SPECIFIC AIMS

Although G protein-coupled receptors (GPCRs) are the largest class of drug targets in the human genome the functions of many GPCRs are unknown or understudied. Our goal is to illuminate the pharmacology and create chemical probes (e.g. molecules) or genetic constructs (e.g. mice) to specifically modulate these orphan or understudied G protein coupled receptors (oGPCRs). The chemical probes and genetic constructs will also be directly useful to community investigators for interrogation of individual oGPCRs. This IDG Data and Resource Generating Center will thus create a "*research consortium to … unveil the functions*" of the oGPCR-ome. We will interrogate the full set of oGPCRs specified in RFA-RM-16-026, using the scalable technologies established in the Phase I period. By revealing the pharmacology, testing the chemical probes and constructs in cellular, tissue, and *in vivo* models, we and community investigators will illuminate the physiological functions and cell-type expression of the oGPCR-ome (**Fig 1**).

To accomplish these aims, we will create and administer an integrated infrastructure for computational, pharmacological, chemical, genetic experiments between UNC-Chapel Hill (UNC), UCSF, and Icahn School of Medicine at Mount Sinai (ISMMS). UNC will provide administrative, pharmacological and genetic infrastructure, UCSF will provide structure-based docking and compound optimization, and will coordinate informatics, data and resource sharing, while ISMMS will provide chemical synthesis and medicinal chemistry support. The three groups collaborated extensively in Phase I of the IDG [1 2] and in other projects [3,4 5,6 7 8,9 10 11-16 17] and previously have enjoyed fruitful collaborations with others within the Phase I network [18,19 20 21 22 23 24 25 26]

1. Illuminate the pharmacology and discover chemical probes for oGPCRs, and use these to interrogate their function, signaling, pharmacology and physiology. As no single technology can interrogate all oGPCRs, we will use several platforms we optimized and scaled in the Phase I period including: arrestin-based reporters, yeast-based platforms, reporter gene readouts, and panels of transducer-deficient CRISPR lines. We will screen most oGPCRs to illuminate their pharmacology and to discover and optimize specific probes by a combination of computational, physical screening and directed synthetic approaches, as in Phase I period of this project (**Fig 1**).

2. Create engineered CRISPR-tagged mice that, combined with DREADD technology, will reveal the function, signaling, physiology, cell-type and regional expression of oGPCRs. We will create a panel of as many as 60 engineered lines of mice suitable for illuminating crucial functions of oGPCRs, and will use chemogenetic and reporter gene technologies to reveal their cell-type-specific distributions and functions *in vivo* (**Fig 1**).

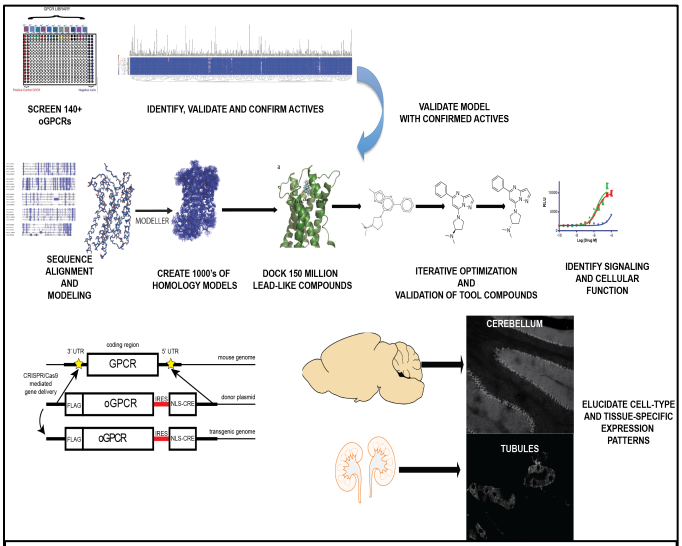

Figure 1. Illuminating the druggable GPCR-ome. Shown are the combined physical screening, computational and genetic approaches for fulfilling the aims of the RFA. We combine parallel screening of oGPCRs with modeling, docking and iterative rounds of medicinal chemistry to identify tool compounds to illuminate signaling, pharmacology, functionality and chemistry of oGPCRs. The genetic approach uses CRISPR and DREADD technology to illuminate cell-type expression and functionality of oGPCRS.

3. Provide integrated infrastructure—computational, pharmacological, chemical, genetic and administrative—to coordinate collaborations, assemble and integrate large datasets, and to disseminate this information openly to the community. All resources (probes, cell lines, computational tools, chemo- and optogenetic tools and genetically engineered mice) will be shared openly (ADDGENE for plasmid and virally-based resources; JAX for mice; on-line computational databases, docking programs, and results, as at http://zinc15.docking.org, http://blaster.docking.org; we will continue to collaborate with Millipore-Sigma to make our chemical probes widely available; all detailed in the Data Sharing section). The administrative unit will coordinate the 10% of the award designated to fund collaborations with other IDG groups. Significantly, although this proposal is ambitious in scope, extensive preliminary results from the Phase I period support feasibility, significance and ultimate impact.

RESEARCH STRATEGY

A. SIGNIFICANCE: G-protein coupled receptors (GPCRs) represent the single largest class of druggable targets in the human genome.[27-29] Of the 390 or so druggable and non-olfactory human GPCRs, many are orphan or understudied [see ref [28] for recent review]; we refer to these as "oGPCRs" [28 30 2]. The current RFA lists 143 oGPCRs; our goal is to illuminate the pharmacology, signaling pathways, chemical biology, distribution and/or function of all 143 oGPCRs. Given the central importance of GPCRs for all areas of biomedical research, illuminating the pharmacology, function, signaling and/or chemical biology of these oGPCRs will have far-reaching impact for both therapeutics and basic biomedical science.

B. INNOVATION: This project seeks to discover and develop specific, community accessible tools—chemical probe molecules and engineered animals—that enable investigators to interrogate the biological functions of oGPCRs. The apparent simplicity of these goals is belied by the extensive technological innovations that undergird it. Building upon our recent published and unpublished techniques, this collaboration introduces the following innovations:

- Seeking chemical matter useful as probe or 'tool' molecules for oGPCRs, we use novel and unprecedented (by scale) *in silico* [1,2,9,31] and physical screening approaches [1 2,30,32] to discover and optimize tool molecules suitable for illuminating the signaling pathways and functionalities of oGPCRs. Lead compounds are further optimized by end-stage medicinal chemistry.
    - We know of no group that has physically screened oGPCRs as systematically or as comprehensively as are we. Such screening is enabled by two new platforms we developed, validated, and made open access over the last period: The first exploits arrestinergic recruitment (Presto-Tango)[30 2] while the second exploits yeast[1].
    - We leverage the hits from these first-pass small molecule screens to build structural models of the targeted oGPCRs, which are then used to discover and optimize specific chemical probes using large-library molecular docking [see refs [1,2,31] for examples].
    - In the next period, we expand our docking libraries from 3.5 million, available, physically well-behaved molecules, to >150 million never-before interrogated, but interesting and readily available molecules. The technology that enables this is new to this project.

  We illustrate allied technological innovations in the main application.
- The probe molecules that emerge are not only primary outcomes but also key points of innovation. Each probe enables experiments on particular oGPCRs that have previously been impossible, owing to lack of specific reagents. The new probes are all open access via a collaboration with Sigma-Millipore, as in been the case with the probes developed for GPR68 (Huang et al, **Nature** 2015) and MRGPRX2 (Lansu et al, **Nature Chemical Biol** *in press*) (letter from Dr. Shari Spector, & e.g., http://www.sigmaaldrich.com/catalog/product/sigma/sml1482?lang=en&region=US).
- As not all oGPCRs are amenable to chemical tool development, we will combine CRISPR and DREADD technologies to generate mouse models to illuminate the distribution, function and physiology of selected oGPCRs. DREADDs were invented by Dr. Roth[33] and are listed a potential approach in the RFA. This application may take advantage of new conditional DREADD mice[34], novel DREADD chemical actuators[35 36] and new DREADDs[37 36], in addition to novel CRISPR-tagging approaches piloted in this application. Here too, we sketch directions for further innovation in the application.

C. APPROACH

Specific Aim #1. 1. Illuminate the pharmacology and discover chemical probes for oGPCRs, and use these to interrogate their function, signaling, pharmacology and physiology..

Background, preliminary findings and statement of problem: As GPCRs as a class are druggable, a fruitful way to reveal their function is to illuminate their pharmacology and discover potent and selective chemical probes. We define a probe as a ligand with: (1) >30-fold specificity vs related targets; (2) affinity in the sub-μM range; (3) relevant cellular functional activity; and where possible (4) pairing with a close inactive analog, making a probe-pair (**Figure 7** shows a probe pair for MRGPRX2). This definition resembles that adopted by the Structural Genomics Consortium (http://www.thesgc.org/chemical-probes) for understudied kinases and epigenetic targets.

In many research projects, probes are discovered from high-throughput screens (HTS) followed by cycles of optimization. Most GPCR HTS campaigns demand agonists and antagonists as positive controls, but for many

oGPCRs even non-selective agonists are unavailable[28,30]; furthermore many of the 'selective' ligands have been revealed to be non-selective or inactive by ourselves[1,2,32] and others[38 39 40]. Finally, for most screens it is useful, even critical, to know the G proteins with which GPCRs couple; for many oGPCRs this too is unknown.

To overcome these hurdles, we adapted two approaches that afford scalable and HTS-like platforms for discovering agonists[1,30], inverse agonists (antagonists with negative intrinsic activity)[1], and allosteric modulators[1] for oGPCRs. Both platforms will be used in this application, as each has advantages and disadvantages. As we have published extensively on, and fruitfully exploited both the yeast-[1,33,41] and arrestin–based approaches[2,3,8,17,30,32,42,43], only their key features will be summarized here.

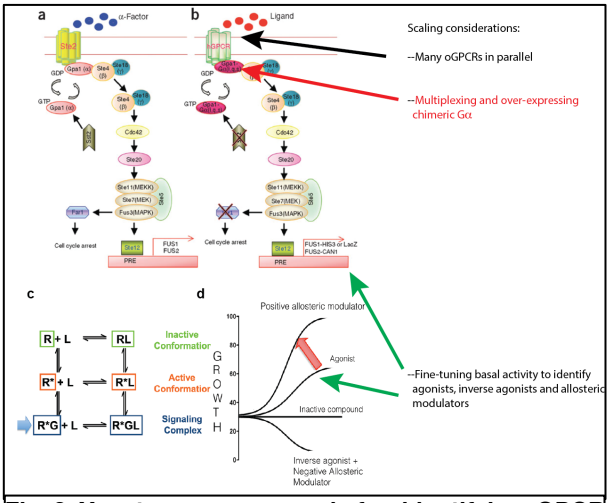

Fig 2 Yeast as an approach for identifying GPCR modulators. See text for details.

The first approach—which relies on genetically-engineered yeast that can be transduced with human GPCRs and chimeric G proteins—was perfected by Mark Pausch's group[44-48] and shared with the Roth lab many years ago[33] (**Fig 2a-b)**. The platform has been used by Pausch,[49] ourselves,[1] and others[50,51] to discover endogenous and exogenous ligands for oGPCRs. Our major innovations were to: (a) ***scale*** the approach to screen dozens of oGPCRs simultaneously and (b) ***to over-express*** in a multiplexed manner ***chimeric G*** proteins (**Fig 2b,c,d**) to provide sufficient basal activity to facilitate the simultaneous identification of agonists, inverse agonists (e.g antagonists), and negative and positive allosteric modulators (**Fig 2c,d**).

Pros and cons of Yeast platform: The yeast screen has several *advantages*:

--Deletion of endogenous GPCRs and G proteins that could otherwise interact with human oGPCRs.
--Cheap, scalable assays (¢/well)
--Does not require agonist as positive control (owing to high basal signaling)
--Can identify NAMs, PAMs, agonists and inverse agonists

Admittedly, yeast also suffer from potential *disadvantages*:

--Cell wall and export pumps that can, if not controlled, inhibit access of small molecules.
--Inefficient coupling with some GPCRs
--Poor expression for some human GPCRs.

<u>*Use of yeast platform for discovering pharmacology and developing proof-of-concept probe for the orphan GPR68.*</u> The yeast platform, and its integration with structure-based docking, has been published (Huang, Karpiak et al, Shoichet & Roth, ***Nature*** 2015) and is summarized here. We initially screened 24 oGPCRs in parallel with three over-expressed yeast-human Gα chimeric proteins (Gpa1-i, Gpa1-s, Gpa1-q) or empty vector (96 combinations), to find the Gα chimeras with the largest screening window. These were then used to physically screen a small proof-of-concept library (NIH Clinical Collection #1). The sparse matrix of actives (**Fig 3** heat map) was confirmed by concentration-response studies with authentic resupplied compounds (**Fig 3a,b**). Although actives were found for several oGPCRs, the activity of benzodiazepines as positive allosteric modulators (PAMs) of GPR68—also known as OGR1—stood out (**Fig 3a-d**) revealing GPR68 as a receptor for N-unsubstituted benzodiazepines (**Fig 3b,c**).

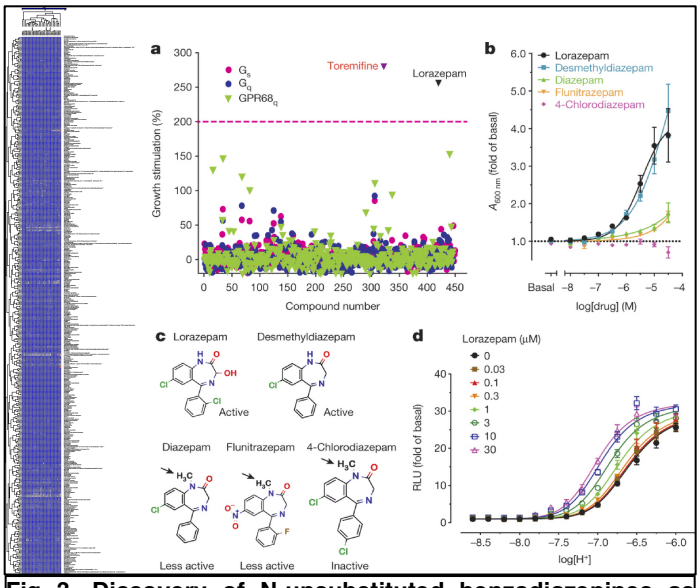

Fig 3. Discovery of N-unsubstituted benzodiazepines as allosteric potentiators for GPR68. See text for details.

