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TITLE: Identification of G-Protein-Coupled Receptors (GPCRs) in Pulmonary Artery Smooth Muscle Cells as Novel Therapeutic Targets

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14. ABSTRACT
Pulmonary arterial hypertension (PAH) is associated with increased vascular resistance, sustained contraction, and enhanced proliferation of pulmonary arterial smooth muscle cells (PASMCs). The underlying idea of this project is that the currently limited treatments for PAH represent an unmet medical need for soldiers, veterans and those in the general population. We are testing the hypothesis that the discovery of "novel" (i.e., not previously recognized) G-protein-coupled receptors (GPCRs) and their functional activity in the PASMCs from subjects with PAH can reveal new insights into pathophysiology and new therapeutic targets for PAH. Our approach is to isolate PASMCs from PAH subjects and controls, to define the expression and function of their complement of GPCRs, with the goal of identifying GPCRs that have known physiologic agonists and are uniquely expressed and/or prominently up-regulated in PAH-PASMCs and to define their potential as novel therapeutic targets for PAH.

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1. INTRODUCTION

Pulmonary arterial hypertension (PAH) is associated with increased vascular resistance, sustained contraction, and enhanced proliferation of pulmonary arterial smooth muscle cells (PASMCs). The underlying idea of this project is that the currently limited treatments for PAH represent an unmet medical need for soldiers, veterans and those in the general population. We are testing the hypothesis that the *discovery of "novel" (i.e., not previously recognized) G-protein-coupled receptors (GPCRs) and their functional activity in the PASMCs from subjects with PAH can reveal new insights into pathophysiology and new therapeutic targets for PAH.* Our approach is to isolate PASMCs from PAH subjects and controls, to define the expression and function of their complement of GPCRs, with the goal of identifying GPCRs that have known physiologic agonists and are uniquely expressed and/or prominently up-regulated in PAH-PASMCs and to define their potential as novel therapeutic target*s* for PAH*.*

2. KEYWORDS

Pulmonary arterial hypertension (PAH), pulmonary artery smooth muscle cells (PASMCs), G-protein-coupled receptors (GPCRs), cyclic AMP, hypoxia.

3. ACCOMPLISHMENTS

- **What were the major goals of the project?**

The 3 Aims/goals of the project are to: 1) Define the expression of GPCRs that have known physiologic agonists in human, rat and mouse PASMCs. 2) Determine if the expression profile of such PASMC-GPCRs is altered in PAH and if GPCRs with altered expression contribute to the pathophysiology of PAH. 3) Determine the therapeutic potential of such PAH-PASMC-expressed GPCRs.

- **What was accomplished under these goals? (in this reporting period)**

1) Major Activities:

Aim 1's major task is to isolate control PASMCs from humans, rats and mice and identify GPCRs with known physiologic agonists expressed by the PASMCs, to independently confirm the mRNA expression and analyze protein expression and responses mediated by a subset of those GPCRs. We also propose to determine if the profile of PASMC-expressed GPCRs differs from that of coronary artery and aortic smooth muscle cells (SMCs). This major task has several subtasks, to which we have devoted efforts during this reporting period, including:

a) Isolation and preparation of primary cultures of PASMCs from the lungs of subjects who do not have PAH; **b**) Preparation of RNA and cDNA from PASMCs and use of Taqman GPCR arrays to identify and quantify GPCR expression; **c**) Preparation of RNA/cDNA from commercially obtained coronary artery and aortic SMCs and use of Taqman GPCR arrays to identify/quantify GPCR expression; **d**) Isolation and culture of PASMCs from Sprague Dawley rats and C57/BL6 mice prior to preparing RNA/cDNA and use of GPCR arrays to assess GPCR expression in the PASMCs; and **e)** Confirmation of mRNA expression by independent qPCR analyses of highly expressed GPCRs from pulmonary artery, coronary artery and aortic SMCs.

The major task of Aim 2 is to isolate PASMCs from subjects with PAH and determine if those PASMCs have altered expression of GPCRs with known physiologic agonists and that may contribute to the pathophysiology of PAH. The primary subtask of this reporting period has been to isolate PASMCs and prepare PASMC cultures from PAH subjects, including human subjects and animal subjects. In addition, we established rat and mouse models of PAH (chronic hypoxia, including the Sugen model in mice) and isolated PASMCs for primary culture, RNA/cDNA isolation and GPCR array analysis.

The major task of Aim 3 (to be undertaken later in the project) is to determine the therapeutic potential of preferentially expressed PAH-PASMC GPCRs that have physiologic agonists. Criteria we plan to use in **c**hoosing GPCRs to target include GPCRs with greatest increase in expression in PAH-PASMCs, similarity of GPCR changes with PAH in humans, rats and mice, GPCRs predicted to impact on pathophysiology and for which drugs (in particular, FDA-approved drugs) are available.

2) Specific objectives:

a) Obtain approval of the proposed experiments that involve use of animal and human subjects from the UCSD Institutional Animal Care and Use Committee (IACUC) and UCSD Institutional Review Board (IRB), respectively, followed by approval from reviews by DoD staff, including from the Human Research Protection Office (HRPO) Office of Research Protections (ORP) U.S. Army Medical Research and Materiel Command (USAMRMC);

b) Establish a protocol to obtain lung samples for studying GPCR expression from human PAH and non-PAH subjects;

c) Initiate the collection of lung samples and then isolate and grow PASMCs;

d) Isolate RNA and prepare cDNA from PASMCs; identify and quantify GPCR expression using Taqman GPCR arrays;

e) Obtain and culture commercially available human coronary artery and aortic SMCs, isolate RNA and prepare cDNA from those cells and assay GPCR expression using Taqman GPCR arrays;

f) Isolate and grow PASMCs from rats and mice, prepare RNA/cDNA from those PASMCs and assay GPCR expression using Taqman GPCR arrays;

g) Establish rat (chronic hypoxia) and mouse (chronic hypoxia and Sugen) models of PAH, isolate PASMCs from the animals for primary culture, prepare RNA/cDNA from those PASMCs and assay GPCR expression using Taqman GPCR arrays.

h) Isolate PASMCs from human subjects with PAH and determine if those PASMCs have altered GPCR expression, including of GPCRs that may contribute to the pathophysiology of PAH.

3) Significant results:

During this reporting period, we have made progress on each of the Specific objectives above:

a) We obtained approval from the UCSD IACUC and IRB for the proposed studies involving animal and human subjects, respectively, and subsequent approval by HRPO ORP USAMRMC. However, HRPO ORP USAMRMC approval for the human subject studies did not occur until 5-29-15. Thus we were delayed in undertaking such studies. Even so, as shown below, we were able to make progress in obtaining samples and generating data related to other objectives.

b) At the beginning of the funding of this grant, we participated in a meeting with UCSD colleagues, including our co-investigator, Dr. Patricia Thistlethwaite, a cardiothoracic surgeon, and with pulmonologists and pathologists, who all sought to obtain lung samples from patients undergoing surgery. An action plan was developed, under the auspices of approved IRB protocols, for pathologists to obtain intra-operative samples and then provide tissue to “on call” laboratory personnel, who would immediately receive the samples for the preparation of cells (in our case, PASMCs) for tissue culture.

c) The plan established (under **b**) has proved to be highly effective and been aided by the frequency with which lung resection is undertaken at UCSD on patients with various pulmonary disorders, including PAH and in addition, by the cooperative interaction with physicians and staff in pulmonary medicine, thoracic surgery,

and pathology. We have thus far obtained samples and grown PASMCs from 3 PAH patients, 4 non-PAH subjects, and 1 patient with pulmonary vascular occlusive disease (PVOD).

d) We isolated RNA and generated cDNA from each patient sample noted in c) and then used Taqman GPCR arrays (Life Technologies) to identify and quantify expression of ~355 non-chemosensory (other than odorant, taste, visual) GPCRs and certain additional mRNAs, including ones (e.g., GAPDH, 18S rRNA) for normalization of GPCR expression results. The assay uses an ABI Prism 7900HT system (Applied Biosystems) with accompanying data analysis software. We find that Taqman GPCR Arrays are more sensitive and accurate for quantifying GPCR mRNA than are arrays (e.g., Affymetrix) that assess overall gene expression. Normalized data for GPCR expression allows the calculation of ΔCt (difference in cycle threshold) values relative to 18S rRNA (which we find is well-suited for such normalization) and ΔΔCt values, so as to define differences in GPCR expression between PAH-PASMCs and PASMCs from control subjects. We cluster GPCR expression data based on receptor linkage to heterotrimeric G-proteins (G_s, $G_{i/o}$, $G_{q/11}$, $G_{12/13}$) and rank GPCRs on the basis of expression level. In addition, we developed a way to "weight" the relative contribution of individual GPCRs to the overall pool of cellular GPCRs. As shown in **Table 1**, PASMCs from non-PAH subjects express a mean of 123 GPCRs (range 112-144); 73 of the GPCRs are shared among the 4 samples. **Table 2** shows the levels of expression (ΔCt values) of the 50 highest expressed GPCRs in these PASMCs. **Table 3** shows the G-protein linkages of those 50 GPCRs; because certain GPCRs have multiple such linkages, the total number of GPCRs in **Table 3** is more than 50. The highest number of GPCRs link to Gq or have unknown linkages. **Table 4** shows the known physiologic agonist and G-proteins linkages of the highest expressed GPCRs for control PASMCs for which that information is known.

