Bioluminescence imaging of G protein-coupled receptor activation in living mice

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G protein-coupled receptors (GPCRs), a superfamily of cell-surface receptors involved in virtually all physiological processes, are the major target class for approved drugs. Imaging GPCR activation in real time in living animals would provide a powerful way to study their role in biology and disease. Here, we describe a mouse model that enables the bioluminescent detection of GPCR activation in real time by utilizing the clinically important GPCR, sphingosine-1-phosphate receptor 1 (S1P1). A synthetic S1P1 signaling pathway, designed to report the interaction between S1P1 and β-arrestin2 via the firefly split luciferase fragment complementation system, is genetically encoded in these mice. Upon receptor activation and subsequent β-arrestin2 recruitment, an active luciferase enzyme complex is produced, which can be detected by in vivo bioluminescence imaging. This imaging strategy reveals the dynamics and spatial specificity of S1P1 activation in normal and pathophysiologic contexts in vivo and can be applied to other GPCRs.
protein-coupled receptors (GPCRs) (also known as seven-transmembrane-domain receptors) are the largest and most diverse gene superfamily in the human genome, comprising greater than 3% of the protein coding genes. GPCRs are widely expressed, initiate cellular signal transduction by a diverse array of extracellular ligands, and are involved in virtually all physiological functions. They are also extremely important in clinical medicine as a major drug target class. It is estimated that ~30% of approved pharmaceutical drugs are directed to GPCRs. Despite their enormous biological and clinical importance, technologies to image GPCR activation in their native physiologic environment in real time are lacking, hampering a full understanding of the spatiotemporal dynamics of their activation in vivo by endogenous or exogenous ligands.

Sphingosine-1-phosphate receptor 1 (SIP1) is one of a five-member GPCR family with high affinity for the bioactive sphingolipid, sphingosine-1-phosphate (SIP). It is ubiquitously expressed among tissues and is highly enriched in endothelial cells, where it serves as a key regulator of vascular barrier function. SIP also performs important actions in the immune and nervous systems. Its endogenous sphingolipid ligand, SIP, is produced by all cells and is carried by lipoproteins and albumin in the circulation. SIP–SIP1 signaling has been linked to diverse disease processes, including infection, multiple sclerosis, atherosclerosis, inflammatory bowel disease, and cancer. FT7270 (fingolimod/Gilenya), an SIP1 ligand, is an FDA-approved drug for the treatment of remitting-relapsing multiple sclerosis.

Ligand-activated GPCRs stimulate intracellular signaling pathways via G proteins and other effectors, including β-arrestins. β-Arrestin binding to activated GPCRs also mediates receptor desensitization, internalization, and recycling. The near-universal interaction between GPCRs and β-arrestins following agonist binding has been employed in assays for quantifying receptor activation. We recently developed a mouse model for the detection of activated SIP1 in which a green fluorescent protein (GFP) reporter gene was activated. This model enabled the identification of cells in which receptor activation has occurred in a cumulative manner, but it is not suited for intravital imaging of real-time activation of GPCR signaling.

Here, we describe a genetic model in mice that enables the imaging of SIP1 signaling in real time in the native physiological environment of the receptor. These SIP1 signaling mice were genetically designed to report the interaction between SIP1 and β-arrestin2 upon receptor activation via firefly split luciferase fragment complementation, which produces an active enzyme complex whose activity can be detected by bioluminescence imaging. The SIP1 signaling mice respond to synthetic ligands in accordance to the specificity of the native receptor and the stability of pharmacologic agonists. The SIP1 signaling mice also reveal the timing and anatomical localization of receptor activation by the endogenously synthesized SIP ligand during endotoxin-induced systemic inflammation. This paradigm can be applied to other members of the clinically important GPCR family to enable the study of receptor activation in their in vivo settings.

Results

Generation of SIP1 luciferase signaling mice. Our strategy for imaging the activation of a GPCR in real time in living mice was to adapt a firefly split luciferase complementation system for the detection of the interaction between ligand-activated SIP1 and β-arrestin2 (Fig. 1a). Both SIP1 and β-arrestin2 were genetically modified as individual fusion proteins to carry inactive, but complementary, fragments of luciferase. The SIP1 C-terminus was linked to the C-terminal fragment of firefly luciferase (CLuc: amino acids 394–550), and the β-arrestin2 N-terminus was linked to the N-terminal fragment of firefly luciferase (NLuc: amino acids 1–416). In this scheme, SIP1 activation will promote its interaction with β-arrestin2, facilitating the association of the inactive luciferase fragments and producing an active enzyme complex that, in the presence of substrates ATP and D-luciferin, generates light that can be detected by bioluminescence imaging (BLI).

The efficacy and specificity of the split luciferase complementation system for detection of SIP1 activation was tested by co-transfection of expression vectors containing the SIP1-CLuc and NLuc–β-arrestin2 fusion genes into U2OS cells. As a negative interaction control, NLuc was fused to herpes simplex virus thymidine kinase (HSV-tk) in place of β-arrestin2, and co-transfected with SIP1-CLuc. In transfected cells treated with vehicle, minimal luciferase activity was detected (Supplementary Fig. 1a, b). However, addition of SIP or RP-001, a synthetic SIP1-selective agonist, caused a significant increase in luciferase activity in the SIP1-CLuc/NLuc–β-arrestin2-transfected cells compared with the SIP1-CLuc/NLuc–HSV-tk-transfected cells. These results demonstrate that the split luciferase complementation system, utilizing SIP1 and β-arrestin fusions, can specifically detect SIP1 activation.

