

Lakshmi Anantharaman and Neil Charter
DiscoverX Corporation, Fremont, CA 94538

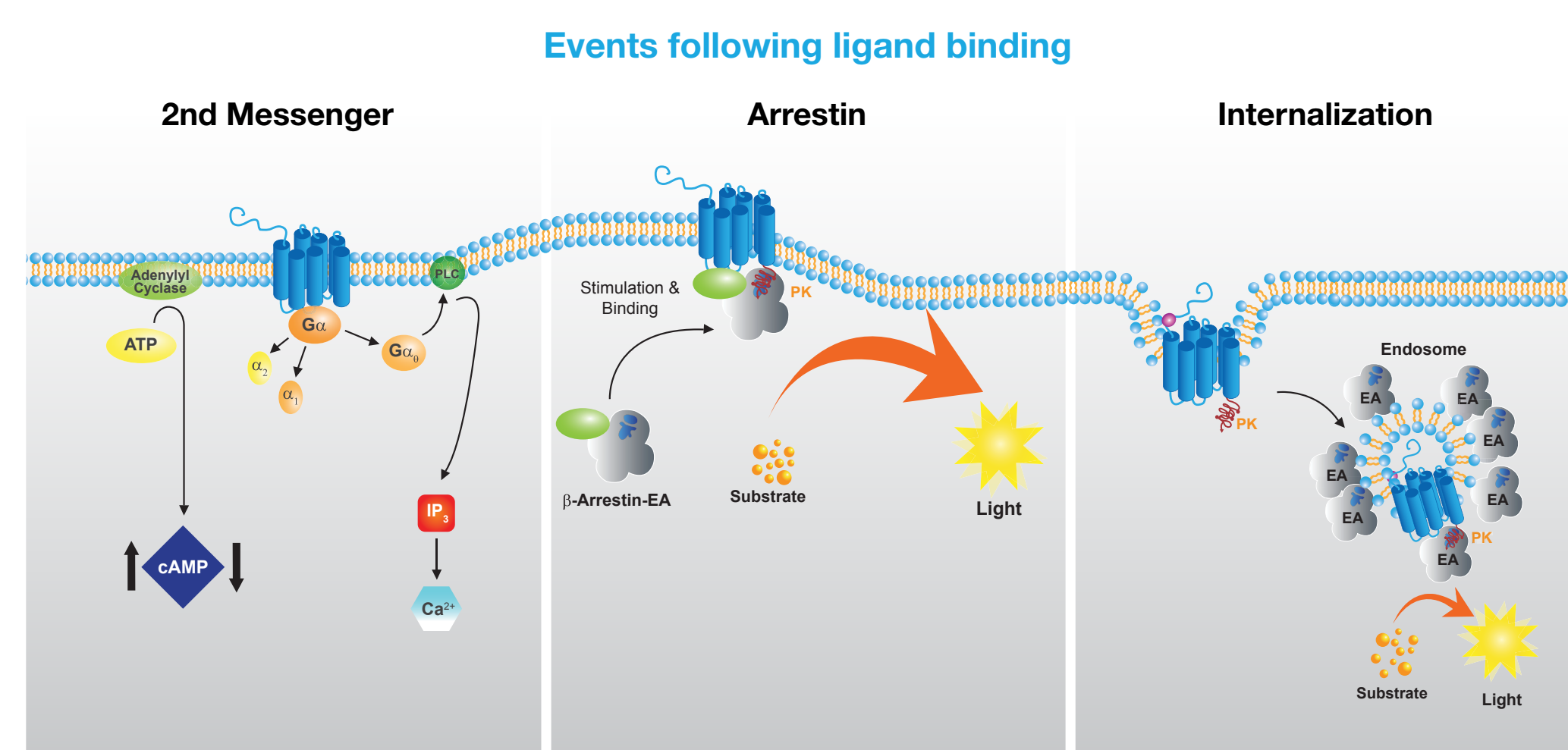
Abstract

Compounds that bind to allosteric sites offer many benefits over those which interact orthosterically. These include enhanced specificity, ability to operate only on receptor activation and reduced risk of overdose. A number of allosteric compounds have been identified in multiple disease areas including CNS (physiological and neuropathic disorders), metabolic disorder (diabetes and weight control), immunomodulation and cardiovascular indications. Allosteric approaches provide opportunities for new drug discovery by overcoming ligand incompatibility with certain drug scaffolds and improving the ability of small molecules drugs to modulate peptide ligand activity. In this study, we investigate the efficacy of a range of allosteric compounds using multiple GPCR readouts including arrestin recruitment, calcium mobilization, cAMP modulation and receptor internalization. Compounds were tested for ability to potentiate or down-regulate agonist responses using different approaches including EC₅₀ shift analysis and residual agonist activity. Interactions were obtained in all three GPCR classes. Responses were obtained for multiple pathways demonstrating the utility for readouts involving either arrestin or second messenger signaling. Allosteric activity was also determined for receptor internalization and provides the means to examine the effect of allosterics to alter receptor cell surface population and activation kinetics.

Methods

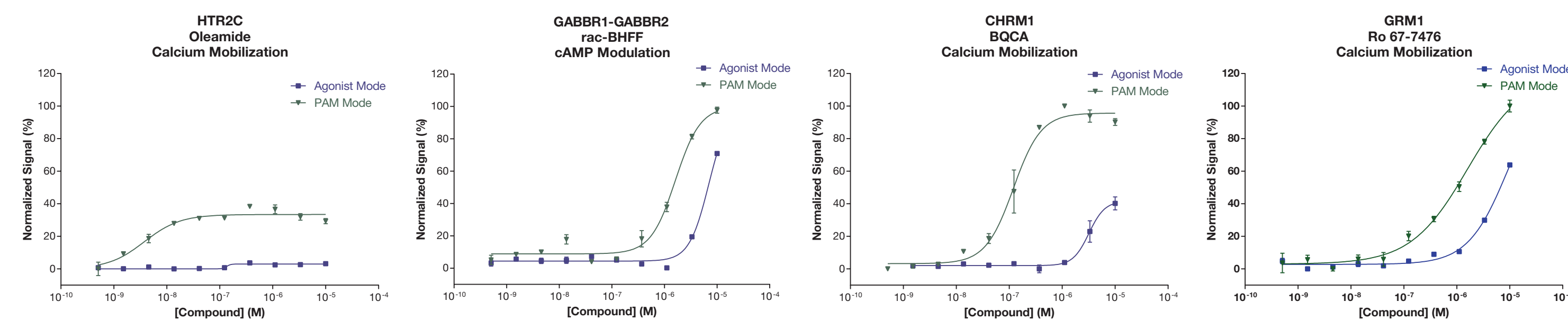
All cell lines used were from DiscoverX. For Arrestin recruitment assays, cell lines stably express various GPCR tagged with ProLink and EA-Beta-Arrestin. For second messenger assays, cell lines stably express GPCR with no additional modification. In some cases, Calcium mobilization assays were performed in Arrestin cell lines. (1,2)
