Evolving the lock to fit the key to create a family of G protein-coupled receptors potently activated by an inert ligand

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We evolved muscarinic receptors in yeast to generate a family of G protein-coupled receptors (GPCRs) that are activated solely by a pharmacologically inert drug-like and bioavailable compound (clozapine-*N*-oxide). Subsequent screening in human cell lines facilitated the creation of a family of muscarinic acetylcholine GPCRs suitable for *in vitro* and *in situ* studies. We subsequently created lines of telomerase-immortalized human pulmonary artery smooth muscle cells stably expressing all five family members and found that each one faithfully recapitulated the signaling phenotype of the parent receptor. We also expressed a G_i-coupled designer receptor in hippocampal neurons (hM_4D) and demonstrated its ability to induce membrane hyperpolarization and neuronal silencing. We have thus devised a facile approach for designing families of GPCRs with engineered ligand specificities. Such reverse-engineered GPCRs will prove to be powerful tools for selectively modulating signal-transduction pathways *in vitro* and *in vivo*.

cell engineering | molecular evolution | receptorome

Because of the assorted cellular responses directed by them, their number, and the ease of which they are pharmacologically screened, the superfamily of G protein-coupled receptors (GPCRs) is one of the most therapeutically important targets in the proteome (1). However, the potential of this family is restricted by our ability to assess their function, which currently involves transgenic, knockout, and/or *in vivo* studies with selective drugs. Genetic studies are frequently limited to loss-of-function phenotypes, whereas nonselectiveness of a drug often interferes with interpretation of pharmacological studies. Knowledge of the roles of the individual family members is being bolstered by the ongoing creation of knockout mice for many GPCRs. Selective activation of individual GPCR subtypes in a defined tissue, in either a knockout or wild-type animal, is currently problematic but, if possible, would serve to complement present findings by providing novel insights into disease states resulting from overstimulation of certain signaling pathways.

One approach to this problem has been to rationally modify receptors to favor synthetic over natural substrate/ligand recognition, and subsequently, these mutant proteins have been used as bio-tools to study protein function in complex biological environments (2, 3). At the forefront of such modified GPCRs is Ro1, a $G_{i/o}$-coupled κ opioid receptor activated by a synthetic but not a native ligand, which has been conditionally expressed in transgenic mice to study cardiac function after its selective activation (4). Such mutant receptors, like Ro1, have been classified as receptors activated solely by synthetic ligands (RASSLs), because they are activated by synthetic ligands but not by their endogenous ligands (5). RASSLs, as in the case of Ro1, have been demonstrated to be valuable tools (4, 6); however, because the synthetic ligand frequently has high affinity and/or potency at the native receptor (5, 7, 8), this potentially limits their usefulness *in vivo*, at least in tissues with a wild-type receptor present. In this context, we sought to develop designer receptors exclusively activated by a designer drug (DREADD), or simply "designer" receptors, which represent receptors that are activated solely by a synthetic ligand(s) possessing minimal or no biologic activity.

In this study, we present a directed molecular evolution approach, which facilitated the creation of a family of muscarinic receptors that is potently activated by the pharmacologically inert compound clozapine-*N*-oxide (CNO) but not by its native ligand acetylcholine (ACh). We further demonstrate that such designer muscarinic receptors are active in a variety of native and artificial cellular contexts. We thus provide a validated and unbiased approach for generating GPCRs with defined ligand specificities and for proof of concept have created a family of muscarinic ACh receptor (mAChR) DREADDs having promise as unique biological tools to study either receptor-specific functions [e.g., , human mAChR DREADD subtype 3 (hM_3) vs. hM_1] or general downstream signaling (e.g., $G_{q/11}$ vs. $G_{i/o}$) emanating from the activated GPCR.

Results

Directed Molecular Evolution of Rat M_3 Receptor in Yeast. We set out to develop an M_3 DREADD that was responsive to a synthetic ligand of choice by using a directed molecular evolution approach. CNO was selected as the synthetic ligand because: (*i*) its parent compound, clozapine, has high affinity to M_3 receptors and, therefore, we predicted few mutations would be required to permit CNO to be a potent agonist; (*ii*) CNO is highly bioavailable in rodents and humans (9, 10); and (*iii*) importantly, CNO is a pharmacologically inert molecule lacking appreciable (<1 μM) affinity for receptors [ref. 11 and supporting information (SI) Fig. 6]. For initial studies, a modified rat M_3 receptor containing a sizable deletion in its third intracellular loop [(i3); $rM_3\Delta i3$] was used (SI Fig. 7). The rat $M_3\Delta i3$ receptor has been previously demonstrated to be functionally expressed in *Saccharomyces cerevisiae* genetically modified to enable ligand activation of heterologously expressed mammalian GPCRs to engage the pheromone signaling pathway to promote growth on selective medium (12).

By means of random mutagenesis, we created a large library of

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Abbreviations: GPCR, G protein-coupled receptor; DREADD, designer receptors exclusively activated by a designer drug; CNO, clozapine-*N*-oxide; ACh, acetylcholine; CCh, carbachol; mAChR, muscarinic ACh receptor; hM_{1-5}, human mAChR subtypes 1–5; $hM_{1-5}D$, human mAChR DREADD subtypes 1–5; $rM3\Delta i3$, rat M_3 receptor containing a third intracellular loop deletion; hPASMC, human pulmonary artery smooth muscle cell; PI, phosphatidylinositol; GIRK, G protein inward-rectifying potassium channel.