Gene targeting in embryonic stem cells was employed to produce two individual mouse lines, each carrying one of the fusion constructs (Supplementary Fig. 2a, b). One mouse line was generated with the SIP1-CLuc fusion knocked in to the SIP1 coding region. In this configuration, the native SIP1 promoter elements were maintained to allow for expression of the SIP1 fusion gene in the endogenous context. The other mouse line was established with the NLuc–β-arrestin2 fusion knocked in to the safe harbor Rosa26 locus under control of the Rosa26 promoter elements to provide a ubiquitous expression pattern, ensuring that NLuc–β-arrestin2 would be co-expressed along with SIP1-CLuc. The SIP1-CLuc knock-in mice were crossed with the NLuc–β-arrestin2 knock-in mice to derive mice carrying both alleles (Fig. 1a), which are termed SIP1 luciferase signaling mice.

To determine whether the synthetic SIP1 signaling pathway genetically encoded within the SIP1 luciferase signaling mice was responsive to ligands with specificity similar to native SIP1, primary mouse embryonic fibroblasts (MEFs) derived from SIP1 luciferase signaling mice were utilized. Addition of SIP but not vehicle rapidly induced bioluminescence in the MEFs (Fig. 1b). Bioluminescence activity peaked within 3 min after addition of SIP to the MEFs and was rapidly lost thereafter (Fig. 1b). This loss of activity indicates that luciferase fragment complementation is transient and may reflect the rapid kinetics of SIP1 desensitization after activation and the subsequent dissociation of the receptor–β-arrestin2 complex. SIP and RP-001, a potent synthetic SIP1-selective agonist, induced luciferase activity in the SIP1 luciferase signaling MEFs, but not in MEFs carrying only the SIP1-CLuc or the NLuc–β-arrestin2 alleles (Fig. 1b, c), showing that both components of the genetically encoded split luciferase complementation system were necessary to report the ligand-activated SIP1–CLuc interaction with NLuc–β-arrestin2. Two structural analogs of SIP, lysophosphatidic acid (LPA) and sphingosine, that are not ligands for SIP1 did not induce bioluminescence when added to SIP1 luciferase signaling MEFs at a concentration of 10−6 M (Fig. 1d, e).

The EC50 values for the activation of bioluminescence by SIP and RP-001 in SIP1 luciferase signaling MEFs were determined to be 19.4 ± 0.8 and 0.46 ± 0.02 nM, respectively (Fig. 1f). The natural ligand dihydro-SIP (dSIP), exhibited an EC50 for the activation of bioluminescence of 32.4 ± 0.5 nM.
**Fig. 1** Generation of S1P1 luciferase signaling mice and detection of S1P1 activation. 

**a** (top), Schematic of the firefly split luciferase complementation design to monitor S1P1–β-arrestin2 interactions. 

**b** S1P1–CLuc luciferase signaling mice were exposed to S1P, sphingosine (Sph), or LPA (10 μM) in triplicate, and immediately subjected to BLI. Imaging was for sequential 3-min periods. Data represent the mean ± SEM. 

**c** Bar graphs showing the total flux (p/s; cm²) for 1st, 2nd, and 3rd acquisitions in 10⁻⁶ M S1P, RP-001, or Vehicle. Data represent the mean ± SEM.

**d** Bar graphs showing the total flux (p/s; cm²) for 10⁻⁶ M S1P, LPA, or Sph in 1st, 2nd, and 3rd acquisitions. Data represent the mean ± SEM.

**e** Bar graphs showing the total flux (p/s; cm²) for 10⁻⁶ M S1P, RP-001, or Vehicle in 1st, 2nd, and 3rd acquisitions. Data represent the mean ± SEM.

**f** Bar graphs showing the total flux (p/s; cm²) for 10⁻⁶ M S1P, LPA, or Sph in 1st, 2nd, and 3rd acquisitions. Data represent the mean ± SEM.

**g** Bar graphs showing the total flux (p/s; cm²) for 10⁻⁶ M S1P, LPA, or Sph in 1st, 2nd, and 3rd acquisitions. Data represent the mean ± SEM.

**h** Bar graphs showing the total flux (p/s; cm²) for 10⁻⁶ M S1P, LPA, or Sph in 1st, 2nd, and 3rd acquisitions. Data represent the mean ± SEM.

**i** Bar graphs showing the total flux (p/s; cm²) for 10⁻⁶ M S1P, LPA, or Sph in 1st, 2nd, and 3rd acquisitions. Data represent the mean ± SEM.

**j** Bar graphs showing the total flux (p/s; cm²) for 10⁻⁶ M S1P, LPA, or Sph in 1st, 2nd, and 3rd acquisitions. Data represent the mean ± SEM.
To determine if the genetically encoded split luciferase complementation system could detect basal S1P₁ activation in vivo, S1P₁ luciferase signaling mice, as well as mice carrying only the S1P₁–CLuc or NLuc–β-arrestin2 alleles, were subjected to BLI while under anesthesia. Significantly higher levels of bioluminescence activity were detected in the S1P₁ luciferase signaling mice compared with the mice carrying only the S1P₁–CLuc or NLuc–β-arrestin2 alleles (Fig. 1g). We quantified bioluminescence activity in the cephalic, thoracic, and epigastric regions, as the activity varied between different body regions. The highest activity was observed in the epigastric region, followed by the thoracic and cephalic regions (Fig. 1h). To identify the anatomical sites of basal S1P₁ activation in S1P₁ luciferase signaling mice, S1P₁ luciferase signaling mice were subjected to BLI after surgically exposing the thoracic region and abdominal cavity. The highest bioluminescence signals were detected in lungs, in lymph nodes, and in the splenic region. Notably, no signal was detected in the liver (Fig. 1i).