Table 1: Number of GPCRs detected in human SMCs

Sample	# of GPCRs
Control 1 (commercial) PASMC	112
Control 2 (commercial) PASMC	120
Control 3 (commercial) PASMC	116
Control 4 (patient 1) PASMC	144
Coronary artery 1 (commercial) SMC	114
Aortic (commercial) SMC	129
Aortic (commercial) SMC	129
IPAH, pt1 PASMC	126
IPAH, pt2 PASMC	103
IPAH, pt3 PASMC	126
IPVOD PASMC	132

Table 2: 50 highest expressed GPCRs in control human PASMCs

Gene Name	Avg ΔCt vs 18s	Gene Name	Avg ΔCt vs 18s	Gene Name	Avg ΔCt vs 18s
EDG2	14.12	PTGFR	17.99	LGR4	19.89
FZD6	14.29	GPR161	18.10	MRGPRX3	19.92
LPHN2	14.57	EDNRA	18.14	EDG1	20.10
GPR176	14.95	FZD2	18.21	GPR51	20.26
ELTD1	15.01	GPR153	18.32	GPR1	20.32
FZD4	15.62	GPR68	18.42	ADORA2A	20.47
F2R	15.70	EDG3	18.42	MRGPRF	20.55
CD97	15.71	FZD8	18.61	CELSR1	20.66
GPR124	15.77	FZD7	18.62	PTGIR	20.72
BDKRB1	15.81	HTR2A	18.73	P2RY11	20.82
C11ORF4	16.03	GPR115	18.99	OXTR	20.83
GPRC5B	16.10	F2RL1	19.11	GPR39	20.98
GPRC5A	16.29	GPR173	19.11	GPR3	20.98
BDKRB2	16.30	GPR56	19.18	ADORA1	21.07
GPR125	17.21	FZD1	19.26	SMO	21.08
ADORA2B	17.50	CALCRL	19.38	GPR21	21.48
GABBR1	17.73	BAI2	19.51		

Table 3: G-protein linkages of the 50 highest-expressed GPCRs in control PASMCs

Primary Transduction Mechanism	# of GPCRs
Gi/Go	13
Gq/G11	18
Gs	8
G12/13	6
Unknown/Unresolved	18

Table 4: Highest Expressed GPCRs with known G-protein linkages and known endogenous agonists (endoGPCRs) in control PASMCs

Primary Transduction Mechanism	Gene Name	Avg ΔCt vs 18S	Endogenous Agonist
Gi/Go, Gq/G11, G12/13	EDG2 (lysophosphatidic acid receptor 1)	14.12	lysophosphatidic acid
Gi/Go, Gq/G11	FZD6 (frizzled class receptor 6)	14.29	Wnt-4
Gi/Go, Gq/G11, G12/13	F2R (coagulation factor II (thrombin) receptor)	15.70	thrombin
Gi/Go, Gq/G11	BDKRB1 (bradykinin receptor B1)	15.81	bradykinin
Gi/Go, Gq/G11	GPR68 (G protein-coupled receptor 68)	18.42	protons
Gi/Go, Gq/G11	EDG3 (sphingosine-1-phosphate receptor 3)	18.43	Sphingosine 1-phosphate
Gq/G11	HTR2A (5-hydroxytryptamine (serotonin) receptor 2A)	18.73	5-hydroxytryptamine
Gq/G11, G12/13	GPR56 (adhesion G protein-coupled receptor G1)	19.18	Collagen3A1
G12/13	FZD4 (frizzled class receptor 4)	15.62	Wnt
Gs	ADORA2B (adenosine A2b receptor)	17.50	adenosine
Gs	CALCRL (calcitonin receptor-like)	19.38	adrenomedullin
Gs	LGR4 (leucine-rich repeat containing G protein-coupled receptor 4)	19.89	R-spondins

e) We obtained human coronary arterial (n=1) and aortic SMCs (n=2) from a commercial source, cultured these cells, prepared RNA, generated cDNA and used the Taqman GPCR arrays to identify and quantify GPCRs expressed by the cells. As shown in **Table 1**, both the coronary arterial and aortic SMCs express a similar number of GPCRs as do the PASMCs. Many of the most highly expressed GPCRs with known agonists were similar in PA, coronary arterial and aortic SMCs (**Tables 4-6**).

Table 5: Highest Expressed GPCRs with known G-protein linkages and known endogenous agonists (endoGPCRs) in hCASMCs

Primary Transduction Mechanism	Gene Name	Avg ΔCt vs 18S	Endogenous Agonist
Gi/Go, Gq/G11, G12/13	EDG2 (lysophosphatidic acid receptor 1)	14.65	lysophosphatidic acid
Gi/Go, Gq/G11	FZD6 (frizzled class receptor 6)	14.58	Wnt-4
Gi/Go, Gq/G11, G12/13	F2R (coagulation factor II (thrombin) receptor)	16.66	thrombin
Gq/G11	OXTR (Oxytocin Receptor)	16.51	oxytocin
Gi/Go, Gq/G11	GPR68 (G protein-coupled receptor 68)	17.81	protons
Gi/Go, Gq/G11	EDG3 (sphingosine-1-phosphate receptor 3)	18.16	Sphingosine 1-phosphate
Gq/G11	HTR2A (5-hydroxytryptamine (serotonin) receptor 2B)	17.35	5-hydroxytryptamine
Gq/G11, G12/13	GPR56 (adhesion G protein-coupled receptor G1)	20.35	Collagen3A1
G12/13	FZD4 (frizzled class receptor 4)	15.17	Wnt
Gs	ADORA2B (adenosine A2b receptor)	17.43	adenosine
Gs	ADORA2A (adenosine A2a receptor)	18.11	adenosine
Gs, Gi/Go, Gq/G11	BDKRB2 (bradykinin receptor, B2)	17.06	bradykinin

Table 6: Highest Expressed GPCRs with known G-protein linkages and known endogenous agonists (endoGPCRs) in human aortic SMCs

Primary Transduction Mechanism	Gene Name	Avg ΔCt vs 18S	Endogenous Agonist
Gi/Go, Gq/G11, G12/13	EDG2 (lysophosphatidic acid receptor 1)	15.49	lysophosphatidic acid
Gi/Go, Gq/G11	FZD6 (frizzled class receptor 6)	13.51	Wnt-4
Gi/Go, Gq/G11, G12/13	F2R (coagulation factor II (thrombin) receptor)	14.90	thrombin
Gq/G11	OXTR (Oxytocin Receptor)	14.69	oxytocin
Gi/Go, Gq/G11	SUCNR1 (succinate receptor 1)	18.80	succinic acid
Gi/Go, Gq/G11	EDG3 (sphingosine-1-phosphate receptor 3)	15.21	sphingosine 1-phosphate
Gq/G11	HTR2A (5-hydroxytryptamine (serotonin) receptor 2B)	15.68	5-hydroxytryptamine
Gq/G11, G12/13	GPR56 (adhesion G protein-coupled receptor G1)	16.78	Collagen3A1
G12/13	FZD4 (frizzled class receptor 4)	13.26	Wnt
Gs	CALCRL (calcitonin receptor-like)	16.51	adrenomedullin
Gs	ADORA2A (adenosine A2a receptor)	17.95	adenosine
Gs, Gi/Go, Gq/G11	ADRB2 (adrenoreceptor beta 2)	17.87	adrenaline

f) We obtained lungs from 3 month old Sprague-Dawley rats (one healthy female and male) and one healthy male mouse (C57/BL), isolated and cultured PASMCs, have prepared RNA/cDNA and are in the midst of assaying their GPCR expression using Taqman GPCR arrays.

g) We subjected rats and mice to chronic hypoxia as a means to induce PAH. In some experiments, mice were treated with Sugen (dose) as an additional means to induce PAH (as recommended by a DoD staff reviewer of our grant application). We obtained evidence that the protocols used to induce PAH produced this response (**Figures 1 and 2**). Following the chronic hypoxia studies with the rats, PASMCs and aortic smooth muscle cells were isolated. We cultured these cells, isolated RNA and generated cDNA from them and are currently assessing GPCR expression using Taqman GPCR arrays.

Figure 1. *Pulmonary artery (PA) remodeling of rats exposed to chronic hypoxia (CH) for 2 weeks*. PA remodeling is characterized by endothelial injury, smooth muscle cell proliferation, and infiltration of inflammatory cells (as shown in the H&E staining in the left panels of **A** and **B**, obtained from lungs from CH-Rats female and male respectively compared to normoxic controls (right panels). Lung tissue (left lobe) was cryosectioned at 10 micron thickness and H&E stained. Images are from 20X objective of an inverted Nikon microscope.