With several active and inactive benzodiazepines in hand (affording SAR, **Fig 3c, d**), we sought specific molecules to modulate GPR68; benzodiazepines are not useful due to their potent GABA-A receptor activity. Our structure-inspired approach began with an ensemble of

3300 models of the receptor calculated using Modeler[52] and the elastic network modeling program ENS.[53] Because the template crystal structure on which this models were based, CXCR4, bore only 29% sequence identity to the TM-region of GPR68, we needed a non-structural criterion to select which of the models was best suited to prospective compound discovery. We docked the active benzodiazepines along with the 460 known inactives and about 500 property matched decoys[54] against each of the models ($3x10^{12}$ complexes calculated). Our goal was to find the model that best enriched lorazepam and active analogs over the decoys. Cycles of optimization led to two candidate models, which were tested by site-directed mutagenesis to settle on the most predictive one (**Fig 4A**).

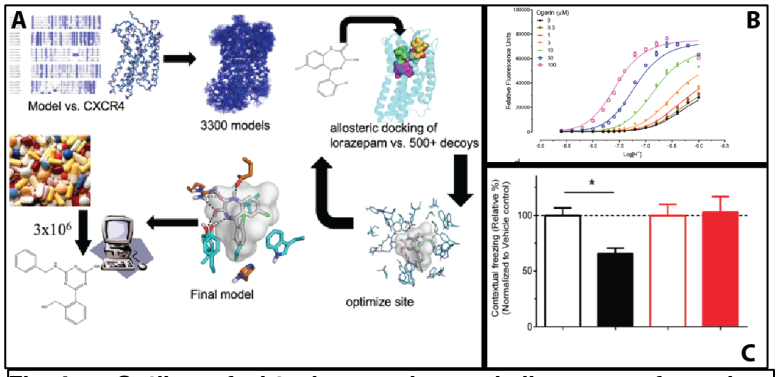

Fig 4. Outline of virtual screening and discovery of ogerin a Gpr68 PAM with activity in vitro and in vivo. See text and Huang et al, Nature 2015 for details

With this model in hand, we docked 3 million lead-like commercially available molecules[55,56] from our ZINC library against the putative GPR68 PAM site, using DOCK3.6.[57,58] From this virtual screen, 15 high-ranking compounds were selected for testing, 5 of which were active as PAMs. A cycle of analog docking and testing resulted in a molecule dubbed "ogerin" (for OGR1 ligand) that is not a benzodiazepine and was 30-fold more potent as a PAM than lorazepam. Encouragingly, ogerin had no activity on the GABA channel and was inactive as an agonist on 316 other GPCRs, including the related GPR4 and GPR65. Re-synthesis of ogerin in the Jin lab (Mt. Sinai), and synthesis of analogs with even greater potency, confirmed the chemical structure of the molecule and its potential for further optimization

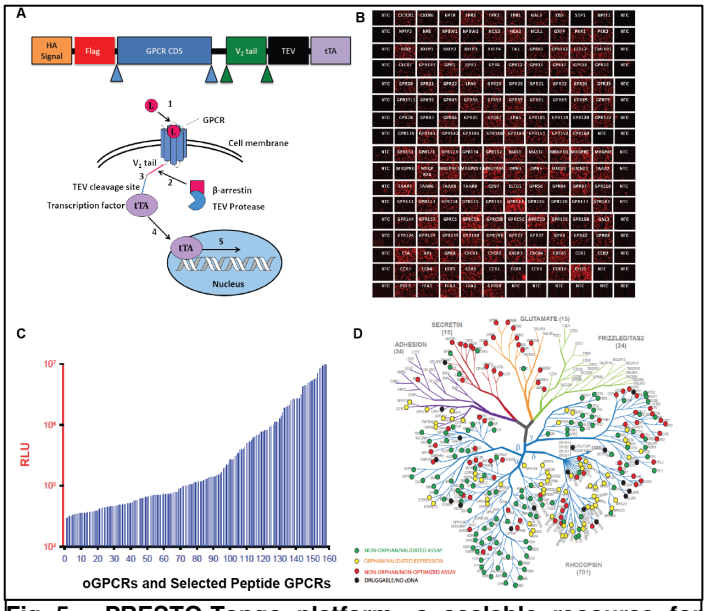

Fig 5. PRESTO-Tango platform: a scalable resource for illuminating the GPCR-ome. (A) shows modular design strategy; (B) shows surface expression for large number of oGPCRs; (C) shows basal activity for oGPCRs and (D) shows coverage of resource; see Kroeze et al, 2015 for details and text.

These observations encouraged us to use ogerin to probe the vivo function of GPR68. Based on its high expression in the brain, especially the hippocampus, we put WT and GPR68 KO mice through a battery of behavioral tests, either with ogerin or a close but inactive analog. In WT mice, ogerin reduced hippocampal context-based learning, while no such effect was observed in the KO mice, nor with the inactive analog against the WT mice; ogerin itself had no effect on cue-based learning, which is not hippocampus-based (**Fig 4C** and ref[1]). This **chemical-genetic epistasis** supports a role for GPR68 in hippocampal learning, among the first *bona fide* functions for this orphan receptor. We anticipate that ogerin and its inactive analog will be a useful probe pair for further functional interrogation; both molecules are made available to the community via our collaboration with Sigma-Millipore (http://www.sigmaaldrich.com/catalog/product/sigma/sml1482 and …sml1483; see letter). In the same **Nature** paper we also identified NAMs and PAMs for another oGPCR listed in the RFA: GPR65[1].

We next developed a ***second, G-protein independent platform*** to find pharmacologically active compounds at oGPCRs, modifying an approach invented in Richard Axel's lab: Tango (transcriptional activation following arrestin translocation)[59] (**Fig 4**). Our three labs have used this approach to discover novel ligands and signaling pathways for D3-[3] and D2-dopamine[17 43], κ-[42], δ-[60] and μ–[9] opioid receptors, various serotonin receptors[61 62 8] and other GPCRs[63 64]; it is a reliable and scalable screening platform.

We modified the Tango platform to create a set of 120 oGPCRs, spanning most of those specified in the RFA, and published this resource in ***Nature Structure and Molecular Biology*** [see **Fig 5** and ref [30]]. The assay was extensively validated for surface expression and functionality with known ligands at well-annotated

GPCRs[30] and **made open source via ADDGENE**.

Relevant to this application, we performed an initial screen of 91 oGPCRs against the NIH Clinical Collection #1 in a parallel fashion (**Fig 6a,b**) and identified several bona fide and replicable agonists for oGPCRs (**Fig 6c-f**). Among were agonists for the RFA-defined oGPCR MRGPRX4, which we found signaled through Gq (**Fig 6b-d**), and exogenous agonists and the signaling pathway for the bombeisin-receptor related oGPCR BB3 (**Fig 6e-f**). We have also used this platform to discover novel pharmacology for GPR39[32] and to verify the specificity of chemical probes [see [32] and [9]]. For reasons of space, we illustrate only one example here.

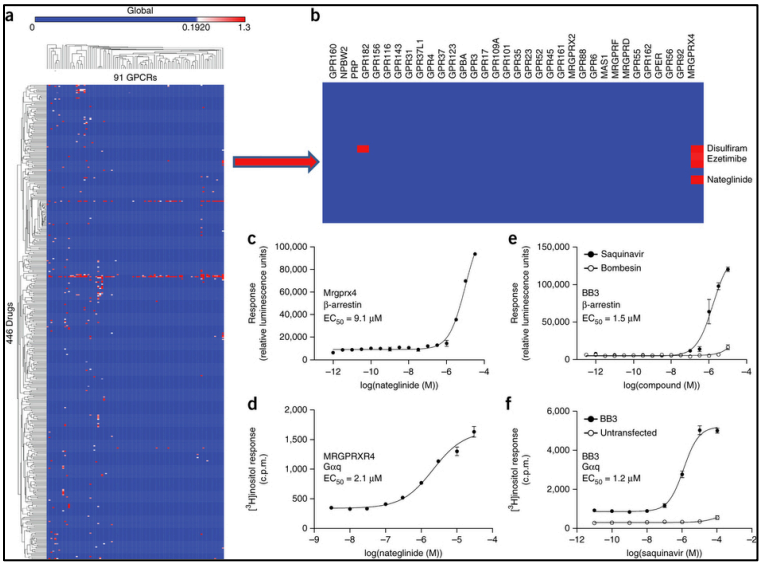

Fig 6. Parallel screening and ligand discovery via PRESTO-Tango resource. Details in text and Kroeze et al, Nat Mol Struct Biol, 2015

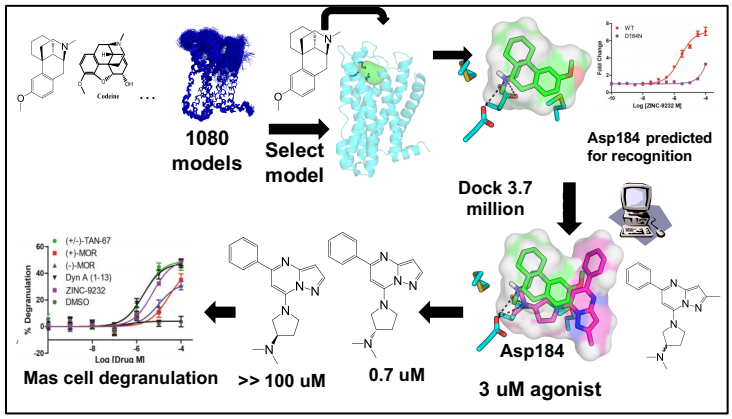

Fig 7. Pipeline to discovery probes for MRGPRX2. See Lansu et al, Nat Chem Biol, *in press* and text for details.

Proof-of-concept probe discovery for MRGPRX2: The RFA-listed oGPCR MRGPRX2 is exclusively expressed in dorsal root ganglia and primate mast cells[65] and has been associated with mast-cell based inflammation and pain.[66,67 68] Although some 'selective' MRGPRX2-agonists have been reported, we and others have struggled to replicate most of those findings [see [2] for discussion]. *To illuminate pharmacology and discover tool molecules* for MRGPRX2, we first screened our library of 5,695 privileged compounds (world and FDA drugs, reagents, IND molecules, and probes) against the receptor using the Tango platform. From this emerged opioid-like drugs ADL-5859, sinomenine, dextromethorphan, dextrorphan--and a previously reported MRGPRX2 non-selective agonist TAN-67[69]. Further studies revealed that N-unsubstituted morphinan-based opioid agonists were active while canonical opioid antagonists like naltrexone and naloxone were inactive, affording SAR. The agonism of these opioids support MRGPRX2's role as the long-sought receptor mediating opioid-induced itch[2] thereby *revealing novel MRGPRX2 pharmacology and function*.

As exciting as these results were, they did not reveal a suitable probe for MRGPRX2 and we again turned to structure-based docking. As the κ-opioid receptor had 23.3% sequence identity in the TM region to MRGPRX2, we used it as a structural template, generating 1080 MRGPRX2 models (**Fig. 7**). A cycle of docking the opioid agonists vs the 5650 decoys molecules from the Tango-screen led to a model that correctly predicted that Glu164$^{4.60}$ and Asp184$^{5.36}$ were crucial for opioid recognition (tested, again, by mutagenesis). Buttressed by this success, we docked our ZINC[70] lead-like library, which by now had grown to 3.7 million molecules, against the high-performing MRGPRX2 model. From the top 0.13% of the docking-ranked library, 20 molecules were purchased and tested in the Tango and in calcium release assays vs MRGPRX2, revealing ZINC-72469232 as a μM agonist; this compound was selective with no agonist activity against 320 other GPCRs, including MRGPRX1 and MRGPRX4, and had no activity better than 20 μM against a panel of 97 human kinases. We optimized the molecule by analog docking leading to ZINC-72453573 (EC_{50} 0.76 μM). Re-synthesis of stereochemically pure R- and S-isomers of '3573 in the Jin lab (Mt. Sinai) found that only the former is active, effectively revealing a probe-pair of physically matched compounds. Encouragingly, R-'3573 is a potent agonist of mast cell degranulation [**Fig. 7** and ref [2]]. This probe pair will, by the time this application is reviewed, be publically available via our collaboration with Sigma-Millipore.

Additional key resource available for SA#1. In addition to TANGO- and yeast-based platforms, we have also obtained *CRISPR-KO HEK* cell lines *for each of the individual G proteins and arrestins* from Asuka Inoue

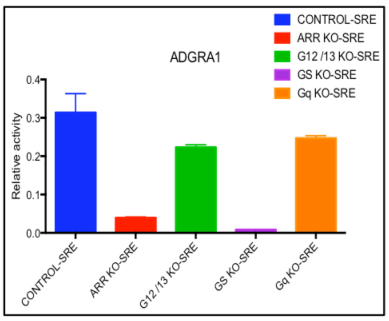

Fig 8. Use of CRISPR KO cells to define G protein and arrestin requirements for signaling. Shown is a typical experiment (N=18-24 replicates) for the adhesion GPCR ADGRA1's constitutive SRE-luciferase activity. The Arr KO and Gs KO are significantly diminished.