For Beta-Arrestin recruitment and Internalization assays, 5000 cells per well were seeded in 20 µL media and incubated overnight prior to assay. Agonist responses were induced by addition of 5µL 5 X compound and PAM responses were obtained by co-incubation of 5µL 6 X compound plus 5µL 6 X EC₂₀ ligand. EC₅₀ shift analysis was achieved by performing agonist dose responses in the presence and absence of compound at varying concentrations. Incubation time was 120 minutes for Arrestin and 180 minutes for Internalization assays. Beta-Arrestin recruitment or Internalization was detected after 1 hour room temperature incubation with 50 % (v/v) of PathHunter Detection Reagent (Dx 93-0001) and chemiluminescence read using a PerkinElmer Envision reader. Data was plotted with GraphPad Prism using Sigmoidal dose-response (variable slope) or Allosteric EC₅₀ shift fits.
For second messenger assays, 10,000 cells per well were seeded in 20 µL media and incubated overnight prior to assay. For cAMP modulation assays, media was exchanged with 10 µL 1:1 HBSS/10mM Hepes / cAMP Ab reagent. Agonist responses were induced by addition of 5µL buffer plus 5µL 4 X compound. Positive Allosteric Modulator (PAM) responses were obtained by co-incubation of 5µL 4 X compound plus 5µL 4 X EC₂₀ ligand. EC₅₀ shift analysis was achieved by performing agonist dose responses in the presence and absence of compound at varying concentrations. For Calcium mobilization assays, media was exchanged with 20 µL Dye loading buffer (Calcium No Wash™ kit). Agonist responses were induced by addition of 10µL 3 X compound. PAM responses were obtained by addition of 10µL 4 X EC₂₀ ligand. Calcium mobilization was monitored for 2 minutes using a FLIPR Tetra (MDS, CA) after compound and EC₂₀ addition to determine agonist and PAM responses. EC₅₀ shift analysis was achieved by performing agonist dose responses in the presence and absence of compound at varying concentrations. Data was plotted with GraphPad Prism using Sigmoidal dose-response (variable slope) or Allosteric EC₅₀ shift fits.