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mutant $rM_3\Delta i3$ receptors and screened them initially for activation by clozapine, which has high affinity but which is an exceedingly weak partial agonist at native M_3 receptors. Ten independent clones, eight of which contained, among other mutations, a $Y148X^{3.33}$ mutation, were isolated from this initial screen to identify $rM_3\Delta i3$ receptor mutants activated by clozapine (see *Material and Methods* for details). Whereas yeast expressing wild-type $rM_3\Delta i3$ receptor responded only to either ACh or carbachol (CCh), an ACh analog, in liquid growth assays, yeast expressing any of the 10 clones were efficaciously activated by clozapine within ≈10-fold range of 10 μM concentration used to screen the library (Fig. 1 and SI Table 1). Strikingly, all mutants were defective in their response (reduced potency) to CCh and/or ACh, with one clone (G6) unable to be activated by 10 mM ACh (Fig. 1 and SI Table 1). This loss in ACh potency was serendipitous, because diminished response to the native ligand is one requirement of a DREADD. Although most clones tested were not activated by up to 100 μM CNO, a single clone, G2, was found to be mildly responsive to CNO (Fig. 1 and SI Table 1). This was a promising finding, because we anticipated that clones selected to be activated by clozapine may be prone to CNO activation as well.

A subset of clones were selected to be remutated to manufacture a second-generation library to select for mutants activated by 1 μM CNO (SI Table 1 and *SI Materials and Methods*). The second-generation $rM_3\Delta i3$ receptor mutants were efficaciously activated by CNO with a broad range of potencies (35–5,000 nM), which invariably was accompanied by a ≈100-fold increase in clozapine vs. CNO potency (Fig. 1 and SI Table 1). Again all mutants, with a single exception, had ≥1,000-fold reduction in ACh potency (SI Table 1).

A final library was constructed by using a subset of the second-generation CNO-responsive $rM_3\Delta i3$ receptor mutants as the mutagenesis template and screened with ≈5 nM CNO to select for potently activated mutants (SI Table 1 and *SI Materials and Methods*). Two clones were isolated that were activated by CNO and clozapine with potencies of <10 nM and ≈0.1 nM, respectively (Fig. 1 and SI Table 1).

Engineering an hM_3 Receptor to Be Potently and Efficaciously Activated by CNO in Human Cells. Using the yeast-based screen, we were able to greatly enhance the pharmacological attributes of CNO at the M_3 receptor. However, the ultimate goal was to create a full-length M_3 receptor suitable for mammalian use. We next determined whether the mutants obtained by the yeast screen would be directly useful in human cells. In mammalian cells, activation of $G_{q/11}$-coupled receptors, like M_3 receptor, can be measured by the accumulation of inositol monophosphate (IP_1), a metabolite of inositol trisphosphate (IP_3) produced by phospholipase C β catalyzed hydrolysis of phosphatidylinositol (PI).

CNO was essentially inactive at wild-type $rM_3\Delta i3$ transiently expressed in HEK T cells, whereas selected mutant $rM_3\Delta i3$ receptors displayed robust responses to CNO as well greatly diminished ACh potency (Fig. 2*A* and SI Table 2). Because many of the mutants with the highest CNO potencies had high levels of constitutive activity (Fig. 2*A* and SI Table 2), we next screened a focused library of hM_3 receptor mutants in HEK T cells to generate a receptor that was potently activated by CNO with minimal constitutive activity.

We chose the $Y149C^{3.33}$ mutant as an initial template because, (*i*) the $rM_3\Delta i3$ $Y148^{3.33}$ was the most consistently mutated residue to arise in the yeast screen, (*ii*) the $Tyr^{3.33}$ residue is known to be important in ligand binding of muscarinic ligands (13–15), and (*iii*) preliminary experiments showed that mutation of $Tyr\text{-}149^{3.33}$ in hM_3 receptor to either Cys or His similarly increased CNO responsiveness without increasing basal activity, yet the Y149C mutant had ≈100-fold better reduction in ACh potency compared with Y149H when expressed in HEK T cells (Fig. 2 and SI Table 2). Ultimately, we found the double mutant $Y149C^{3.33}/A239G^{5.46}$ to be the best possible combination of mutations to generate a hM_3 DREADD (hM_3D) (Fig. 2 and SI Table 2).

