of mice are created, one harboring the tet-O

transgene construct, and the other harboring the tissue-specific promoter tTA construct, and then bred together. Using the tet system to drive expression of the RASSL allows control of where the RASSL is expressed (by choice of tTA line) and when it is expressed (by administration or withdrawal of doxycycline). Two forms of tTA exist, one which binds DNA and activates transgene expression in the presence of doxycycline (the so called tet-on system) and one which represses expression in the presence of doxycycline (the tet-off system). The tTA (tet-off) system is generally preferred for *in vivo* studies because doxycycline is absent during the experimental period, removing this experimental variable. Doxycycline is known to have many (generally benign) biological effects that are not a problem for breeding mice, but could complicate an experiment if a response only occurs with the application of doxycycline (as in the tet-on system). To date, our collaborators have generated tTA lines that drive the expression of RASSLs in a wide variety of tissues, including heart, brain, salivary gland, liver, kidney, and brown fat (see Table 1 and more details, below). Since our TetO-Ro1 line (available from Jackson Labs, Bar Harbor, ME, [www.jax.org](http://www.jax.org)) is well characterized and known to be regulated by doxycycline, this line can be used as a reporter line to test expression patterns obtained with new tTA lines.

We first used the tet system to examine RASSL signaling in the heart. Maximal Ro1 transgene expression in the heart is reached approximately 10 days after withdrawal of doxycycline, when the drug has been washed out of the animal's system completely.<sup>7</sup> Because doxycycline levels can be quickly raised after readministration, subsequent suppression of transgene expression is rapid and largely dependent on the natural half-life of the protein being expressed. In most cases, transgene expression can be suppressed far more quickly than it can be completely induced. We have observed transgene suppression within 24 hours after doxycycline administration.<sup>8</sup> In contrast, the tet-on system would be expected to have a rapid onset, but a relatively slow suppression of expression *in vivo*.

### *Doxycycline Administration Protocols for Mice*

To avoid expression of the transgene during early development, it is important to maintain mothers on doxycycline during gestation and nursing of litters. Sufficient doxycycline is available to offspring via the placenta or milk to suppress transgene expression.

In Drinking Water: Make a 100X stock solution of 20 mg/ml doxycycline (Sigma, St. Louis, MO) in water. It will take about two minutes of shaking to dissolve doxycycline. This solution can be frozen in 5-ml aliquots, wrapped in aluminum foil. Add the stock to mouse drinking water bottles for a final concentration of 200 µg/ml. Because doxycycline is light sensitive, use amber colored bottles (for instance, Wheaton 900 RediPak Amber Glass Packers with Caps, 250 ml). It is not necessary to add sucrose to the drinking water. For most purposes, the 200 µg/ml dose is more than adequate to suppress gene expression in peripheral organs and in the brain. Fresh doxycycline should be added to the water weekly; more frequent changes are unnecessary.

In Mouse Chow: Doxycycline-containing food pellets can be obtained by custom order from Bio-Serv (Frenchtown, NJ) with quote #908-996-2155. The pellets are made by adding 200 mg of doxycycline per kg of regular mouse chow; green food coloring is added to the chow to distinguish it from normal chow. Doxycycline administration in food is less labor-intensive—simply replenish the food as needed.

By Intraperitoneal Injection: If transgene expression prevents the animals from eating or drinking normally, it may be necessary to administer an initial dose of doxycycline by intraperitoneal (IP) injection. In a single injection, 10 µg of doxycycline can be administered in 0.5 ml of sterile water.

### Controlling RASSL Signaling *in Vivo*

When the receptor is expressed, it should be functionally silent until the administration of spiradoline stimulates the RASSL and activates signaling pathways

rapidly and specifically.  $G_i$  signaling in heart reduces heart rate.<sup>18</sup> Therefore, when Ro1 is expressed in the heart (using the alpha-myosin heavy chain promoter), receptor activation can be studied by simply measuring changes in heart rate. Wild-type animals have few KORs in the heart and therefore show no change in heart rate in response to spiradoline administration. In animals expressing Ro1 in the heart, however, heart rate decreases within 30 seconds after drug administration (Fig. 4). This bradycardia is reversible within 10–15 minutes and can be blocked by antagonists.<sup>7</sup> These experiments indicate that a RASSL can be used to modulate a physiological response that requires  $G_i$  signaling *in vivo*. Expression of RASSLs in other tissues will enable researchers to regulate many other physiological and behavioral responses.

