HHS Public Access

Author manuscript

Neuron. Author manuscript; available in PMC 2017 February 17.

Published in final edited form as:

Neuron. 2016 February 17; 89(4): 683–694. doi:10.1016/j.neuron.2016.01.040.

DREADDs for Neuroscientists

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Abstract

To understand brain function, it is essential that we discover how cellular signaling specifies normal and pathological brain function. In this regard, chemogenetic technologies represent valuable platforms for manipulating neuronal and non-neuronal signal transduction in a cell-type-specific fashion in freely moving animals. Designer Receptors Exclusively Activated by Designer Drugs (DREADD)-based chemogenetic tools are now commonly used by neuroscientists to identify the circuitry and cellular signals that specify behavior, perceptions, emotions, innate drives, and motor functions in species ranging from flies to nonhuman primates. Here I provide a primer on DREADDs highlighting key technical and conceptual considerations and identify challenges for chemogenetics going forward.

Chemogenetics has been defined as a method by which proteins are engineered to interact with previously unrecognized small molecule chemical actuators (Forkmann and Dangelmayr, 1980; Sternson and Roth, 2014; Strobel, 1998). Over the past two decades, a large number of chemogenetic (also known as “chemical genetic”; (Bishop et al., 1998; Strader et al., 1991; Chen et al., 2005; Sternson and Roth, 2014) platforms have been invented that have been useful for biologists in general and most especially for neuroscientists.

A number of protein classes (Table 1) have been chemogenetically engineered including kinases (Bishop et al., 1998; Bishop et al., 2000; Chen et al., 2005; Cohen et al., 2005; Dar et al., 2012; Liu et al., 1998), non-kinase enzymes (Collot et al., 2003; Häring and Distefano, 2001; Klein et al., 2005; Strobel, 1998), G protein-coupled receptors (GPCRs) (Alexander et al., 2009; Armbruster and Roth, 2005; Armbruster et al., 2007; Redfern et al., 1999; Redfern et al., 2000; Vardy et al., 2015), and ligand-gated ion channels (Arenkiel et al., 2008; Lerchner et al., 2007; Magnus et al., 2011; Zemelman et al., 2003) (for recent review, see Sternson and Roth, 2014). Of these various classes of chemogenetically engineered proteins, the most widely used to date have been Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) (Armbruster and Roth, 2005; Armbruster et al., 2007), and this *Neuron* Primer is devoted to them.

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How an Understanding of GPCR Molecular Pharmacology Facilitates the Appropriate Use of DREADD Technology

Before discussing DREADDs in detail, I will first summarize essential foundational concepts of GPCR molecular pharmacology and signaling. This background information is essential for all readers so that they may understand how DREADDs may be most effectively used. According to classical models of GPCR action GPCRs exist in multiple ligand-dependent and -independent states. These multiple GPCR states range from "fully inactive" to "partially active" to "fully active" to "signaling complexes" (Roth and Marshall, 2012; Samama et al., 1993). As depicted in Figure 1, GPCRs (R) are modulated by ligands (L) and can interact with both hetereotrimeric G proteins (G) and β-arrestins (βArr). According to the most recent findings, multiple inactive (e.g., "ground") states exist that can be stabilized by ligands (R_1L, R_2L, and so on) or can even occur in the absence of ligands (R). Sodium ions stabilize the ground state by exerting a negative allosteric modulation via a highly conserved allosteric site (Fenalti et al., 2014; Katritch et al., 2014). Drugs that stabilize the R_1L, R_2L ground states function as inverse agonists (Samama et al., 1993, 1994). Inverse agonists are also known as "antagonists with negative intrinsic activity" (Costa and Herz, 1989). The evidence for multiple GPCR states is supported by classical molecular pharmacological (Samama et al., 1993, 1994), biophysical (Gether et al., 1995), and structural studies (Manglik et al., 2015).

Both full and partial agonists stabilize the active state (R*L) and promote the formation of a signaling complex (e.g., the "ternary complex") consisting of (1) the active receptor, (2) an agonist, and (3) the heterotrimeric G protein (R*LG) (De Lean et al., 1980; Samama et al., 1993). In addition to the ligand-induced activation and inactivation of GPCRs, GPCRs can also spontaneously isomerize to an active state (R*) in the absence of ligand. Further, this active state can spontaneously interact with G proteins to yield a binary signaling complex in the absence of ligand (R*G) (Samama et al., 1993). This active state in the absence of ligand is termed "constitutive activity."