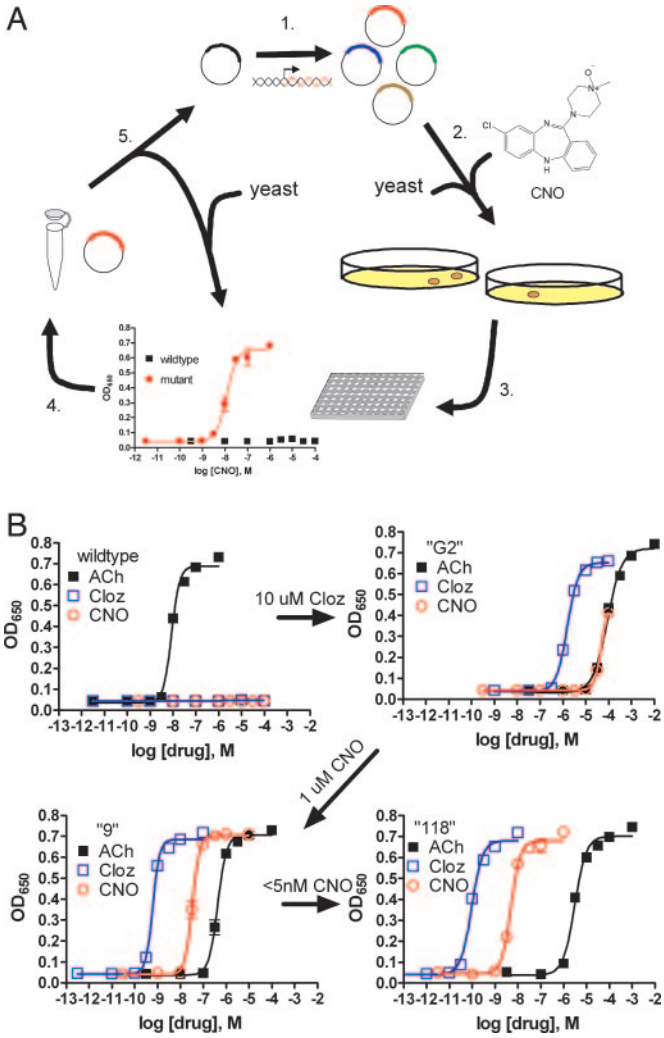

Fig. 1. Pharmacological profiles of an $rM_3\Delta i3$ receptor mutant selected during directed molecular evolution for CNO responsiveness. (*A*) Experimental design for directed evolution of mammalian GPCRs in yeast to create DREADDs. (*1*) Libraries of randomly mutated $rM_3\Delta i3$ receptors were produced by mutagenic PCR; (*2*) yeast-expressing mutant receptors activated by synthetic ligands (e.g., CNO) were selected for by growth on nutrient deficient medium; (*3*) mutants were verified by secondary liquid growth assays in 96-well plates; (*4*) plasmid DNA was isolated from yeast; (*5*) clones were retransformed into yeast to pharmacologically profile mutants by liquid growth assays, and those with desirable properties were sequenced and remutagenized for subsequent rounds of selection to yield receptors with higher potency. (*B*) Optical density at 650 nm of liquid cultures of yeast transformed with either wild type, clone ''G2'' (first library, 10 μM clozapine screen), clone ''9'' (second library, 1 μM CNO screen), or clone ''118'' (third library, 5-nM CNO screen) $rM_3\Delta i3$ receptors incubated with ACh (■), clozapine (□), or CNO (○). Data shown are mean ± SEM values of a representative experiment performed with two independent yeast transformants grown for each clone.

Characterization of hM_3 DREADD in Immortalized Human Pulmonary Artery Smooth Muscle Cells (hPASMC). Although smooth muscle cells endogenously express M_3 receptors (16), we found a particular human hPASMC line, devoid of endogenous muscarinic receptors, which would allowed us to investigate hM_3D receptor function in a null but native environment. We were able

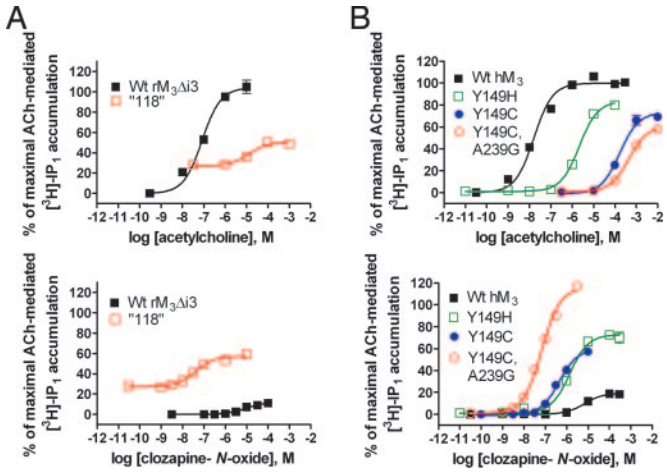

Fig. 2. Focused screening of hM_3 receptor mutants to optimize CNO stimulation of PI hydrolysis in HEK T cells. (*A*) Receptor-activated PI hydrolysis in HEK T cells transfected with a third-generation clone "118" (□) identified by the yeast screen and wild-type $rM_3\Delta i3$ receptor (■) treated with ACh (*Upper*) or CNO (*Lower*). (*B*) Similarly, wild-type (■) human M_3 receptors with the indicated single Y149H (□) and Y149C (●) or multiple mutations [Y149C, A239G (○); hM_3 DREADD], found in yeast screen clones, were transiently expressed in HEK T cells to measure receptor activation after treatment with either ACh (*Upper*) or CNO (*Lower*). Data of accumulated radiolabeled inositol 1-phosphate ([3H]-IP_1) are normalized to maximal ACh-mediated response in HEK T cells expressing either wild-type $rM_3\Delta i3$ (*A*) or wild-type hM_3 (*B*) receptors. Values shown are mean ± SEM from representative assays performed in duplicate.