**Treatment of S1P₁ signaling mice with antagonist.** To determine if the bioluminescence activity detected in S1P₁ luciferase signaling mice was the result of endogenous S1P₁ activation, the mice were injected with W146, a potent S1P₁-selective antagonist (Fig. 2a). W146 treatment administered 30 min prior to BLI significantly lowered the bioluminescence in the S1P₁ luciferase signaling mice by 70–80% in a dose-dependent manner in the cephalic, epigastric, and thoracic regions compared with the same mice treated with vehicle (Fig. 2b–d). The basal bioluminescence activity was completely restored in these S1P₁ luciferase signaling mice 1 day post-W146 injection (Fig. 2b–d), which is consistent with the rapid clearance reported for this compound. The inhibition of bioluminescence activity by an S1P₁ antagonist indicates that the basal activity detected in the live mice is primarily the result of endogenous S1P₁ activation.

**Treatment of S1P₁ signaling mice with agonists.** FTY720 (fingolimod/Gilenya\(^{29}\)，a sphingosine analog, is phosphorylated by sphingosine kinases in vivo, and converted to a form that, at nanomolar concentrations, activates S1P₁, as well as S1P₃, S1P₄, and S1P₅.\(^{30}\) Its half-life in blood after oral administration in rats is ~24 h. To determine the time course of S1P₁ activation by FTY720 in the S1P₁ luciferase signaling mice, the mice were imaged prior to the administration of compounds and then imaged serially at 1.5, 6, 24, and 48 h after intraperitoneal injection of each compound (Fig. 3). FTY720 concurrently induced peak levels of bioluminescence activity in the cephalic, thoracic, and epigastric regions at 6 h after injection. Significant bioluminescence activity continued to be detected at 24 and 48 h after injection of FTY720, commensurate with the relatively long half-life of the drug. BLI performed on FTY720-treated S1P₁ luciferase signaling mice (5 h after FTY720 treatment) with their internal organs surgically exposed showed highly elevated bioluminescence signals over lung, lymph nodes, spleen, and liver (Supplementary Fig. 3a). Compared with FTY720, RP-001 is a very short-acting S1P₁ agonist that decreases to undetectable levels in blood by 8 h after administration in mice\(^{25}\) but is substantially more potent, being effective at picomolar concentrations. RP-001

![Figure 2](image-url)

**Fig. 2** S1P₁ activation after treatment with an S1P₁ antagonist in live mice. a S1P₁ luciferase signaling (S1P₁LS) mice were serially injected with vehicle, followed by the S1P₁ antagonist W146 (5 or 10 mg/kg, ip injection). b Representative repeat bioluminescence images of the same mice (top, supine view of mouse #1, 2, 3; bottom, prone view of mouse #4, 5, 6) comparing the effects of vehicle with those of W146 (10 mg/kg), 0.5 h after injection. BLI was also performed 1 day after the W146 injection (post W146). Red open rectangles representing ROI were positioned around cephalic, thoracic, and epigastric regions. c, d Total flux (p/s) in each ROI. Data acquired from supine (c) and prone (d) views are shown. Data represent the mean ± SEM. n = 6 for each group. P values were determined by one-way ANOVA followed by Bonferroni’s multiple comparisons test; **P ≤ 0.01, ***P ≤ 0.001.
induced a peak of bioluminescence activity 1.5 h after administration to S1P1 luciferase signaling mice in the cephalic, thoracic, and epigastric regions. The bioluminescence signal was most intense in the epigastric region. By 24 and 48 h after dosing, the bioluminescence activity in RP-001-treated S1P1 luciferase signaling mice declined to baseline levels (Fig. 3b–e).

**LPS-induced systemic inflammation in S1P1 signaling mice.** S1P1 signaling is activated during systemic inflammation induced by bacterial lipopolysaccharide (LPS); however, the timing and anatomical distribution of receptor activation is not well established. In order to define these parameters, S1P1 luciferase signaling mice were intraperitoneally injected with a sublethal dose of LPS and then subjected to serial BLI at 2, 6, 24, 48, 72, and 96 h (Fig. 4). At 2 h after injection, a weak increase in bioluminescence was observed primarily in the epigastric region, which returned to baseline levels at 6 h. At 24 h after administration, the bioluminescent signal was significantly increased in the cephalic and thoracic regions (Fig. 4b, c and Supplementary Fig. 5). At 48 h after administration, bioluminescent signal was elevated in the thoracic region. At 72 h after administration, the strongest signal was observed in the epigastric region. At 24 h after LPS administration, BLI performed in S1P1 luciferase signaling mice with surgically exposed internal organs demonstrated bioluminescent signals induced over lungs, lymph nodes, spleen, and liver (Supplementary Fig. 3b). These results show that LPS induces heightened S1P1 activation in a sustained manner systemically. In addition, the timing of S1P1 activation was distinctive for specific anatomical locations.

**LPS-induced S1P1 activation by hematopoietically derived S1P.** A major fraction of circulating S1P is produced by hematopoietically derived cells. To determine if hematopoietic cell-derived S1P is critical for systemic S1P1 activation during LPS-induced inflammation, irradiated S1P1 luciferase signaling mice were transplanted with sphingosine kinase-deficient bone marrow from plasmaS1Pless mice, which lack the ability to produce S1P in the hematopoietic system, and then subjected to BLI (Fig. 5a). Plasma S1P and dhS1P levels were significantly decreased in the plasmaS1Pless bone marrow-transplanted mice to ~20 and 10%, respectively, of the levels observed in control bone marrow-transplanted mice (Fig. 5b). The residual S1P and dhS1P in the plasmaS1Pless bone marrow-transplanted mice are likely produced by the sphingosine kinase-replete endothelial cells of the recipient mice. After LPS treatment, mice transplanted with control bone marrow exhibited significantly increased bioluminescence activity compared with the plasmaS1Pless bone marrow-transplanted mice in the thoracic region at 2, 6, and 24 h.
and in the epigastric region at 24 h (Fig. 5c–f). The residual bioluminescence activity in plasmaS1Pless bone marrow-transplanted mice at 24 h after LPS treatment was significantly inhibited by the S1P1 antagonist W146 (Supplementary Fig. 4). These results suggest that the low levels of dhS1P and S1P remaining in these mice may provide some signaling activity. A high degree of mortality occurred 24 h after LPS treatment in S1P1 luciferase signaling mice transplanted with plasmaS1Pless bone marrow, precluding further measurements. These results indicate that hematopoietically derived S1P is responsible for a significant portion of the S1P1 activation that occurs in vivo during LPS-induced inflammation.