GPCRs (R) also interact with arrestins (βArr) to form alternative signaling complexes (R**L and R**L βArr) (Luttrell et al., 1999; Wacker et al., 2013; Kroeze et al., 2015). GPCRs with high levels of basal (e.g., constitutive) activity can spontaneously interact with βArr to form an R**βArr complex in the absence of agonist (Marion et al., 2004; Kroeze et al., 2015). Based on high-resolution crystal structures of GPCR-arrestin complexes, the R*L βArr state appears to sterically occlude G-protein interactions with the receptor thereby abolishing G-protein signaling (Shukla et al., 2014; Kang et al., 2015). Accordingly, the interaction of GPCRs with βArr also represents a "desensitized" or inactive G-protein state of the complex. At the single molecule level, when GPCRs are activated by agonists, they can couple to either G-proteins or arrestins, but not both. At the cellular level conformational ensembles of all of the states identified above exist. Biasing for one particular state is dependent upon both the cellular context and the available ligand (Vardy and Roth, 2013; Wacker et al., 2013).

A clear understanding of the implications of this extended and modified ternary complex model—for which there is now compelling biochemical (Strachan et al., 2014), biophysical

(Sounier et al., 2015; Nygaard et al., 2013), pharmacological (Weiss et al., 2013; Fenalti et al., 2014), and structural evidence (Fenalti et al., 2014; Manglik et al., 2015; Rasmussen et al., 2011; Wacker et al., 2013)—is crucial for understanding how GPCR-based chemogenetic technologies can be harnessed in neuroscience. Thus, for instance, a major concern for chemogenetic technologies is the possibility that high levels of expression of an engineered protein might have effects in the absence of chemical activation (Conklin et al., 2008). Indeed, many of the second-generation chemogenetic GPCRs (e.g., receptors activated solely by synthetic ligands [RASSLs]) had high basal levels of activity leading to phenotypes in the absence of chemical actuators (Hsiao et al., 2008; Sweger et al., 2007).

As depicted in Figure 1, a GPCR with constitutive activity would be more likely to exist in the R* state and thus to spontaneously interact with G proteins to yield a signaling complex in the absence of ligand (R*G). As shown in Figure 2A, high levels of expression of a GPCR with constitutive activity leads to signaling in the absence of ligand. Although no studies to date have demonstrated a basal phenotype for any of the known DREADDs, it is important to express DREADDS at the lowest level consistent with experimental design. For hM3Dq (Alexander et al., 2009) and hM4Di (Zhu et al., 2014), life-long and extremely high levels of expression were attained using a genetically encoded tetracycline-sensitive induction system without basal electrophysiological, behavioral, or anatomical abnormalities being observed. More modest life-long expression of the Gs-DREADD (GsD) also was attained without any detectible electrophysiological, behavioral, or anatomical phenotype (Farrell et al., 2013). High levels of virally mediated expression of various DREADDs have yet not been reported to yield any significant basal phenotypes (Urban et al., 2015; Vardy et al., 2015; Denis et al., 2015; Isosaka et al., 2015; Hayashi et al., 2015). Of course, the absence of reports of basal activity does not imply the absence of basal activity. Going forward, if basal activity is observed, it would be prudent to simply lower the level of DREADD expression using (1) a lower titer of virus, (2) a weaker promotor, or (3) modifying post-transcriptional expression (e.g., deleting a woodchuck hepatitis virus [WPRE] element from the 3′ end of the construct). Thus, based on the law of mass action, decreasing [R] decreases the probability of [R]→[R*]→[R*G] (e.g., inactive, active, and signaling state) transitions.

An additional concern with DREADD technologies relates to issues of desensitization and subsequent receptor downregulation. Thus, following repeated dosing with a DREADD chemical actuator, one might observe diminished responses due to receptor desensitization and downregulation. This diminished response might be predicted because it is well known that GPCRs can be desensitized and subsequently internalized and downregulated following agonist-induced activation (DeWire et al., 2007).

As depicted in Figure 2A, the degree of desensitization depends greatly on the extent to which receptors are over-expressed and the subsequent amount of “receptor reserve.” “Receptor reserve” is a pharmacological term that describes the phenomenon whereby a maximum agonist response can be achieved with less than full occupancy of all of the receptors by agonists (Ruffolo, 1982). From a practical perspective, the concept of receptor reserve predicts that when DREADD expression is quite high, lower concentrations of the chemical actuator are needed to achieve a maximal response (Figure 2A). Additionally,

when receptors are desensitized or downregulated, there may be no change in the maximum response elicited by the agonist, but there may be a shift in the dose-response curve to the right because of receptor reserve (2A). Thus, when DREADDs are expressed at high levels relative to native GPCRs via viral or transgenic approaches, the cellular and behavioral responses will be less sensitive to repeated dosing than when they are expressed at lower levels. This phenomenon might explain why no significant desensitization was seen when DREADDs were virally or transgenically expressed (Alexander et al., 2009; Krashes et al., 2011)