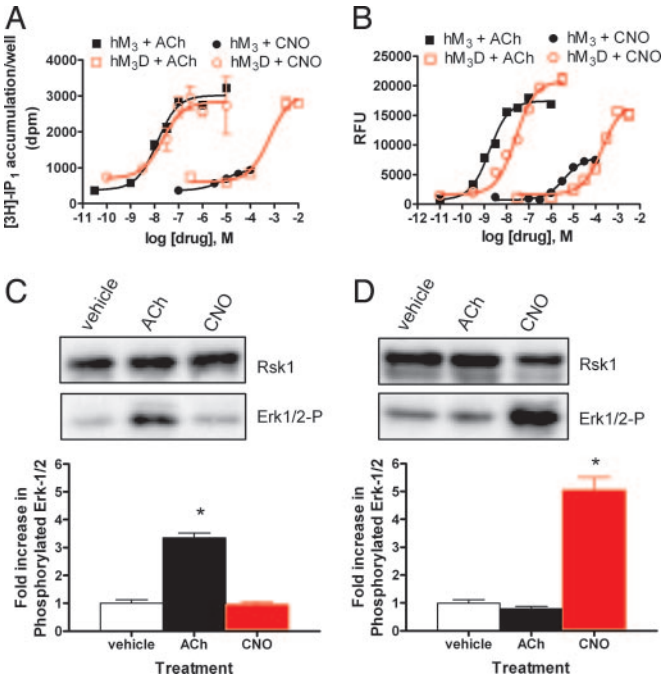

Fig. 3. Functional characterization of HA-epitope tagged wild-type and DREADD hM_3 receptors in immortalized hPASMC. (*A*) Drug-induced PI hydrolysis in immortalized hPASMCs stably expressing wild-type (hM_3) or DREADD (hM_3D) receptors. Shown are mean ± SEM values of a representative experiment performed in duplicate comparing [3H]-IP_1 accumulation after ACh or CNO treatment of hM_3 (■ or ●, respectively) or hM_3D (□ and ○, respectively) cells. (*B*) Calcium mobilization resulting from delivery of ACh or CNO to hM_3 (■ or ●, respectively) or hM_3D (□ or ○, respectively) expressing immortalized hPASMCs. A representative experiment, performed in quadruplicate, with mean values of Ca^{2+} mobilization in relative fluorescent units (RFU), is shown. (*C* and *D*) A representative experiment determining change in ERK-1/2 phosphorylation compared with p90 ribosomal S6 kinase loading control after incubating 1 μM of the indicated drugs for 5 min with immortalized hPASMCs expressing either wild-type (*C*) or DREADD hM_3 (*D*) receptors by immunoblot (*Upper*) with quantification of ERK-1/2 phosphorylation (*Lower*) from three independent experiments with significant differences (*, $P < 0.001$) between drug and vehicle treatment as determined by one-way ANOVA is shown.

to immortalize hPASMCs, as determined by propagating cells well past senescence, by the stable introduction of the gene encoding the human catalytic subunit of telomerase, hTERT (SI Fig. 8). When wild-type hM_3 receptors were stably expressed in immortalized hPASMCs, they faithfully elicited PI hydrolysis, Ca^{2+} mobilization and ERK-1/2 phosphorylation (see below and *SI Materials and Methods* for assay description) after ACh treatment (Fig. 3 and SI Tables 3–5). Significantly, the hM_3D receptor stably expressed in immortalized hPASMCs was potently (≈20–30 nM) and efficaciously activated by CNO (Fig. 3 *A* and *B* and SI Tables 3 and 4). The hM_3D was found to have a severe reduction (>40,000-fold) in ACh potency compared with the wild-type receptor (Fig. 3 and SI Tables 3 and 4).

Because some interactions between drug and receptor result in the onset of some but not all GPCR-related functions (17), we wanted to test whether hM_3D receptors could stimulate distinct downstream pathways after CNO treatment. A common signaling event radiating from activated GPCRs is the downstream phosphorylation of the MAPK proteins, ERK-1/2, resulting from the association of the MAPK-β-arrestin complex with the activated receptor (1). Treatment with 1 μM ACh but not CNO resulted in an enhanced level of ERK-1/2 phosphorylation in hPASMCs expressing hM_3 receptors (Fig. 3*C*). Conversely, hPASMCs expressing hM_3D receptors responded only to CNO under the same conditions (Fig. 3*D*). Together, these experiments demonstrate that the hM_3D receptor, created through the mutation of $Y149C^{3.33}$/$A239G^{5.46}$, fits the definition of a DREADD receptor.

Creation of a Family of Designer Muscarinic Receptors. Examination of protein alignments of mammalian mAChR family members revealed that $Y^{3.33}$ and $A^{5.46}$ are strictly conserved (data not shown). We also showed mutation of $Y^{6.51}$, a similarly strictly conserved residue, enhances the agonistic properties of clozapine-like drugs in two distinct family members (SI Table 2 and ref. 18). Taken together, we hypothesized that introduction of $Y^{3.33}C$ and $A^{5.46}G$ mutations in the other mAChR family members would allow CNO to activate these as well.