S1P1 activation in the brain by inflammation. During LPS-induced inflammation, bioluminescence activity was significantly increased in the cephalic region at 24 h (Fig. 4b, c and Supplementary Fig. 5), raising the possibility that S1P1 may be activated in the central nervous system (CNS). To determine if S1P1 signaling was activated within the CNS and, if so, in which cell types, we utilized the previously described S1P1 GFP signaling mice. In these mice, S1P1 activation is also based on interaction of the GPCR with β-arrestin2; however, in this reporter system the interaction leads to the proteolytic release of a transcription factor tethered to S1P1, that enters the nucleus and activates a histone-GFP reporter gene. The cells in which S1P1 activation occurs are then stably marked with GFP-labeled nuclei (Fig. 6a). LPS was administered to S1P1 GFP signaling mice and, 7 days later, brain tissue was collected and sectioned, then the cortex, cerebellum, and brainstem were examined for GFP-labeled cells (Fig. 6b, c).

Compared with vehicle-treated mice, LPS-treated mice exhibited increased numbers of GFP-positive cells in each of these regions, indicating that S1P1 activation had taken place in the CNS. The GFP-positive cells appeared to be associated with the vasculature and were CD31 positive, establishing their identity as endothelial cells (Fig. 6c). Next, brain endothelial cells were isolated from S1P1 luciferase signaling mice, treated with S1P or RP-001, and examined by BLI. Bioluminescent activity was induced in the brain endothelial cells in a dose-dependent manner, with RP-001 exhibiting a higher potency than S1P (Fig. 6d–f). Collectively, the results indicate that S1P1 is activated in endothelial cells of the neurovascular unit of the brain during systemic inflammation induced by LPS and illustrate that the two different S1P1 signaling mouse models provide complementary information for defining S1P1 activation in vivo.

Discussion

The luciferase signaling model for GPCR activation described here affords a way to study GPCRs that was not previously possible, yielding spatial and temporal information on the status of endogenous receptor activation in real time in a living animal. The nearly universal interaction between GPCRs with β-arrestin13 was exploited to induce assembly of inactive fragments of luciferase, producing an active enzyme complex and enabling BLI upon activation of a GPCR, S1P1, in living mice. The in vivo S1P1 signaling detection system was shown to be activated by both synthetic and natural ligands of S1P1. BLI of the luciferase signaling mice revealed the anatomical locations of homeostatic S1P1 signaling, as well as the dynamics of S1P1 signaling under...
inflammatory conditions. Although the resolution of the luciferase signaling model was limited to the identification of signaling at an organ or tissue level, the associated use of the previously described GFP signaling model for S1P1 activation, which relies on transcription factor reporter gene activation, provided a means to ultimately define S1P1 signaling sites at a cellular level. While visualizing the source of the bioluminescent signal with single cell resolution is not possible at present, detection of signaling in real-time in vivo at a single cell level would be an important goal to achieve.

We have demonstrated that the S1P1 luciferase signaling mice can be used for the characterization of the in vivo signaling activity of the receptor-active pharmacological compounds FTY720 and RP-001 (Fig. 3). FTY720, an approved drug (Gilenya™) used for the treatment of multiple sclerosis, targets S1P receptors 1, 3, 4, and 5. It has a relatively long half-life in blood and has been demonstrated to have diverse physiologic effects in the immune, cardiovascular, and nervous systems. RP-001 is an S1P1-selective agonist that is more potent than FTY720, but with a much shorter half-life in blood. Similar to FTY720, RP-001 has been shown to trigger lymphopenia. Administration of these compounds to the S1P1 luciferase signaling mice enabled determination of the timing of signaling and anatomical location of signaling events, which indicates that this model will be generally useful for characterization of S1P1 modulators. The signaling induced by RP-001 was relatively intense but decayed rapidly, commensurate with its high potency and short half-life (Fig. 3b–e). The very intense epigastic signal induced by RP-001 may reflect its rapid clearance in the liver. In contrast, the signaling induced by FTY720 in vivo persisted over a long duration, lasting up to 48 h (Fig. 3b–e). While consistent with the relatively long half-life of the compound, prolonged signaling, and the apparent absence of rapid desensitization would be compatible with the existence of a receptor reserve. Under normal conditions, a receptor reserve may underpin the long-term tonic S1P signaling in the vascular endothelium. Compartmentalization of S1P1 receptors may be a mechanism underlying such a receptor reserve. However, a different mechanism must operate on