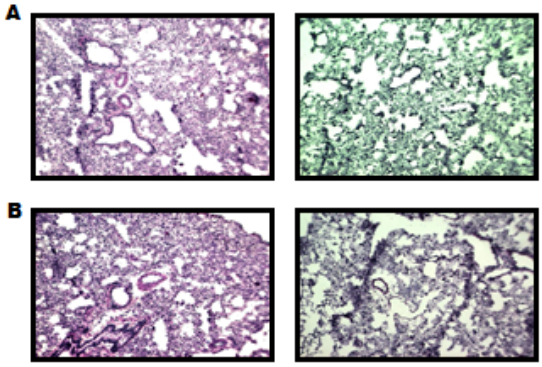
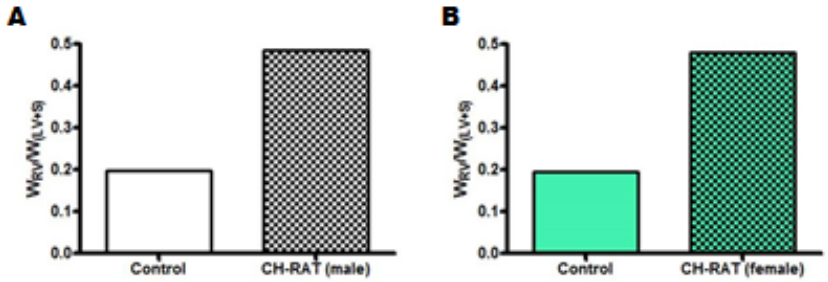


Figure 2. *Right ventricular hypertrophy (RVH) of male and female rats in the Chronic Hypoxia (CH)-PAH model*. 3 month old male Sprague Dawley Rats were subjected to CH for 2 weeks (CH-RAT) or normoxia (Control). RVH was assessed by the Fulton index (measured as the ratio of the RV weight to the LV+septum weight). **A.)** Male, *n=2* **B.)** Female, *n=2*



h) As noted in **c)** above, we obtained lungs samples and have grown PASMCs from 3 (female) patients with PAH, 1 (female) patient with PVOD in addition to the 4 non-PAH subjects. The primary cultures of PASMCs have been used at low passage for studies of expression of GPCRs and other mRNAs. As shown in **Table 1**, the overall number of GPCRs was similar in the non-PAH-, PVOD- and PAH-PASMCs. **Table 7** lists the 50 highest expressed GPCRs in the diseased PASMCs. Of note, the most highly expressed GPCRs are generally similar to those in control PASMCs (**Table 2**) and the G-protein linkage patterns of the GPCRs is similar to that of control PASMCs (**Table 8** and not shown).

Table 7: The 50 highest expressed GPCRs in diseased PASMCs

Gene Name	Avg ΔCt vs 18s	Gene Name	Avg ΔCt vs 18s	Gene Name	Avg ΔCt vs 18s
LPHN2	14.55	TM7SF1	15.16	FZD5	20.04
EDG2	14.97	HTR2B	15.45	CELSR1	20.05
F2R	15.14	BDKRB2	15.47	MRGPRF	20.24
GPR176	15.20	FZD2	15.48	GPR50	20.52
FZD6	15.43	ADORA2B	15.71	GPR65	20.58
CD97	15.59	GPR68	15.79	CALCRL	20.66
C11ORF4	15.77	GPR1	15.80	MC1R	20.81
FZD4	15.88	GPR56	19.06	SSTR1	20.99
GPR124	15.91	GPR161	19.08	P2RY11	21.11
GPRC5A	16.05	GPR175	19.13	GPR126	21.12
OXTR	16.14	HTR2A	19.16	BAI2	21.20
ELTD1	16.45	GPR39	19.24	LGR7	21.25
GPRC5B	17.03	GABBR1	19.54	ADORA1	21.27
GPR155	17.26	F2RL1	19.50	CR2A4	21.45
EDG5	17.48	FZD1	19.59	EDNRA	21.47
FZD7	17.68	GPR115	19.95	C5AR1	21.52
GPR125	18.04	BDKRB1	20.00		

Table 8: Highest Expressed GPCRs with known G-protein linkages and known endogenous agonists (endoGPCRs) in diseased hPASMCs

Primary Transduction Mechanism	Gene Name	Avg ΔCt vs 18S	Endogenous Agonist
Gi/Go, Gq/G11, G12/13	EDG2 (lysophosphatidic acid receptor 1)	14.97	lysophosphatidic acid
Gi/Go, Gq/G11	FZD6 (frizzled class receptor 6)	15.43	Wnt-4
Gi/Go, Gq/G11, G12/13	F2R (coagulation factor II (thrombin) receptor)	15.14	thrombin
Gq/G11	OXTR (Oxytocin Receptor)	16.14	oxytocin
Gi/Go, Gq/G11	GPR68 (G protein-coupled receptor 68)	18.79	protons
Gi/Go, Gq/G11	EDG3 (sphingosine-1-phosphate receptor 3)	17.48	Sphingosine 1-phosphate
Gq/G11	HTR2B (5-hydroxytryptamine (serotonin) receptor 2B)	18.43	5-hydroxytryptamine
Gq/G11, G12/13	GPR56 (adhesion G protein-coupled receptor G1)	19.18	Collagen3A1
G12/13	FZD4 (frizzled class receptor 4)	15.88	Wnt
Gs	ADORA2B (adenosine A2b receptor)	18.71	adenosine
Gs	CALCRL (calcitonin receptor-like)	19.38	adrenomedullin
Gs, Gi/Go, Gq/G11	BDKRB2 (bradykinin receptor, B2)	18.47	bradykinin

- **What opportunities for training and professional development has the project provided?**

Nothing to report

- **How were the results disseminated to communities of interest?**

Nothing to report

- **What do you plan to do during the next report period to accomplish the goals?**

We plan to undertake further efforts on each of the 3 Aims and to expand the number of subjects for studies in Aims 1 and 2. A key goal is to confirm and extend our initial results, for example, to determine if we can identify a “PAH-specific GPCR expression profile”. In addition, we will undertake studies to confirm the expression of GPCRs identified by the Taqman GPCR arrays. These studies will include independent qPCR analyses, assays of GPCR proteins (using antibodies and other proteomic techniques), and studies of functional

activities (e.g., signaling events, DNA synthesis, effects on cell growth and cell death, caspase 3 activation, expression of cell cycle proteins [p21/p27], cell migration) that PASMC-GPCRs may regulate.

4. IMPACT

- **What was the impact on the development of the principal discipline(s) of the project?**

Our findings thus far imply that there are many more G-protein-coupled receptors (GPCRs), which are recognition sites on the cell surface for hormones, proteins, neurotransmitters and other "messengers" than were previously known. Potentially these GPCRs, which that are the focus of this project, may play important roles in healthy and diseased pulmonary blood vessels and may be new targets for treating high blood pressure in the circulation of the lung, i.e. pulmonary arterial hypertension (PAH), in particular GPCRs that are more highly expressed in PAH-PASMC.

- **What was the impact on other disciplines?**

The notion that individual cell types express many more types of GPCRs than were previously known is potentially important for the regulation of cells and tissues in health and disease. The results have impact on cell biology, biochemistry, physiology, pharmacology and pathology, as well as clinical medicine, especially if the newly recognized GPCRs can further understanding of pathophysiology and be used to aid in diagnosis, assessing prognosis and/or serve as therapeutic targets in disease states.

- **What was the impact on technology transfer?**

Nothing to report

- **What was the impact on society beyond science and technology?**

Nothing to report

5. CHANGES/PROBLEMS

Nothing to report

6. PRODUCTS

- **Publications, conference papers and presentations**
  Journal publications:

Insel PA, Wilderman A, Zambon AC, Snead AN, Murray F, Aroonsakool N, McDonald DS, Zhou S, McCann T, Zhang L, Sriram K, Chinn AM, Michkov AV, Lynch RM, Overland AC, Corriden R. G Protein-Coupled Receptor (GPCR) Expression in Native Cells: "Novel" endoGPCRs as Physiologic Regulators and Therapeutic Targets. *Molecular Pharmacology*. 88: 2015; 181-187. Published; Acknowledgement of federal support (yes)

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**

Name: Paul A. Insel
Project Role: Principal Investigator
Researcher Identifier: 402799
Nearest person month worked: 2
Contribution to Project: Directed all phases of the project
Funding Support: this project

Name: Nakon Aroonsakool

Project Role: Lab Assistant
Researcher Identifier: 93600
Nearest person month worked: 6
Contribution to Project: Obtained lung samples, prepared and cultured PASMCs, assisted with preparation of RNA, cDNA and with GPCR arrays; set up animal models of PAH
Funding Support: this project

Name: Krishna Sriram
Project Role: Post-doctoral fellow
Researcher Identifier: 791836
Nearest person month worked: 4
Contribution to Project: Performed GPCR arrays, analyzed all GPCR data
Funding Support: this project and a contract from Bristol-Myers-Squibb

Name: Anna Busija
Project Role: Graduate student
Researcher Identifier: 114428
Nearest person month worked: 1
Contribution to Project: Assisted with GPCR analysis
Funding Support: this project and an American Heart Assn predoctoral fellowship

- **Has there been a change in the active other support of the PD/PI or senior/key personnel since the last reporting period?**

New support for the PI, Dr. Insel, other than this grant, is as follows:

1R56AI110505 (NIH) Raz, Eyal (PI) 2015/03/10-2016/02/29
"The inductive role of Gs/Gi signaling in dendritic cell polarization and function".
This one-year study initiates studies of Gs and Gi in regulating dendritic cell function and asthma.
Role: Co-PI

1 R21 CA189477 (NIH) Insel, Paul A. (multi-PI with A. Lowy) 2014/07/01-2016/06/30
"GPCRs: novel targets in cancer-associated fibroblasts"
This study is assessing GPCR expression in pancreatic cancer-associated fibroblasts (CAFs) and seeks to ascertain the functional contribution of GPCRs with altered expression in CAFs.
Role: Co-PI