We individually expressed human $M_{1,\ 2,\ 4,\ and\ 5}$ DREADDs in immortalized hPASMCs to test whether CNO could similarly activate these receptors. Like the hM_3 receptor, we found that substitution of $Tyr^{3.33}$/$Ala^{5.46}$ into the other mAChR family members successfully transformed these receptors into CNO-activated DREADDs (Fig. 4*A* and SI Tables 3 and 4). Receptor activation remained sensitive, albeit with reduced potency, to the nonselective mACh receptor antagonists, atropine and (±)-quinuclidinyl benzilate, as monitored by Ca^{2+} mobilization (SI Table 6). We found treatment of immortalized hPASMCs with pertussis toxin, an irreversible $G_{i/o}$ inhibitor, selectively abolished the Ca^{2+} response in those cells expressing hM_2 and hM_4 compared with $hM_{1,\ 3,\ and\ 5}$ receptors (Fig. 4*B* and data not shown). Additionally, treatment of CNO selectively reduces forskolin-stimulated cAMP formation in hPASMCs expressing hM_2D receptors with a potency similar to that seen by Ca^{2+} mobilization assays (*SI Materials and Methods* and SI Tables 4 and 7). Therefore, hM_2 and hM_4 receptors retain their coupling properties when expressed in hPASMCs and presumably activate phospholipase C β by $G_{\beta\gamma}$ subunits (19).

CNO Silences Hippocampal Neurons Expressing hM_4D Receptors. M_4 receptors coupled to $G_{i/o}$ and $G_{i/o}$-coupled responses are increasingly used for neuronal silencing; we next determined whether the hM_4D could induce a G protein inward-rectifying

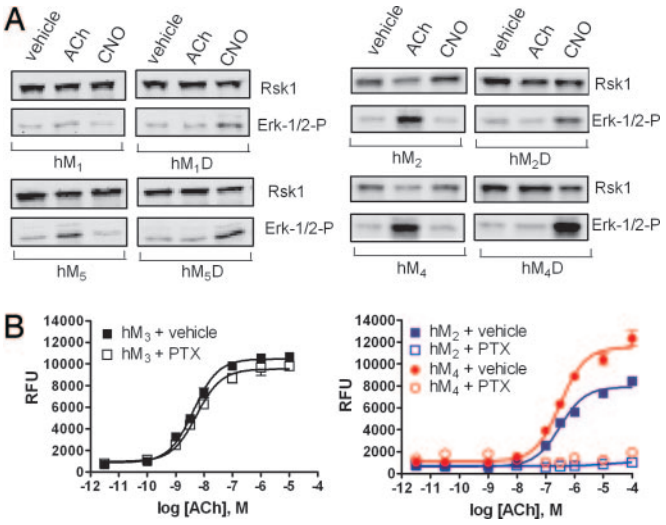

Fig. 4. Transformation of CNO response in mACh receptor family members. (*A*) Representative Western blots detecting either phosphorylated ERK-1/2 or, as a loading control, p90 ribosomal S6 kinase 1, after a 5-min application of a 1 μM final concentration of the indicated drugs to immortalized hPASMCs expressing wild-type or DREADD hM_1, hM_2, hM_4, and hM_5 receptors, as indicated. (*B*) Ca^{2+} mobilization response in immortalized hPASMCs expressing either the $G_{q/11}$-coupled hM_3 (black symbols; *Left*) or the $G_{i/o}$-coupled receptors hM_2 and hM_4 (blue and red symbols, respectively; *Right*) is shown. Cells were treated with ACh after overnight incubation with either vehicle control (solid symbols) or pertussis toxin or pertussis toxin (open symbols) on receptor-mediated Ca^{2+} release. Values shown are mean ± SEM from a representative experiment performed in triplicate.