Signaling of KOR-based RASSLs is controlled by spiradoline administration. Most of the parameters relating to spiradoline administration have been worked out in mice expressing Ro1 in the heart. Receptor activation decreases heart rate, which can be measured using implantable telemetry units as described below. It should be noted that repeated drug administration desensitizes the response to spiradoline. This is a characteristic of the desensitization mechanisms native to the KOR. This desensitization demonstrates that signaling via a RASSL displays some of the normal physiological characteristics of the native, unengineered KOR. We are currently creating RASSL variants with altered desensitization kinetics to further explore the mechanisms and physiological relevance of receptor desensitization for  $G_i$  signaling *in vivo*.

#### *Administration of Spiradoline*

Spiradoline (U-62066E) can be purchased from Research Biochemicals International (Natick, MA), which is now a division of Sigma-Aldrich. In mice expressing Ro1 in the heart, the drug can be administered IP in a volume of 10  $\mu$ l/g body weight. We found that single injections of 0.05–5 mg/kg body weight are sufficient to reduce heart rate in a dose-dependent manner.

### *Heart Rate Monitoring in Mice*

To measure heart rate and wave form, we implanted cardiac telemetry units under sterile conditions into mice expressing Ro1. The mice were anesthetized with Avertin (10  $\mu$ l/ml). The peritoneum was opened, and a PhysioTel Implant (Model TA10EA-F20, Data Sciences International, St. Paul, MN) was inserted; monopolar electrodes were then tunneled subcutaneously across the precordium. Heart rate was monitored with AcqKnowledge III software (BIOPAC Systems, Santa Barbara, CA). For all baseline heart rate measurements, mice were injected with water (10  $\mu$ l/g, IP) and monitored for 20 min. After treatment with spiradoline, heart rate was monitored for 20 more minutes. More extended measurements of heart function can be made by sampling heart function at fixed time points.

### *Eliminating Unwanted Signaling by Using KOR Knockout Mice*

In a tissue like the heart, where expression of the endogenous KOR is extremely low, the actions of spiradoline at the native receptor are not a major concern. However, for experiments that use RASSLs in KOR-rich tissues, like the brain, experimental results could be complicated by the actions of spiradoline at those receptors. Stimulation of neural KORs results in sedation, an effect that could mask many interesting behavioral responses caused by the action of spiradoline at RASSLs. We therefore used the tet system to express the RASSL in KOR knockout mice<sup>19</sup> (generously provided by Dr. J. Pintar, Rutgers University). With the proper controls, any behavioral or physiological effects of spiradoline administration can then be attributed to the effect of spiradoline at the RASSL, rather than endogenous KORs.

### Detecting RASSLs *in Vivo*

For many experiments that use RASSLs to study the role of  $G_i$  signaling *in vivo*, it is essential to know precisely where the RASSLs are localized, perhaps even at a subcellular level. For this reason, RASSLs contain an N-terminal FLAG epitope tag, recognized by commercially available FLAG M1 antibody. We have used the FLAG tag to detect Ro1 and Ro2 expression by a variety of methods: immunoprecipitation or western blotting of homogenized tissue, FACS sorting or ELISA of whole cells, and immunocytochemistry on whole tissue sections. We have not detected any changes in receptor activity resulting from the FLAG tag.

### *Solubilizing and Homogenizing RASSLs from Whole Tissue*

- Place 1 ml of homogenization buffer (50 mM Tris-HCl, pH 7.4, 1x Complete Cocktail (Boehringer), 1 mM dithiothreitol (DTT); 1 mM phenylmethylsulfonyl fluoride (PMSF) in distilled H<sub>2</sub>O) in a round-bottom 14-ml tube. Since DTT and PMSF are unstable in water, it is important to use the buffer within 30 minutes after the addition of these reagents.
- Transfer frozen (−90°C) tissue into the 14-ml tube. Homogenize with a homogenizer at maximum speed until tissue is fully ground (usually about 30–60 seconds).
- Pipette the homogenate into an eppendorf tube and place on ice immediately. Sonicate for 3–5 seconds if the homogenate is still too viscous.
- Normalize the samples by tissue weight and use approximately 10–50 mg of tissue for solubilization.
- Solubilize each sample in an eppendorf tube under the following conditions:
  - 1x solubilization sample buffer (100 mM Tris-HCl, pH 7.4, 1.5 M NaCl)
  - 1x Triton-X 100
  - 1x Complete Cocktail
- Incubate for 30 min at 4°C on a rotator.

- Spin for 5 min at 4°C at maximum speed.
- Transfer supernatant to new eppendorf tube.