Bristol Myers Squibb Insel, Paul A. (PI) 2014/07/01-2016/06/30
"Novel therapeutic targets in cardiac and extra-cardiac fibroblasts in the treatment of fibrotic diseases"
This study seeks to identify GPCRs and validate them in signaling and functional studies of cardiac and lung fibroblasts with the goal of identifying new therapeutic targets for tissue fibrosis
Role: PI

- **What other organizations were involved as partners?**

None

8. SPECIAL REPORTING REQUIREMENTS

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G Protein–Coupled Receptor (GPCR) Expression in Native Cells: "Novel" endoGPCRs as Physiologic Regulators and Therapeutic Targets

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ABSTRACT

G protein–coupled receptors (GPCRs), the largest family of signaling receptors in the human genome, are also the largest class of targets of approved drugs. Are the optimal GPCRs (in terms of efficacy and safety) currently targeted therapeutically? Especially given the large number (~120) of orphan GPCRs (which lack known physiologic agonists), it is likely that previously unrecognized GPCRs, especially orphan receptors, regulate cell function and can be therapeutic targets. Knowledge is limited regarding the diversity and identity of GPCRs that are activated by endogenous ligands and that native cells express. Here, we review approaches to define GPCR expression in tissues and cells and results from studies using these approaches. We identify problems with the available data and suggest future ways to identify and validate the physiologic and therapeutic roles of previously unrecognized GPCRs. We propose that a particularly useful approach to identify functionally important GPCRs with therapeutic potential will be to focus on receptors that show selective increases in expression in diseased cells from patients and experimental animals.

Introduction

G protein–coupled receptors (GPCRs, also termed 7-transmembrane or heptahelical receptors) have been of major interest for investigators in many disciplines, including molecular pharmacology. Early studies on GPCRs assessed the action in cells and plasma membrane preparations of neurotransmitters, hormones, and pharmacological agents in terms of their ability to regulate the generation of second messengers (e.g., cAMP, Ca^{2+}) and, in turn, cellular events via enzymes (e.g., protein kinases) and ion channels. Results obtained by the Human Genome Project and for the genomes of other eukaryotes have revealed that GPCRs are the largest family of signaling receptors in humans and other species (Fredriksson et al., 2003; Vassilatis et al., 2003; Insel et al., 2012; Foster et al., 2014b). The receptors include those that interact with endogenous ligands (endoGPCRs); GPCRs regulated by exogenous factors, such as photons of light, odorants, and tastants (chemosensory receptors); and GPCRs that lack known physiologic ligands (termed orphan receptors). It is estimated that among the approximately 800 GPCRs in humans, ~380 are endoGPCRs, of which about one-third are orphan receptors, even though there have been substantial efforts at deorphanization (Fredriksson et al., 2003; Kroeze et al., 2003; Ozawa et al., 2010; Amisten et al., 2013; Civelli et al., 2013).

In parallel with work that has involved the cloning, genomic characterization, heterologous expression, and studies of GPCR actions and regulation, other efforts have emphasized the utility of GPCRs as therapeutic targets. Indeed, GPCRs are the largest class (~30%) of the targets of approved drugs (Overington et al., 2006; Lundstrom, 2009; Rask-Andersen et al., 2014). Reasons for the utility of GPCRs as therapeutic targets include the many different types of chemical entities with which they interact, the accessibility of GPCRs on the plasma membrane from the extracellular milieu, their ability to initiate signaling pathways that undergo amplification in target cells, and the selectivity in their expression by different types of cells. This latter property aids in facilitating tissue- and cell-selective actions of GPCR-targeted drugs.

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ABBREVIATIONS: endoGPCR, GPCR activated by endogenous ligands; GPCR, G protein–coupled receptor; PCR, polymerase chain reaction.

Despite the widespread use of GPCRs as therapeutic targets, one can ask the following: Are the optimal GPCRs (in terms of efficacy and safety) targeted by current therapeutic approaches? This question derives in part from the current therapeutic targeting of only a fraction of the endoGPCRs. Moreover, orphan GPCRs have largely been ignored as therapeutic targets. It is thus necessary to identify the repertoire of GPCRs—in particular, endoGPCRs—expressed by individual tissues and, more importantly, native cells. Studies to assess this gap in knowledge test the hypothesis that certain GPCRs are enriched in native cells, regulate cellular (and tissue) function, and can be targeted therapeutically. In this article, we review the approaches and data that have begun to provide information to test this hypothesis. In addition, we discuss problems and limitations of available data and future directions that may help definitively answer the question posed earlier.

Methods and Approaches to Assess GPCR Expression

Analyses of functional responses, second messengers, or other signaling events represent hypothesis-testing approaches by asking if a particular receptor is biologically active and provide indirect ways to assess GPCR expression by tissues and cells. Radioligand binding assays facilitate the direct identification and quantification of GPCRs. However, functional, signaling, and radioligand binding assays are biased approaches: one chooses a GPCR of interest and then uses agonists, antagonists, and radioligands for the receptor being assessed. Thus, one can only study receptors for which appropriate reagents are available.

By contrast, hypothesis-generating approaches are not based on prior knowledge of a GPCR being present, but instead rely on unbiased analyses of the expression of receptor mRNA or protein. Such approaches can define the GPCR expression profile/repertoire and can quantify receptor expression. Table 1 lists several approaches used to assess GPCR expression.

Numerous studies have used DNA microarrays ("DNA chips") to define the transcriptomes of cells and tissues. Such microarrays contain probes (specific DNA sequences) that hybridize with the genes of humans, mice, or other species. Hybridization of the probes to target genes is quantified by chemiluminescence, fluorescence, or another method, facilitating quantitation of the abundance of individual mRNAs/cDNAs. Commercially available microarrays that assess most or all genes in a transcriptome are not optimized to detect GPCRs, but such arrays have been used to characterize GPCR expression.

Proprietary and commercial GPCR microarrays, to be discussed later, and real-time polymerase chain reaction (PCR) analyses for individual GPCRs offer an alternative approach to identify chemosensory and nonchemosensory GPCRs. For example, Regard et al. (2008) used Taqman quantitative real-time PCR to quantify the transcripts of 353 nonodorant GPCRs in 41 adult mouse tissues and predicted previously unanticipated roles for less well studied receptors—an idea consistent with our hypothesis that previously unrecognized GPCRs are enriched in native cells, contribute to physiology, and are potential therapeutic targets.

With improvement in the ability to perform sequencing and a rapid decrease in its cost, new techniques such as high-resolution RNA sequencing have begun to be used to identify and quantify expression of GPCRs and other members of the transcriptome. Such studies have recently included the profiling of GPCRs expressed in single cells (Manteniotis et al., 2013; Spaethling et al., 2014).

An alternative to assessing the expression of GPCR mRNA is the use of unbiased proteomic approaches, such as mass spectrometry. Although such technology has not, as yet, been used to define and quantify overall GPCR expression in cells and tissues, initial results suggest this may be a feasible approach (Eisen et al., 2013; Feve et al., 2014).

TABLE 1
Unbiased methods to assess expression of GPCRs

DNA microarrays that assess entire transcriptomes
Real-time PCR analysis with primers for each GPCR
Targeted DNA microarrays that assess expression of nonchemosensory GPCRs
High-resolution RNA sequencing
Proteomic approaches

Microarrays for the Detection of GPCRs

Commercial microarrays have been created that are optimized to detect and quantify the mRNA of ~350 nonchemosensory GPCRs of mice, rats, and humans. We and others have found these arrays to be quite useful to assess GPCR expression. Figure 1 shows a comparison of the detection of GPCRs by such a targeted array (a Taqman GeneSignature array; Life Technologies, Carlsbad, CA) with results obtained using an Affymetrix Mouse Genome 430A microarray (Affymetrix, Santa Clara, CA) (which detects ~14,000 mouse genes). We found that highly expressed GPCRs on the latter microarray show a positive correlation (and an R^2 of 0.37) with the GPCR array data. However, assessment of GPCRs expressed at lower levels reveals that the Affymetrix array has many false-positive and false-negative results (and $R^2 = 0.01$). GPCR arrays thus

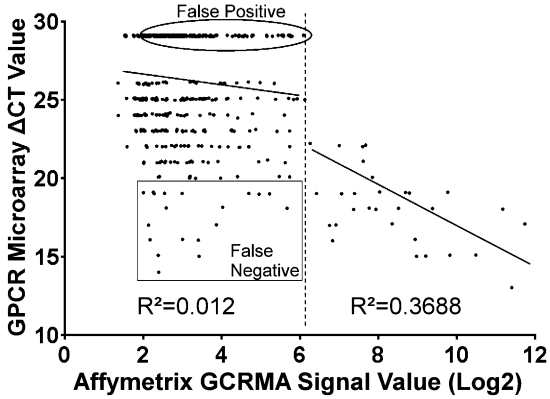

Fig. 1. Comparison of GPCR expression data obtained using a GPCR-specific array and a generic cDNA array. The data were collected in two experiments, with data obtained using cDNA prepared from murine wild-type S49 lymphoma cells and that could be compared for GPCRs present on an Affymetrix Mouse Genome 430A microarray and a Taqman GeneSignature array. The 32 GPCRs highly expressed ($>\log2^6$) on the Affymetrix array show a positive correlation ($R^2 = 0.37$) between the two arrays. GPCRs expressed at lower levels ($<\log2^6$) show numerous false-positive and false-negative results in the detection of GPCRs by the Affymetrix array compared with the GPCR array ($R^2 = 0.01$). ΔCT, cycle threshold relative to the mRNA used to normalize expression of each GPCR; GCRMA, guanine cytosine robust multiarray analysis.

seem to be more useful to detect and quantify GPCRs than nontargeted arrays. Data from others support this conclusion (Maurel et al., 2011).