**Fig. 5** S1P1 activation during LPS-induced systemic inflammation in live mice transplanted with plasmaS1Pless bone marrow. a Bone marrow cells from control or plasmaS1Pless bone marrow mice were transplanted (BMT) into irradiated S1P1 luciferase signaling (S1P1LS) mice to produce control transplanted (Control-T) or pS1Pless transplanted (S1Pless-T) mice, respectively. LPS was injected intraperitoneally into the stably transplanted mice 20 weeks later. Mice were subjected to BLI prior to the LPS injection (baseline, B/L) and 2, 6, and 24 h after LPS injection. b Plasma S1P and dhS1P levels in S1P1 luciferase signaling mice, transplanted with control (Control-T) or pS1Pless (S1Pless-T) bone marrow. Data represent the mean ± SEM. n = 6 for Control-T mice, n = 8 for S1Pless-T mice. c Representative bioluminescence images of the same S1P1 luciferase signaling mice, transplanted with control (Cont-T) or pS1Pless (S1Pless-T) bone marrow, at the specified time points. Mice were imaged in the supine position. Red open rectangles representing ROI were positioned around cephalic, thoracic, and epigastric regions. d–f The bioluminescence activity was quantified by determining the total flux (photons/sec; p/sec) in the cephalic (d), thoracic (e), and epigastric (f) ROI. Data represent the mean ± SEM. n = 5 for control BM-transplanted S1P1 luciferase signaling (Control-T) mice and n = 7 for pS1Pless BM-transplanted S1P1 luciferase signaling (S1Pless-T) mice at the baseline, 24 h, and 6 h time points. n = 4 for control BM-transplanted S1P1 luciferase signaling (Control-T) mice and n = 6 for pS1Pless BM-transplanted S1P1 luciferase signaling (S1Pless-T) mice at 24 h. P values were determined two-tailed Student’s t-test; *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.
other cell types, such as lymphocytes, that rapidly downmodulate S1P1 upon exposure to agonist\(^\text{25}\).

The S1P1 luciferase signaling mice provide the unique capability to detect the signaling activity of the endogenously produced natural ligand under basal and pathophysiological conditions in real time. Under basal conditions, lung and lymphoid tissues showed the highest levels of signaling (Fig. 1i). This is compatible with ample evidence for a functional S1P1 signaling requirement in these tissues. In lung, blocking S1P1 signaling—either by removal of S1P from the blood\(^\text{35}\) or by S1P1 antagonism\(^\text{28,29}\)—induced vascular leakage, indicating that S1P1 signaling is critical to maintain vascular integrity in lung. In lymphoid tissues, S1P1 signaling has been shown to be necessary for lymphocyte egress\(^\text{36}\) and for the positioning of cells, such as splenic marginal zone B cells\(^\text{37}\). S1P1 signaling is also required in endothelial cells in lymph nodes, notably on high endothelial venules, which serve as the portals for lymphocyte entry to maintain their vascular integrity\(^\text{38}\).

Under conditions of systemic inflammation induced by LPS exposure, the S1P1 luciferase signaling mice reported region-specific dynamics in S1P1 activation (Fig. 4). Liver showed little or no signaling activity under basal conditions (Fig. 1i), but exhibited dramatically increased S1P1 signaling during systemic inflammation (Supplementary Fig. 3b). Hepatocytes have been shown to express abundant S1P1, that is accessible for signaling in response to FTY720 administration\(^\text{22}\). Based on the findings reported here, they are apparently not exposed to sufficient S1P under basal conditions to stimulate signaling. However, during conditions that induce vascular leakage, such as inflammation, S1P derived from blood sources can stimulate S1P1 signaling in hepatocytes and endothelial cells of liver, and endothelial cells in lung, as has been demonstrated in an S1P1 GFP signaling mouse model\(^\text{32}\). We have extended these findings here by showing increased S1P1 signaling in brain endothelial cells during systemic inflammation (Fig. 6).

The strategy used to produce the S1P1 luciferase signaling mice provides a template for the development of a library of other GPCR-signaling models. Creation of models for other GPCRs that similarly interact with β-arrestin2 should only require the derivation of a mouse with a receptor knock-in of the C-terminal Luc fragment (amino acids #394–550). Subsequent cross-breeding with the NLuc–β-arrestin2 mouse described here, which carries the complementary luciferase N-terminal fragment (amino acids #1–416), would yield unique GPCR-signaling models. These

![Diagram](https://example.com/diagram.png)

**Fig. 6** S1P1 activation by LPS in the brain. a Schematic of the design to monitor S1P1 activation in S1P1 GFP signaling mice. S1P1 is fused to a transcription factor (TF) via a protease recognition sequence. Ligand binding to the receptor stimulates the recruitment of a β-arrestin2–TEV protease fusion protein, triggering the release of the transcription factor from the C terminus of modified S1P1. The transcription factor stimulates expression of a histone-GFP reporter gene, fluorescently labeling cell nuclei. b LPS or vehicle was injected intraperitoneally into S1P1 GFP signaling (S1P1GS) mice, and the brains were harvested 7 days after injection and examined by immunofluorescence. The tissues of three mice injected with LPS and three mice injected with vehicle were examined. c Representative histological sections were immunostained with antibodies to CD31, and the images of cerebral cortex, cerebellum, and brainstem were captured using an inverted laser scanning confocal microscope. Scale bar, 50 μm. d Brain endothelial cells (EC) were isolated from S1P1 luciferase signaling (S1P1,LS) mice, grown in triplicate in 24-well plates, treated with S1P or RP-001 and D-luciferin, and immediately subjected to BLI. e Bioluminescence images of the cells exposed to the indicated concentrations of S1P or RP-001 represent a 15-min acquisition. The experiment was repeated twice. f The bioluminescence activity was quantified by determining the total flux (photons/sec/p/sec). Data represent the mean ± SEM, n = 6.
models can be used to gain new understandings of GPCR signaling in normal and disease-specific biological contexts, and enable in vivo analysis of GPCR-signaling perturbation by receptor-active compounds, thereby facilitating drug development for this important target class.