(Tohoku University). This is a set of individual clonal HEK lines where individual G proteins (e.g. Gq, G11, Gas, and so on), G protein families (e.g. Gq/11; G12/13, etc) and arrestins (e.g. βArr1/2 KO) have been deleted by CRISPR-based editing. We[8] and others[71,72] have used these lines to verify the G-protein or arrestin-necessity and specificity for many downstream GPCR signaling events. Here we will use them to validate the G protein(s) essential for oGPCR functions as outlined further in the application. For example, we assayed Adhesion GPCR (ADGRA) constitutive SRE-luciferase expression as a generic down-stream readout of ADGRA signaling. The RFA-specified ADGRA1's constitutive SRE-luc activity is nearly abolished by β-arrestin1/2 or Gs KO and relatively unaffected by G12/13 or Gq/11 KO (**Fig 8**). These preliminary results support signaling via Gs and then β-arrestin.

Proposed experiments: Revealing pharmacology and identifying tool compounds for oGPCRs as specified in the RFA.

Our publications demonstrate that we have scalable physical and computational assays suited for this RFA, and a productive work flow for integrating these assays with late-stage chemical optimization. SA#1's strategy is likely to be successful for nearly all of the Rhodopsin (Family A) oGPCRs, as most of them have sequence identities that make them modelable against templates GPCRs whose crystal structures have been solved as of Feb 2017 (**Fig 9**). Based on results on MRGPRX2 and GPR68 in the last period, we anticipate that modeling-and-docking is plausible down to 35% sequence similarity in the TM region; this amounts to over 60% of the oGPCR identified in the RFA. Among each major group of templates, bona fide ligand binding sites have been identified.

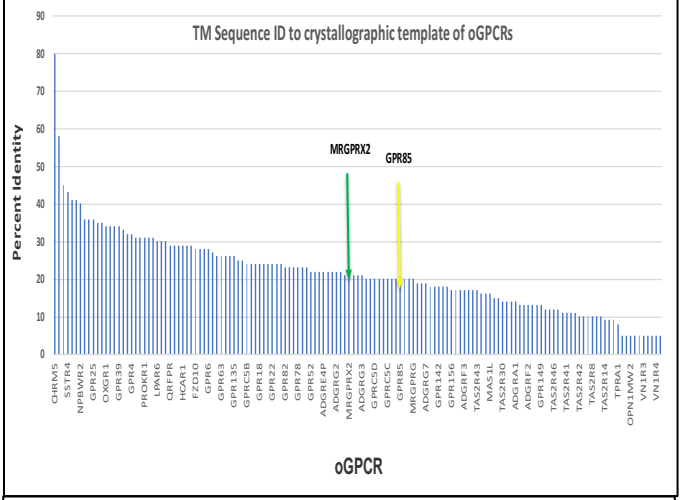

Fig 9 RFA-defined oGPCRs, plotted by sequence identity in the TM region to template GPCRs with x-ray structures. MRGPRX2 and GPR85 are marked for reference. We have successfully modeled and docked against MRGPRX2 (Lansu et al., *Nature Chem Biol* 2017) and current modeling suggests that we can go at least as far down as GPR85 to MRGPRG, covering, today, at least 60% of the oGPRC's specified in the RFA by modeling and docking.

Statistical and technical considerations for oGPCR physical screening: Before beginning the screens, Z'-scores, day-to-day and plate-to-plate variability, and signal/noise are calculated to ensure that the assays are robust for small molecule screening.[73] Fortunately, for many of the RFA-listed GPCRs at least some actives are available for Z'-score calculations (mostly from our own initial screens in the first period, but also from the literature) (**Fig 10**). In the many cases where agonists are unavailable, or untrustworthy, we will perform quadruplicate determinations for all compounds screened to calculate sample variability (similar in concept to a strictly standardized mean difference statistic[74]), and simultaneously evaluate the fold-change from baseline in a positive or negative direction for each compound. Typically, we use a 2-fold cut-off for the initial identification of actives—an approach we have validated in prior papers where positive control compounds were unavailable for formal Z'-score calculations.[1,2,30] As the total number of compounds evaluated is relatively small (<10,000), putatively active compounds emerge above the variability and are further tested as outlined below.

Outline of proposed pipeline to fulfill aims of this RFA: For most of the RFA-liste GPCRs we have assays in hand. Our approach for discovering and validating probes for oGPCRs is documented in prior publications[1,2,30] and outlined below and in **Fig 1**. We have already performed proof-of-concept screens against 100+ oGPCRs to verify robustness, reproducibility, day-to-day variability and suitability for moderate-scale screens.

1. Perform initial small molecule screen to reveal pharmacology and identify actives (~10,000 compounds; Roth lab)
    - To date have performed initial small, validation-scale screens for >100 oGPCRs; results and validation for 93 oGPCRs has been published.[30] **Fig 10** shows our progress in identifying initial

chemical matter for the oGPCRs in the RFA; *we note that several of those in the RFA are pseudogenes in humans and we assume that these will not be of interest going forward*. Confirmed actives (e.g. dose-response curves with fresh and authentic powder) are highlighted in green while screening hits (e.g. initial actives found in quadruplicate determinations) are in yellow. Targets with reported agonists and/or antagonists (typically non-selective) are underlined.

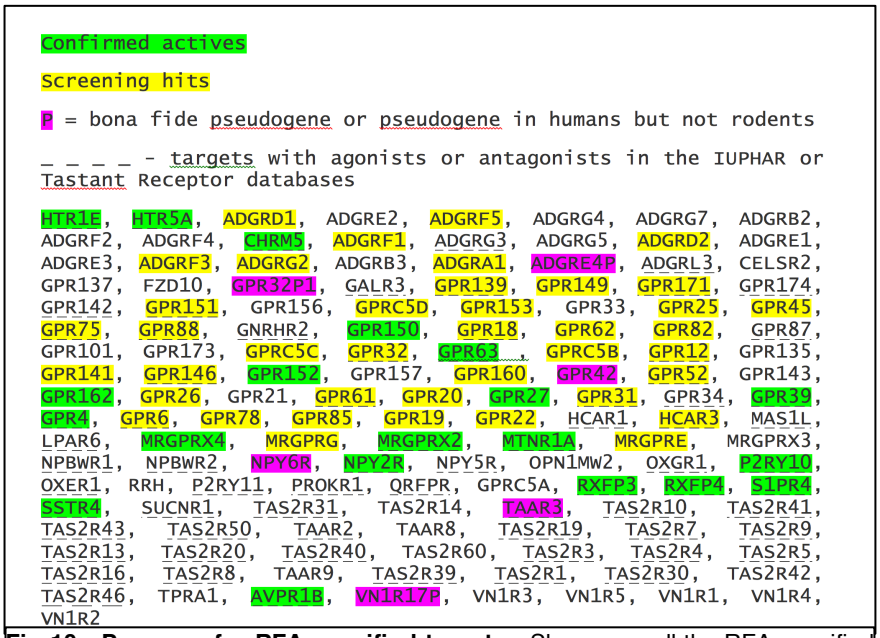

Fig 10. Progress for RFA-specified targets. Shown are all the RFA-specified targets; those for which chemical matter has been reported in the IUPHAR or tastant receptor databases are underlined. Purple represent pseudogenes in humans while green and yellow represent confirmed actives and screening hits respectively. See text for details.

2. Follow-up validation in dose-response with fresh compound (identity & purity verified by LC-MS; Jin lab).
   - Actives will be used to devise orthogonal screens in mammalian cells when yeast-based platform was used in initial screen.
   - Actives will be used to devise orthogonal G-protein-based screens where TANGO-platform was used in initial screen.
   - CRISPR KO cells used to validate G protein signaling pathway(s) identified above.
3. "Analog by catalog" (Shoichet, Jin and Roth labs) with chemically validated compounds.
   - The Shoichet lab's ZINC (http://zinc15.docking.org) platform allows multiple analogs searches (by topology, by structure or substructure), and accesses over 300 million commercially available molecules from reputable vendors (the docked 150 million "lead-like" and a larger list of Ro5-compliant molecules).
4. Homology model construction and docking (Shoichet)
   - Testing final models by site-directed mutagenesis, as in GPR68 and MRGPRX2[75,76] (& below).
   - Hit-picking (Roth and Shoichet).
5. Testing initial predicted hits (Roth)
6. Cycles of testing, predicting new actives, and further testing.
7. Limited medicinal chemistry optimization (Jin) and synthesis of inactive analogues as previously.[75,76]
   - **For at least four of the oGPCRs, we will work with investigators at Pfizer and at Takeda to chemically optimize probes (see letters from Drs. John Matthias and Derek Cole).** Pfizer will optimize hits for GPR4 and GPR177; the two initial Takeda targets remain to be picked. These collaborations are still fluid, as noted in the letters, and while they are not crucial to this work, they will help to engage Pharma in this enterprise, which can only increase its impact.
8. GPCR-ome profiling and PDSP-profiling (NIMH-PDSP; Dr. Roth is PI); targeted interrogation of other druggable targets (e.g. kinome; coordinated by Shoichet lab); PK, microsome stability, hERG on selected compounds (NIMH-PDSP will perform hERG; Jin and Shoichet labs to coordinate PK and microsome stability, where relevant, via CRO as we have done in the past[9,10,77]).
9. For selected compounds testing in vivo with WT and KO mice to verify on-target activity.

We note that we seek to illuminate the pharmacology and identify chemical probes for oGPCRs, not to purify and identify their endogenous agonists, which is a very different enterprise. Sometimes, such endogenous agonists emerge from the direct screens, as with the discovery of H^+ for GPR68 (Huang et al, **Nature** 2015) and dynorphins for MRGPRX2 (Lansu, **Nature Chem. Biol.** 2017), or from chemoinformatic similarity to screening hits. Naturally, we are interested in the endogenous agonists of oGPCRs, and will pursue them when they emerge from this pipeline although they are not our primary goal.

Details of modeling & Docking Pipeline: Docking for specific probes of oGPCRs is ambitious. Docking, after all, can struggle even against known structures—as we ourselves have argued[78-80]—and here we target homology

models. Four aspects of this enterprise make it pragmatic, and explain its successes against GPR68, GPR65, and MRGPRX2 in the last period, and recently against 5HT5a (below):

(1) GPCRs are more readily modeled than most targets, with topological constraints and conserved through-space interactions that allow one to confidently model them at relatively low sequence similarities. **(2)** The hits and inactive "decoys" from the physical screen in the Roth lab, are wonderful restraints against which to test our models; in docking screens of the Roth library molecules, a competent model must highly rank the actives and deprioritize the decoys. **(3)** The models that emerge predict specific binding site residues. For instance, in MRGPRX2 Glu164$^{4.60}$ and Asp184$^{5.36}$ were predicted to be interacting residues, which was tested and confirmed by mutagenesis. **(4)** Finally, the docking screens interrogate a far larger library of molecules, with far greater diversity, than is accessed by any other screen.

In the last period, we routinely docked over 3 million "lead-like", available small molecules, and *in the next period this number will rise to over 150 million*. These molecules are readily accessed, have favorable physical properties (e.g., amu ≤ 350, cLogP ≤ 3.5), and are diverse. They derive from new "make-on-demand" libraries that are now being made available by vendors such as Enamine. We have tested the pragmatic availability of these molecules, successfully sourcing over 50 diverse ones within five weeks of order placement. We would add that we are painfully aware—perhaps more than most groups—of the potential pitfalls of large library expansions, both in the exploration of diverse-but-uninteresting chemotypes, and in the generation of the pathological molecules that so plagued combichem—this is an area where we are expert. The new libraries, however, represent specific, often unexplored chemotypes, and are ***not*** sourced as large libraries, but as individual molecules that are only made, one-at-a-time, as justified by docking. Chemoinformatic analysis (not shown, for space) suggests that the new libraries are diverse but nevertheless bio-relevant, retaining well-established chemotypes displayed in new ways. A breakdown and enumeration of the expanded library begins at http://zinc15.docking.org/tranches/home/. Here we summarize the docking pipeline, already described in detail in papers from the last period[75,76] (see also **Figures 4** and **7**, above):

1. We typically begin with actives and inactives from the physical screen in the Roth lab (e.g., lorazepam for GPR68 and morphine for MRGPRX2). Based on template crystal structures, thousands of models are using Modeler[52] followed by conformational expansion by elastic network models[53] and residue rotamer generation in the putative ligand binding sites, again by Modeler.
2. Against the thousands of models that emerge, we dock the several actives and the thousands of inactives identified in the Roth lab screen. Each is evaluated for electrostatic and van der Waals complementarity to the site, corrected for ligand and protein desolvation.[57,58] This is done against each of the models, and those models that best separate the true actives vs the decoys are advanced.
3. These prioritized models make specific predictions about how the active molecules interact with the oGPCR. Their predictions are tested by site directed mutagenesis. The model that best predicts the effects of these mutants is advanced to a full docking screen.
4. A library of 150 million "lead-like", commercial molecules are screened against the model, each in about 10^6 configurations, over 10^{14} complexes are calculated. At this size, we retain the capacity to prosecute these screens on our lab 2018 core cluster, but we anticipate increasingly doing so on the Amazon and Google clouds; in full test screens this has cost <$700/screen and falling, making this an efficient choice, especially as we anticipate further library growth. Once docked, the top-scoring hits are clustered by chemotype. Finally, a set of diverse scaffolds are picked for experimental testing, beginning the cycle of testing and optimization described above. The ultimate optimization step is often chemical synthesis, though sometimes this can be a matter of re-synthesis or synthesis of stereochemically pure molecules, as in the last period for the probes Ogerin and R-'3573, which came directly from docking.

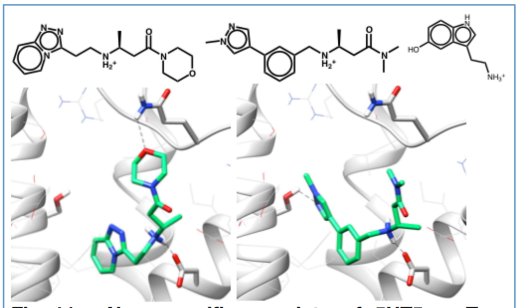

Fig 11. New specific agonists of 5HT5a. Top. Compounds **1** (left) and **8** (center), two high-ranking docking hits dissimilar to 5HT itself (right). **Bottom.** Their docked geometries; predicted interactions with Asp$^{3.32}$, Ser$^{5.42}$ & Gln193 are shown.