*Immunoprecipitation of FLAG-tagged RASSLs*

- Add the following to the supernatant of solubilized sample (use 50 ng of a FLAG-tagged protein):
  - 2 µl of 1 M CaCl<sub>2</sub>
  - 2 µl of FLAG M1-antibody (1 µg/µl; Sigma)
  - 15 µl of 50% suspension Protein-A agarose beads (Sigma)
- Incubate overnight at 4°C on a rotator.
- Spin down beads for 1 min at 4°C at 3,000 rpm.
- Remove supernatant with a pipet or vacuum (being careful not to disturb beads) and discard.
- Add 1 ml of TBS-T Ca<sup>2+</sup> wash buffer (1x TBS; 0.1% Tween-20; 1 mM CaCl<sub>2</sub>) to beads. Invert tube several times, and then spin for 1 min at room temperature at 3,000 rpm.
- Discard supernatant.
- Repeat wash.
- Add 50 µl of 1x electrophoresis sample buffer (2x Tris-glycine SDS sample buffer (Novex), and 5% beta-mercaptoethanol, diluted to 1x with ddH<sub>2</sub>O) to beads. Vortex and boil sample for 5 min.
- Vortex again. Spin for 2 min at room temperature at 5,000 rpm.
- Using flat tips, transfer supernatant to new tube or gel. Pipet carefully to avoid disturbing the beads.

*Immunoblotting of FLAG-tagged RASSLs*

- Remove precast 10% Tris-Glycine gel (Novex) from refrigerator and warm to room temperature.
- Prepare controls and/or standards and adjust to the same volume as samples:
  - Standard:
    - 5  $\mu$ l Kaleidoscope prestained standard (BioRad)
    - 5 ng FLAG-tagged protein
    - 40  $\mu$ l of 1x sample buffer
- Remove precast gel from bag. Remove tape from bottom of gel. Place gel into apparatus (Novex: X ClI II Mini-Cell). Remove comb. Fill the inside chamber with running buffer (1x Tris/glycine/SDS buffer; Bio-Rad). Using a pipet, rinse wells with buffer. Make sure that there is no leakage from the inside to the outside chamber. Fill the outside chamber with running buffer until the buffer covers the opening on the bottom of the gel.
- Using flat tips, load 45–50  $\mu$ l of each sample into well. Fill blank wells with buffer to minimize curving of bands along the sides of the gel.
- Run gel at 125 constant volts for 90 min or until blue dye front has exited gel.
- While gel is running, prewet nitrocellulose, filter paper, and blotting pads in blotting buffer (1x blotting buffer and 25x Tris-glycine Transfer Buffer for blotting, Novex; 20% methanol).
- Remove gel from apparatus. Using a razor blade, slice the lower opening of the gel and remove gel pieces in the slot. Pop open plastic plates with a knife. Remove the top portion (1 cm) of the gel.
- Assemble gel transfer sandwich as follows:
  - (facing + electrode) top pad

filter paper  
nitrocellulose  
gel  
filter paper  
(facing – electrode) bottom pad

- Fill the inside of the transfer chamber with blotting buffer and the outside with water.
- Transfer for 1–2 hours at 30 constant volts.
- Disassemble apparatus. Place nitrocellulose in container with blocking solution (50 ml of 5% milk in TBS). Incubate for 0.5–3.0 hours at room temperature, with gentle shaking.
- Discard blocking solution. Rinse nitrocellulose for 5 min in TBS.
- Place nitrocellulose in container with FLAG M1 antibody directly conjugated to horseradish peroxidase (Chromaprobe Inc.; custom conjugation). Incubate for 1–3 hours at room temperature, with gentle shaking. Cover container with foil.

Incubation mix:

1 mM CaCl<sub>2</sub>

1% BSA

0.25 µg/ml M1-HRP

in 1x TBS and 0.1% Tween-20

- Rinse blot with TBS-T Ca<sup>2+</sup> wash buffer. Then, wash twice for 15 min each.
- Remove nitrocellulose from buffer and place on plastic wrap.
- From the Amersham ECL kit, combine 4 ml of solution 1 with solution 2. Immediately pour mixture onto the surface of the nitrocellulose. Allow reaction to proceed for exactly 1 min at room temperature.

- Pour off developing mixture. Enclose nitrocellulose in plastic wrap and place a fluorescent marker next to nitrocellulose. Transfer to a film cassette.
- In dark room, place a piece of film on top of plastic wrap. Expose for 1 min at room temperature and develop film. If signal is low, increase exposure time.

*Detection of FLAG-tagged RASSLs in Suspended Cells by Flow Cytometry*

This method is for detecting RASSLs on lymphocytes of transgenic mice expressing Ro1. With modifications, it can be used to detect RASSLs on a variety of suspended cells. We use a similar protocol to monitor RASSL expression on cultured mammalian cell lines (HEK 293 and Rat1a).