Methods

Reagents. SIP (d181), sphingosine (d181), and LPA (181) were purchased from Avanti Polar Lipids; dhSIP (d180), LPS (Escherichia coli 055:B5; L2880), and polyisopropylpolyglycyclic ribonucleic acid (pIpgP) (P9913) from Sigma-Aldrich; FITC720 and W146 from Cayman; and RP-001 from Tocris.

Generation of S1P1 luciferase signaling mice. A split firefly luciferase enzyme complementation system23-25 (Fig. 1a) was designed for detection of the SIP-β-arrestin2 interaction after receptor activation. The fragments were fused to the mouse S1pr1 (Accession: NM_007901.5) or Arrb2 (Accession: NM_145249.5) coding regions via a linker (GGGGS/SSGGGS); the S1pr1 coding region was linked to the firefly luciferase fragment (amino acids 1-416) to produce the NLuc-β-arrestin2 fusion. Targeting vectors for mouse embryonic stem cells were constructed to insert the SIP-CLuc or NLuc-β-arrestin2 fusions into the S1pr1 or Rosa26 locus, respectively (Supplementary Fig. 2a, b). In the S1pr1-targeting vector, the gene in-frame with the stop resistance gene (NeoR), flanked by loxp sites, was inserted downstream of the fusion gene. In the Rosa26-targeting vector39, NeoR and a stop cassette (NeoR Stop), flanked by loxp sites, was placed upstream of the fusion gene. Gene targeting in embryonic stem cell lines and generation of chimeric and heterozygous mice were conducted as described previously42. The genotypes were determined by PCR analysis of genomic DNA isolated from mouse tails. For genotyping of the S1pr1–CLuc fusion knock-in mice carrying NeoR, three primers were used: 5′-AGAGGATAATGCGCCTGTGATCCT-3′ (Primer 1); 5′-GGTGAACATCACCACCATATACCA-3′ (Primer 2); and 5′-CCAAATATAGGCGCATGTCCTCG-3′ (Primer 3). Primers 1 and 2 detected the wild-type (WT) allele and amplified a 290-bp fragment. Primers 2 and 3 detected the knock-in allele carrying NeoR and amplified a 400-bp fragment. Forty cycles of 94 °C (1 min), 60 °C (1 min), and 72 °C (1.5 min) were used for PCR. For genotyping of the NLuc–β-arrestin2 knock-in mice bearing NeoR stop, three primers were used: 5′-CGAAGTGGAAGTTCAGACT-3′ (Primer 4); 5′-GGGATTACACGGCATGTAAT-3′ (Primer 5); and 5′-TACCGGTGATGGATGAA-3′ (Primer 6). Primers 4 and 5 detected the WT allele and amplified a 450-bp fragment. Primers 4 and 6 detected the knock-in allele bearing NeoR stop and the stop codon and amplified a 580-bp fragment. Forty cycles of 94 °C (1 min), 63 °C (1 min), and 72 °C (1.5 min) were used for PCR.

An Ella Cre recombinase transgenic line48 (stock #000724, The Jackson Laboratory) was crossed with the knock-in mice to remove the loxp-flanked genes in the germline. For genotyping of the S1pr1–CLuc knock-in mice with NeoR excised (−NeoR), three primers were used. Primers 1 and 7 (5′-AGAGGATAATGCGCCTGTGATCCT-3′) detected the WT allele and amplified a 400-bp fragment. Primers 7 and 8 (5′-TAGTGGCAAGCATCCGTGTTG-3′) detected the S1pr1–CLuc (−NeoR) knock-in allele and amplified a 510-bp fragment. Forty cycles of 94 °C (1 min), 63 °C (1 min), and 72 °C (1.5 min) were used for PCR. For genotyping of the NLuc–β-arrestin2 (−NeoR Stop) knock-in mice, three primers were used: 5′-TACCGGTGATGGATGAA-3′ (Primer 4); 5′-CCAAATATAGGCGCATGTCCTCG-3′ (Primer 5); and 5′-CCAAATATAGGCGCATGTCCTCG-3′ (Primer 6). Primers 4 and 5 detected the WT allele and amplified a 450-bp fragment. Forty cycles of 94 °C (1 min), 63 °C (1 min), and 72 °C (1.5 min) were used for PCR.

The scheme to derive SIP, luciferase signaling mice is shown in Fig. 1a. Mice heterozygous for S1pr1–CLuc (−NeoR) knock-in mice were crossed with mice heterozygous for NLuc–β-arrestin2 (−NeoR) to obtain mice heterozygous for S1pr1–CLuc (−NeoR) and homozygous for NLuc–β-arrestin2 (−NeoR) to obtain mice heterozygous for S1pr1–CLuc (−NeoR) and homozygous for NLuc–β-arrestin2 (−NeoR) allele; and heterozygous for the NLuc–β-arrestin2 allele (−NeoR Stop) allele showed a higher bioluminescence signal after FTY220 treatment than mice heterozygous for both of these alleles (Supplementary Fig. 6). For this reason, mice heterozygous for the S1pr1–CLuc (−NeoR) allele and homozygous for the NLuc–β-arrestin2 (−NeoR Stop) allele were used in S1pr1 luciferase signaling mouse studies. Animals were housed in a specific pathogen-free facility and provided food and water ad libitum. Age-matched offspring of both sexes (2–5 months old) were used for experiments. The mice were at the initiation of experiments. The sample size was selected based on expected effect size. No randomization method was employed. Investigators were blinded to the genotype of animals during data analysis. No experimental animals were excluded from the analysis.