A variation is when the target may be confidently modeled without initial ligands from the Roth lab screens. Among the RFA-defined oGPCRs are ten with TM sequence *identities* of ≥36% to GPCRs of determined structures, including 5HT5a, CHRM5, and 5HT1e; these are candidates for direct-to-docking screens. In a proof-of-concept study, we have modeled the RFA-listed 5HT5a on 5HT1b, with

which it shares 41% TM sequence identity. From a preliminary screen of 6 million "lead-like" molecules, 20 diverse molecules have been selected for testing; for several, interactions with a specificity Gln193 were prioritized (**Fig 11**). On testing, 6 of the 20 act as agonists, with binding confirmed by radio-ligand displacement and functionality by reversal with the 5-HT5a-specific antagonist SB-699551.[81] Several, such as compounds **1** and **8** (**Fig 11**)**,** have efficacies approaching that of serotonin itself, though binding for these initial hits remains micromolar. The compounds were chosen for their *dissimilarity* to any known serotonergic ligand, to enable their optimization for selective agonism of 5HT5a, something that has eluded the field.[82,83] Doing so will require rounds of computational and perhaps synthetic optimization, but our ability to rapidly find these 5HT5a agonists—beginning less than 10 weeks ago after RFA publication—makes us sanguine about progress. It also suggests that the ten oGPCRs of high sequence identity to known structures are accessible by docking alone; they will receive early attention in the project.

Time line for SA#1: Once fully operational, we anticipate finding 30 validated screening hits, 15 docking actives, and 8 optimized probes/year. Over the project's course we thus expect to illuminate new pharmacology and discover new chemical matter for more than 100 oGPCRs and probes for 45 of them, even assuming that no advances occur between now and the project's end. The probes will fulfill the essential elements of the RFA as follows:

- Place oGPCRs into relevant signaling cascades (e.g. specific G-protein and role of arrestin-ergic signaling) and identify initial pharmacology and SAR
- Identify oGPCRs as important or potential off-targets for the pharmacological actions of known drugs, thereby illuminating function
- Identify signaling events modulated by the oGPCRs, including down-stream second messengers.
- Provide tool compounds with which the community can interrogate the oGPCRs
- Probe-modulation of the *in vitro* signaling of the oGPCRs; where a KO mouse control is available and the probes have suitable PK, we may interrogate the *in vivo* functions the of oGPCRs, as with GPR68.

Thus in SA#1 we fulfill the RFA-defined requirements of adapting and enabling technologies and approaches, demonstrating the ability to tackle diverse GPCRs within and outside the consortium, and placing the data in the context of physiological pathways.

RFA target name	Other IUPHAR names	TANGO-IZED Version In-Hand	Likely G-alpha partner (from literature reports)	Initial screens performed and actives discovered?	RFA target name	Other IUPHAR names	TANGO-IZED Version In-Hand	Likely G-alpha partner (from literature reports)
ADGRA1	GPR123	YES		YES	ADGRB2	BAI2	NO	Gq/11
ADGRD1	GPR133	YES	Gs, Gi/o	YES	ADGRB3	BAI3	NO	
ADGRD2	GPR144	YES		YES	ADGRE1	TM7LN3	NO	
ADGRF1	GPR110	YES	Gq/11	YES	ADGRE2		NO	Gq/11
ADGRF2	GPR111	YES			ADGRE3		NO	
ADGRF3	GPR113	YES		YES	ADGRG4	GPR112	NO	
ADGRF4	GPR115	YES	Gq/11		ADGRL3	LPHN3; latrophilin 3	NO	
ADGRF5	GPR116	YES	Gq/11	YES	ADGRE4P	PSEUDOGENE		
ADGRG2	GPR64	YES	Gs, G12/13, Gq	YES				
ADGRG3	GPR97	YES	Gi/o, G12/13					
ADGRG5	GPR114	YES	Gs					
ADGRG7	GPR128	YES						

Table 1. Progress on Adhesion GPCRs to date. See text for details.

Potential pitfalls and solutions for atypical GPCRs listed in the RFA

Adhesion GPCRs: Several adhesion-family receptors (ADGRG) are listed in the RFA and most are already being successfully interrogated by ourselves [28,30] and others [84-86] (**Fig 7** and **Table 1**). ADGRG's have complex signaling including via canonical G-proteins [84-86] and, in some cases, via arrestin [86]. Indeed, arrestin interactions with adhesion GPCRs are unusually stable, at least for the handful of adhesion GPCRs studied to date in this regard[87,88] [89], suggesting that the Presto-TANGO assay may be especially useful for screens involving adhesion GPCRs. Most of the RFA-listed ADGRG's have annotated G-protein signaling partners but lack chemical probes, detailed descriptions of cell- and tissue-distribution, and have opaque pharmacology and physiology [although this is a rapidly expanding field[84-86]] (**Table 1**). Several ADGRG's can be activated by *Stachel*-sequence derived peptides which are liberated following cleavage[84] and are likely druggable. Indeed, a recent report identifies dihydromunduletone as a selective inhibitor for the RFA-enumerated GPR56/ADGRG1 and GPR114/ADGRG5 [85]. We have already obtained many of the ADGRGs listed in the RFA (**Fig 7** and **Table 1**) and can readily obtain the others (see Letter of Support from Randy Hall, Emory). We have begun screening ADGRGs in the TANGO system (**Table 1**) and in reporter gene assays (**Fig 7**) and have identified small molecule modulators for the majority of those we have in hand.

FZD family: For only one member of the FZD family-- Smoothened (SMO)—are chemical tools available; FDA approved medications and high resolution structures are also available only for SMO. SMO's 7-TM structure was elucidated by the Stevens and Roth labs[90,91] and has been used as a template by the Shoichet group to discover novel chemotypes.[31] The RFA specifies only FZD10 for which there are no known small molecules and as it is not feasible to create homology models against FZD10 (although as this is a rapidly progressing

field it may be possible in the near future), we will utilize the genetic approach outlined below.

TAS2R and VNR families: TAS2R-Tastant- and vomeronasal (VNR)-family receptors are specified in the RFA. TAS2Rs function as bitter taste receptors[92] and VNRs are pheromone receptors in the vomeronasal organ (VNO).[93-96] Presumably, the RFA is more interested in the potential role of these receptors outside the tongue and VNO; indeed VNRs[97] and TAS2Rs such as TAS2R1[98] and other tastant receptors[99] have been reported to be expressed in the gut and other organs.[100,101] Recent reports[102,103] suggest that only 2 TAS2R's lack agonists (TAS2R42 and TAS2R60); the remainder have at least one annotated and confirmed small molecule bitter tastant agonist. For those listed in the RFA with already annotated small molecules, we have begun validation assays (**Fig 12**) in transiently transfected HEK cells containing engineered gustucin, TASR2's and β/γ subunits as described by others using Ca++ mobilization as a read-out.[104] Although here we used transiently transfected cells, going forward we could make stable TASR2 lines [as described for other GPCR projects[2]] and perform targeted optimization via a combination of analogue-by-catalog [via ZINC] and focused medicinal chemistry. Selectivity will be assessed by counter-screening against the collection of TAS2Rs we are currently assembling. For VNR family of oGPCRs, Stephen Liberles (Harvard; see Letter of Support)—an expert on this family of receptors—has agreed to share resources and expertise and we will likely use the approach outlined in SA#2. *For those oGPCRs for which too few structures are available for homology modeling and are not able to be interrogated by our combined approach, we will employ the strategy outlined in SA#2.*

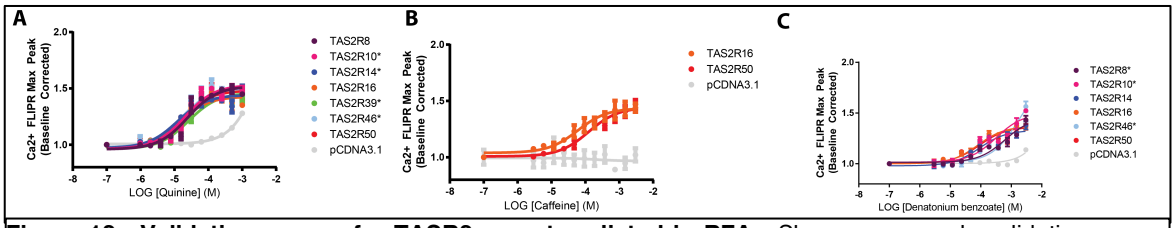

Figure 12. Validation assays for TASR2 receptors listed in RFA. Shown are sample validation assays with known TASR2 agonists. An asterisk--*-- indicates newly validated agonist activity; see text for details.

Prioritization of oGPCRs for probe development: **(1)** Begin with targets on which we can make rapid progress, such as the ten of high similarity to GPCRs of known structure (above), and others for which we already have validated hits from our physical screen (e.g., GPR150, GPR152, GPR27, MRGPRG, GPR4). **(2)** Next, focus on oGPCRs with a regular family A fold, that have sequence identities at least as good as MRGPRG (**Figure 9**, above), and have higher biological novelty in the IDG Pharos database. (**3**) Finally, those family A oGPCRs that are less novel and do not have the other advantages sketched above (e.g., 5HT5a is among the less novel oGPCRs in Pharos, but is prioritized by high do-ability—i.e, simple pragmatism plays a role in prioritization). Two gaps should be mentioned: **(a)** while we will seek ligands for all RFA-oGPCRs, for some actual probe discovery will inevitably fail (e.g., the right chemistry is simply not in the libraries); **(b)** several oGPCRs are unsuited to this plan, because of modeling and assaying difficulties. Both cases are addressed in **SA#2**, where we develop genetic tools for oGPCRs for which the chemical approach fails, or which are from the start inaccessible.

SA#1 Summary: From a pipeline of privileged library screening, model-building, and ultra-large scale docking, we will illuminate pharmacology and identify early chemical matter for about 100 RFA-defined oGPCRs (e.g., **Figure 10**, above), and develop probes for at least 35 and ideally 45 of these. For these probes, we will demonstrate cellular and, occasionally, in vivo receptor functions. The probes will be made openly available to the community via our collaboration with Millipore-Sigma (see Letter of Support from Dr. Sheri Spector), guaranteeing a ready supply well-past the end of this project (see also Resource Sharing). We expect these probes will be used by others to further illuminate the functions of the oGPCRs in research programs to which they bring their own special expertise. That fulfills a central goal and premise of the IDG program.

Specific Aim #2 Create engineered CRISPR-tagged mice that, combined with DREADD technology, will reveal the function, signaling, physiology, cell-type and regional expression of oGPCRs.

Background and rationale for approach: As some oGPCRs will likely prove intractable to probe discovery, we have devised a complementary strategy to fulfill the RFA's aims. This approach exploits CRISPR and DREADD technologies, a combination that is particularly useful for oGPCRs in the Adhesion, Frizzled, Tastant and Vomeronasal receptor families. In all four, the tissue distribution for oGPCR protein, physiology, signaling and function remain largely unknown.

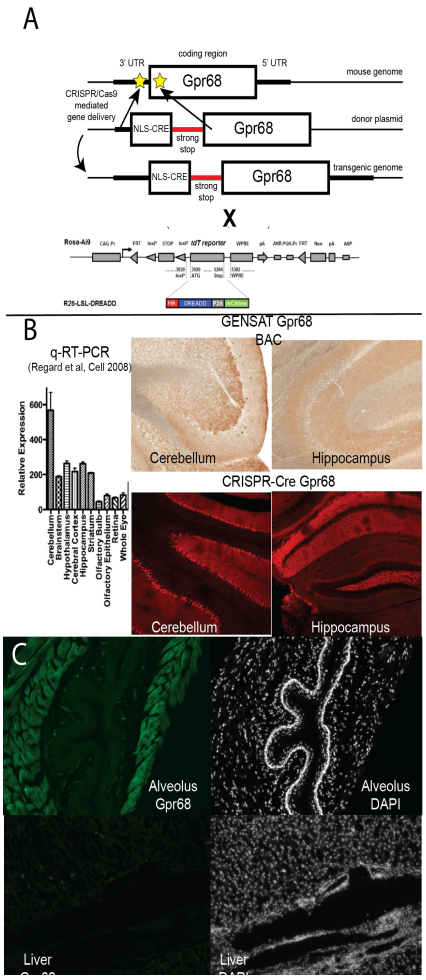

Figure 13. Gpr68-Cre validation data. (A) shows CRISPR-insertion strategy; (B) shows known Gpr68 mRNA distribution as well as distribution with Gpr68-Cre mice vs GENSAT (which are negative); (C) shows distribution in alveolar muscle layer as well as lack of expression in liver.

A main goal of the RFA is to identify the cell- and tissue-specific expression of oGPCRs; unfortunately, antibodies for even well-annotated and extensively studied GPCRs are rare and promiscuity is the rule rather than the exception.[105-109] For the oGPCRs we have studied, we have not found any antibodies useful for fulfilling the aims of this RFA. A standard, albeit time-consuming, approach to identify cell- and tissue-specific GPCR expression is to knock-in reporter constructs into the endogenous locus. Previously this was accomplished by homologous recombination and typically took 1-2 years to obtain mice with germ-line transmission of reporters in the endogenous locus (e.g. for Gpr65[110], Gpr182[111] and Gpr151[112] among others). However, CRISPR genome editing technology now affords a scalable platform to find the endogenous cell- and tissue-type expression patterns of oGPCRs. Here we modify the original procedure described by Jaenisch's group[113-115] and this modified approach is now used routinely to create multiple engineered mice/year by the UNC mouse genetics core facility (see Letter of Support).