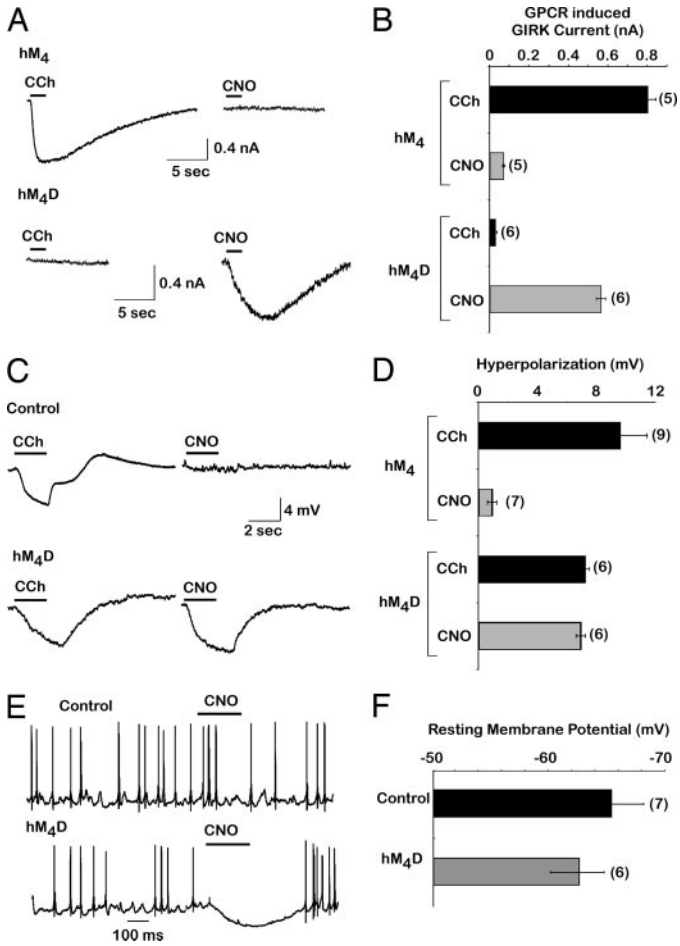

Fig. 5. Characterization of hM_4 DREADD on GIRK channel activation, membrane hyperpolarization, and neuronal silencing. (*A*) Sample traces of receptor-induced GIRK currents in HEK293 cells cotransfected with GIRK1/2 channel subunits and either hM_4 (*Upper*) or hM_4D (*Lower*) receptors and treated with either 10 μM CCh or CNO at a holding potential of −60 mV as described in *SI Materials and Methods*. (*B*) Comparison of induced GIRK channel currents at −60 mV when coexpressed with either hM_4 or hM_4D receptors. (*C*) Sample traces of CCh- and CNO-induced voltage changes in cultured hippocampal neurons infected with either hM_4 (*Upper*) or hM_4D (*Lower*). (*D*) Summary of the hM_4- and hM_4D-induced voltage changes by CCh and CNO. (*E*) Sample traces of hippocampal neurons spontaneously firing action potentials. In the presence of hM_4D receptors (lower trace), application of CNO induces hyperpolarization and neuronal silencing. (*F*) Comparison between the resting membrane potential of hM_4D receptor-expressing and control hippocampal neurons, indicating that the expression of hM_4D receptors does not change the resting membrane potential without activation of the receptor. Number of cells tested is indicated in parentheses with mean ± SEM shown.

potassium channel (GIRK) response in hippocampal neurons. Additionally, because mAChR family members are present, to a varying extent, in the brain and have been implicated in many processes, mACh DREADDs, expressed individually in isolated neuronal regions would be ideal tools to evaluate their roles in this multifarious background. Therefore, we investigated hM_4D receptor functionality in hippocampal neurons in which $G_{i/o}$ coupled receptors are known to induce neuronal silencing by activation of GIRK (20).

We first verified that the hM_4D receptor could modify GIRK currents in HEK cells transiently expressing GIRK1/2 subunits in combination with hM_4D receptor. As a control, we found that 10 μM CCh could induce currents in HEK cells transfected with GIRK1/2 subunits and wild-type hM_4 receptors, whereas as little to no current was found in cells treated with the same concentration of CNO (Fig. 5 *A* and *B Upper*). As anticipated, CNO compared with CCh, which had no effect, selectively activated GIRK channels in HEK cells that hM_4D receptors were coexpressed (Fig. 5 *A* and *B Lower*).

We next examined whether this behavior was recapitulated in cultured hippocampal neurons. When wild-type hM_4 receptors were transiently expressed in hippocampal neurons, we found that CCh, but not CNO, induced hyperpolarization (Fig. 5 *C* and *D Upper*). Significantly, CNO treatment of culture hippocampal neurons transiently expressing hM_4D receptor also resulted in neuronal hyperpolarization (Fig. 5 *C* and *D Lower*). Expression of hM_4D receptors in these neurons did not prevent CCh from modulating hyperpolarization through endogenous receptors (Fig. 5*C* and data not shown), suggesting that expression of hM_4D does not impede native receptor function. Additionally, CNO can selectively prevent action potential firing in cultured hippocampal neurons expressing hM_4D receptors (Fig. 5*E*) without significantly altering resting membrane potential in untreated infected neurons (Fig. 5*F*). These experiments clearly establish the unique ability of CNO to exclusively activate a cellular response in cells exogenously expressing hM_4D receptors and demonstrate their utility as a tool for *in vivo* neuronal silencing.

Discussion

Here we demonstrate that directed molecular evolution can be used to engineer a GPCR to be potently and efficaciously activated by a synthetic ligand that is pharmacologically inert. We extend this approach to show that an entire family of GPCRs, in this case the human muscarinic receptor family, can be created to be activated by an inert ligand. We also show that the signal transduction pathways and novel pharmacologies are faithfully recapitulated in a variety of cellular contexts including smooth muscle cells and hippocampal neurons. We suggest that at least one of these designer receptors, hM_4D, will prove useful for

neuronal silencing *in* vitro and *in vivo*. Using this general approach, it should be possible to eventually evolve GPCRs to bind any arbitrary drug-like small molecule by essentially designing the "lock" (GPCR) to fit the "key" (the ligand).