- Cut mouse tail with sharp razor.
- Collect 200  $\mu$ l of blood in 15-ml Falcon conical tube containing 12 ml of fresh PBS and heparin (1–2 units/ml).
- Invert tube several times and store on ice while cutting other tails.
- Spin Falcon tubes in centrifuge at 1000–1200 rpm for 5 min.
- Pour off supernatant (careful, the pellet is not tight) and resuspend in 3 ml of 1x lysis buffer (10x lysis buffer: 80.2 g  $\text{NH}_4\text{Cl}$ , 8.4 g  $\text{NaHCO}_3$ , 3.7 g EDTA in 1L distilled  $\text{H}_2\text{O}$ ).
- Let sample stand in lysis buffer for 5–10 min at room temperature.
- Spin for 3 min at 1000 rpm.
- Resuspend in medium (Delbecco's Modified Eagle's medium + 2% fetal calf serum) and spin again for 3 min at 1000 rpm.
- Resuspend in antibody staining solution (for each reaction: 1 ml of medium solution plus 1  $\mu$ g FLAG M1 antibody. It requires custom conjugation to phycoerythrin by Molecular Probes, Eugene, OR). Keep protected from light. Shake gently on ice for 20–30 min.
- Pellet cells by spinning; wash in 2 ml of medium.

- Pellet cells again; wash in 1% paraformaldehyde in PBS.
- Pellet cells again; resuspend in 500  $\mu$ l of 1% paraformaldehyde/PBS in a 15-ml Falcon tube (2054).
- Cover with aluminum foil and refrigerate until time for FACS analysis.

In theory, immunolabeling of the same FLAG epitope can be used to localize the RASSL *in vivo* by using immunohistological techniques on tissue sections. However, the FLAG M1 antibody is notoriously difficult to use on tissue sections. We are making efforts to improve labeling specificity for the FLAG tag in whole tissue. However, we do have some alternative methods that can be used to visualize RASSL expression in tissue sections.

#### *Beta-galactosidase Staining*

An indirect approach to detecting RASSL expression in tissue section relies on the coinjection of tetO-lacZ along with tetO-RASSL DNA into transgenic mouse lines. This allows both the RASSL and lacZ to integrate stably into the genome at the same location.<sup>20</sup>  $\beta$ -Galactosidase activity can then be used to indicate the expression of lacZ. Although it is not possible to estimate the precise expression level of the RASSL from the intensity of  $\beta$ -galactosidase staining,  $\beta$ -galactosidase is useful for rapid, clear visualization of transgene expression. Typically, we inject three times the amount of RASSL construct relative to the quantity of lacZ. This ratio results in cointegration of the constructs in > 90% of our founder lines.

We have used the following protocol for a variety of fresh frozen tissue samples, including heart, brain, liver, and whole embryo.

- Cut 10- $\mu$ m cryostat sections from fresh-frozen tissues onto pap-penned slides. Immerse immediately in cold formaldehyde/glutaraldehyde for 5 min; then rinse in ddH<sub>2</sub>O for 1 min.

- Let section dry completely onto slide.
- Rinse with 1x PBS.
- Apply final x-gal solution (dilute 40X stock of 40 mg/ml x-gal in DMSO into a solution of 5 mM potassium ferricyanide crystalline, 5 mM potassium ferricyanide trihydrate, 2 mM MgCl in PBS) to sections and incubate at 37°C for 30 min to 24 hours. Check sections under microscope regularly; stop reaction when deep blue staining is observed.
- Rinse with PBS.
- Wash twice for 2 min each with distilled H<sub>2</sub>O.
- Counterstain for 3 min with nuclear fast red.
- Wash twice for 2 min each with distilled H<sub>2</sub>O.
- Coverslip with Gel Mount (Biomedica).

#### *RASSLs Tagged with Green Fluorescent Protein*

The immunological and histological techniques described above typically require that the tissue be fixed or lysed before processing. Sometimes, though, it is useful to visualize the location of the RASSL in living cells. For this purpose, we have tagged our Ro2 RASSL with green fluorescent protein (emerald GFP from Packard). We placed the GFP tag on the N-terminus of the RASSL to avoid potential interference between GFP and the cellular sorting of the RASSL via the cytoplasmic surface of the receptor. GFP is located after the signal sequence and the FLAG tag, but before the coding region of the receptor. This GFP-RASSL fusion protein can be expressed in mammalian cell lines. In preliminary studies of HEK 293 and Rat1a cells, the fusion protein was sorted to the membrane correctly and signaled normally. One advantage that GFP has over  $\beta$ -galactosidase is that in very large cells, like neurons, the location of  $\beta$ -galactosidase staining might not correspond directly to the location of the receptor. The portions of the cells where protein is translated will stain positive for  $\beta$ -galactosidase, but neurons may

transport the receptor protein to distal regions that do not have enough  $\beta$ -galactosidase for detection.

