Multi-Parameter GPCR Screening: An Essential Tool for Uncovering GPCR Mutant Phenotypes

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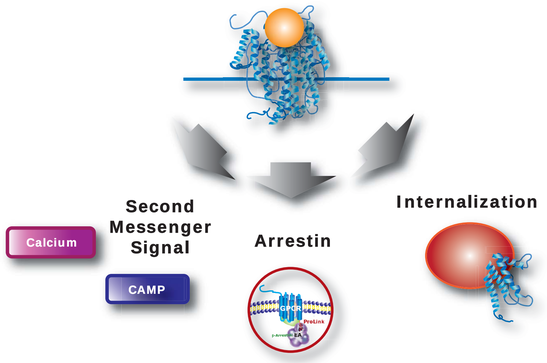
Abstract

The recent appreciation of functional selectivity/ligand bias in GPCR signaling has uncovered new opportunities for therapeutic discovery and continues to increase our understanding of compound function. We reasoned that since ligands and hence receptor conformation can differentially stimulate particular signaling pathways, variations in amino acid composition could similarly bias receptors and their responses to ligand. To test this hypothesis, we have compared a number of common GPCR variants and their wild-type counterparts in their ability to traffic to the cell surface and signal through g-proteins, arrestin, and internalization. This comprehensive suite of assays appropriately described mutants that caused GPCR misfolding and ER retention, as well as detailed changes in the functional selectivity of compounds. Thus the combination of these platforms provides an unprecedented view of GPCR variant activity in a quantifiable and comparable manner amenable to paneling large numbers of variants. These tools can be used identify new prospects for existing compounds and stratify patient populations for similar responses to drug candidates.

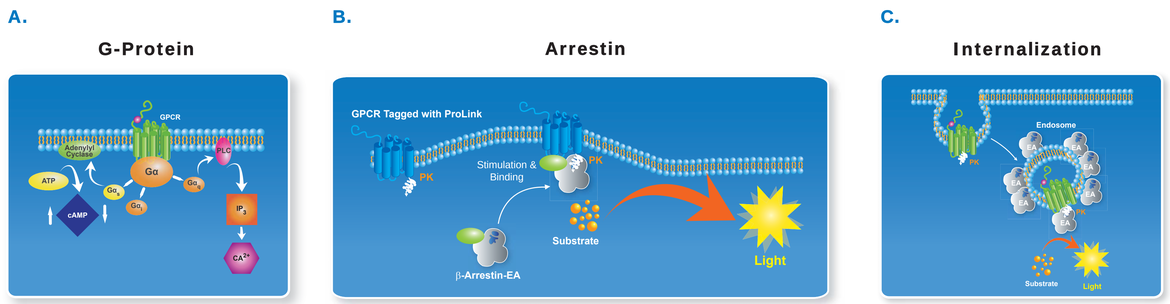
Model System



Edited forms of 5-HT2CR:

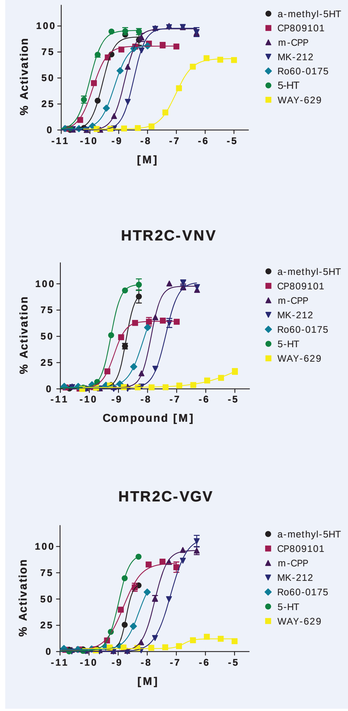
The messenger RNA encoding the 5-HT2c Receptor undergoes adenosine deamination to produce a variety of edited variants. These variants can encode up to 24 different proteins. Utilizing two of these variants in comparison to the wild-type, we surveyed a panel of serotonin receptor agonists to uncover mutant specific biases in the signaling pathways. For each receptor variant, we generated assays for calcium, arrestin, and internalization such that the responses to compound could be quantified and compared across mutants and across assays. The internalization and arrestin assays are ideal for mutant comparisons because they generate signal in a stochiometirc fashion, where the signal does not saturate until all of the cell-surface receptors are engaged. This is in stark contrast to second messenger assays where only a small fraction of receptor need to be activated to realize full signal. For this reason,calcium assays are prone to signal saturation that can erroneously characterize partial agonists and overestimate the potency of full agonists. In addition, since target protein expression influences potency in these systems, it is difficult to compare across cell lines or across panels of mutant receptors. Thus systems such as the Pathhunter® Arrestin and Internalization platforms provide an ideal solution to investigating GPCR variants.