BLI of S1P1, activation in cells and mice. S1pr1–CLuc, NLuc–β-arrestin2, and NLuc–HSV-tk (accession: Q9QNP7.1) fusions were produced using a linker (GGGSGGGGGG) sequence as described above and cloned into pcDNA3.1 (Thermo Fisher Scientific). U2OS cells (American Type Culture Collection) were plated in 24-well plates (1.5 × 10⁵ cells/well) and transfected by pcDNA3.1 (SIP) or pcDNA3.1 (NeoR stop) together with pcDNA3.1-NLuc–HSV-tk (500 ng) using Lipofectamine 3000 (Thermo Fisher Scientific). The media was replaced 24 h after transfection with DMEM containing 10% charcoal-stripped FBS and the cells incubated for 16 h. The media was then changed to DMEM with 0.1% FBS for 4 h, and then treated simultaneously with 125 nM D-luciferin (K + Salt Bioluminescent Substrate) or S1P, RP-001, or vehicle. Bioluminescence activity was immediately detected in an in vivo imaging system (IVIS Lumina II, PerkinElmer).

SIP, luciferase signaling MEFs were isolated from E12.5 embryos with three different genotypes: heterozygous for the NLuc–β-arrestin2 allele; and heterozygous for the S1pr1–CLuc allele and homozygous for the NLuc–β-arrestin2 allele (SIP, luciferase signaling mice). The MEFs were plated in 24-well plates (2 × 10⁴ cells/well) and incubated for 16 h in DMEM containing 10% charcoal-stripped FBS for 16 h followed by DMEM with 0.1% FBS for 4 h. After treatment with agonists, bioluminescence activity was measured immediately as described above.

SIP, luciferase signaling mouse brain endothelial cells were prepared as previously described45. The endothelial cells in 24-well plates (10⁴/well) were incubated in medium (DMEM high glucose [12–70 F, LONZA], 5 μg/ml endothelial cell growth factor [BT-203, Biochem Tech], 100 μg/ml heparin, 100 U/ml penicillin, and 100 U/ml streptomycin) containing 10% charcoal-stripped FBS for 16 h followed by medium containing 0.1% BSA for 4 h. After treatment with agonists, bioluminescence activity was immediately detected as described above.

For BLI in live mice, the hair was removed with a clipper and hair removal cream. Xenogen Light Red Detect D-luciferin (PerkinElmer) (150 mg/kg BW) was injected intraperitoneally into mice subjected to BLI imaging system (IVIS Lumina II) with four acquisitions lasting 5 min each.

In some mouse studies, prior to imaging, mice were intraperitoneally injected with FTY720 (1 mg/kg BW; vehicle, 43% ethanol-7% DMSO in PBS), RP-001 (1 mg/kg BW; vehicle, 43% ethanol-7% DMSO in PBS), W146 (5 and 10 mg/kg BW; vehicle, 10 mM sodium carbonate-2% (2-hydroxypropyl)-β-cyclodextrin in water), or LPS (16 mg/kg BW; vehicle, PBS).

Analysis of S1P1, activation in brain. S1P, GFP signaling mice have been previously described22. LPS (16 mg/kg BW; vehicle, PBS) was injected intraperitoneally into S1P, GFP signaling mice. Seven days after the injection, the mice were perfused with normal saline followed by chilled 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) and the brains were harvested. The tissues were post fixed in 4% paraformaldehyde for 4 h and incubated in 20% sucrose in 0.1 M phosphate buffer (pH 7.4) at 4 °C for 2 days, then embedded in OCT (Sakura) and sectioned at 7-μm thickness. For immunostaining, nonconjugated primary antibody for mouse CD31 (clone SZ31, Dianova) and fluoroscence labeled secondary antibody (donkey anti-rat IgG Cyanine Cy3; Jackson ImmunoResearch Laboratories) were used. The images were captured with an inverted laser scanning confocal microscope (LSM 780, Carl Zeiss Microscopy) using Zeiss Zen 2012 software.

Preparation of plasma S1P-deficient S1P1; luciferase signaling mice. Sphk1fl/+; Sphk2−/− mice (stock #003008-UCD, MMRRC), Sphk2−/+ mice42 and Mx1-c1 mice43 (stock #003556, The Jackson Laboratory). Plasma S1P-deficient (plasmaS1Pless) mice were prepared by injection of pLP.C into Sphk1fl/+; Sphk2−/− mice using an in vivo imaging system (IVIS) with four acquisitions lasting 5 min each.

Spingolipid analysis. SIP and dhSIP were measured by HPLC-tandem MS by the Lipidomics Core at the Medical University of South Carolina on a Thermo Finnigan TSQ 7000 triple quadrupole mass spectrometer (Thermo Fisher
3. Ezkurdia, I. et al. Multiple evidence strands suggest that there may be as few as 10,000 genes involved in diseases and performed in accordance with the National Institutes of Health (NIH) and the Food and Drug Administration. The study is available within this paper or its Supplementary Information.

4. Roth, B. L. & Kroeze, W. K. Integrated approaches for genome-wide phylogenetic analysis, paralogon groups, and fingerprints. Mol. Pharmacol. 63, 1256–1277 (2003).

5. Lefkowitz, R. J. & Shenoy, S. K. Transduction of receptor signals by β-arrestins. Annu. Rev. Pharmacol. Toxicol. 55, 117–144 (2011).

6. Allen, J. A. & Roth, B. L. Strategies to discover unexpected targets for drugs active at G protein-coupled receptors. Annu. Rev. Pharmacol. Toxicol. 51, 117–144 (2011).

7. Sheny, S. K. & Lefkowitz, R. J. β-arrestin-mediated receptor trafficking and signal transduction. Trends Pharmacol. Sci. 32, 521–533 (2011).

8. Lee, M. H. et al. The conformational signature of β-arrestin2 predicts its trafficking and signalling functions. Nature 531, 665–668 (2016).

9. Wils, J. W., Xiao, K., Thomsen, A. R. & Lefkowitz, R. J. Recent developments in biased agonism. Curr. Opin. Cell Biol. 27, 18–24 (2014).