Functional GPCR Signaling



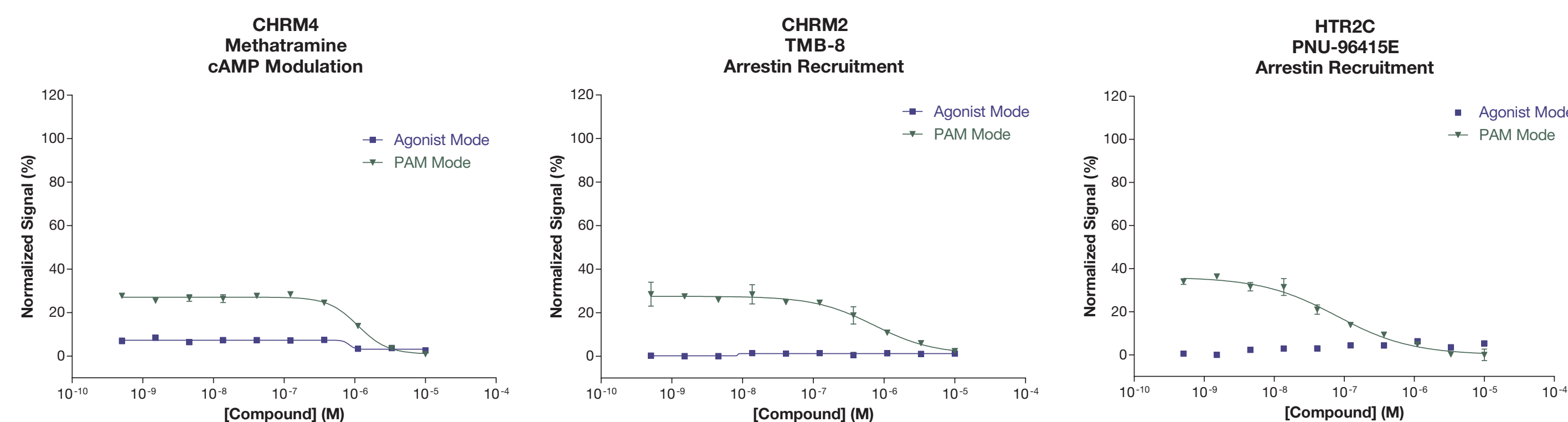
DiscoverX offers multiple approaches for interrogating compound activity for GPCRs. This enables the ability to examine compound efficacy from receptor activation to internalization and transport to the endosome.

Positive Allosteric Modulators (PAM)



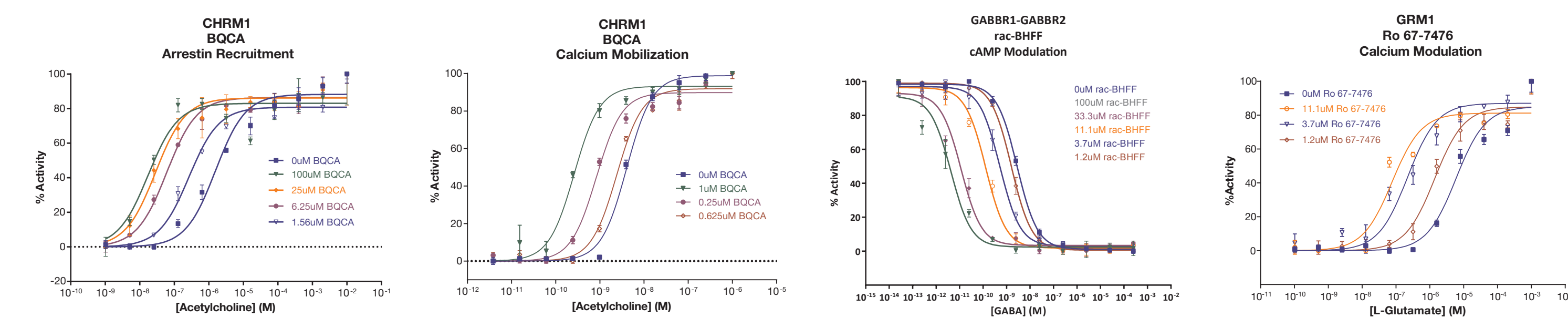
A number of known allosteric compounds were screened for activity in agonist and PAM mode. Compounds ranged in level of allosteric agonist activity and produced significant potentiation in the presence of EC₂₀ ligand.