Currently, we are using GFP-Ro2 to monitor receptor internalization after agonist stimulation. We expect to be able to use this protein to assay for localization of the RASSL both *in vitro* and *in vivo* in transgenic mice.

#### Applications of RASSL Technology

Acute activation of RASSLs expressed in discrete tissues or cell types will allow researchers to probe the role of  $G_i$  signaling in the normal function of those cells. Correspondingly, overexpression or extended activation of RASSLs can be used to explore disease models of pathologies related to hyperactive G protein signaling.

#### *Tissue-specific Expression and Activation of RASSLs*

RASSLs have been successfully expressed in a variety of tissues and organs of transgenic mice, including heart, liver, salivary glands, smooth muscle, adipose tissue, specific brain regions, and the vomeronasal organ (see Table 1). This offers several opportunities for new research programs. Our laboratory is particularly interested in using RASSLs to study the control of electrically active tissues by G protein signaling.<sup>7</sup> We have already demonstrated that RASSL activation in the heart can slow heart rate. A number of mouse lines that express the RASSL in specific brain regions are being used to study how activation of  $G_i$  signaling in a given brain nucleus can affect the activity of downstream neural circuits.

#### *Long-term Expression and Activation of RASSLs*

RASSLs can also be used to probe the role of G protein signaling in a number of long-term biological changes. RASSLs that are overexpressed or activated chronically will yield changes in gene expression, and long-term adaptive shifts in the cells

expressing the receptors. Long-term changes in cellular function underlie such important, yet poorly understood, biological functions as neuronal plasticity, cytoskeletal remodeling, apoptosis, proliferation, and differentiation. By using RASSLs to induce chronic shifts in G protein signaling, scientists can better understand how G protein signaling mediates both adaptive and pathological changes in cell function.

#### *Reversible Models of Disease States*

The ability to modulate both RASSL expression (by the tetracycline-inducible expression system) and signaling (by administration of spiradoline) offers the opportunity to create reversible models of disease caused by abnormal G protein signaling.

Our laboratory has developed a mouse model of dilated cardiomyopathy by overexpressing Ro1 in the heart.<sup>8</sup> Mice that express the RASSL for more than 3 weeks begin to develop abnormal heart function, including decreased contractility and wide QRS complexes on electrocardiograms, even in the absence of synthetic ligand. The hearts of these animals have enlarged ventricles and elevated levels of fibrosis and collagen. This phenotype appears to be caused by increased basal  $G_i$  signaling due to the overexpression of Ro1, since the cardiomyopathy occurs without the addition of ligand and it can be blocked by suppressing Ro1 expression. Similarly, blocking signaling by administering antagonists to the receptor (nor-binaltorphimine dihydrochloride) or inhibitors of  $G_i$  (pertussis toxin) can prevent the development of the phenotype or partially rescue it, depending on the time of administration (N. Cotte, T. Nanevicz, and B. Conklin, unpublished observations). This work suggests that hyperactive  $G_i$  signaling can disrupt heart function and lead to dilated cardiomyopathy.

Potentially, RASSLs could be used to study other diseases that may be related to hyperactive  $G_i$  signaling. In the brain, a number of pathologies may be caused by signaling abnormalities. For instance, seizures may be induced by asynchronous activation of G proteins in a particular population of neurons.<sup>21</sup> Dementias and

neurodegenerative disorders have been associated with abnormal G protein signaling.<sup>22</sup> Psychiatric disorders, like schizophrenia, may be related to hyperactive G protein signaling through dopamine receptors.<sup>23</sup> Other diseases that may be tied to abnormal G protein signaling include osteoporosis, vasospasm, and immune disorders like lupus and Crohn's disease.

#### *Gene Expression Fingerprints for Signaling Pathways and Disease States*

RASSLs can be combined with cDNA microarray technologies to monitor the changes in gene expression induced by G protein signaling. This type of study will allow researchers to create gene expression "fingerprints" that identify genes whose expression is regulated by G protein activation in different tissues or at different times in development. This approach is a potentially powerful means for improving our understanding of both normal biological function and pathological states. Data from gene expression experiments based on Ro1-induced cardiomyopathy can be viewed in the sample data provided with a new bioinformatics software program developed in the Conklin lab called GenMAPP ([www.GenMAPP.org](http://www.GenMAPP.org)).