Another conceptual issue specific to DREADD technology relates to whether the effects observed regarding neuronal output and behavior occur due to canonical or non-canonical GPCR signaling. As shown in Figure 1, agonists may activate multiple downstream effector pathways, and it is likely that actions other than simply enhancing or silencing neural activity can result when DREADDs are activated. Specifically, one might be concerned about conditions in which βArr signaling is activated. To date, there have been no reports suggesting that the actions of the silencing (e.g., Gi-based DREADDs) or activating (e.g., Gq-based DREADDs) DREADDs on neuronal activity and subsequent physiological readouts could be explained by any mechanism other than altered neuronal firing. Pertinent to this issue, many studies have used DREADD and optogenetic technologies on the same neuronal populations. These studies have invariably identified essentially equivalent effects in terms of both the valence and magnitude of the effect on the physiological readout, although the duration is typically longer with DREADDs (Table 2 for representative examples). Indeed, many investigators now use both DREADD and optogenetic technologies to provide independent and converging lines of evidence in terms of both sufficiency and necessity when deconstructing neural circuits.

Current DREADDs

As shown in Table 1 (for recent reviews, see Sternson and Roth, 2014;Urban and Roth, 2015; English and Roth, 2015), there now exist many GPCR-based chemogenetic tools. These include first- (“Alelle-specific GPCRs”; Strader et al., 1991), second-(RASSLs and “Engineered GPCRs”; Coward et al., 1998; Westkaemper et al., 1999), and third-generation (DREADDs; Armbruster and Roth, 2005; Armbruster et al., 2007) platforms. Currently, DREADDs are the most widely used chemogenetic tool; the available DREADDs are shown in Figure 3A.

Gq-DREADDS, CNO Analogues, and Basal Activity

For enhancing neuronal firing and activating Gq signaling in neuronal and non-neuronal cells, the hM3Dq DREADD is typically used (Alexander et al., 2009; Armbruster et al., 2007). hM3Dq can be activated by clozapine-N-oxide (CNO)—a pharmacologically inert metabolite of the atypical antipsychotic drug clozapine (Armbruster et al., 2007; Roth et al., 1994). When the original DREADDs were invented, three Gq-coupled DREADDs were created, each of which was based on a different human muscarinic receptor: hM1Dq, hM3Dq, and hM5Dq (Armbruster et al., 2007). All three Gq-DREADDs are activated by low nM concentrations of CNO and mobilize intracellular calcium (Armbruster et al., 2007).

All three Gq-DREADDs could be used as excitatory DREADDs, although hM3Dq appears to be most frequently used.

CNO (Figure 3B) represents the prototypical chemical actuator for Gq-DREADDs. Based on many reports, CNO appears to be pharmacologically and behaviorally inert in mice (Alexander et al., 2009; Krashes et al., 2011; Farrell et al., 2013; Guettier et al., 2009; Urban et al., 2015; Zhu et al., 2014) and rats (Ferguson et al., 2011, 2013) when administered at the recommended doses (generally 0.1–3 mg/kg). CNO may be metabolized via back-transformation to clozapine—especially in guinea pigs, humans (Jann et al., 1994), and nonhuman primates (unpublished obsrevations). Although the amount of back-metabolism to clozapine is low even in humans (10% or less by mass; Jann et al., 1994), care should be taken to ensure that clozapine-like side-effects (e.g., hypotension, sedation, and anticholinergic syndrome) do not occur by keeping the dose as small as possible and by always performing appropriate controls (e.g., administering CNO to animals expressing GFP or similarly irrelevant protein).

CNO has excellent drug-like properties with rapid CNS penetration and distribution in mice (Bender et al., 1994). CNO appears to have at least a 60 min residence in vivo in mice following intraperitoneal administration (Bender et al., 1994). Given the long residence time of CNO, it is not surprising that the in vivo effects of CNO-mediated activation of hM3Dq can be both robust and prolonged (Alexander et al., 2009; Krashes et al., 2013). Clearly, unless long-term activation of Gq signaling and neuronal firing is needed, it is recommended that the lowest effective dose of CNO be administered so that only peak CNO concentrations activate the DREADD (Figures 2B and 2C). As can be seen in Figure 2B, when a large dose of CNO is administered, the effects of CNO may be greatly prolonged because brain levels of CNO remain higher than the threshold for activation of the DREADD receptor. By contrast, lower systemic doses of CNO (Figure 2C) would result in transient peak activation and then a relatively rapid decay of activity.