Negative Allosteric Modulators (NAM)



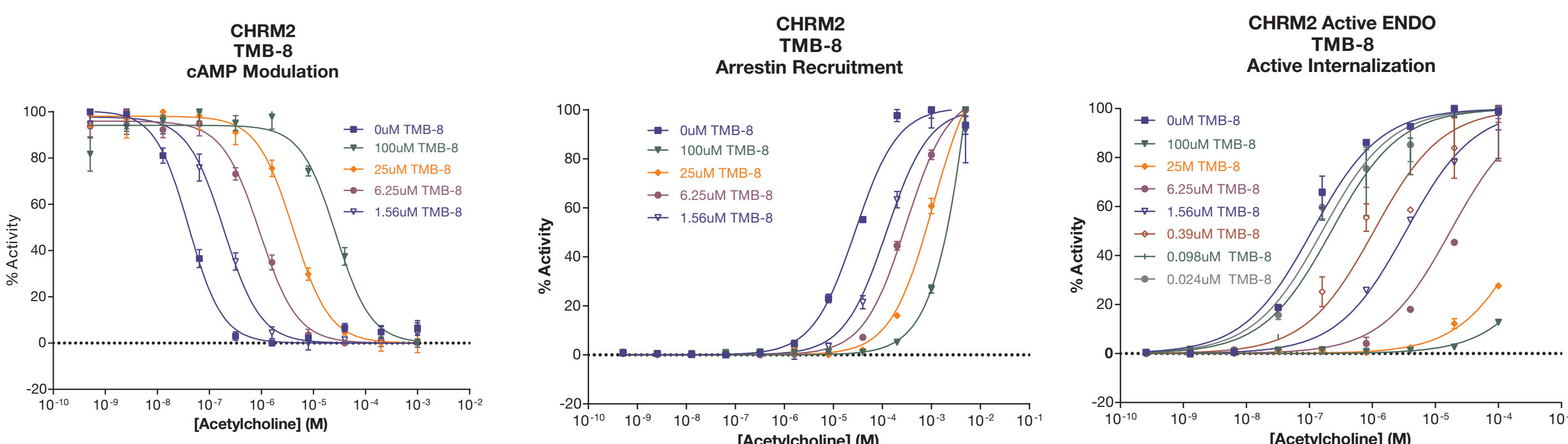
Negative allosteric modulators were also identified by the ability to reduce ligand efficacy. In these examples EC₂₀ responses are sufficient to pick up potential NAM activity.

PAM EC₅₀ Shift Analysis



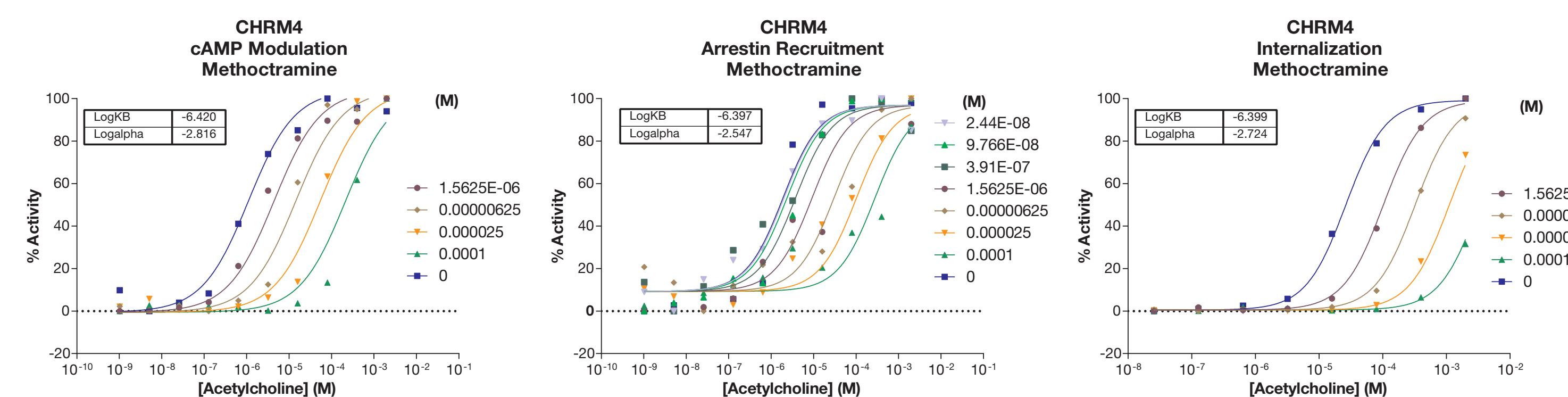
EC₅₀ shift analysis provides an ideal method for examining the ability of PAMs to increase receptor binding affinity. Compounds identified in the initial potentiation experiments were tested for the effect of enhancing ligand potency. Significant shifts to the left of the agonist dose responses were observed with increasing modulator concentration.