#### Future Directions: New RASSLs

Existing RASSLs will allow study of the effects of  $G_i$  signaling in specific tissues under specific circumstances. Of course, it would be even more valuable to extend these studies to include other G protein signals, like  $G_s$  and  $G_q$ . New RASSLs can be developed by using many of the same principles used to develop the existing  $G_i$ -coupled RASSLs. The ideal RASSL activator should be a small-molecule drug that is readily available from commercial sources. Its binding site should be well characterized. It must be highly specific for a single receptor subtype to minimize effects of the drug at non-RASSL receptors. Ideally, this could be a synthetic system, in which the ligand would not activate endogenous receptors. Failing that, the ligand should not cause significant side effects,

and the effects of activation of its natural receptor should be relatively minor in humans or rodents. As small-molecule ligands developed by the pharmaceutical industry are approved for human use and become freely available to researchers, there will be new opportunities to develop RASSLs that signal through other G protein pathways.

The combination of RASSL technology with the tet system provides a reversible molecular switch that allows researchers to control the timing, location, and specificity of G protein signaling *in vivo*. With the completion of the Human Genome Project, all GPCRs will soon be identified. We can then focus on the significant challenge of understanding how this diverse family of proteins modulates physiological processes. Recent findings revealing the significance of dimerization,<sup>24</sup> protein–protein interactions,<sup>25</sup> and alternative splicing of GPCRs<sup>26</sup> suggest that the signaling and modulation of these proteins is even more complex than previously believed. RASSLs provide a new tool to help study these processes *in vivo* and provide specific functional data for a variety of physiologically important signaling pathways.

## References

- 1 K. Scearce-Levie, P. Coward, C. H. Redfern, and B. R. Conklin, *Trends Pharmacol. Sci.* (In press)
- 2 C. D. Strader, T. M. Fong, M. R. Tota, and D. Underwood, *Annu. Rev. Biochem.* **63**, 101–132 (1994).
- 3 A. M. Spiegel, A. Shenker, and L. S. Weinstein, *Endocr. Rev.* **13**, 536–565 (1992).
- 4 D. Hoyer, D. E. Clarke, J. R. Fozard, P. R. Hartig, G. R. Martin, E. J. Mylecharane, P. R. Saxena, and P. P. A. Humphrey, *Pharmacol. Rev.* **46**, 157–203 (1994).
- 5 A. Bishop, O. Buzko, S. Heyeck-Dumas, I. Jung, B. Kraybill, Y. Liu, K. Shah, S. Ulrich, L. Witucki, F. Yang, C. Zhang, and K. M. Shokat, *Annu. Rev. Biophys. Biomol. Struct.* **29**, 577–606 (2000).
- 6 P. Coward, H. G. Wada, M. S. Falk, S. D. H. Chan, F. Meng, H. Akil, and B. R. Conklin, *Proc. Natl. Acad. Sci. USA* **95**, 352–357 (1998).
- 7 C. H. Redfern, P. Coward, M. Y. Degtyarev, E. K. Lee, A. T. Kwa, L. Hennighausen, H. Bujard, G. I. Fishman, and B. R. Conklin, *Nat. Biotechnol.* **17**, 165–169 (1999).

- 8 C. H. Redfern, M. Y. Degtyarev, A. T. Kwa, N. Salomonis, N. Cotte, T. Nanevycz, N. Fidelman, K. Desai, K. Vranizan, E. K. Lee, P. Coward, N. Shah, J. A. Warrington, G. I. Fishman, D. Bernstein, A. J. Baker, and B. R. Conklin, *Proc. Natl. Acad. Sci. USA* **97**, 4826–4831 (2000).
- 9 G. H. Rimoy, D. M. Wright, N. K. Bhaskar, and P. C. Rubin, *Eur. J. Clin. Pharmacol.* **46**, 203–207 (1994).
- 10 P. A. Reece, A. J. Sedman, S. Rose, D. S. Wright, R. Dawkins, and R. Rajagopalan, *J. Clin. Pharmacol.* **34**, 1126–1132 (1994).
- 11 M. J. Millan, *Trends Pharmacol. Sci.* **11**, 70–76 (1990).
- 12 J.-C. Xue, C. Chen, J. Zhu, S. P. Kunapuli, J. K. de Riel, L. Yu, and L.-Y. Liu-Chen, *J. Biol. Chem.* **270**, 12977–12979 (1995).
- 13 E. V. Varga, X. Li, D. Stropova, T. Zalewska, R. S. Landsman, R. J. Knapp, E. Malatynska, K. Kawai, A. Mizusura, H. Nagase, S. N. Calderon, K. Rice, V. J. Hruby, W. R. Roeske, and H. I. Yamamura, *Mol. Pharmacol.* **50**, 1619–1624 (1996).
- 14 H. Kong, K. Raynor, H. Yano, J. Takeda, G. I. Bell, and T. Reisine, *Proc. Natl. Acad. Sci. USA* **91**, 8042–8046 (1994).
- 15 F. Meng, Y. Ueda, M. T. Hoversten, R. C. Thompson, L. Taylor, S. J. Watson, and H. Akil, *Eur. J. Pharmacol.* **311**, 285–292 (1996).