Because of the potential for back-metabolism of CNO to clozapine and other clozapine metabolites in non-rodent species—including the pharmacologically diverse compound N-desmethyl-clozapine (NDMC) (Davies et al., 2005)—we have developed new non-CNO chemical actuators (Chen et al., 2015). The first of these—Compound 21 (Figure 3B)—has minimal off-target activity and exquisite selectivity for activating hM3Dq versus muscarinic and other GPCRs (Chen et al., 2015). Preliminary studies indicate that Compound 21 has equivalent potency in studies in vivo when compared with CNO (unpublished data). Compound 21 likely cannot be metabolized via normal routes to clozapine or any related compound and thus represents an alternative to CNO for studies in which back metabolism of CNO to clozapine is problematic.

An additional compound especially suited for translational studies is perlapine (Figure 3B), a drug that is approved for use in Japan for insomnia. Perlapine has >10,000-fold selectivity for activating hM3Dq versus muscarinic receptors with an EC_{50} at hM3Dq of 2.8 nM (Chen et al., 2015). Given perlapine's modest affinity for some biogenic amine receptors (e.g., 5-HT2A, 5-HT6, 5-HT7, and D4) (Davies et al., 2005; Roth et al., 1992, 1994, 1995), it is essential to test perlapine at the lowest possible dose in animals not expressing DREADDs

before embarking on studies involving DREADDs. These preliminary studies would ensure that off-target actions of perlapine do not interfere with the phenomena studied. Perlapine will likely find its greatest utility in translational studies of DREADDs in primates and, perhaps, in humans given that it is approved for use in humans. It is likely that further investigation of the scaffolds for compound 21 and perlapine will yield even more effective, potent, and selective chemical actuators for muscarinic-based DREADDs.

The first report that CNO-induced activation of hM3Dq depolarized and excited genetically defined neurons appeared in 2009 (Alexander et al 2009). Since then, hM3Dq has been widely used to enhance neuronal firing, and I here cite only representative examples in which feeding (Krashes et al., 2011; Atasoy et al., 2012), energy expenditure (Kong et al., 2012), locomotion and striatal synaptogenesis (Kozorovitskiy et al., 2012), memory (Garner et al., 2012), and social behaviors (Peñagarikano et al., 2015) have been modulated by hM3Dq in vivo. Because hM3Dq activation induces intracellular calcium release, it has also been used to “activate” astrocytes (Agulhon et al., 2013; Bull et al., 2014; Scofield et al., 2015), hepatocytes (Li et al., 2013), pancreatic βcells (Guettier et al., 2009; Jain et al., 2013), vascular smooth muscle cells (Armbruster et al., 2007), and iPS-derived neuroblasts (Dell’Anno et al., 2014).

Multiple options are currently available for expressing hM3Dq in genetically specified cells. Thus, genetically engineered mice are now available for expressing hM3Dq under control of tetracycline (*tet-off*) promotor (Alexander et al., 2009; Garner et al., 2012) and via Cre-mediated recombination (Teissier et al., 2015) (Table 3); and some of these are available from Jackson Labs (Table 3). Many labs are using the FLEX switch (Schnütgen et al., 2003) as adapted by the Sternson lab for AAV (Atasoy et al., 2008) that allows for Cre-mediated cell-type-specific expression in any cell type for which there is a Cre-driver line available (Figure 4A). A key innovation for the development of AAV- and lentiviral-based FLEX switch vectors (also known as double-floxed inverse open reading frame [DIO]) (Gradinaru et al., 2010) was the use of separate antiparallel loxP-type recombination sites (especially loxP and lox2272) that allow for homotypic but not heterotypic recombination (Lee and Saito, 1998). In addition, a growing number of promotors have been characterized that allow for cell-type-specific expression using many viral vectors, including modified herpes simplex viruses (HSVs) (Ferguson et al., 2010), AAV (Zhu et al., 2014; Scofield et al., 2015), and lentivirus (Mahler et al., 2014; Vazey and Aston-Jones, 2014). Finally, the use of canine adenovirus (CAV) expressing Cre-recombinase (CAV-Cre) allows for the projection-specific expression of DREADDs. Projection-specific expression of DREADD is possible because CAV-Cre is preferentially retrogradely transported to neuronal somas. In the neuronal cell bodies, recombination of AAV-FLEX-DREADD constructs can occur to allow expression of DREADDs in a projection-specific fashion (Boender et al., 2014) (Figure 4B). The use of CAV-Cre and FLEX-DREADD constructs has been dubbed the “Retro-DREADD” technique (Marchant et al., 2016) and in theory could be used for intersectional and multiplexed applications.