Preliminary findings: As an initial proof-of-concept study we knocked-in Cre-recombinase into Gpr68 (**Fig 13**) via two guide RNAs; we had previously identified PAMs and NAMs for this oGPCR in our 2015 **Nature** paper.[1] Over 10 germ-line founders were obtained and, after validation of the insert specificity, localization and integrity, one was crossed to our DREADD reporter mice.[34] GPR68 was well expressed in the cerebellum and with moderate expression in hippocampus by mRNA[116] (**Fig 13B**). Robust and cell-type specific expression was observed in the resulting Gpr68-Cre crossed with our hM3Dq-DREADD reporter mice[34] (**Fig 13B** & **C**), while none was seen in either reporter mice minus Cre nor Gpr68-Cre mice [not shown; see [34] for extensive validation of DREADD-reporter mice]. As an aside, we note that GENSAT provides GPCR bacterial artificial chromosome-Cre (BAC-Cre) mice for several of the oGPCRs in the RFA although we frequently find that the expression patterns reported do not conform to the known patters of mRNA expression of these oGPCRs (see **Fig 13B** for example).

We have used mice from the Gpr68-Cre X DREADD reporter mice to provide a preliminary survey of Gpr68 expression in several peripheral organs. Gpr68—reported by mCitrene—was expressed in a number of peripheral organs including alveolar smooth muscle cells (**Fig 13C**), stomach epithelial mucosal cells, renal tubular cells, and splenic white pulp (not shown). No expression was seen in hepatocytes (**Fig 13C**), although occasional stellate cells displayed Gpr68 expression. Encouraged by these results, we have begun creating further mice using a slightly different approach where we both 'tag' the endogenous receptor (using a SIGNAL-FLAG epitope) and simultaneously introduce Cre-recombinase via an IRES sequence downstream of the oGPCR (**Fig 14**). As many of the RFA oGPCRs are intron-less, we can easily create synthetic open-reading frames (**Fig 14**); this allows us to visualize both endogenous oGPCR expression (via anti-FLAG immune-fluorescence) and reporter-gene expression when crossed with the appropriate reporter line (e.g. DREADD or Ai9 lines). For oGPCRs without introns or long open-reading frames, we would either utilize N-terminal CRISPR-tagging or insert IRES-Cre as in Fig 14.

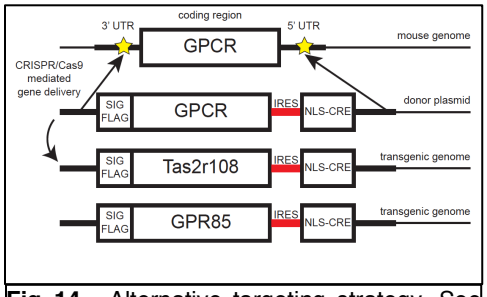

Fig 14. Alternative targeting strategy. See text for details

How this approach fulfills aims of RFA: We will use approaches similar to those outlined in **Figs 13** and **14** to create multiple oGPCR reporter lines. W*e will map the tissue- and cell-type-specific expression of oGPCRs in major mouse organs and tissues, revealing potential physiological functioning of oGPCRs as specified in the RFA.*

Proposed studies: Over the six years of the initiative, we will create between 18 and 60 lines of GPCR reporter mice using the approach (or improved variant) sketched above; this represents 3 to 10 mouse lines/year. We will use these mice to characterize the tissue and cell-type distribution those oGPCRs not easily interrogated by chemical and computational technologies in SA#1. Initially, we will focus on the TAS2R, VNR, ADGRG and FZD family receptors for which (a) bona fide mouse homologues exist and (b) for which no endogenous or exogenous agonist has been identified. We anticipate that we can create and characterize as many as 10 individual lines/year. Many line can be crossed with an appropriate reporter line [e.g. Ai9[117]] and selected lines could be crossed with our floxed-DREADD-mCitrene reporter line.

The tagged oGPCRs (**Fig 14**) will be used to visualize their endogenous cell- and tissue-type expression patterns by anti-FLAG immunofluorescence. Expression patterns can be verified by in situ hybridization or crossing with a tdTomato reporter line Ai9[117] (which is now being used by both the Roth and Kash labs). Interrogation of behavioral phenotypes mediated by down-stream signaling can be accomplished by expressing Gs, Gi or Gq-DREADDs in the oGPCR locus by crossing with one of our FLOXED-DREADD mice [Gq & Gi-DREADD described in ref[34]; Gs-DREADD mice *publication in preparation*[36]]. New microscopy and tissue-clearing strategies have become available, including CLARITY[118] and iDISCO,[119] which may allow light-sheet microscopy enabled whole-body visualization of oGPCR expression (Ariel Letter of Support). We have access to both via collaborations at UNC (Kash lab; Letter of Support) and Cal Tech (http://www.beckmaninstitute.caltech.edu/clover.shtml Gradinaru lab[118]; Letter of Support). Each technique has advantages and disadvantages,[120] both work well with soft tissues like brain while iDISCO may have advantages for connective tissues.[121]

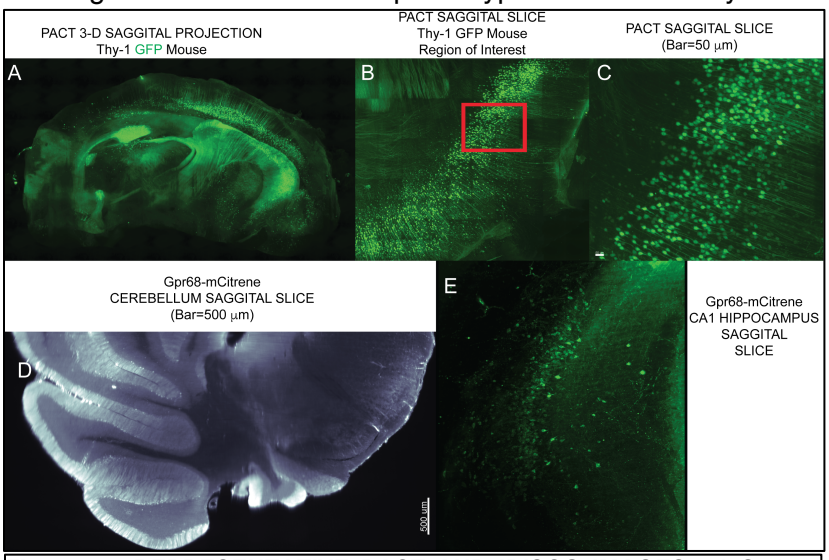

Figure 15. REPRESENTATIVE PACT AND iDISCO IMAGES. A-C show representative image quality using genetically-encoded reporter while D and E show iDISCO images of Gpr68-mCitrene reporter in (D) Cerebellum and (E) Hippocampus.

Pipeline for SA#2. We adopt a sequential approach to illuminating the distribution and potential functioning of oGPCRs using these engineered mice as follows:

- Survey of oGPCR expression in major organ systems: to determine tissue and cell-type specific expression of oGPCRs (brain, heart, liver, lungs, kidneys, spleen, testis, ovary, stomach, small and large intestines) via immune-fluorescence and standard immuno-histochemistry of CRISPR-tagged oGPCRs (**Fig 15,** above). We will be assisted here by the UNC pathology core (Letter of Support). Images will be shared publically (see Data and Resource Sharing) and mice will be shared without restrictions via JAX. Validation of expression in selected tissues such as brain will be via *in situ* hybridization which the Roth[122], Kash[123] and Gradinaru[118] labs have experience.
- Whole organ imaging to illuminate and clarify oGPCR cell-type expression: iDISCO or similar tissue-clearing technique may also be used for selected oGPCRs to visualize entire organs by light-sheet microscopy (see Kash and Gradinaru letters of support). PACT (Passive Clarity Technique; **Fig 15 A-C**)[124] is suited for whole-organ clearing and affords visualization of cell-type specific GFP- or tdTomato reporter expression patterns[124]. Viviana Gradinaru who developed PACT and CLARITY will be collaborating with us to initially perform clearing and microscopy at Cal Tech and ultimately will serve as a consultant as we transport the protocols here at UNC. iDISCO has been adapted by Tom Kash (UNC), a Rothlab's long-time collaborator[37,125,126]; for validation we provide representative iDISCO images for Gr68-mCitrene showing robust expression in cerebellum (**Fig 15D**) and hippocampus (**Fig 15E**). We anticipate that raw images as well as reconstructed 3-D images will be shared openly following quality control [see Data and Resource Sharing].
- Illumination of oGPRC behavioral phenotypes via DREADDs: With selected oGPCRs for which down-stream signaling is known and for which defined behavioral phenotypes might be elucidated, we will obtain

crosses of FLOXED-DREADD x oGPCR-Cre mice and monitor the previously described generic behaviors (with assistance of UNC mouse behavioral core facility; see S. Moy, Letter of Support) following CNO administration as described previously[126 127 37 128 129]. For all behavioral studies we use a 2 x 2 design (CNO or Vehicle to DREADD or GFP-expressing mice).[126 127 37 128 129] We also have created non-CNO chemical actuators [e.g. Compound 21[35]] that represent metabolically-stable alternatives to CNO. Given the enormity of this type of undertaking for even one engineered mouse and overall budgetary constraints, it is likely that we will elucidate these sorts of *in vivo* functions for 1-3 oGPCRs/year. Our behavioral phenotyping would be guided by the expression patterns visualized, for instance: in striatum we would examine locomotor behavior, as described by Roth lab previously[128,130-132 133]; for oGPCRs with high expression in cerebellum we would evaluate coordination via rotor rod[134]; for oGPCRs with high expression in hippocampus or amygdala we would examine conditioned fear,[126] and so on.

3. Integrated infrastructure to coordinate collaborations, assemble and integrate large datasets, and to disseminate this information openly to the community.

Background and rationale: The RFA specifies an administrative infrastructure to coordinate this U24. Dr. Roth's has substantial experience coordinating and administering these initiatives being PI of: a U19MH82441 for 10 years; the NIMH PDSP for 19 years; and P01DA035764 for 3 years as Co-PI. The scheduled end of the U19 will release sufficient effort for Dr. Roth's efforts as PI of this U24. The overall organization is sketched below with multi-PI considerations in the designated portion of the grant.

Dr. Roth will be overall PI and will have particular responsibilities related to screening, mouse genetics, and administrative guidance. *Screening* will be coordinated by Dr. Roth with input Randy Hall who will serve as a consultant for the studies involving Adhesion GPCRs (see Letter of Support). Roth's group has nearly 20 years of experience screening GPCRs with small molecules, including Phase I of this initiative, as Director of the PDSP, and other initiatives [135 136 137 4,138 5 1,2,8,9,131]

Mouse genetics will be performed in collaboration with the UNC core facility (see Letter of Support); this core creates about 70 lines of engineered mice/yr of which 50% use the CRISPR approach (or variant) outlined in this grant. Additionally, Dr. Roth has a long track record studying and creating engineered mice [139 4 140 141 17,142 133 143 37,128,129,134] including the conditional DREADD mice in this grant.[34]

Overall administration which includes coordinating budgets and face-to-face and on-line bi-weekly meetings will be coordinated by Ms Estela Lopez, who has nearly 20 years experience with these by virtue of her work with the NIMH-PDSP, U19MH82441 and Dr. Roth's lab.

Dr. Shoichet will serve as co-PI and will coordinate chemistry efforts, both virtual and physical. Dr. Shoichet has decades of experience with structure-guided drug discovery [144 145 4,25,146-148 149-152 54,153-156 2,8,9,31] and the creation and curation of virtual libraries.[70 54] He and Dr. Roth have collaborated for 11 years and15 collaborative papers [3-8,64,75,131,138,141,154,157-159] and were co-PIs of Phase I of this project. Collaborating with Dr. Shoichet is *Jian Jin, PhD* a long-time collaborator with both the Roth and Shoichet labs [1,2,10,35,43,160 161 16 17]. Dr. Jin has extensive expertise in medicinal chemistry and in chemical probe development. Dr. Shoichet will also coordinate small molecule and data sharing and will coordinate with the data management centers.

SUMMARY. GPCRs respond to signals from light to adrenaline, lipids to chemokine proteins, and control physiology ranging from vision to respiration, heart rate to learning and memory; they are the family of proteins most targeted by therapeutic drugs. Astonishingly, 1/3rd of the pharmacologically relevant GPCRs remain orphans, without specific reagents to control their activity. The advent of new technologies enables the development of chemical probes, genetic reporters and perturbants for the oGPCRs. The proof-of-concept studies that led to probes for GPR68 and for MRGPRX2 in the last period demonstrate the pragmatism of the physical screening and computer-based docking platforms, while the CRISPR and DREADD technologies are well-in-hand in the Roth lab, who invented the latter. In the upcoming period, these platforms will be deployed at scale. The probes and tools that result will be made available to all investigators, who can use them to interrogate oGPCRs of specific interest to themselves for functions that we can only begin to anticipate.

Vertebrate Animals

University of North Carolina Chapel Hill School of Medicine

1. Provide a detailed description of the proposed use of the animals in the work outlined in the Research Design and Methods section. Identify the species, strains, ages, sex, and numbers of animals to be used in the proposed work.

Male and female mice (C57/BL6 background) will be used for all studies of receptor distribution and for routine behavioral studies will include baseline measurements of behavior including locomotion in the open field, assessment of cerebellar function using rotorod and conditioned avoidance responding.

Species Strain Age/Weight Number

Mouse C57BL/6J Adult 100 mice (25 male; 25 female for WT and CRISP-tagged mice) for breeding and assessment of baseline expression for each oGPCR. Up to 1000 mice/yr (10 strains). We will need to have colonies of sufficient size to ensure adequate numbers of animals for baseline expression studies as well as sufficient breeders to ensure back-up breeders prior to transfer to Jackson Labs which will serve as the repository for the mice.

Mouse C57BL/6J Adult 252 (32 male; 32 female for WT and CRISPR-tagged; DREADD expressing) for follow-up behavioral studies. Up to 2520 mice/yr (10 strains). Our estimates of animal use include experiments for completion of the aims of the proposed grant. The animal estimates are based on prior experience with these types of experiments and are reflective numbers of mice required for statistical significance. Generally, an estimate of the expected experimental variability can be made based on pilot studies and published results. All of our study parameters are designed to minimize the number of animal subjects required. This number of animals will ensure adequate numbers for statistical comparison (generally 12/genotype with adequate numbers for one independent replication with both sexes if male/female differences are obtained in pilot studies).