What Do Available Data Reveal about GPCR Expression in Tissues and Native Cells?

GPCR expression has thus far been determined in a number of tissues (Table 2) and a smaller number of individual cell types (Table 3). Our laboratory has assessed the expression of nonchemosensory GPCRs by numerous individual cell types, including lymphoid cells, dendritic cells, neutrophils, vascular smooth muscle cells, several types of fibroblasts, adipocytes, renal epithelial cells, trigeminal neurons, and several types of cancer cells.

The notion that GPCRs and GPCR signaling pathways are altered (and are potential therapeutic targets) in cancer in addition to various endocrine tumors (for which GPCR-targeted drugs are commonly used) has been largely ignored by investigators in oncology. Even so, several recent reviews have emphasized the importance of GPCRs and GPCR signaling in cancer (Lappano and Maggiolini, 2011; Feigin, 2013; O'Hayre et al., 2013, 2014). O'Hayre and colleagues (2013) noted that nearly 20% of human tumors have GPCR mutations, 4% of tumors have activating mutations of the $G\alpha_s$ gene, and activating mutations of $G\alpha_q$ family members occur in melanomas. GPCRs may contribute to cancer not only by effects on the growth, death, metabolism, and function of malignant cells, but also by actions on cells of the tumor microenvironment, including vascular cells, immune cells, and cancer-associated fibroblasts. Although not widely explored, such actions may have functional importance for the malignant phenotype (Hanahan and Weinberg, 2011) and thus have therapeutic potential.

TABLE 2
GPCR expression in tissues

Tissue	Species	Methods and Results	Reference
Various: neurons, pancreas, liver, etc.	Human, Mouse	Proprietary arrays; assessed known, orphan, and odorant receptors in human tissues and blood cells	(Hakak et al., 2003)
Various: neurons, kidney, liver, etc.	Human, Mouse	RT-PCR; assessed 100 endoGPCRs (as no, low, moderate, or strong expression) in 17 tissues and 9 brain regions	(Vassilatis et al., 2003)
Lung, breast, prostate, melanoma, and gastric cancers; B cell lymphoma	Human	In silico analysis of GPCR expression in human tumors (eight microarray data sets of non–small-cell lung cancer, breast cancer, prostate cancer, melanoma, gastric cancer, and diffuse B cell lymphoma); found several GPCRs overexpressed in the cancers	(Li et al., 2005)
Various: eye, cardiovascular, pulmonary, etc.	Mouse	Transcript analysis of 353 nonodorant GPCRs in 41 mouse tissues; predicted functional roles for previously unrecognized GPCRs	(Regard et al., 2008)
Squamous cell carcinoma and adenocarcinoma	Human	Laser capture microdissection and GPCR-focused DNA microarrays; assessed 929 GPCR transcripts in patient-derived squamous cell lung carcinoma or adenocarcinoma; found 51 GPCRs overexpressed plus many with decreased expression	(Gugger et al., 2008)
Cardiac chambers	Mouse	Real-time RT-PCR; evaluated nonchemosensory endoGPCRs in the four cardiac chambers; focused on 128 GPCRs and chamber-specific expression	(Moore-Morris et al., 2009)
Adrenal gland	Human	cDNA chip with nucleotide sequences of 865 GPCRs to assess adrenals and adrenal cortical tumors; tumors had higher expression of several GPCRs	(Assie et al., 2010)
Melanoma metastases and nevi	Human	Quantitative PCR; assessed expression of 75 orphan and 19 chemokine GPCRs in melanoma metastases and benign nevi; found several orphan GPCRs with higher expression in the metastases	(Qin et al., 2011)
Urothelial tissue	Human	RT-PCR array; evaluated 40 GPCRs in human urothelium and urothelium-derived cell lines; cell lines had lower expression of most GPCRs	(Ochodnicky et al., 2012)
Pancreas, small intestine	Human	Taqman GPCR arrays; compared small bowel and pancreatic neuroendocrine tumors (SMNET, PNET) and normal tissue; found altered expression of 28 and 18 GPCRs in SMNET and PNET, respectively	(Carr et al., 2012)
Pancreatic islets	Human	Data from the IUPHAR GPCR database; GeneCards.org, ingenuity.com, PubMed.gov used to define a human GPCRome and then qPCR primers for these 384 GPCRs; found 293 GPCRs expressed predicted to be activated by 271 ligands and identified 107 drugs predicted to stimulate and 184 drugs predicted to inhibit insulin secretion	(Amisten et al., 2013)
Cerebellum	Human	GPCR RT-PCR arrays; four normal pediatric cerebellums and 41 medulloblastomas; numerous GPCRs had increased expression and clusters of tumors had particular patterns of GPCR expression	(Whittier et al., 2013)
Eye	Human, Mouse	RNA sequencing of cDNA of eyes and retinas of mice and a human donor eye; 165 GPCRs identified	(Chen et al., 2013)
Adipose tissue	Human	Quantitative PCR of 384 GPCRs; found 163 GPCRs in subcutaneous adipose tissue, 119 drugs (acting on 23 GPCRs) that may stimulate lipolysis, and 173 drugs (acting on 25 GPCRs) that may inhibit lipolysis	(Amisten et al., 2015)
Hypothalamus	Mouse	Taqman GPCR arrays; examined hypothalamic arcuate nucleus of female mice; identified 292 GPCRs (including 109 orphan GPCRs)	(Ronnekleiv et al., 2014)

IUPHAR, International Union of Basic and Clinical Pharmacology; qPCR, quantitative PCR; RT-PCR, reverse-transcription PCR.

TABLE 3
GPCR expression in cells

Cell	Species	Methods and Results	Reference
Bone marrow stromal cell lines	Human	GPCR RT-PCR array; showed relationship between number of GPCRs detected versus amount of total RNA in samples; detected mRNA for 199 GPCRs; highly expressed GPCRs detected with ~1 ng total RNA.	(Hansen et al., 2007)
Macrophages (bone marrow and peritoneal)	Mouse	DNA microarray data from NCBI (accession number GSE10246); assessed expression in bone marrow- and peritoneal-derived macrophages, microglia, and macrophage-like cells, RAW264; 67 GPCRs expressed constitutively or induced by lipopolysaccharide	(Lattin et al., 2008)
Embryonic stem cells (ESCs)	Mouse	GPCR RT-PCR arrays; examined ESCs cultured 4 or 20 days; ~200 GPCRs had low, high, or moderate expression	(Layden et al., 2010)
BV2	Rat	DNA high-density arrays and GPCR-specific qPCR; assessed 20,000 genes of HEK293, AtT20 (pituitary), BV2 (microglial), and N18 (neuroblastoma) cells; found 73, 79, 108, and 105 nonchemosensory GPCRs, respectively, in those cells and related signaling proteins	(Atwood et al., 2011)
N18	Mouse		
HEK293, AtT20	Human		
Cerebral neurons	Mouse	Customized quantitative real-time RT-PCR; studied cerebellar granule neurons (CGNs); identified 38 highly and 46 intermediately expressed GPCRs; expression of some GPCRs changed during CGN development	(Maurel et al., 2011)
Cardiac fibroblasts	Rat	GPCR RT-PCR arrays; assessed cardiac fibroblasts; identified 190 GPCRs; highest expressed, protease-activated receptor 1, was shown to be profibrotic	(Snead and Insel, 2012)
Monocytes, macrophages	Human	GPCR arrays; assessed alveolar macrophages (AMs); phorbol ester–treated THP-1, HL60, and U937 cells; peripheral blood monocytes and monocyte-derived macrophages; 164 GPCRs identified; highest expressed in AM was complement 5a receptor (C5R1)	(Groot-Kormelink et al., 2012)
Lung fibroblasts	Human	GPCR RT-PCR array; defined GPCR expression differences in normal lung fibroblasts and a gefitinib-resistant non–small-cell lung cancer cell line, H1975	(Kuzumaki et al., 2012)
Monocytes, macrophages	Human	GPCR RT-PCR arrays; evaluated GPCR expression changes during differentiation of monocytes to macrophages and by TLR4 activation, which altered expression of 101 GPCRs	(Hohenhaus et al., 2013)
Ghrelin cells	Mouse	GPCR arrays; found 90 GPCRs in gastric ghrelin cells; validated functional activity of several GPCRs	(Engelstoft et al., 2013)
Glioblastoma cancer stem-like cells, U-87 MG cells, astrocytes, fetal neural stem cells	Human	GPCR RT-PCR array; assessed glioblastoma cancer stem-like cells, glial tumor cells, a glioblastoma cell line (U-87 MG cells), astrocytes, and fetal neural stem cells; eight GPCRs were specific to glioblastoma cells; 17 GPCRs specific to cells with stem properties	(Feve et al., 2014)

HEK293, human embryonic kidney 293; NCBI, National Center for Biotechnology Information; qPCR, quantitative PCR; RT-PCR, reverse-transcription PCR.