Gi-DREADDs

To date there are three Gi-DREADDs: hM2Di, hM4Di, and KORD. Of these, hM2Di and hM4Di can be activated by CNO (Armbruster et al., 2007), compound 21, and perlapine (B.L. Roth, unpublished data). Currently, hM4Di is the most commonly used inhibitory DREADD (for review, see Urban and Roth, 2015). Many labs have reported successful neuronal silencing with hM4Di, and only representative reports are cited wherein DREADDs silenced neurons (Armbruster et al., 2007), modulated amphetamine sensitization (Ferguson et al., 2011) and synaptic plasticity (Kozorovitskiy et al., 2012), regulated breathing (Ray et al., 2011), feeding (Carter et al., 2013), itching (Bourane et al., 2015), and emotional(Teissier et al., 2015) behaviors.

The κ-opioid-derived DREADD (KORD) is a new chemogenetic GPCR that is activated by the pharmacologically inert compound. Thus, salvinorin B has no activity at any other tested molecular target (>350 GPCRs, ion channels, transporters, and enzymes evaluated) and thus has no apparent off-target activity (Figure 3C) (Chavkin et al., 2004; Vardy et al., 2015). Salvinorin B does retain modest affinity for KOR (>100 nM) so that investigators using the KORD should use the lowest dose possible and verify no effects of salvinorin B in the absence of KORD. Several labs have reported successful inhibition of neural activity with KORD (Marchant et al., 2016; Vardy et al., 2015; Denis et al., 2015).

Both hM4Di and KORD appear to inhibit neuronal activity via two mechanisms: (a) induction of hyperpolarization by Gβ/γ-mediated activation of G-protein inwardly rectifying potassium channels (GIRKs) (Armbruster et al., 2007; Vardy et al., 2015) and (b) via inhibition of the presynaptic release of neurotransmitters (e.g., synaptic silencing) (Stachniak et al., 2014; Vardy et al., 2015). Thus, unlike bacterial opsins, which silence neurons via a strong hyperpolarization and with millisecond precision, DREADDs induce a modest hyperpolarization and an apparently strong inhibition of axonal release of neurotransmitter (Stachniak et al., 2014; Vardy et al., 2015) in the s-min-hr time frame. Because of the dependence upon Gβ/γ-mediated activation of GIRKs for inducing hyperpolarization, it is possible that hM4Di and KORD might not hyperpolarize all neurons. To date, there have been no reports that hM4Di or KORD fail to silence or inhibit neuronal activity.

Given that the Gi-coupled DREADDs have effects on terminal release the possibility that CNO (or an analogue) or SalB (or analogue) could micro-infused to locally suppress neural activity has been tested (Figure 4C). Thus, at least two groups (Stachniak et al., 2014; Mahler et al., 2014) have successfully silenced terminal projections via local infusion of CNO. Terminal axons have also been activated by local CNO infusion into rats expression hM3Dq (Vazey and Aston-Jones, 2014). For selective axonal silencing, an hM4Di variant has been created (Figure 3A; $hM4D^{nrxn}$) that is targeted preferentially to axons and axon terminals (Stachniak et al., 2014).

The availability of a new inhibitory DREADD—KORD—activated by a ligand orthogonal to CNO now allows for the multiplexed and bidirectional chemogenetic modulation of neural activity and behavior (Vardy et al., 2015). Thus, we recently demonstrated that KORD may be expressed simultaneously with hM3Dq to allow for the sequential

chemogenetic activation (with hM3Dq and CNO) and inhibition (with SalB and KORD) (Vardy et al., 2015) of neuronal activity (Figure 4D). It is likely that KORD and hM3Dq could be combined in a combinatorial fashion with various opsins and other chemogenetic tools (e.g., PSEM and PSAMs) to afford highly multiplexed control of neuronal activity with millisecond precision (e.g., with opsins) and for long periods of time for behavioral studies (e.g., with DREADDs or PSAMs).