Perfusion of mice for anatomical studies. Perfusion of mice for anatomical studies. For perfusion and fixation: mice will be anesthetized with tribromoethanol (250 mg/kg using a 1.25 percent solution, administered I.P.). Following loss of toe and tail pinch reflexes, a short incision will be made to open the chest cavity and expose the heart. A needle which is attached to an infusion pump will be inserted into the left cardiac ventricle and 1x PBS, followed by 4 percent paraformaldehyde, will be infused to fix the brain tissue. Following fixation, the brain will be collected for immunohistochemistry. For collection of fresh tissue: mice will be anesthetized as described above. When anesthesia is sufficient, mice will be decapitated and the brains will be collected from the skull for biochemical analysis

Locomotion studies. Locomotor activity is measured in an open-field activity monitoring device. For these studies, mice are individually removed from their cages and placed in the test apparatus by the experimenter and their activity is tracked by evenly spaced infrared transmitters and receivers for up to 3 hrs. During the locomotor activity procedure, effects are measured after administration of drugs. At the end of the experimental session, mice are removed by the experimenter and returned to their home cage. The first set of behavioral experiments involves locomotor activity in which the locomotor effects of selective neuronal modulation with DREADD expression are examined In order to obtain

statistical significance, each experimental manipulation requires an N of 8-10 mice. Experimental manipulations include dosage, with each experiment including 2-3 doses of CNO and vehicle in both DREADD and non-DREADD expressing mice Taken together, each experiment that examines 3 doses of CNO would yield 4 experimental manipulations, each requiring an N of 12 mice for each sex with replications, or a total mouse count of as many as 252, per experiment.

Rotarod experiments. Balance and motor coordination will be measured on an accelerating rotarod after drug treatment (Ugo-Basile, Stoelting Co., Wood Dale, IL). Briefly, the rod initially rotated at 3 rpm, gradually increasing to a maximum of 30 rpm over a 5-minute period, which is also the maximum length of the trial. Two days before the experiment, mice are trained on the apparatus in two or three trials, with a 1-minute break between trials. The latency to fall off the rod is measured by the rotarod timer. Additionally, testing is stopped for mice that rotated off the top of the rod. On testing days, each mouse first completed a drug-free trial to determine baseline performance before administration of drug. Rotarod performance is assessed 10, 20, and 30 minutes after drug administration. Experimental manipulations include dosage, with each experiment including 2-3 doses of CNO and vehicle in both DREADD and non-DREADD expressing mice Taken together, each experiment that examines 3 doses of CNO would yield 4 experimental manipulations, each requiring an N of 12 mice for each sex with replications, or a total mouse count of as many as 252, per experiment.

Conditioned fear: In the fear conditioned response paradigm, mice are placed in conditioning chamber with a floor grid that delivers a foot shock. During conditioning, mice are exposed to a tone that coincides with a 2 second 0.6 miliamp foot shock, and this is done three times. Mice are then returned to their home cage. 24 hours and 48 hours later for contextual and cue-related memory tests, mice are placed in the conditioning chamber and exposed to either no tone or tone. The amount of time the mice freeze during this period is recorded by a video camera and processed by video analysis software. Each mouse is conditioned only once. Experimental manipulations include dosage, with each experiment including 2-3 doses of CNO and vehicle in both DREADD and non-DREADD expressing mice Taken together, each experiment that examines 3 doses of CNO would yield 4 experimental manipulations, each requiring an N of 12 mice for each sex with replications, or a total mouse count of as many as 252, per experiment.

2. Justify the use of animals, the choice of species, and the numbers used. If the animals are in short supply, costly, or to be used in large numbers, provide an additional rationale for their selection and their numbers.

The complexity of the processes being studied cannot be duplicated or modeled in simpler systems i.e. computer or mathematical models. Further, there is not enough information known about the processes being studied to design nonliving models. Mice are the lowest sentient species that rovides adequate size, tissue,or anatomy for the proposed study. We have modeled the maximum number of mice to be used based on our prior publications and experience and actual numbers of mice to be used is likely to be less than the maximum anticipated.

Locomotion studies. Experimental manipulations include dosage, with each experiment including 2-3 doses of CNO and vehicle in both DREADD and non-DREADD expressing mice Taken together, each experiment that examines 3 doses of CNO would yield 4 experimental manipulations, each requiring an N of 12 mice for each sex with replications, or a total mouse count of as many as 252, per experiment.

Rotarod studies: Experimental manipulations include dosage, with each experiment including 2-3 doses of CNO and vehicle in both DREADD and non-DREADD expressing mice Taken together, each experiment that examines 3 doses of CNO would yield 4 experimental manipulations, each requiring an N of 12 mice for each sex with replications, or a total mouse count of as many as 252, per experiment.

Conditioned fear studies: Experimental manipulations include dosage, with each experiment including 2-3 doses of CNO and vehicle in both DREADD and non-DREADD expressing mice Taken together, each experiment that examines 3 doses of CNO would yield 4 experimental manipulations, each requiring an N of 12 mice for each sex with replications, or a total mouse count of as many as 252, per experiment.

3. Minimization of pain and distress:

A staff veterinarian is available to provide professional health care at all times. In addition, routine animal maintenance and care are provided by animal care technicians under the supervision of the staff veterinarian. Deviations from normal health are immediately reported to the veterinarian who will initiate appropriate therapeutic measures including medication and dietary supplements. Animals will not be used for scheduled experiments unless they are in good health and there is no risk for unnecessary discomfort. All aspects of the program for procurement, breeding, phenotyping conditioning/ quarantine, housing, management, veterinary care and disposal of carcasses follow the guidelines set down in the NIH Guide for the Care and Use of Laboratory Animals. Discomfort and injury to animals will be limited to that which is unavoidable in the conduct of scientifically valuable research. Animals are euthanized using CO2 overdose and death is confirmed by lack of any respiratory activity for 2 minutes

4. Describe any euthanasia method to be used and the reasons for its selection.

There should be little or no pain or distress in these studies. The methods of euthanasia (CO2 overdose) are consistent with the recommendations of the American Veterinary Medical Association Panel on Euthanasia and follow the NIH Guide on Laboratory Animal Welfare.

VERTEBRATE ANIMALS

Icahn School of Medicine at Mount Sinai

Standard mouse pharmacokinetic (PK) studies will be conducted by Agilux, a Contract Research Organization based in Worcester, Massachusetts. The animal facilities of Agilux are AAALAC (Association for the Assessment and Accreditation of Laboratory Animal Care) and OLAW (Office of Laboratory Animal Welfare) accredited with the certification number of 001475 and A4645-01, respectively.

1. Description of Procedures.

Intended studies. Standard pharmacokinetic (PK) studies will be performed in mice to determine drug exposure and PK properties of test compounds.

Description and estimated usage. Both male and female CD-1 mice (also known as Swiss mice) at 8-10 weeks of age will be utilized. This strain is commonly used for standard PK studies. All animals will be acquired from approved vendors and be housed and used in the animal facilities of Agilux. Initial animal quarantine and husbandry care procedures will be conducted. All animals will be allowed for physical, environmental, nutritional and physiological stabilization for at least three days in designated quarantine room, and will be carefully observed by an experienced veterinarian. We plan to evaluate 3 compounds per year in mouse PK studies using the intraperitoneal administration route. Blood and brain samples will be collected from each test animal at 6 time points (in hours: 0.5, 1, 2, 4, 8, and 24). We will use 2 male and 2 female mice per compound per time point. A total of 72 mice (3 compounds x 6 time points x 4 mice/compound/time point) will be used per year and **a total of 432 mice (216 male and 216 female) will be used for the 6-year project period.**

2. Justification.

No alternative test systems exist which have been adequately validated to permit replacement of the use of live animals in the *in vivo* PK studies. Every effort has been made to obtain the maximum amount of information while reducing to a minimum number of animals required for these studies. The proposed studies will eventually improve human and animal health, and advance scientific knowledge. Mice are the most common species for determining drug exposure and PK properties of test compounds.

3. Minimization of Pain and Distress.

Treatment of animals will be in accordance with the study protocol and also in accordance with Agilux standard operating procedures which adhere to the regulations outlined in the USDA Animal Welfare Act (9 CFR Parts 1, 2 and 3) and the conditions specified in the Guide for the Care and Use of Laboratory Animals (ILAR publication, NRC, 2011, The National Academies Press). The assessment of pain and distress in study animals and the use or non-use of pain alleviating medications will be in accordance with Standard Operating Procedures. The study will be terminated in part or whole for humane reasons if unnecessary pain occurs. A member of the veterinary staff or other authorized personnel is available at Agilux to monitor the daily activities of the animal house and to take care of animals.

4. Euthanasia.

CO_2 Asphyxiation: Euthanasia for mice will be performed using CO_2 euthanasia chamber placed in a hood having exhaust. CO_2 induces rapid unconsciousness and death without pain to the laboratory animals. Euthanasia will be carried out in isolated area by an experienced and trained scientist. The death of animals will be confirmed by a trained scientist or veterinarian. All procedures will be carried out in accordance with Test Facility Standard Operating Procedures and the American Veterinary Medical Association (AVMA) Guidelines on Euthanasia 2007.

Multiple PD/PI Leadership Plan

As each component of the proposed studies requires specialized expertise, we propose a team approach, leveraging a diverse group of highly qualified individuals at the University of North Carolina (UNC) and the University of California San Francisco to jointly undertake this complex basic research study. This collaborative team consists of the contact PI, Bryan Roth with expertise in cellular biochemistry/pharmacology and drug screening and Dr. Brian Shoichet with extensive *in silico* and computational approaches for drug screening.

The PIs all have extensive experience in screening, profiling and drug discovery, and accordingly we anticipate that the work will flow smoothly if an award is granted as the result of this application.

Dr. Bryan Roth at UNC will be responsible for all of the physical screening based studies and mouse genetics studies.

Dr. Brian Shoichet at UCSF will coordinate and execute all of the computational studies, resource sharing and data handling.

Dr. Roth will serve as the corresponding PI and ensure the timely submission of reports to the NIH.

All data of a chemical, pharmacological or behavioral nature will be shared freely among all collaborators working on this project with communication taking place by phone, email, and a secure website.

Consortium/ Contractual Arrangements

The appropriate programmatic and administrative personnel of each organization involved in this grant application are aware of the NIH consortium agreement policy and are prepared to establish the necessary inter-organizational agreement(s) consistent with that policy.

Intellectual Property

We anticipate no new intellectual property as all results will be freely shared with the scientific community.

Conflict Resolution

If a potential conflict does develop, the PIs shall meet and attempt to resolve any dispute. If they fail to resolve the dispute, the disagreement shall be referred to an arbitration committee consisting of one impartial senior administrator from each of the PI’s institutions and a fourth impartial senior administrator mutually agreed upon by all PIs. No members of the arbitration committee will be directly involved in the research grant or disagreement.

Change in PI Location

If a PI moves to a new institution, attempts will be made to transfer the relevant portion of the grant to the new institution. In the event that a PI cannot carry out his/her duties, a new PI will be recruited as a replacement at one of the participating institutions.

Additionally, in accordance with NIH policy any changes in key personnel will be submitted to the NIH program officer for approval of a replacement.

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February 22, 2017

Proposal No. P0522261

Office of Sponsored Research
The University of North Carolina at Chapel Hill
104 Airport Drive, Ste 2200, CB 1350
Chapel Hill, NC 27599-1350

Dear Administrator,

We are presenting for your review a request for support of the following project:

PROJECT TITLE:	Scalable technologies for illuminating the GPCR-ome
PRINCIPAL INVESTIGATOR:	Brian K. Shoichet, Ph.D.
TYPE OF PROPOSAL:	New Research Subcontract
PRIME SPONSOR:	NIH
INDIRECT COST RATE:	58.5% MTDC

Included, please find:

(i) Supplemental Information Sheet containing relevant information that is necessary to administer a subcontract as a result of this proposal; and
(ii) UCSF Facilities & Administrative Rate Agreement, dated May 23, 2012.

Your favorable consideration will be appreciated. If this application is favored with an award, the Regents of the University of California reserve the right to negotiate the terms and conditions of the award.

Any award documentation or correspondence should be sent directly to my attention at the address listed in the upper-right corner of this letter, or by e-mail to: CGAwardTeam@ucsf.edu.

If you have any questions or concerns regarding this proposal, please contact me by phone at (415) 502-1841 or by e-mail at anne.crosthwaite@ucsf.edu.

Sincerely,

Anne Crosthwaite
Contracts and Grants Officer

Icahn School of Medicine at Mount Sinai

Grants and Contracts Office
One Gustave L. Levy Place Box 1075
New York, NY 10029-6574
Phone: 212.824-8300
Facsimile: 212.241-3294
Email: grants@mssm.edu

STATEMENT OF INTENT TO ESTABLISH A CONSORTIUM AGREEMENT

PRIME INSTITUTION
INSTITUTION NAME & ADDRESS: University Of North Carolina Medical School At Chapel Hill
TITLE OF APPLICATION: Scalable technologies for illuminating the GPCR-ome
PD/PI NAME: Bryan Roth, PhD
FUNDING AGENCY NAME: NIH
FUNDING AGENCY NUMBER (if applicable):
PROPOSED PROJECT PERIOD DATES: 11/01/2017 – 10/31/2023

SUB-AWARDEE INSTITUTION
INSTITUTION NAME& ADDRESS: **Icahn School of Medicine at Mount Sinai (ISMMS)**
One Gustave L. Levy Place, Box 1075
New York, New York 10029-6574

DUNS NO.: **078861598**
SUBAWARD PD/PI NAME: Dr. Jian Jin
IS SUBAWARD PD/PI A MULTIPLE PD/PI? [ ] Yes [x] No

	INITIAL PROPOSED AMOUNT	TOTAL PROPOSED AMOUNT
DIRECT COSTS:	$ 175,573	$ 1,053,438
INDIRECT COSTS:	$ 122,023	$ 732,138
TOTAL COSTS:	$ 297,596	$ 1,785,576

The appropriate programmatic and administrative personnel involved in this sponsored project application are aware of all applicable Federal regulations and policies, and will establish the necessary inter-institutional agreement(s) consistent with those policies. In signing below, ISMMS certifies that it has implemented and is enforcing the new PHS regulations on Conflict of Interest as of August 24, 2012 and is in compliance with the updated provisions of 42 CFR Part 50, Subpart F. ISMMS confirms that it participates in the FDP Clearinghouse of PHS-COI Compliant Institutions (http://sites.nationalacademies.org/PGA/fdp/PGA_070596).