- 16 S. A. Hjorth, K. Thirstrup, D. K. Grandy, and T. W. Schwartz, *Mol. Pharmacol.* **47**, 1089–1094 (1995).
- 17 M. Gossen, and H. Bujard, *Proc. Natl. Acad. Sci. USA* **89**, 5547–5551 (1992).
- 18 S. R. Holmer, and C. J. Homcy, *Circulation* **84**, 1891–1902 (1991).
- 19 F. Simonin, O. Valverde, C. Smadja, S. Slowe, I. Kitchen, A. Dierich, M. Le Meur, B. P. Roques, R. Maldonado, and B. L. Kieffer, *EMBO J.* **17**, 886–897 (1998).
- 20 R. R. Behringer, T. M. Ryan, M. P. Reilly, T. Asakura, R. D. Palmiter, R. L. Brinster, and T. M. Townes, *Science* **245**, 971–973 (1989).
- 21 N. G. Bowery, K. Parry, A. Boehrer, P. Mathivet, C. Marescaux, and R. Bernasconi, *Neuropharmacology* **38**, 1691–1697 (1999).
- 22 A. J. Berger, A. C. Hart, and J. M. Kaplan, *J. Neurosci.* **18**, 2871–2880 (1998).
- 23 A. Cravchik, D. R. Sibley, and P. V. Gejman, *J. Biol. Chem.* **271**, 26013–26017 (1996).
- 24 H. Möhler, and J.-M. Fritschy, *Trends Pharmacol. Sci.* **20**, 87–89 (1999).
- 25 Y. Tang, L. A. Hu, W. E. Miller, N. Ringstad, R. A. Hall, J. A. Pitcher, P. DeCamilli, and R. J. Lefkowitz, *Proc. Natl. Acad. Sci. USA* **96**, 12559–12564 (1999).
- 26 G. J. Kilpatrick, F. M. Dautzenberg, G. R. Martin, and R. M. Eglen, *Trends Pharmacol. Sci.* **20**, 294–301 (1999).

## Figure Legends

Fig. 1. Construction of an opioid RASSL. Replacing the third extracellular loop of the human KOR with the corresponding sequence from the delta opioid receptor significantly attenuates binding to endogenous peptides, while maintaining affinity for small-molecule kappa agonists. The specificity of the RASSL for small-molecule agonists was further enhanced by mutating the glutamic acid at 297 to glutamine. K-WT = wild-type KOR.

Fig. 2. Prototype RASSL signals specifically in response to the synthetic drug spiradoline. Agonist-mediated changes in intracellular calcium flux were measured with a fluorometric imaging plate reader assay. Curves are sample tracings of actual calcium fluorescence in response to 1  $\mu$ M doses of agonist. The arrow indicates addition of the agonist.

Fig. 3. Tissue-specific gene expression using the tet-tTA system. Two transgenes are required. First, the gene of interest (Ro1, in this case) is placed under the control of the tet operon sequence. Expression of this transgene requires the tetracycline transactivator (tTA). The gene for the tTA can be placed under the control of a tissue-specific promoter to ensure that tTA, and therefore Ro1, are expressed only in a particular tissue. Expression of Ro1 can be suppressed by the addition of doxycycline, which binds to tTA and inactivates it. For gene expression in mice, this system requires two transgenic lines: one with the promoter-tTA transgene, the other with tetO-Ro1. When these mice are mated together, 25% of the offspring will have both transgenes. However, even in these bigenic mice, there will be no Ro1 expression as long as the animals are fed doxycycline. (Reprinted with the permission of Nature America, Inc.)