NAM EC₅₀ Shift Analysis



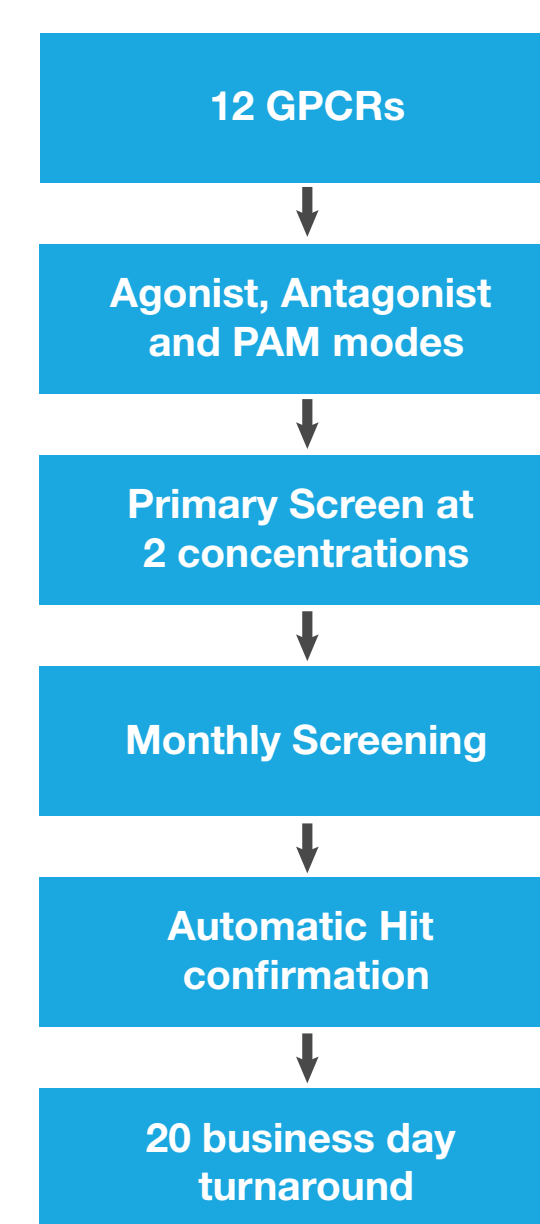
Negative allosteric modulators reduce receptor affinity for ligands and their effects are readily examined by agonist shift assays. In this example the effect of TMB-8 on the M2 receptor is shown for 3 signaling events, Gs-coupled cAMP second messenger modulation, arrestin recruitment and subsequent internalization of the receptor to the endosome.

Determination of Modulator Kb and Alpha



Methoctramine is a negative allosteric modulator of the M4 muscarinic receptor. The compound significantly reduces acetylcholine affinity for cAMP modulation, arrestin recruitment and receptor internalization. Values for the modulator affinity constant and alpha constant were obtained by fitting the data to an allosteric EC₅₀ shift model. A Kb of 400 nM was generated for all three assay readouts. Alpha values ranged from 0.0015 to 0.003 and were consistent with the observation that methoctramine greatly reduces ligand binding.