ISMMS AUTHORIZING ORGANIZATION REPRESENTATIVE (AOR)
NAME: MR. MICHAEL KING
TITLE: DIRECTOR, GRANTS AND CONTRACTS
TEL #: (212) 824-8300 E-MAIL: grants@mssm.edu
SIGNATURE: DATE: 02/17/2017

rev. 7/14

CALIFORNIA INSTITUTE OF TECHNOLOGY

From: Viviana Gradinaru, PhD
Caltech MC 156-29
Pasadena, CA 91125

To: Dr. Bryan Roth
UNC School of Medicine, NC

Date: February 15th, 2017

Dear Bryan,

As we discussed, it is my pleasure to support your proposed U24 work on "Illuminating the druggable GPCR-ome" by facilitating the implementation of tissue clearing techniques we have working in our laboratory at Caltech.

As you know, my work has focused on developing and using tools for neuroscience such as optogenetics (Gradinaru et al., *Cell*, 2010) and CLARITY and variants (Chung et al., *Nature*, 2013; Yang et al., *Cell*, 2014; Treweek et al, *Nat.Prot*, 2015; Shah et al, *Development*, 2016; Greenbaum et al, in press, *Science Translational Medicine*) to dissect the circuitry underlying movement and mood disorders (Gradinaru et al., *Science*, 2009; Xiao et al, *Neuron*, 2016).

I am also the principal investigator of a Beckman Institute Resource center for optogenetics, CLARITY, and vector engineering (http://www.beckmaninstitute.caltech.edu/clover.shtml). The center aims to support tissue clearing and imaging projects, optogenetics studies, and custom gene delivery vehicle development through technology and methodology innovation, training, infrastructure and resource sharing.

As discussed, you are welcomed to use the Center facilities and resources for your tissue clearing work. Specifically, we can train your lab members on current CLARITY practices (the PACT method that ensures gentle clearing), they can clear samples at Caltech and we will help you setup the method in your laboratory at UNC.

Your proposed work is very exciting and I am looking forward to our ongoing collaboration. Best of luck!

Sincerely yours,

Assistant Professor of Biology and Biological Engineering
Investigator, Heritage Medical Research Institute

PASADENA, CALIFORNIA 91125 *USA*
E-MAIL viviana@caltech.edu
WEB glab.caltech.edu

UNC Animal Models Core Facility

Transgenics, Gene-Targeting and Associated Biotechnologies

February 16, 2017

Bryan Roth MD, PhD
Department of Pharmacology
UNC Chapel Hill School of Medicine

Dear Bryan,

As director of the Animal Models Core, I am pleased to offer the Core's CRISPR/Cas9 services to support your proposal, entitled "Illuminating the druggable GPCR-ome". As you are aware, we have fully implemented the CRISPR/Cas9 system and have developed deep expertise in the use of CRISPR/Cas9 for engineering the mouse genome. Our services span the full range of CRISPR/Cas9 needs including guide RNA design, production and validation, embryo microinjection, founder genotyping, off-target mutation screening and founder breeding for germline transmission. We have developed efficient systems for development and validation of CRISPR reagents as well as screening and validation of founder animals. Our experience has also enhanced our ability to troubleshoot in cases where the CRISPR system has not worked optimally. This has enabled us to achieve success in cases where projects proved challenging due to limited guide RNA options, mutational lethality or working in difficult strains. Thus, I am confident in our ability to successfully generate the models you propose.

We have the capacity and experience to provide the models needed for your program. We have successfully completed 65 projects using embryo pronuclear microinjection of CRISPR reagents since 2013. Approximately half of these projects have focused on targeted insertion of tags, cDNAs or reporter genes similar to the models you are proposing to generate. Our typical project throughput for a year is around 60-80 total projects including transgenes, BACs, CRISPR and ES cell targeted models. I do not anticipate any challenge with completing up to 10 additional projects per year as you are proposing.

I look forward to a productive collaboration.

Best regards,

Dale Cowley, Ph.D.
Director, UNC Animal Models Core
502 Taylor Hall
109 Mason Farm Road
University of North Carolina at Chapel Hill
Chapel Hill, NC 27599-7295
(919) 843-9125
dcowley@med.unc.edu

EMORY UNIVERSITY SCHOOL OF MEDICINE
Department of Pharmacology

February 15th, 2017

Bryan L. Roth, M.D., Ph.D.
Michael Hooker Distinguished Professor
University of North Carolina School of Medicine
Chapel Hill, NC, 27599

Dear Bryan,

I would like to express my enthusiasm for serving as a consultant for your project entitled "Interrogating the Druggable GPCRome". As you know, my lab has made seminal contributions to understanding the activation mechanisms of adhesion GPCRs and the signaling pathways downstream of these receptors (Paavola et al., *J. Biol. Chem*., 2011; Paavola & Hall, *Mol. Pharmacol.*, 2012; Stephenson et al., *J. Biol. Chem*., 2013; Kishore et al., *J. Biol. Chem.*, 2016). Thus, I will bring extensive expertise to guiding the screens focused on adhesion GPCRs that will be part of the Druggable GPCRome project. Moreover, as a Board member of the Adhesion GPCR Consortium, I can help connect the Druggable GPCRome project with other world leaders in the adhesion GPCR field in order to gather any additional technical advice or perspectives that might be needed to address specific issues.

In my view, the Druggable GPCRome project is incredibly timely and important because the adhesion GPCR field is currently being held back due to a near-complete lack of agonists, antagonists and modulators to control the activity of these receptors. The Druggable GPCRome project will provide an arsenal of novel ligands for adhesion GPCRs, and these compounds will be extremely important as basic research tools that will allow many labs around the world to shed new light on the physiological functions of this fascinating family of receptors.

Good luck with this exciting proposal!

Randy Hall

Randy A. Hall, Ph.D.
Professor of Pharmacology
Emory University School of Medicine
Atlanta, GA, 30322

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An equal opportunity, affirmative action university

February 5, 2017

Dear Bryan,

I am very happy to offer this letter in support of your U24 “Scalable technologies for illuminating the GPCR-ome”. Spefically, I am happy to serve as a consultant to bring iDISCO and the associated image analysis methods to your as part of our ongoing successful collaborations. We have already obtained some very promising results using this approach on the GPR68-cre mouse line that we jointly made.

Please do not hesitate to contact me at any point during this research. I look forward to seeing the results of these exciting studies.

Sincerely,

Thomas L. Kash, Ph.D.
Associate Professor
Bowles Center for Alcohol Studies
Department of Pharmacology
UNC Chapel Hill

University of North Carolina at Chapel Hill, CB 7178, Chapel Hill, NC 27599
Phone (919) 966-5678. Fax (919) 966-5679. Treatment (919) 402-1644. Email: ftcrews@med.unc.edu
http://www.med.unc.edu/alcohol

Pfizer Inc.
610 Main Street
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John Mathias, PhD
Senior Director
Head of Inflammation & Immunology Design
Pfizer, Cambridge MA

Professors Brian Shoichet & Bryan Roth
Dept. of Pharmaceutical Chemistry, UCSF
& Dept. of Pharmacology, UNC

Feb 13, 2017

Dear Bryan & Brian,

I am keen to confirm my strong interest to collaborate with you on discovering open access tool molecules for orphan GPCRs, as part of your NIH grant on *Illuminating the Orphan GPCR-ome.*

As you know, we have a keen interest in de-orphanizing understudied, potentially druggable targets, and have collaborated effectively to do so against several kinases and epigenetic targets with the Structural Genomics Consortium, an open access enterprise. With the renewed interest in GPCRs in our field, and with your own success developing tool molecules for the orphan receptors GPR68, GPR65, and MRGPRX2, it seems an opportune time to extend this sort of collaboration to orphan GPCRs with your two labs. As we have discussed over the last year, your role would be initial discovery of hit matter (Roth lab) followed by optimization for specificity by structure-based docking (Shoichet lab), while we would contribute medicinal chemistry optimization for final specificity and other probe-like qualities, and perhaps counsel on initial hit picking, and resources from chemical libraries. We believe that the ability to synthetically modify your lead matter will substantially improve their properties over what you have been able to access by analog-by-catalog alone. There is also the possibility of extending some of the initial screening with our own chemogenomics library, a collection of several thousand highly active and interesting molecules that we have put together over the last decade at Pfizer, and that may well-complement the library already in hand in the Roth lab.

I think this is a great opportunity for us all to do some exciting science, and impact the field with new open access probes. Our first focus in this collaboration will be optimization of compounds you beginning to investigate for GPR4 and GPR174. I must state that I cannot commit Pfizer to this collaboration until a formal agreement is in place, but can confirm that such an agreement, which is not for funding, just for collaborative effort, is strongly supported and being finalized.

We would look forward to an exciting collaboration; good luck with the grant!

John Mathias

John Mathias, Ph.D.
Senior Director
Head of Inflammation & Immunology Design
Pfizer

Professors Brian Shoichet and Bryan Roth
Department of Pharmaceutical Chemistry, UCSF
and Department of Pharmacology, UNC

Date: February 10, 2017

Dear Brian and Bryan,

I am happy to confirm our ongoing collaboration with you as part of your NIH grant, *Illuminating the Orphan GPCR-ome.* We will evaluate the commercial viability of the new molecule probes you are developing, with the possibility of making them widely available as MilliporeSigma chemical probes. As you know, we have done this already with two probe pairs from your project, ogerin and its inactive analog for GPR68 (e.g., http://www.sigmaaldrich.com/catalog/product/sigma/sml1482?lang=en&region=US and http://www.sigmaaldrich.com/catalog/product/sigma/sml1483?lang=en&region=US), and for R- and S-'3573 for MRGPRX2. We are therefore optimistic that your newly developed probes will also be of commercial interest.

We have a longstanding commitment to making chemical probes available at MilliporeSigma, and we are excited continue to doing so with the molecules you are developing to illuminate the orphan GPCR-ome.

Best wishes for the grant,

Shari Spector, PhD
Business Development Manager, Chemical Biology and Bioactives
shari.spector@emdmillipore.com

MilliporeSigma

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UNC
CAROLINA INSTITUTE FOR
DEVELOPMENTAL DISABILITIES

February 16, 2017

Bryan L. Roth, M.D., Ph.D.
Professor, Department of Pharmacology
Director, NIMH Psychoactive Drug Screening Program
University of North Carolina at Chapel Hill

Dear Bryan,

I am pleased to confirm access to the Mouse Behavioral Phenotyping Core for your highly innovative grant application, "*Illuminating the druggable GPCR-ome*," focused on the identification and characterization of novel targets for new interventions in human disorders. As you know, I am Director of the behavior core, a research facility of the UNC Carolina Institute for Developmental Disabilities. This laboratory was designed to evaluate mouse behavior across a broad range of domains, including sensory and motor ability, activity and exploration, anxiety- and depression- like behavior, social interaction, and learning and memory. For your proposed studies, we can provide resources for behavioral testing, as well as expertise for experimental design, data analysis, and interpretation of results. Our past history of collaboration has included early work with the DREADD mouse lines, which have become a widely-used tool in the study of neural circuitry and function (Alexander et al. 2009, Neuron 63(1): 27-39; Farrell et al. 2013, Neuropsychopharmacology 38(5): 854-862), and research on the role of specific hippocampal regions in conditioned fear (Zhu et al. 2014, Neuropsychopharmacology 39(8): 1880-1892). More recently, our core collaborated with your laboratory on the first characterization of two novel G-protein-coupled receptors (Huang et al. 2015, Nature 527(7579): 477-483), a project which clearly demonstrated the remarkable promise of GPCRs for pharmaceutical discovery. Your proposal could significantly advance our understanding of these potential new drug targets, and I am delighted that our core could contribute to this highly important work.

With best regards,

Sheryl Moy

Sheryl S. Moy, Ph.D.
Research Professor, Department of Psychiatry
Director, Mouse Behavioral Phenotyping Core
Carolina Institute for Developmental Disabilities
University of North Carolina School of Medicine
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February 9, 2017

Dear Dr. Roth:

The Animal Histopathology Core lab (AHC) would be delighted in offering histology and immunohistochemistry support for the transgenic-cre-recombinase project. The project involves the investigation of the anatomical and cell-type selective expression of the orphan GPCRome. Through the UNC transgenics core, the investigators plan to create a multitude of mice expressing cre following the promoter of orphan GPCRs, as well as tagging the orphan GPCR with an easily identifiable epitope (FLAG). The goal is to identify where the orphan is expressed and utilizing another technology developed by the researchers' lab (DREADDs) to "mimic" the activity of these receptors by replacing them with artificially constructed receptors with known specific ligands.

The AHC currently provides a variety of routine histologic services for investigators that utilize animal models of human disease. Currently, we employ 2 full-time histotechnologists experienced in routine histologic techniques and myself, a certified histotechnologist, who splits time in managing the facility and bench. Our current services also include immunohistochemistry (single and multiplex) and immunofluorescence (single and multiplex). Additionally, our Faculty Director, Stephanie Montgomery, is a board-certified veterinary pathologist. She would be happy in assisting with pathologic assessment of the animal tissues that are generated.

We offer a platform of services and stains to include cryosectioning and special staining.

Given the Animal Histopathology Core's equipment, personnel, and experience, I am confident that our facility is fully prepared to support this study. Please do not hesitate to contact me regarding further services that our lab can provide to enable the research.