Gs- and β-Arrestin-DREADDs

The only DREADD coupled to Gs was created by swapping the intracellular regions of the turkey erythrocyte β adrenergic receptor for equivalent regions of a rat M3 DREADD to create a rat Gs-DREADD (Guettier et al., 2009) (Figure 3A). Unlike the current Gq and Gi-DREADDs, the Gs-DREADD (GsD) has a small degree of constitutive activity in transfected cells (Guettier et al., 2009) leading to a modest basal phenotype in pancreatic β cells (Guettier et al., 2009; Jain et al., 2013). Given that $G\alpha_{olf}$ is the major Gs-like Gα protein in some brain regions (Zhuang et al., 2000), it was critical to determine if GsD can also activate $G\alpha_{olf.}$ Importantly, we demonstrated that GsD efficiently couples to $G\alpha_{olf}$ in vitro and in vivo (Farrell et al., 2013) and that GsD had minimal constitutive activity for $G\alpha_{olf}$-mediated signaling in vitro and in vivo (Farrell et al., 2013). GsD has been used by several groups to probe the role(s) of Gs-like signaling for a number of behaviors including ethanol consumption (Pleil et al., 2015), reward (Ferguson et al., 2013), locomotor sensitization (Farrell et al., 2013), and circadian rythmns (Brancaccio et al., 2013).

Finally, a DREADD that apparently signals exclusively via β-arrestin has been developed (Nakajima and Wess, 2012) (Figure 3A). This β-arrestin-specific DREADD (Rq(R165L) has not yet been used in vivo but has the potential to illuminate specific behaviors downstream of β-arrestin signaling (for discussion, see Allen and Roth, 2011; Allen et al., 2011).

Areas for Enhancement of DREADD Technologies

Chemogenetic technologies are now widely used neuroscientists with publications appearing at the rate of one to two per day. To date, hM4Di (for silencing) and hM3Dq (for activating) neurons have been the most frequent used DREADDs. DREADDs have been used to modulate neural activity and behavior in flies (Becnel et al., 2013), mice (Alexander et al., 2009), rats (Ferguson et al., 2011), and nonhuman primates (Eldridge et al., 2016). Although DREADD technology has clearly been useful, there are several ways in which the technological platform could be enhanced.

Outlook for New DREADDs and Chemical Actuators

In terms of creating new DREADDs, we have described a generic platform wherein human GPCRs can be expressed in yeast with engineered selectable markers and chimeric G proteins (Armbruster et al., 2007; Dong et al., 2010) and have used this platform to express dozens of human GPCRs (Huang et al., 2015b). In theory it should be possible to create new DREADDs by directed molecular evolution of human GPCRs using the prior yeast-based platforms (Armbruster et al., 2007; Dong et al., 2010; Huang et al 2015c).

An alternative approach is to use structure-guided docking of drug-like and pharmacologically inert small molecules against GPCRs for which there are available structures. This structure-guided approach was used by us to create KORD (Vardy et al., 2015). Currently there are many high-resolution structures of GPCRs including a 1.8 Å structure of the human δ-opioid receptor (Katritch et al., 2014), two serotonin receptors in partially active states (Wang et al., 2013), active and coupled states of the μ-opioid (Huang et al., 2015a), M1-muscarinic (Kruse et al., 2013), β2-adrenergic receptors (Rasmussen et al., 2011), and many other inactive state structures (Katritch et al., 2014). Additionally, my lab and collaborators have used these structures for the successful structure-guided discovery of novel small molecule modulators (Weiss et al., 2013; Carlsson et al., 2011; Shoichet and Kobilka, 2012; Huang et al., 2015c). It is thus possible that new DREADDs could be created using these sorts of approaches.

With regard to new small molecule actuators, it would be useful to identify other CNO- and salvinorin B analogues with (a) good drug-like properties, (b) excellent CNS penetrability, (c) clean off-target pharmacology, and (d) favorable pharmacokinetic and toxicological features (Arrowsmith et al., 2015). Additionally the availability of salvinorin B analogues which are water soluble—as salvinorin B is typically dissolved in dimethylsulfoxide—would also be useful. The development of these sorts of tool compounds could be achieved via conventional medicinal chemistry approaches (Chen et al., 2015) and by new technologies developed by my lab and my collaborators. These new chemical biology technologies allow for the design and validation of novel drug-like molecules using a combination of in silicio and in vitro approaches (Keiser et al., 2009; Besnard et al., 2012; Huang et al., 2015c; Kroeze et al., 2015). Additionally, new chemical biology platforms that allow for the unbiased assessment of on- and off-target pharmacologies of chemical actuators across the GPCR-ome (Kroeze et al., 2015), kinome (Elkins et al., 2015), and other targets (Arrowsmith et al., 2015) are key to validating the specificity of DREADD ligands.