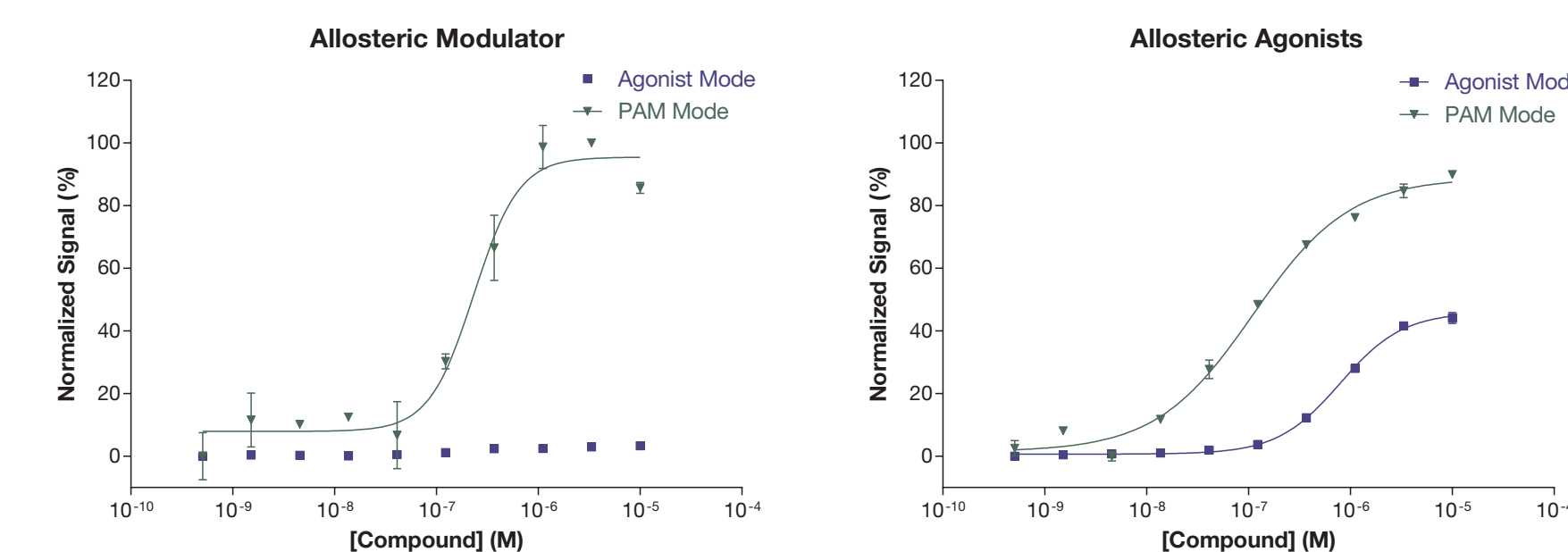
Functional Panel Screening



Liability screening typically employs testing compounds for affinity to ligand binding domains in high risk targets. This approach does not provide mechanism of action in general and specifically does not address whether compounds bind to allosteric sites.

We developed a functional panel for a client to screen compounds against selected targets in agonist, antagonist and PAM mode. The program incorporates an automatic hit confirmation for actives.

- Over 750 compounds screened to date
- >75% Confirmation Rate
- Examples of allosteric modulator and allosteric agonist identified in screen



Summary

Allosteric modulators provide an alternative approach for controlling GPCR activity. Here we demonstrate that allosteric regulation can be identified using multiple functional readouts from secondary messenger signaling, arrestin recruitment and receptor internalization. DiscoverX offers the largest commercially available library of functional cell-based assays for GPCRs, including Calcium, cAMP, β-Arrestin, and receptor internalization. These assays are available to clients as continuous culture cell lines, ready-to-assay kits, and PathHunter Services. The ability to test these assays with a wide variety of readouts such as these enables the identification of allosteric compounds as well as biased ligands.

References

- A homogeneous enzyme fragment complementation-based beta-arrestin translocation assay for high-throughput screening of G-protein-coupled receptors. Zhao X, Jones A, Olson KR, Peng K, Wehrman T, Park A, Mallari R, Nebalasca D, Young SW, Xiao SH. *J Biol Chem*. 2008 Sep;283:13737
- Characterization of G-protein coupled receptor modulators using homogeneous cAMP assays. Bassoni DL, Jafri Q, Sastry S, Mathrubutham M, Wehrman TS. *Methods Mol Biol*. 2012;897:171