There are a number of technical considerations that should be borne in mind when embarking on a campaign to reverse engineer a GPCR. We found, in general, that most of the key "transforming" residues were found in the initial screen and, compared with those mutants found in later screens, more of these mutants had unchanged basal activity when expressed in human cells. Therefore, it is advisable to screen a large initial library, which is likely to have the highest impact in unearthing important and easily identifiable residues, as well as counter screen promising mutants in mammalian cells early on to reduce the risk of evolving constitutively active receptors. A distinct advantage to directed evolution by using randomized libraries is that is unbiased. However, the screen can be significantly enhanced with prior knowledge of residue importance. Not surprisingly, four residues ($Y^{3.33}$, $T^{5.42}$, $W^{6.48}$, and $Y^{6.51}$) involved in binding to ACh and other mAChR ligands (13, 21) were mutated in our screen, illustrating that special attention should be given to these residues or local helical regions. Intriguingly, this lends evidence to the notion that clozapine-related ligands, such as clozapine, CNO, and olanzapine (data not shown), which readily activate the hM_3D receptor, may either bind distinctly within the ACh orthosteric site or, as previously suggested, are allosteric ligands (18). Similarly, it is potentially worthwhile to perform saturating mutagenesis at residue "hot spots" to optimize functionality, because we found multiple substitutions at one residue (e.g., $Y148C/H/N^{3.33}$) enhanced $rM_3\Delta i3$ receptor activation by clozapine/CNO in yeast and considering most single-nucleotide mutations within a codon will not change the translated amino acid to all of the other possible amino acids. For example, in our initial library, if all possible single-nucleotide mutations were introduced within the Y148 codon, only 7 of 19 possible amino acid substitutions would have been achieved. In this way, subsequent libraries would be seeded with an optimized receptor while avoiding propagation of unimportant secondary mutations.

The first reported receptor that can be considered a DREADD is a β_2-adrenergic receptor rationally mutated at the highly conserved residue $D113S^{3.32}$. This mutant had a severe reduction in both potency and efficacy to catecholamine agonists but instead was fully activated, albeit with low potency (>40 μM), by compounds foreign to the wild-type receptor (22). More recently, rational mutation of $F435A^{6.55}$ in the histamine H_1 receptor (23) and $D100A^{3.32}$ of the 5-HT_4 receptor (8) [the first $G_{q/11}$ and G_s-coupled receptors activated solely by synthetic ligands (RASSLs), respectively] enhanced the potency for synthetic ligands and reduced affinity and potency for their native ligands, although not nearly to same extent as the mACh DREADDs described here.

Here, guided by mutations arising from directed molecular evolution in yeast, we discovered $Y^{3.33}C$ and $A^{5.46}G$ mutations to be sufficient to generate a human hM_3 DREADD that responded potently, efficaciously, and selectively to CNO vs. ACh by PI hydrolysis, Ca^{2+} mobilization, and ERK-1/2 phosphorylation in a physiologically relevant background. Fortuitously, mutations at these same two conserved residues effectively switched the responsiveness of all other human mAChR family members so that CNO selectively activated them in two independent cellular settings by using a variety of signaling readouts. Interestingly, this dramatic enhancement of functional activation is accompanied by a minimal (<5-fold) increase in CNO affinity, which remains to be explored (not shown).

Knockout of individual or combinations of mACh receptors in mice has given enormous insight into function and possible pathophysiologies resulting to disruption of these receptors (16). However, these studies provide limited insight into what role either acute or chronic receptor overstimulation plays in disease states. For example, studies have shown that M_1 and M_4 knockout mice have increased locomotor activity and modified dopamine levels and/or release (24–26); however, it is difficult to decipher whether and which receptors exert a tonic check for movement or whether overstimulation of either receptor may participate in Parkinson's disease. The unique ability of CNO to induce neuronal silencing in neurons that express hM_4 DREADD will facilitate studies aimed at investigating the role of defined neuronal populations in a large number of physiological and pathological processes.

Materials and Methods

Plasmid Construction and Materials. p416GPD was purchased from American Type Culture Collection. p416GPD-$rM_3\Delta i3R$ and pMP290 were previously described (12). pcDNA3.1(+) containing inserts of each human hM_{1-5} receptor was obtained from (University of Missouri Rolla cDNA Resource Center, Rolla, MO). Single codon mutations were sequentially introduced into pcDNA3.1(+) constructs containing the human muscarinic receptors by Quikchange Site-directed mutagenesis kit (Stratagene, La Jolla, CA). hM_1 and hM_3 receptors were subcloned into the BamHI and XbaI sites of a modified pcDNA3.1(+), pcDNA3.1(+)-EcoRI, which contains an additional EcoRI site 3′ of the XbaI site. Retroviral vectors pBabepuro (27) and pHA-Babepuro were generated by the addition of a Kozak sequence and sequence encoding an HA epitope tag with an initiating methionine (MYPYDVPDYA) into the BamHI site of pBabepuro. pcDNA3.1(+)-EcoRI-hM1 and pcDNA3.1(+)-EcoRI-hM_3 wild-type and mutant receptors were digested with BamHI and EcoRI and inserted into pBabepuro and, in the case of hM_3, also pHA-Babepuro digested with the same enzymes. pcDNA3.1(+)-hM_2 was digested with EcoRI and XhoI and inserted into EcoRI and SalI sites of pBabepuro. pcDNA3.1(+)-hM_4 and pcDNA3.1(+)-hM_5 were digested with BamHI and XhoI and inserted into BamHI and SalI sites of pBabepuro. GIRK1/2 subunits were cloned into pcDNA3.1. Sindbis virus vector SinRep(nsP2S^{726}) and helper DH-BB were kindly provided by P. Osten (Max Planck Institute for Medical Research, Heidelberg, Germany) (28). Human M_4 was cloned into the PmlI site of SinRep(nsP2S^{726}) after digesting of pcDNA3.1(+)-hM_4 with PmeI. pBabehygro-flag-hTERT was previously described (29). Sequence identity was determined by automated sequencing (Cleveland Genomics, Cleveland, OH).