Several general conclusions regarding GPCR expression in tissues and cells can be drawn from published findings and our unpublished results:

1) Most tissues and individual cell types express at least 100 different GPCRs, including GPCRs that link to each of the major classes of heterotrimeric G proteins (G_s, $G_{i/o}$, $G_{q/11}$, and $G_{12/13}$).
2) Among the GPCRs with the highest expression are those not previously known to be expressed in the previously mentioned tissues and cells and, thus, not the subject of prior studies.
3) Many of the highest expressed GPCRs are orphan receptors.
4) Cells from animals or patients with particular diseases show prominent changes (increases and decreases) in expression of numerous GPCRs, thus revealing disease-specific changes in GPCR expression.
5) Additional methods [quantitative real-time PCR, antibody-based and functional (including signal transduction and cellular response) assays]) confirm data obtained with GPCR arrays, including, for example, evidence that the expression of several G_s-coupled GPCRs correlates with their ability to maximally stimulate cellular cAMP production.

Such findings thus indicate that analysis of GPCR expression of tissues, and especially of individual cell types, appears to be a highly useful means to identify previously unrecognized GPCRs that may be functional, contribute to pathophysiology, and serve as novel drug targets.

What Are Some of the Problems and Limitations of Efforts to Define GPCR Expression in Tissues and Cells?

Despite the potential importance of the findings related to the discovery of "new GPCRs," i.e., endoGPCRs not previously known to be expressed, in individual tissues and cell types, a number of issues must be considered in studies of GPCR expression:

1) The source of material analyzed for GPCR expression: The results in Table 2 were primarily derived from studies of whole organs, tissues, and tissue biopsies, all of which are heterogeneous cellular preparations. The contribution of the different cell types in such preparations is thus not clear. Studies of individual cell types (Table 3) obviate this concern. Even in such studies, however, one analyzes a population of cells. The use of techniques such as high-resolution RNA sequencing (Manteniotis et al., 2013; Spaethling et al., 2014) and perhaps proteomic methods (Davies et al., 2007; Wu et al., 2012; Eisen et al., 2013) to assess GPCRs in individual cells will thus be an important future direction for

defining the profile and variability in GPCR expression in particular cell types.

2) Reproducibility and consistency of methods used to prepare mRNA, cDNA, or protein and to assess gene and protein expression: Standards have been developed to facilitate reproducibility in microarray (Brazma et al., 2001; Hansen et al., 2007; Rung and Brazma, 2013) and proteomic studies (Davies et al., 2007; Taylor et al., 2007), but results from different laboratories can differ. Such differences are generally attributed to variations in the methods used for the preparation and processing of samples. Primer design may not be ideal for the detection of all relevant GPCRs by arrays. For example, Taqman-based GPCR arrays may not detect certain, including functionally relevant, GPCRs. The use of different approaches for data analysis and statistics by different laboratories can contribute to discordant results (Pan, 2002; Motulsky, 2014). The dearth of well validated antibodies to detect native GPCRs, including for use in proteomic studies, is an important problem in GPCR research (Hutchings et al., 2010; Talmont et al., 2012; Eisen et al., 2013; Marchalant et al., 2014). Use of antibodies to study GPCRs is challenging because the receptors are typically expressed in cells at lower levels than many other cellular proteins. Criteria, including studies with cells or animals engineered to have a knockout of a particular GPCR, have been proposed to help validate GPCR-targeted antibodies (Michel et al., 2009). Moreover, since protein levels in cells are not necessarily predicted by mRNA abundance, differences in protein expression could derive from factors that include altered protein translation and/or degradation (de Sousa Abreu et al., 2009; Maier et al., 2009). Especially given the difficulties in obtaining well validated GPCR antibodies, a possible solution is to measure levels of actively translated mRNA using polysome profile analysis together with DNA high-density arrays or GPCR-specific quantitative PCR (Mašek et al., 2011; Gandin et al., 2014).
3) Normalization of results for GPCR expression: Most assays used to determine mRNA and protein expression rely on normalization to "housekeeping" genes/proteins. An extensive literature has discussed problems related to normalization in such studies. One such problem is a change in expression of the gene/protein used for normalization—a particular concern in studies of development, differentiation, or disease (Khimani et al., 2005; Brattelid et al., 2007). Two issues in the use of arrays to assess GPCR expression are how to define the limit of detection of a receptor in terms of Δcycle-threshold [ΔC(t)] relative to the reference used for normalization, and the most appropriate statistical tests for data analysis (Khimani et al., 2005; Rubie et al., 2005).
4) Approaches used to classify GPCRs in profiles of their expression in tissues and cells: One approach is to cluster results for GPCR expression on the basis of the coupling of receptors to heterotrimeric G proteins. Such information is available in articles and databases, such as the International Union of Basic and Clinical Pharmacology/British Pharmacological Society Guide to Pharmacology (Pawson et al., 2014). The International Union of Basic and Clinical Pharmacology/British Pharmacological Society systematically annotates each GPCR and provides reviews from expert subcommittees for each of the target families in the database. Results that supersede such data are published on an ongoing basis; therefore, one must be vigilant in updating conclusions regarding the coupling of GPCRs to G proteins. Problems associated with classifying results on this basis include the evidence that some GPCRs couple to multiple G proteins, the limited data regarding the G protein linkage of orphan GPCRs, and the ability of GPCRs to act via β-arrestin instead of (or in addition to) G proteins. Other ways to classify GPCRs include the Glutamate, Rhodopsin, Adhesion, Frizzled/taste2, and Secretin system, evolutionary relationships, ligand interactions, structural data, and susceptibility to post-translational modifications (Fredriksson et al., 2003; Davies et al., 2007; Secker et al., 2010; Lin et al., 2013; Venkatakrishnan et al., 2013).
5) Which types of GPCRs should be studied? As shown by the studies in Tables 2 and 3, most efforts have focused on the use of array- or PCR-based methods and have emphasized the expression of nonchemosensory GPCRs. Recent studies, however, have identified chemosensory receptor expression in tissues not typically thought to be in involved in sensation (Reimann et al., 2012; Foster et al., 2014a,b; Pronin et al., 2014; Rajkumar et al., 2014; Malki et al., 2015). With the discovery of small-molecule metabolites [in some cases, products of microbiota (e.g., Natarajan and Pluznick, 2014)] that interact with GPCRs and that may have been considered odorants or tastants, the distinction between chemosensory and nonchemosensory GPCRs is blurring. Thus, future studies may need to incorporate analyses of both types of GPCRs.
6) Prioritization of results regarding GPCR expression to guide subsequent studies that validate the expression and evaluate the functional role of individual receptors: The discovery that native cells typically express >100 GPCRs creates an embarrassment of riches, but also a challenge in terms of choosing individual GPCRs for subsequent studies. A key goal is to identify GPCRs that are important for cellular function and that may be therapeutic targets. The use of RNA interference and gene editing approaches (i.e., CRISPR/Cas9) to knock down receptor expression in cells of interest and then to assess the impact of receptor knock down on functional activity (signal transduction or cellular responses) provides a way to survey a population of GPCRs and identify physiologically (and potentially pharmacologically) important GPCRs (Willets and Nash, 2013). Additional features to help such prioritization include 1) the level of expression (more highly expressed GPCRs are predicted to contribute to a greater extent to cell function and may be easier to study); 2) knowledge of other cell types that express receptors of potential interest [to achieve greater selectivity in the site of action, including by comparing expression in cells that are related to one another, such as, for example, in different types of macrophages (Lattin et al., 2008; Groot-Kormelink et al., 2012; Hohenhaus et al., 2013)]; 3) choosing GPCRs whose expression is shared in a cell type found in humans and experimental animals (e.g., mice, rats); and 4) availability of reagents

(e.g., agonists, antagonists, antibodies) to conduct signaling and functional studies. A useful feature, especially for studies of disease settings, is to focus efforts on GPCRs with prominent differences in expression between the normal cells and cells from diseased animals or patients. Differential GPCR expression can also be helpful in choosing GPCRs for studies of development and differentiation of tissues and cells.

7) Determining the function of highly expressed orphan GPCRs: Some of the most exciting (and unexpected) data that we have obtained from our studies of GPCR expression in native cells is the high expression of a variety of orphan GPCRs. This is perhaps not surprising since orphan receptors represent about one-third of the endoGPCRs, and limited reagents have been available to assess function mediated by most orphan GPCRs. Since the endogenous ligands for such receptors are unknown, investigating their physiologic roles can be difficult (Ahmad et al., 2014). Molecular approaches, such as RNA interference or overexpression in heterologous cells, can help define signaling mechanisms, especially if a GPCR is constitutively active. Antibodies to orphan GPCRs might be used to block signaling and obviate the need for a ligand, although, as noted earlier, validation of GPCR antibodies can be difficult. Another challenge with orphan GPCRs has been the difficulty in setting up screens for ligands, as the G protein coupling of an orphan may not be known; chimeric G proteins may be a way to develop high-throughput screens (Yin et al., 2004).