Other areas of enhancement for DREADD technology would include the design of DREADDs with differential neuronal subdomain-specific targeting. Thus, in addition to the axonal targeting DREADDs previously reported (Stachniak et al., 2014), cell body, dendritically targeted, and spine-specifically targeted DREADDs would all be broadly useful. Enhancing the diversity of signaling cascades to include $G_{12/13}$, G_o, G_{olf}, and so on would also be highly valuable to the community. Here, structure-based approaches might be especially valuable. Finally, improving the temporal resolution via photo-caging DREADDs or via creation of DREADD antagonists would also be potentially highly useful—particularly given the large number of laboratories currently using DREADD technology.

Potential Therapeutic Applications for Chemogenetics

Many therapeutic applications of DREADD-based therapeutics have been suggested, including diabetes (Jain et al., 2013), metabolic disorders (Li et al., 2013), Parkinson's Disease (Dell'Anno et al., 2014), psychostimulant (Ferguson et al., 2011) and ethanol (Pleil et al., 2015) abuse, depression (Urban et al., 2015), post-traumatic stress disorder (Zhu et al., 2014), intractable seizures (Kätzel et al., 2014), inflammatory disorders (Park et al., 2014), autism (Peñagarikano et al., 2015), and many other disorders (English and Roth, 2015).

DREADDs have been successfully expressed in nonhuman primates without apparent toxicity, and an exciting new report demonstrates that CNO-DREADDs can modulate circuitry, electrophysiology, and behavior in nonhuman primates (Eldridge et al., 2016). As AAV is a commonly used gene delivery platform in humans, the most likely viral vector for human studies would be AAV. In terms of the chemical actuator, given the fact that perlapine is an approved medication with a long history of safety in humans, it would be the most likely DREADD ligand for activating CNO-based DREADDs in humans. Although CNO has been given to humans without ill-effects, given its propensity for back-metabolism to clozapine and NDMC in humans, it might not be the first choice for translational studies. Salvinorin B has not been administered to humans, although salvinorin A—it's precursor—has been used in many human studies without any apparent toxicity. Going forward it would be valuable to identify additional drugs that are approved for use in humans to accelerate translation of DREADD technology to humans.

In summary, DREADDs have transformed basic and translational neuroscience research. The availability of multiple DREADDs activated by chemically and pharmacologically distinct actuators will continue to facilitate the multiplexed, chemogenetic interrogation of circuits and cell types involved in behavior, cognition, emotion, memory, and perception.

Acknowledgments

Work involving chemogenetics in my lab is supported by an NIH BRAIN Initiative Award (UO1MH105892), the National Institute of Mental Health Psychoactive Drug Screening Program (271201300017C-1-0-1), and the Michael Hooker Distinguished Professorship in Protein Therapeutics and Translational Proteomics.

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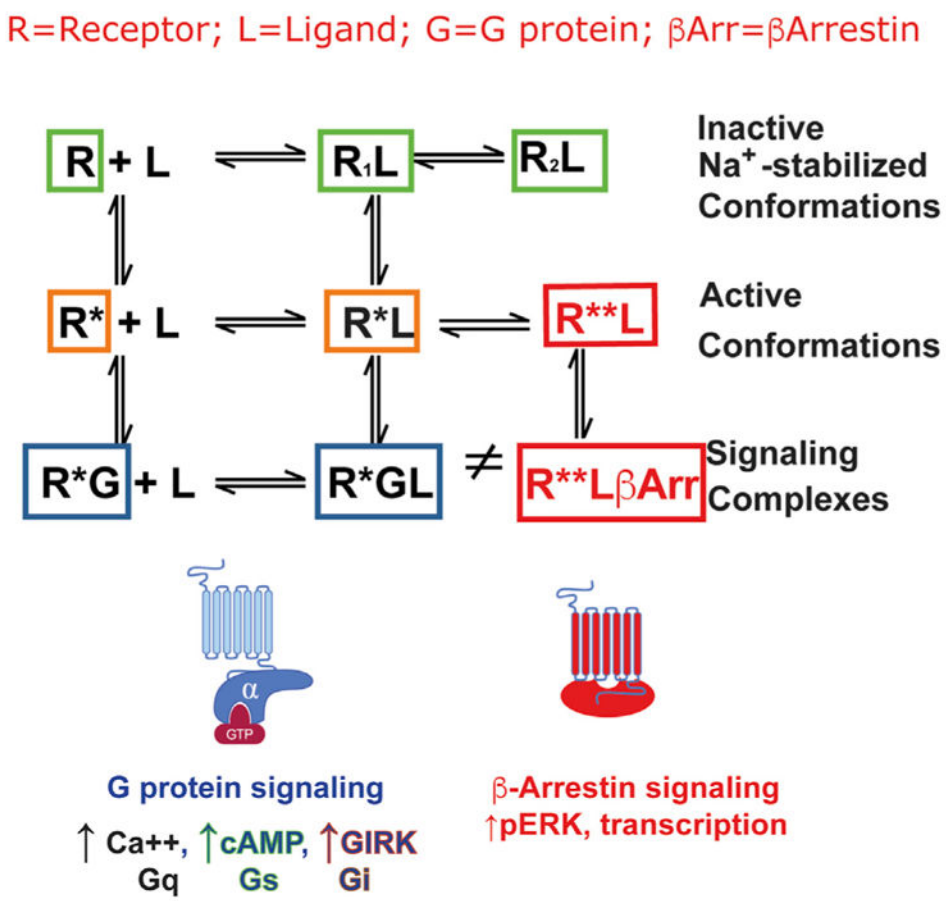