DiscoveRx Assay Formats

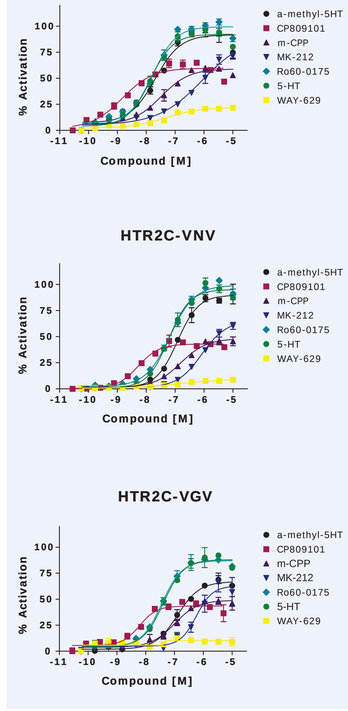


(A) DiscoveRx's HitHunter® Calcium No Wash^{PLUS} kits use a homogenous assay format to detect calcium mobilization. Measurement of intracellular calcium provides valuable information on the activation status of GPCRs and ion channels. Stimulated receptor releases intracellular calcium, causing the calcium-sensitive dye in the kit to fluoresce. **(B)** PathHunter® β-Arrestin GPCR cell lines are engineered to co-express the ProLink™ (PK) tagged GPCR and the Enzyme Acceptor (EA) tagged β-Arrestin. Activation of the GPCR-PK induces β-Arrestin-EA recruitment, forcing complementation of the two β-galactosidase enzyme fragments (EA and PK). **(C)** PathHunter® Total GPCR Internalization cell lines are engineered to co-express the ProLink™ (PK) tagged GPCR, and an Enzyme Acceptor (EA) tag localized to the endosomes. Activation of the GPCR-PK induces internalization of the receptor in EA-tagged endosomes, forcing complementation of the two β-galactosidase enzyme fragments (EA and PK).

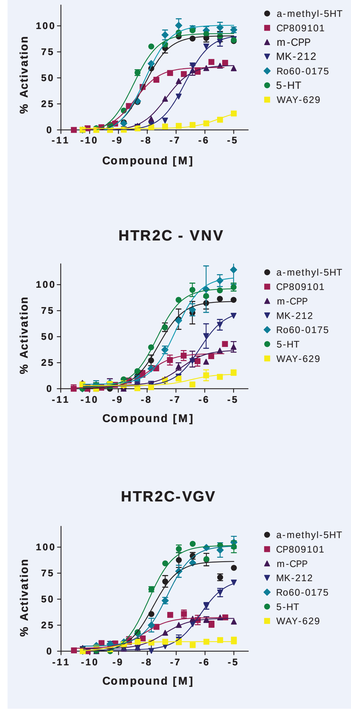
Calcium Mobilization



Arrestin Assay

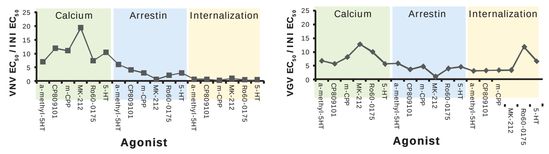


Internalization Assay



HTR2C is a member of the 5-Hydroxytryptamine (5-HT) family of GPCRs, and is a native Gq coupled receptor. PathHunter® Arrestin and Internalization cell lines expressing three variants of HTR2C have been generated: the unedited INI wildtype (WT) HTR2C, and the HTR2C-VNV and VGV variants. Seven agonists, including 5-HT (the endogenous agonist for HTR2C) were used in each of the nine experiments that were run. PathHunter® Arrestin and Internalization assays were run using standard PathHunter® assay protocols for each of the two assay read-outs. The Arrestin cell lines were also used to generate the calcium mobilization data for each HTR2C variant.

EC50 Comparisons by Read-out



EC50s generated by the VNV and VGV versions are compared to the EC50s generated by the wildtype HTR2C for each assay readout. Variant ratios of EC50 were calculated for each agonist and then plotted. Ratios of EC50s generated by the calcium readouts show more variation between the wildtype and mutant receptor variants than do the data generated by the Arrestin and Internalization read-outs. This appears to be particularly evident in the case of the HTR2C-VNV mutant.

Ligand Phenotypes Uncovered Using the PathHunter® System

m-CPP (% of 5-HT Response)			
Assay	WT	VNV	VGV
Calcium	98	97	96
Arrestin	59	48	55
Internalization	62	37	32

MK-212 (% of 5-HT Response)			
Assay	WT	VNV	VGV
Calcium	98	101	108
Arrestin	91	65	75
Internalization	92	75	69

CP809101 (% of 5-HT Response)			
Assay	WT	VNV	VGV
Calcium	81	65	84
Arrestin	59	43	49
Internalization	60	33	32

Efficacies of three compounds are compared across all nine experiments. m-CPP is a reported partial agonists; MK-212 and CP 809,101 are reported full agonists for HTR2C. Here, all three compounds acted as full agonists in the calcium readout, but as partial agonists in the two PathHunter read-outs. CP809101 acted as a partial agonist in all three readouts, but efficacy was significantly lower in the two PathHunter readouts. Among the PathHunter assays, compound efficacy with the two HTR2C mutants was significantly lower than with the WT variant.

Summary

The field of GPCR drug discovery has benefitted from the integration of functional selectivity into the drug screening paradigms. Here we have extended these studies to a multivariate analysis of receptor polymorphisms and selected compounds. This analysis highlights the difficulties in comparing mutant phenotypes using second messenger assays. Alone, calcium and other amplified systems typically suffer from high levels of receptor reserve that masks certain aspects of compound activity and can overestimate potency. As a case in point, the known partial agonist m-CPP is shown to behave as a full agonist in the calcium assays. However now assays are available with limited or no receptor reserve such as the PathHunter arrestin and internalization assays. Using these assays we show that the m-CPP compound consistently behaves as a partial agonist. In addition, the CP 809,101 compound, although described to be 100% efficacious is truly a weak partial agonist in arrestin and internalization assays. Interestingly the MK-212 (also previously characterized as a full agonist) as well as the CP 809,101 compounds both exhibit lower efficacy on the variant receptor populations that the unedited INI version indicating some bias in these compounds that is dependent on receptor sequence. These results suggest that there is a significant added benefit realized from analyzing naturally occurring variants in non-amplified systems and using multiple pathways to define mutant pharmacology.

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