Summary and Conclusions

Even though they have been widely studied and highly useful as therapeutic targets, GPCRs continue to be very important molecular entities. The recognition from genomic studies that there are many more GPCRs than were previously known or characterized, and that many of these are orphan receptors provides opportunities to discover physiologic and therapeutic roles for newly recognized GPCRs. Studies of GPCR expression in tissues, but especially in native cells, can reveal that previously unrecognized GPCRs contribute to cell function in health and disease. Numerous challenges exist in studies of GPCR expression, but if "new" GPCRs can be validated and shown to be functionally active, we anticipate that such GPCRs may prove to be as important—and perhaps even more so—than the GPCRs that have been the focus of efforts in physiology and pharmacology, and that are so valuable as therapeutic targets.

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Authorship Contributions

Conducted experiments: Wilderman, Zambon.
Performed data analysis: Wilderman, Zambon.
Wrote or contributed to the writing of the manuscript: Insel, Wilderman, Zambon, Snead, Murray, Aroonsakool, McDonald, Zhou, McCann, Zhang, Sriram, Chinn, Michkov, Lynch, Overland, Corriden.

References

Ahmad R, Wojciech S, Jockers R (2014) Hunting for the function of orphan GPCRs - beyond the search for the endogenous ligand. *Br J Pharmacol*, in press.
Amisten S, Neville M, Hawkes R, Persaud SJ, Karpe F, and Salehi A (2015) An atlas of G-protein coupled receptor expression and function in human subcutaneous adipose tissue. *Pharmacol Ther* **146**:61–93.
Amisten S, Salehi A, Rorsman P, Jones PM, and Persaud SJ (2013) An atlas and functional analysis of G-protein coupled receptors in human islets of Langerhans. *Pharmacol Ther* **139**:359–391.
Assie G, Louiset E, Sturm N, René-Corail F, Groussin L, Bertherat J, Thomas M, Lefebvre H, Feige JJ, and Clauser E et al. (2010) Systematic analysis of G protein-coupled receptor gene expression in adrenocorticotropin-independent macronodular adrenocortical hyperplasia identifies novel targets for pharmacological control of adrenal Cushing's syndrome. *J Clin Endocrinol Metab* **95**:E253–E262.
Atwood BK, Lopez J, Wager-Miller J, Mackie K, and Straiker A (2011) Expression of G protein-coupled receptors and related proteins in HEK293, AtT20, BV2, and N18 cell lines as revealed by microarray analysis. *BMC Genomics* **12**:14.
Brattelid T, Tveit K, Birkeland JA, Sjaastad I, Qvigstad E, Krobert KA, Hussain RI, Skomedal T, Osnes JB, and Levy FO (2007) Expression of mRNA encoding G protein-coupled receptors involved in congestive heart failure—a quantitative RT-PCR study and the question of normalisation. *Basic Res Cardiol* **102**:198–208.
Brazma A, Hingamp P, Quackenbush J, Sherlock G, Spellman P, Stoeckert C, Aach J, Ansorge W, Ball CA, and Causton HC et al. (2001) Minimum information about a microarray experiment (MIAME)-toward standards for microarray data. *Nat Genet* **29**:365–371.
Carr JC, Boese EA, Spanheimer PM, Dahdaleh FS, Martin M, Calva D, Schafer B, Thole DM, Braun T, and O'Dorisio TM et al. (2012) Differentiation of small bowel and pancreatic neuroendocrine tumors by gene-expression profiling. *Surgery* **152**: 998–1007.
Chen Y, Palczewska G, Mustafi D, Golczak M, Dong Z, Sawada O, Maeda T, Maeda A, and Palczewski K (2013) Systems pharmacology identifies drug targets for Stargardt disease-associated retinal degeneration. *J Clin Invest* **123**:5119–5134.
Civelli O, Reinscheid RK, Zhang Y, Wang Z, Fredriksson R, and Schiöth HB (2013) G protein-coupled receptor deorphanizations. *Annu Rev Pharmacol Toxicol* **53**: 127–146.
Davies MN, Gloriam DE, Secker A, Freitas AA, Mendao M, Timmis J, and Flower DR (2007) Proteomic applications of automated GPCR classification. *Proteomics* **7**: 2800–2814.
de Sousa Abreu R, Penalva LO, Marcotte EM, and Vogel C (2009) Global signatures of protein and mRNA expression levels. *Mol Biosyst* **5**:1512–1526.
Eisen D, Planatscher H, Hardie DB, Kraushaar U, Pynn CJ, Stoll D, Borchers C, Joos TO, and Poetz O (2013) G protein-coupled receptor quantification using peptide group-specific enrichment combined with internal peptide standard reporter calibration. *J Proteomics* **90**:85–95.
Engelstoft MS, Park WM, Sakata I, Kristensen LV, Husted AS, Osborne-Lawrence S, Piper PK, Walker AK, Pedersen MH, and Nøhr MK et al. (2013) Seven transmembrane G protein-coupled receptor repertoire of gastric ghrelin cells. *Mol Metab* **2**:376–392.
Feigin ME (2013) Harnessing the genome for characterization of G-protein coupled receptors in cancer pathogenesis. *FEBS J* **280**:4729–4738.
Fève M, Saliou JM, Zeniou M, Lennon S, Carapito C, Dong J, Van Dorsselaer A, Junier MP, Chneiweiss H, and Cianférani S et al. (2014) Comparative expression study of the endo-G protein coupled receptor (GPCR) repertoire in human glioblastoma cancer stem-like cells, U87-MG cells and non malignant cells of neural origin unveils new potential therapeutic targets. *PLoS ONE* **9**:e91519.
Foster SR, Blank K, See Hoe LE, Behrens M, Meyerhof W, Peart JN, and Thomas WG (2014a) Bitter taste receptor agonists elicit G-protein-dependent negative inotropy in the murine heart. *FASEB J* **28**:4497–4508.
Foster SR, Roura E, and Thomas WG (2014b) Extrasensory perception: odorant and taste receptors beyond the nose and mouth. *Pharmacol Ther* **142**:41–61.
Fredriksson R, Lagerström MC, Lundin LG, and Schiöth HB (2003) The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. *Mol Pharmacol* **63**:1256–1272.
Gandin V, Sikström K, Alain T, Morita M, McLaughlan S, Larsson O, and Topisirovic I (2014) Polysome fractionation and analysis of mammalian translatomes on a genome-wide scale. *J Vis Exp* (87).
Groot-Kormelink PJ, Fawcett L, Wright PD, Gosling M, and Kent TC (2012) Quantitative GPCR and ion channel transcriptomics in primary alveolar macrophages and macrophage surrogates. *BMC Immunol* **13**:57.
Gugger M, White R, Song S, Waser B, Cescato R, Rivière P, and Reubi JC (2008) GPR87 is an overexpressed G-protein coupled receptor in squamous cell carcinoma of the lung. *Dis Markers* **24**:41–50.
Hakak Y, Shrestha D, Goegel MC, Behan DP, and Chalmers DT (2003) Global analysis of G-protein-coupled receptor signaling in human tissues. *FEBS Lett* **550**: 11–17.
Hanahan D and Weinberg RA (2011) Hallmarks of cancer: the next generation. *Cell* **144**:646–674.
Hansen A, Chen Y, Inman JM, Phan QN, Qi ZQ, Xiang CC, Palkovits M, Cherman N, Kuznetsov SA, and Robey PG et al. (2007) Sensitive and specific method for detecting G protein-coupled receptor mRNAs. *Nat Methods* **4**:35–37.
Hohenhaus DM, Schaale K, Le Cao KA, Seow V, Iyer A, Fairlie DP, and Sweet MJ (2013) An mRNA atlas of G protein-coupled receptor expression during primary human monocyte/macrophage differentiation and lipopolysaccharide-mediated activation identifies targetable candidate regulators of inflammation. *Immunobiology* **218**:1345–1353.