Mutant libraries were generated by mutagenic PCR by using GeneMorph II Random Mutagenesis kit (Stratagene) following the manufacturer's protocol by using 30 cycles and ≈250 ng of p416GPD-$rM_3\Delta i3R$ DNA template and primers 5′-ACACCAAGAACTTAGTTTCGACGG and 5′-GGCGTGAATGTAAGCGTGAC, which resulted in an empirical mutational rate of ≈3.5 mutations per kilobase. Mutagenic products were digested with XhoI and XbaI and cloned into the same sites in p416GPD and then transformed into XL10 Gold bacteria (Stratagene) to amplify plasmid library. First-generation library (3×10^4 independent clones, with 6×10^4 colonies screened) used wild-type p416GPD-$rM_3\Delta i3R$ as template. Second-generation library (7×10^4 independent clones, with 2.5×10^4 colonies screened) was amplified from equimolar amounts of plasmid clones "B4," "B11," "G2," "G6," and "G15," chosen for their diversity in mutations and alteration in drug response. Similarly, plasmid clones "3," "7," "8," "9," and "16" were used to produce the third-generation library (6×10^5 independent clones, with 8×10^4 colonies screened).

L-Quinuclidinyl[*phenyl*-4-^{3}H]benzilate (36.5 Ci/mmol) was purchased from GE Healthcare Biosciences (Piscataway, NJ). All other chemicals [(±)-quinuclidinyl benzilate, CNO, ACh, CCh, and atropine, 3-amino-1,2,4-triazole (3-AT)] and amino acids were purchased from Sigma–Aldrich (St. Louis, MO). Synthetic yeast media was purchased from Q-BIOgene (Irvine, CA) and BD Biosciences (San Diego, CA).

Yeast Library Screen and Growth Assays. Plasmid library DNA was transformed into *S. cerevisiae* strain MPY578q5 (12) by a high-efficiency procedure (30) and selected for growth at 30°C for 3–4 days in the presence 10 μM clozapine, 1 μM CNO, or ≈5 nM CNO for first-, second-, and third-generation libraries, respectively, on agar plates containing yeast assay buffer [synthetic complete (SC) media (pH 6.9) lacking uracil and histidine and supplemented with 20 mM 3-AT]. Colonies were then submitted to a secondary liquid-assay screen in which they were grown overnight in SC media without uracil, pelleted, washed with water, then diluted in yeast assay buffer to a final concentration of OD_{650} ≈0.003. Diluted yeast (150 μl) was added to a 96-well plate containing 50 μl of yeast assay buffer supplemented with varying drug concentrations and grown with mild agitation at 25°C for 3 days, at which time the plate was read at OD_{650} on a microplate reader by using SOFTmax Pro 4.3.1 software (Molecular Devices, Sunnyvale, CA). Plasmids were isolated from responsive colonies as described (31) and retransformed into MPY578q5 and/or sequenced. Pharmacological profiling of clones was performed by liquid assay by using two to three independent transformants and typically repeated two to six times.

Viral Production, Cell Line Establishment, and Cell Culture. Human embryonic kidney strain, HEK T, was purchased from American Type Culture Collection (#CRL-11268; Manassas, VA), hPASMCs were purchased from ScienCell (San Diego, CA), and HEK strain 293TS cells were kindly provided by C. M. Counter (Duke University, Durham, NC). Amphotropic retrovirus was produced by cotransfecting a 6-cm plate of 293TS grown in DMEM/10% FBS with 1 μg of pBabehygro-flag-hTERT with 1 μg of the amphotropic packaging plasmid pCL-10A1 (Imgenex, San Diego, CA) by using FuGene6 (Roche, Indianapolis, IN) transfection reagent. Virus-containing medium was collected between 24 and 60 h after transfection, filtered with a sterile 0.45-μm filter, supplemented with a final concentration of 4 μg/ml polybrene (Sigma), and incubated with hPASMCs plated on 0.2% porcine gelatin-coated plates for 6–12 h to infect. After hTERT infection, hPASMCs were then media-changed into Smooth Muscle Cell Medium (ScienCell) and 36–48 h after infection, cells were selected and subsequently grown in Smooth Muscle Cell Medium and 50 μg/ml hygromycin B. The first confluent plate under selection was designated as population doubling 0. hPASMCs were grown on 0.2% gelatin-coated plates in hygromycin B-supplemented Smooth Muscle Cell Medium for immortalization study. Polyclonal hPASMCs stably expressing human mACh receptors were created by retroviral infection, as described above by using pBabepuro or pHA-Babepuro constructs, and grown on 0.2% gelatin-coated plates in DMEM/10% FBS supplemented with 1 μg/ml puromycin. Sindbis virus was produced from electroporated BHK cells grown in MEM/5% FBS according to published procedures (32). All cells were grown at 37°C in 5% CO_2 atmosphere.

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