Sincerely,

Dawud Hilliard
MPM, HTL (ASCP) QIHC
Facility Director
Animal Histopathology Core Lab
Lineberger Comprehensive Cancer Center
University of North Carolina - Chapel Hill
Chapel Hill, North Carolina 27599
(919) 966-3653

February 17, 2017

Professor Brian Shoichet
Dept. of Pharmaceutical Chemistry
University of California, San Francisco

Professor Bryan Roth
Dept. of Pharmacology
University of North Carolina

RE: Letter of Support For NIH Grant Application Entitled "Illuminating the Orphan GPCR-ome"

Dear Dr. Shoichet and Dr. Roth,

With this letter, I would like to express Takeda's support for your NIH grant application titled "*Illuminating the Orphan GPCR-ome*."

Takeda has an active interest in open access probe development for understudied kinases and epigenetic readers and finds your success in developing tool molecules for GPR68 and MRGPRX2 most exciting. Takeda has an active interest in several orphan GPCRs, and it would be most useful Takeda's research if we are able to structurally model orphan GPCRs and develop chemical probes for them.

As discussed during your recent visit to Takeda in San Diego, if your NIH grant application is successful, Takeda would welcome the opportunity to discuss a possible collaboration where the Roth lab conducts initial hit discovery followed by optimization for specificity by structure-based docking by the Shoichet lab. Takeda could then contribute medicinal chemistry optimization for final specificity which is often necessary to achieve true probe-like specificity. Of course, Takeda cannot commit to entering into a collaboration until it knows whether the NIH grant application is funded and until a study plan and agreement is mutually agreed upon.

Takeda looks forward to seeing how your grant application progresses and encourages you to reconnect with Takeda once the grant is awarded.

Sincerely,

Derek Cole, PhD
Director of Medicinal Chemistry

TAKEDA CALIFORNIA, INC.
10410 Science Center Dr. | San Diego, CA 92121-1119 | Phone: 858-622-8528 | Fax: 858-550-0526

Harvard Medical School
DEPARTMENT OF CELL BIOLOGY

Stephen Liberles, Ph.D.
Associate Professor of Cell Biology

February 27, 2017

Bryan L. Roth MD, PhD
Michael Hooker Distinguished Professor
Pharmacology and Chemical Biology and Medicinal Chemistry
Director, National Institute of Mental Health Psychoactive Drug Screening Program
University of North Carolina at Chapel Hill Medical School
Chapel Hill, NC

Dear Bryan:

I am delighted to be a consultant for your U24 entitled: “Illuminating the Druggable GPCR-ome” in the area of vomeronasal sensory receptors. As you know, the primary focus of my lab has been to characterize mammalian sensory receptors. We identified non-classical families of olfactory and vomeronasal receptors (Nature 2006, PNAS 2009), and also published extensively on identifying ligands that activate sensory GPCRs, including behaviorally salient odors, tastes, and pheromones (eLife 2015, Science 2014, Nature 2013, PNAS 2013, Current Biology 2013, ACS Chemical Biology 2012, PNAS 2011).

Despite the fundamental need to understand molecular recognition properties of vomeronasal receptors, to date these receptors have proven recalcitrant to study by in vitro assays involving heterologous expression systems. Your approach to ‘tag’ the appropriate mouse homologues and to express DREADDs in VNR-expressing cells should yield valuable information related to physiological actions outside the vomeronasal organ.

I am very excited for this project. Best wishes for success and I look forward to working with you in the future.

Sincerely,

Stephen Liberles

Associate Professor of Cell Biology
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February 28th, 2016

Dear Bryan,

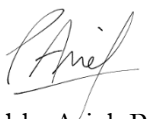
I am writing to express my enthusiasm about assisting you and your lab in your project entitled 'Illuminating the druggable GPCR-ome'. In particular, I will assist you with light-sheet-microscope-based imaging of cleared organs to reveal the cell-type-expression patterns of the GPCRs you study.

This project is an excellent example of the sorts of questions that are readily addressable by light-sheet microscopy of large, cleared samples. We currently have a Lavision Ultramicroscope II in the Microscopy Services Laboratory, which I direct. This system allows extremely fast three-dimensional imaging of large samples, particularly when compared to confocal or multiphoton microscopes. A sample as large as an adult mouse brain hemisphere can be fully scanned in three dimensions in less than ten minutes, which is orders of magnitude faster than the time required on a laser scanning confocal or multiphoton microscope. This state-of-the-art mode of microscopy will allow you to visualize GPCR expression in a comprehensive manner. I look forward to working with you on these important experiments.

Over the past few years I have acquired extensive experience with this light-sheet microscope, as well as with a variety of tissue-clearing methods used to prepare samples. **In fact, as a result of my close collaboration with the Tessier-Lavigne lab at Rockefeller University on the iDISCO protocol, I was a coauthor on a paper describing this method in Cell (Renier *et al*, 2014).** I have also recently published a review about different clearing methods, aimed at researchers new to these techniques (Ariel, 2017). Over the course of my career I have assisted scientists from over 30 labs from more than 11 different institutions in how to prepare samples, optimize imaging parameters, and analyze the resulting data sets. I have hands-on experience with a large variety of samples prepared with the most popular tissue clearing methods (CUBIC, CLARITY, PACT, iDISCO, uDISCO) and will be happy to provide advice on the optimal clearing technique for your application. In fact, I have direct experience with research projects with very similar samples to the ones you will work with: mouse brains where neurons were labelled sparsely with fluorescent markers in volumes spanning many millimeters. In addition to sample preparation and imaging, I am also experienced with commercial software (Bitplane Imaris) that can be used to visualize large three-dimensional data sets. This software is available in the lab on a state-of-the-art workstation and I routinely train researchers on how to use it for light-sheet datasets.

I believe my skills and experience will be of great benefit to your research and look forward to providing all the support necessary to your project. As director of the Microscopy Services Laboratory at UNC, my role is to help people design and execute these types of exciting experiments. I look forward to working with you on this project.

Sincerely,



Pablo Ariel, Ph.D.
Director of the Microscopy Services Laboratory, University of North Carolina at Chapel Hill
pablo_ariel@med.unc.edu

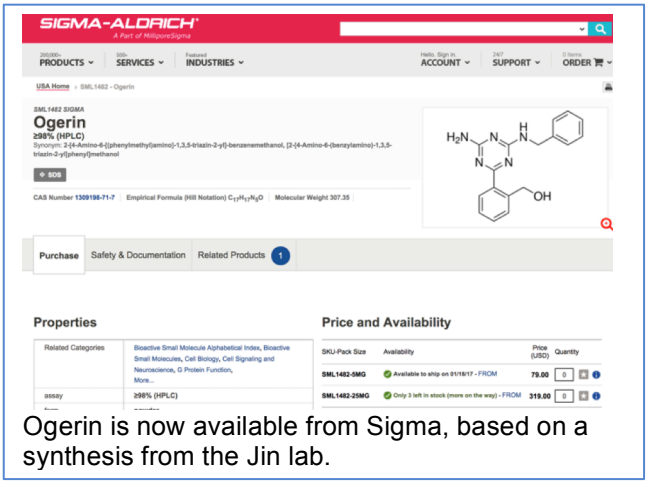
Resource Sharing Plan

The Roth and Shoichet labs have long made reagents, resources, and results open access and readily available to the community. We will abide by all data deposition, quality control metrics, standardization, metadata requirements, data and software release, and public copyright license policies of the IDG program. We will work closely with the knowledge management group to deposit our data and to organize it for ready access and for functional meaning, as we have done in the previous period. We are also committed to working closely with the other IDG groups, as we have done in the previous period.

Our adoption of open access data sharing is attested to by the resources, web-tools, genetic constructs, and probe molecules that we have made available during the last period of the IDG grant, and will continue to do so in the upcoming period.

1. As part of their efforts to interrogate the orphan GPCR-ome, the Roth lab synthesized Tango constructs for 320 GPCRs, including 120 oGPCRs; this is one of two screening platforms used for deorphanization in this project. All of these optimized constructs have been deposited in ADDGENE.

   As new constructs are created for this project, for instance for Adhesion, Tastant, Frizzled and Vomeronasal GPCRs, they will continue to be deposited into ADDGENE.



Ogerin is now available from Sigma, based on a synthesis from the Jin lab.

2. In the last period, two probe pairs were developed, one for GPR68 and one for MRGPRX2. Each consists of an activating molecule and an inactive analog with very similar or identical physical properties. In collaboration with Sigma-Millipore, we have made both the GPR68 PAM ogerin and its inactive analog and MRGPRX2's agonist R-ZINC-'3573 and its inactive S-isomer, openly available to all investigators. The GPR68 probe pair is available at:
   http://www.sigmaaldrich.com/catalog/product/sigma/sml1482 (ogerin)
   http://www.sigmaaldrich.com/catalog/product/sigma/sml1483 (inactive analog)

   The MRGPRX2 probe pair is available at:
   http://www.sigmaaldrich.com/catalog/product/sigma/sml1699 (R-ZINC-'3573)
   http://www.sigmaaldrich.com/catalog/product/sigma/sml1700 (S-ZINC-'3573; inactive analog)

   As new probes are developed for specific oGPCRs, we will continue to make them available to the community via our collaboration with Sigma-Millipore (see letter from Dr. Shari Spector). We believe this is the best way to ensure broad access to these reagents, even after this project is concluded.

3. To prosecute docking screens, the Shoichet lab draws on tools and libraries that it has developed; all are open access to the community (**Table 1**). Several, like ZINC, have become cornerstones of virtual screening; this will continue during the IDG; we believe they will are broadly useful for this enterprise, and are certainly the tools we ourselves use.

Table 1. Shoichet lab open access sites for docking & virtual screening

Website	Unique visits/month (by people)	Page Views per month	Files downloaded per month
ZINC Compound Libraries http://zinc12.docking.org http://zinc15.docking.org	118,000	16 million	16.1 TB
DOCK Blaster docking screens: http://blaster.docking.org	4000	7000	7 GB
DOCKovalent for covalent docking screens: http://dockovalent.docking.org	1000	1600	0.4 GB
Metabolite docking screens http://metabolite.docking.org	500	700	0.2 GB
Similarity Ensemble Approach for target ID http://sea15.docking.org	740	2300	4 GB
Aggregate Advisor for promiscuous hits	900		

http://advisor.bkslab.org			

4. All genetically engineered mice will be shared via Jackson mouse repository (JAX Mice) without restrictions as to use as the Roth lab has done in the past.
5. All mouse oGPCR expression and related images will be openly shared via dedicated website in a variety of formats (non-compressed and compressed image formats). The Shoichet lab routinely makes terabytes of information available to the community via its ZINC platform (**Table 1**), making this a relatively easily manageable enterprise.

AUTHENTICATION OF KEY BIOLOGICAL AND CHEMICAL RESOURCES PLAN

1. Chemicals:

To be tested in biological assays, chemical compounds must meet the purity criteria defined below. We will use well-established analytical methods including ^{1}H and ^{13}C NMR (Nuclear Magnetic Resonance), HPLC (High Performance Liquid Chromatography) and HRMS (High-Resolution Mass Spectrometry) to determine the identity and purity of test compounds. Specifically, we will use ^{1}H and ^{13}C NMR and HRMS to determine compound identity, and ^{1}H and ^{13}C NMR and HPLC to determine compound purity. Compounds with confirmed identity and > 95% purity will be tested in biological assays.

Any specialty chemicals such as GPCR-selective ligands are obtained from vendors and include validation chemical information as part of the package insert. For compounds which are obtained via the Zinc database, we routinely perform LC-MS in house to assess purity and authenticity prior to use. Synthetic compounds are verified by LC-MS, NMR and other standard chemical analysis; synthetic routes are published.

2. Mouse lines:

- We use the Ai9 reporter line which is obtained from JAX Mice (https://www.jax.org/strain/007909) and is maintained on a C57/BL6 background. Mice are genotyped per the JAX protocol prior to use
- The FLOXED-DREADD mice are were created in the Roth lab and the Gi and Gq (B6N.129-*Gt(ROSA)26Sortm1(CAG-CHRM4*,-mCitrine)Ute*/J; B6N.129-*Gt(ROSA)26Sortm1(CAG-CHRM3*,-mCitrine)Ute*/J) have been deposited in JAX and have been backcrossed to C57/BL6 in the Roth lab. The Gs-FLOXED-DREADD mouse has been created and is being backcrossed to C57/BL6 in the Roth lab.
- All engineered mice are validated by sequencing the locus of insertion and verifying no offtarget insertion via PCR- and sequencing-based approaches.

3. Cell lines: The following cell lines are used in this proposal:

- HEK293 (ATCC CRL-1573; Batch 60113019; certified mycoplasma free and authentic by ATCC)
- HEK293-T (HEK293T; ATCC CRL-11268; Batch 59587035; certified mycoplasma free and authentic by ATCC).
- Cells were independently validated by analysis of short tandom repeat (STR) DNA profiles and these profiles showed 100% match at the STR database from ATCC.

cDNA constructs: All engineered cDNA constructs are validated by dsDNA sequencing of both strands at least twice.

Chemicals: Any specialty chemicals such as GPCR-selective ligands are obtained from vendors and include validation chemical information as part of the package insert. For compounds which are obtained via the Zinc database, we routinely perform LC-MS in house to assess purity and authenticity prior to use.
Synthetic compounds are verified by LC-MS, NMR and other standard chemical analysis; synthetic routes are published.

"informatics":

* As part of our project, we use large and ultra-large compound libraries, the contents of which, and the searching of which, is openly available (http://zinc15.docking.org).

* We also use chemoinformtics tools to link targets by their ligands, and to search for endogenous transmitters (http://sea.bkslab.org).

* We make the docking tools that we use openly available for the public (http://blaster.docking.org).

* Chemical tools emerging from the work are sold as part of the Sigma Probe collection, and may be acquired from them.

We'd be happy to have links to the informatics tools from the central KMC site. We will be happy to update that site with our progress on specific targets. We are looking for a temporary coordinator for the IDG informatics...the one we have had is going on mat leave until May.

Brian