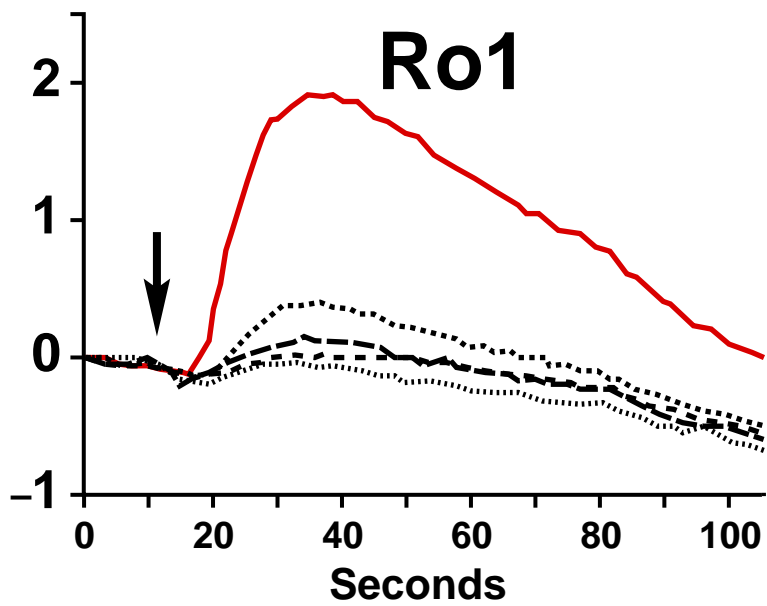
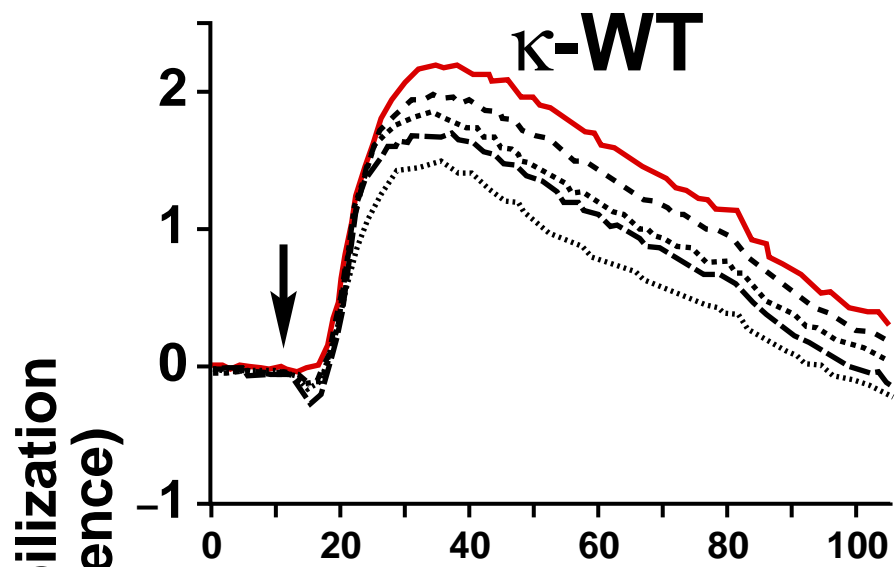
Fig. 4. RASSL-mediated reduction of heart rate (HR). Less than 1 min after spiradoline injection ( $1 \times 10^{-5}$  mol/kg), the heart rate of a mouse expressing Ro1 in the heart decreased to one-third of baseline. Spiradoline had no effect on the control mouse (MHC-tTA). (Reprinted with the permission of Nature America, Inc.)

**Table 1. Physiological effects of  $G_i$  signaling *in vivo* using RASSLs and the tTA-tet system.**

<b>Location of tTA expression</b>	<b>Expected effect of <math>G_i</math> signaling</b>	<b>Laboratories using RASSLs</b>
Vomer nasal organ	Pheromone-induced behavior	Axel (Columbia Univ.) Dulac (Harvard Univ.)
Spinal cord	Analgesia	Iadarola (National Institutes of Health)
Visual cortex	Abnormal vision	Calloway (Salk Inst.)
Arterial smooth muscle	Muscle contraction	Husain (Univ. Toronto)
Kidney, brown fat	Altered mobilization of fat stores	Kopp (National Institutes of Health)
Ventral tegmental area, nucleus accumbens	Motivation, addiction	Conklin/Scarce-Levie (Gladstone/UCSF) Nestler (Univ. Texas, Dallas)
Hippocampus	Abnormal learning and memory	Conklin/Scarce-Levie (Gladstone/UCSF)
Astrocytes	Modulation of neuronal activity	McCarthy (Univ. North Carolina)

Preliminary studies communicated with the permission of Richard Axel, Columbia University; Catherine Dulac, Harvard University; Michael Iadorola, National Institutes of Health; Edward Calloway, Salk Institute; Mansoor Husain, University of Toronto; Jeffrey Kopp, National Institutes of Health; Eric Nestler, University of Texas, Dallas; and Ken McCarthy, University of North Carolina, Chapel Hill.





- **Spiradoline** — **Drug**
  - ..... **DynA1-13**
  - ..... **α-n-End**
  - **Bam-12**
  - ..... **MERF**
- } **Natural opioid peptides**