Hutchings CJ, Koglin M, and Marshall FH (2010) Therapeutic antibodies directed at G protein-coupled receptors. *MAbs* **2**:594–606.
Insel PA, Snead A, Murray F, Zhang L, Yokouchi H, Katakia T, Kwon O, Dimucci D, and Wilderman A (2012) GPCR expression in tissues and cells: are the optimal receptors being used as drug targets? *Br J Pharmacol* **165**:1613–1616.
Khimani AH, Mhashilkar AM, Mikulskis A, O'Malley M, Liao J, Golenko EE, Mayer P, Chada S, Killian JB, and Lott ST (2005) Housekeeping genes in cancer: normalization of array data. *Biotechniques* **38**:739–745.
Kroeze WK, Sheffler DJ, and Roth BL (2003) G-protein-coupled receptors at a glance. *J Cell Sci* **116**:4867–4869.
Kuzumaki N, Suzuki A, Narita M, Hosoya T, Nagasawa A, Imai S, Yamamizu K, Morita H, Suzuki T, and Okada Y et al. (2012) Multiple analyses of G-protein coupled receptor (GPCR) expression in the development of gefitinib-resistance in transforming non-small-cell lung cancer. *PLoS ONE* **7**:e44368.
Lappano R and Maggiolini M (2011) G protein-coupled receptors: novel targets for drug discovery in cancer. *Nat Rev Drug Discov* **10**:47–60.
Lattin JE, Schroder K, Su AI, Walker JR, Zhang J, Wiltshire T, Saijo K, Glass CK, Hume DA, and Kellie S et al. (2008) Expression analysis of G Protein-Coupled Receptors in mouse macrophages. *Immunome Res* **4**:5.
Layden BT, Newman M, Chen F, Fisher A, and Lowe WL, Jr (2010) G protein coupled receptors in embryonic stem cells: a role for Gs-alpha signaling. *PLoS ONE* **5**: e9105.
Li S, Huang S, and Peng SB (2005) Overexpression of G protein-coupled receptors in cancer cells: involvement in tumor progression. *Int J Oncol* **27**:1329–1339.
Lin H, Sassano MF, Roth BL, and Shoichet BK (2013) A pharmacological organization of G protein-coupled receptors. *Nat Methods* **10**:140–146.
Lundstrom K (2009) An overview on GPCRs and drug discovery: structure-based drug design and structural biology on GPCRs. *Methods Mol Biol* **552**:51–66.
Maier T, Güell M, and Serrano L (2009) Correlation of mRNA and protein in complex biological samples. *FEBS Lett* **583**:3966–3973.
Malki A, Fiedler J, Fricke K, Ballweg I, Pfaffl MW, and Krautwurst D (2015) Class I odorant receptors, TAS1R and TAS2R taste receptors, are markers for subpopulations of circulating leukocytes. *J Leukoc Biol* **97**:533–545.
Manteniotis S, Lehmann R, Flegel C, Vogel F, Hofreuter A, Schreiner BS, Altmüller J, Becker C, Schöbel N, and Hatt H et al. (2013) Comprehensive RNA-Seq expression analysis of sensory ganglia with a focus on ion channels and GPCRs in Trigeminal ganglia. *PLoS ONE* **8**:e79523.
Marchalant Y, Brownjohn PW, Bonnet A, Kleffmann T, and Ashton JC (2014) Validating Antibodies to the Cannabinoid CB2 Receptor: Antibody Sensitivity Is Not Evidence of Antibody Specificity. *J Histochem Cytochem* **62**:395–404.
Mašek T, Valášek L, and Pospíšek M (2011) Polysome analysis and RNA purification from sucrose gradients. *Methods Mol Biol* **703**:293–309.
Maurel B, Le Digarcher A, Dantec C, and Journot L (2011) Genome-wide profiling of G protein-coupled receptors in cerebellar granule neurons using high-throughput, real-time PCR. *BMC Genomics* **12**:241.
Michel MC, Wieland T, Tsujimoto G (2009) How reliable are G protein coupled receptor antibodies? *Nauyn Schmiedebergs Arch Pharmacol* **379**: 385–8.
Moore-Morris T, Varrault A, Mangoni ME, Le Digarcher A, Negre V, Dantec C, Journot L, Nargeot J, and Couette B (2009) Identification of potential pharmacological targets by analysis of the comprehensive G protein-coupled receptor repertoire in the four cardiac chambers. *Mol Pharmacol* **75**:1108–1116.
Motulsky HJ (2014) Common misconceptions about data analysis and statistics. *Naunyn Schmiedebergs Arch Pharmacol* **387**:1017–1023.
Natarajan N and Pluznick JL (2014) From microbe to man: the role of microbial short chain fatty acid metabolites in host cell biology. *Am J Physiol Cell Physiol* **307**: C979–C985.
Ochodnický P, Humphreys S, Eccles R, Poljakovic M, Wiklund P, and Michel MC (2012) Expression profiling of G-protein-coupled receptors in human urothelium and related cell lines. *BJU Int* **110** (6 Pt B):E293–E300.
O'Hayre M, Degese MS, and Gutkind JS (2014) Novel insights into G protein and G protein-coupled receptor signaling in cancer. *Curr Opin Cell Biol* **27**:126–135.
O'Hayre M, Vázquez-Prado J, Kufareva I, Stawiski EW, Handel TM, Seshagiri S, and Gutkind JS (2013) The emerging mutational landscape of G proteins and G-protein-coupled receptors in cancer. *Nat Rev Cancer* **13**:412–424.
Overington JP, Al-Lazikani B, and Hopkins AL (2006) How many drug targets are there? *Nat Rev Drug Discov* **5**:993–996.
Ozawa A, Lindberg I, Roth B, and Kroeze WK (2010) Deorphanization of novel peptides and their receptors. *AAPS J* **12**:378–384.
Pan W (2002) A comparative review of statistical methods for discovering differentially expressed genes in replicated microarray experiments. *Bioinformatics* **18**: 546–554.
Pawson AJ, Sharman JL, Benson HE, Faccenda E, Alexander SP, Buneman OP, Davenport AP, McGrath JC, Peters JA, and Southan C et al.; NC-IUPHAR (2014) The IUPHAR/BPS Guide to PHARMACOLOGY: an expert-driven knowledgebase of drug targets and their ligands. *Nucleic Acids Res* **42**:D1098–D1106.
Pronin A, Levay K, Velmeshev D, Faghihi M, Shestopalov VI, and Slepak VZ (2014) Expression of olfactory signaling genes in the eye. *PLoS ONE* **9**:e96435.
Qin Y, Verdegaal EM, Siderius M, Bebelman JP, Smit MJ, Leurs R, Willemze R, Tensen CP, and Osanto S (2011) Quantitative expression profiling of G-protein-coupled receptors (GPCRs) in metastatic melanoma: the constitutively active orphan GPCR GPR18 as novel drug target. *Pigment Cell Melanoma Res* **24**:207–218.
Rajkumar P, Aisenberg WH, Acres OW, Protzko RJ, and Pluznick JL (2014) Identification and characterization of novel renal sensory receptors. *PLoS ONE* **9**:e111053.
Rask-Andersen M, Masuram S, and Schiöth HB (2014) The druggable genome: Evaluation of drug targets in clinical trials suggests major shifts in molecular class and indication. *Annu Rev Pharmacol Toxicol* **54**:9–26.
Regard JB, Sato IT, and Coughlin SR (2008) Anatomical profiling of G protein-coupled receptor expression. *Cell* **135**:561–571.
Reimann F, Tolhurst G, and Gribble FM (2012) G-protein-coupled receptors in intestinal chemosensation. *Cell Metab* **15**:421–431.
Rønnekleiv OK, Fang Y, Zhang C, Nestor CC, Mao P, and Kelly MJ (2014) Research resource: Gene profiling of G protein-coupled receptors in the arcuate nucleus of the female. *Mol Endocrinol* **28**:1362–1380.
Rubie C, Kempf K, Hans J, Su T, Tilton B, Georg T, Brittner B, Ludwig B, and Schilling M (2005) Housekeeping gene variability in normal and cancerous colorectal, pancreatic, esophageal, gastric and hepatic tissues. *Mol Cell Probes* **19**: 101–109.
Rung J and Brazma A (2013) Reuse of public genome-wide gene expression data. *Nat Rev Genet* **14**:89–99.
Secker A, Davies MN, Freitas AA, Clark EB, Timmis J, and Flower DR (2010) Hierarchical classification of G-protein-coupled receptors with data-driven selection of attributes and classifiers. *Int J Data Min Bioinform* **4**:191–210.
Snead AN and Insel PA (2012) Defining the cellular repertoire of GPCRs identifies a profibrotic role for the most highly expressed receptor, protease-activated receptor 1, in cardiac fibroblasts. *FASEB J* **26**:4540–4547.
Spaethling JM, Piel D, Dueck H, Buckley PT, Morris JF, Fisher SA, Lee J, Sul JY, Kim J, and Bartfai T et al. (2014) Serotonergic neuron regulation informed by in vivo single-cell transcriptomics. *FASEB J* **28**:771–780.
Talmont F, Moulédous L, Boué J, Mollereau C, and Dietrich G (2012) Denatured G-protein coupled receptors as immunogens to generate highly specific antibodies. *PLoS ONE* **7**:e46348.
Taylor CF, Paton NW, Lilley KS, Binz PA, Julian RK, Jr, Jones AR, Zhu W, Apweiler R, Aebersold R, and Deutsch EW et al. (2007) The minimum information about a proteomics experiment (MIAPE). *Nat Biotechnol* **25**:887–893.
Vassilatis DK, Hohmann JG, Zeng H, Li F, Ranchalis JE, Mortrud MT, Brown A, Rodriguez SS, Weller JR, and Wright AC et al. (2003) The G protein-coupled receptor repertoires of human and mouse. *Proc Natl Acad Sci USA* **100**:4903–4908.
Venkatakrishnan AJ, Deupi X, Lebon G, Tate CG, Schertler GF, and Babu MM (2013) Molecular signatures of G-protein-coupled receptors. *Nature* **494**:185–194.
Whittier KL, Boese EA, Gibson-Corley KN, Kirby PA, Darbro BW, Qian Q, Ingram WJ, Robertson T, Remke M, and Taylor MD et al. (2013) G-protein coupled receptor expression patterns delineate medulloblastoma subgroups. *Acta Neuropathol Commun* **1**:66.
Willets J and Nash C (2013) Investigation of G Protein-Coupled Receptor Function and Regulation Using Antisense. *Methods Pharmacol Toxicol* **XI**:105–126.
Wu J, Xie N, Zhao X, Nice EC, and Huang C (2012) Dissection of aberrant GPCR signaling in tumorigenesis—a systems biology approach. *Cancer Genomics Proteomics* **9**:37–50.
Yin D, Gavi S, Wang HY, and Malbon CC (2004) Probing receptor structure/function with chimeric G-protein-coupled receptors. *Mol Pharmacol* **65**:1323–1332.

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