10. Barak, L. S., Ferguson, S. S., Zhang, J. & Caron, M. G. A β-arrestin/green fluorescent protein biosensor for detecting G protein-coupled receptor activation. J. Biol. Chem. 272, 27497–27500 (1997).

11. Hattori, M. & Ozawa, T. Live cell bioluminescence imaging in temporal reaction of g protein-coupled receptor for high-throughput screening and analysis. Methods Mol. Biol. 1461, 195–202 (2016).

12. Kono, M. et al. Sphingosine-1-phosphate receptor 1 reporter mouse reveals receptor activation sites in vivo. J. Clin. Invest. 124, 2076–2086 (2014).

13. Paulmurugan, R., Umezawa, Y. & Gambhir, S. S. Noninvasive imaging of protein-protein interactions in living subjects by using reporter protein complementation and reconstitution strategies. Proc. Natl Acad. Sci. USA 99, 15608–15613 (2002).

14. Paulmurugan, R. & Gambhir, S. S. Combinatorial library screening for developing an improved split-firefly luciferase fragment-assisted complementation system for studying protein-protein interactions. Anal. Chem. 79, 2346–2353 (2007).

15. Cahalan, S. M. et al. Actions of a picomolar short-acting S1P(1) agonist in S1P (1)-GFPP knock-in mice. Nat. Chem. Biol. 7, 254–256 (2011).

16. Liu, C. H. et al. Ligand-induced trafficking of the sphingosine-1-phosphate receptor EDG-1. Mol. Biol. Cell 10, 1779–1790 (1999).

17. Van Brocklyn, J. R. et al. Dual actions of sphingosine-1-phosphate: extracellular through the Gi-coupled receptor Edg-1 and intracellular to regulate proliferation and survival. J. Cell Biol. 142, 229–240 (1998).

18. Sanwa, M. G. et al. Enhancement of capillary leakage and restoration of lymphocyte egress by a chiral S1P1 antagonist in vivo. Nat. Chem. Biol. 2, 434–441 (2006).

19. Turason, G. et al. The sphingosine-1-phosphate receptor-1 antagonist, W146, causes early and short-lasting peripheral blood lymphopenia in mice. Int. Immunopharmacol. 11, 1773–1779 (2011).

20. Meno-Tetang, G. M. et al. Physiologically based pharmacokinetic modeling of FTY720 (2-amino-2-[-(4-octylphenyl)ethyl]propane-1,3-diol hydrochloride) in rats after oral and intravenous doses. Drug Metab. Dispos. 34, 1480–1487 (2006).

21. Pappu, R. et al. Promotion of lymphocyte egress into blood and lymph by distinct sources of sphingosine-1-phosphate. Science 316, 295–298 (2007).

22. Fukushima, S. et al. The sphingosine-1-phosphate transporter Spsn2 is expressed on endothelial cells regulates lymphocyte trafficking in mice. J. Clin. Invest. 122, 1416–1426 (2012).

23. Rosen, H., Stevens, R. C., Hansom, M., Roberts, E. & Oldstone, M. B. Sphingosine-1-phosphate and its receptors: structure, signaling, and influence. Annu. Rev. Biochem. 82, 657–662 (2013).

24. Means, C. K., Miyamoto, S., Chun, J. & Brown, J. H. S1P1 receptor localization confers selective for Gi-mediated CAM and contractile responses. J. Biol. Chem. 283, 11954–11963 (2008).

25. Cramer, E. et al. Sphingosine-1-phosphate in the plasma compartment regulates basal and inflammation-induced vascular leak in mice. J. Clin. Invest. 119, 1871–1879 (2009).

26. Cyster, J. G. & Schwah, S. R. Sphingosine-1-phosphate and lymphocyte egress from lymphoid organs. Annu. Rev. Immunol. 30, 69–94 (2012).

27. Arnon, T. I. & Cyster, J. G. Blood, sphingosine-1-phosphate and lymphocyte migration dynamics in the spleen. Curr. Top Microbiol. Immunol. 378, 107–128 (2014).

28. Herzog, B. H. et al. Podoepoanal maintains high endothelial venule integrity by interacting with platelet CLEC-2. Nature 502, 105–109 (2013).

29. Shinivas, S. et al. Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. BMC Dev. Biol. 1, 4 (2001).

30. Lakso, M. et al. Efficient in vivo manipulation of mouse genomic sequences at the zygote stage. Proc. Natl Acad. Sci. USA 93, 5860–5865 (1996).

31. Wu, Z., Hofman, F. M. & Zlokovic, B. V. A simple method for isolation and characterization of mouse brain microvascular endothelial cells. J. Neurosci. Methods 130, 53–63 (2003).

32. Mizugishi, K. et al. Essential role for sphingosine kinases in neural and vascular development. Mol. Cell Biol. 25, 11113–11121 (2005).

33. Kuhn, R., Schwenk, F., Aguet, M. & Rajewsky, K. Inducible gene targeting in mice. Science 269, 1427–1429 (1995).

34. Bielska, J., Szulc, Z. M., Hamann, Y. A. & Bielawska, A. Simultaneous two quantitative analysis of bioactive sphingolipids by high-perform mance liquid chromatography-tandem mass spectrometry. Methods 39, 82–91 (2006).

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Author contributions

M.K. designed and performed experiments, analyzed and interpreted data, and wrote the manuscript. E.G.C. and S.Y.L. performed experiments and analyzed data. K.Y. and T.H. contributed to the brain endothelial cell studies and analyzed data. R.L.P. supervised the
overall project, helped design experiments, analyzed and interpreted data, and wrote the manuscript. All authors read and commented on the manuscript.

**Additional information**

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