Figure 1. A Modified and Extended Ternary Complex Model of GPCR Action

As shown in the top panel GPCRs (R) may interact with ligands (L), hetereotrimeric G proteins (G), and arrestins (βArr) and thereby form a variety of inactive (green boxes), active (orange and red boxes), and signaling complexes (blue and red boxes). The bottom panel shows a cartoon of the various signaling complexes for canonical G protein signaling (L) and β-Arrestin signaling (R).

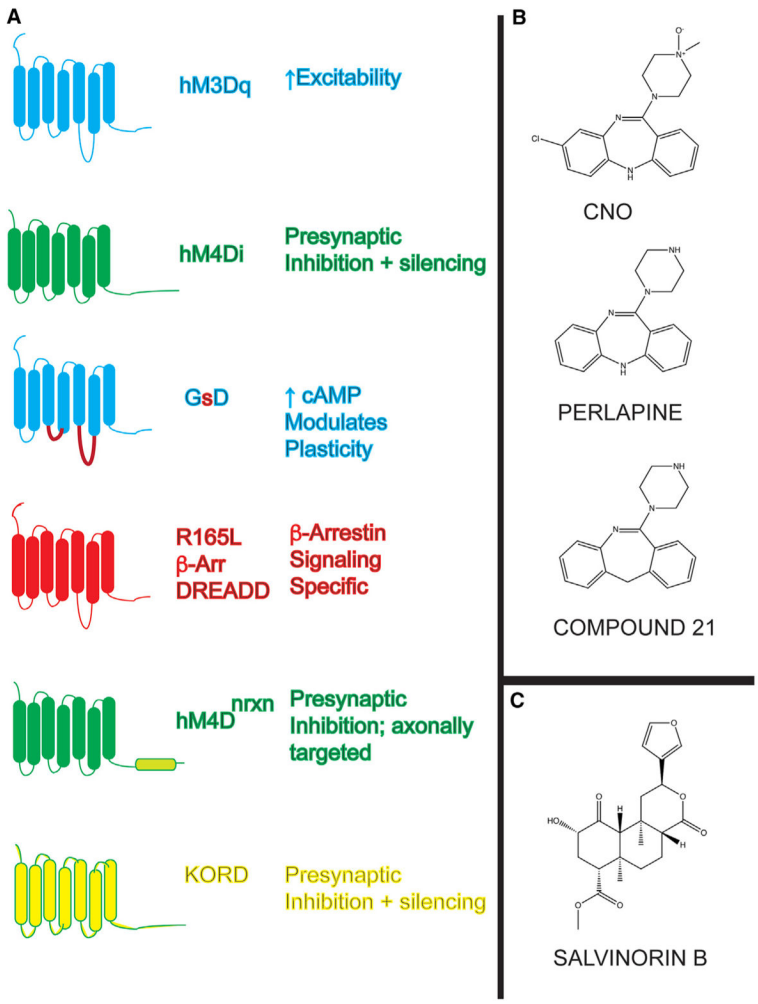

Figure 2. How Receptor Reserve and Constitutive Activity may Modify DREADD Actions In Vitro and In Vivo

(A) Simulations of receptor activity using a standard four-parameter logistic equation for GPCR activation, and variable receptor expression (DeLean et al., 1978) was used to simulate the effects of over-expression of a DREADD with constitutive activity (red circles); high receptor reserve, minimal constitutive activity (blue circles); high receptor reserve + desensitization (green circles); low expression and no receptor reserve (purple circles); and low expression, no receptor reserve, and desensitization (orange circles).

(B and C) Potential pharmacokinetic parameters of CNO following high (B) and lower (C) doses. The dotted red line indicates the threshold concentration required for activation of the DREADD in situ.

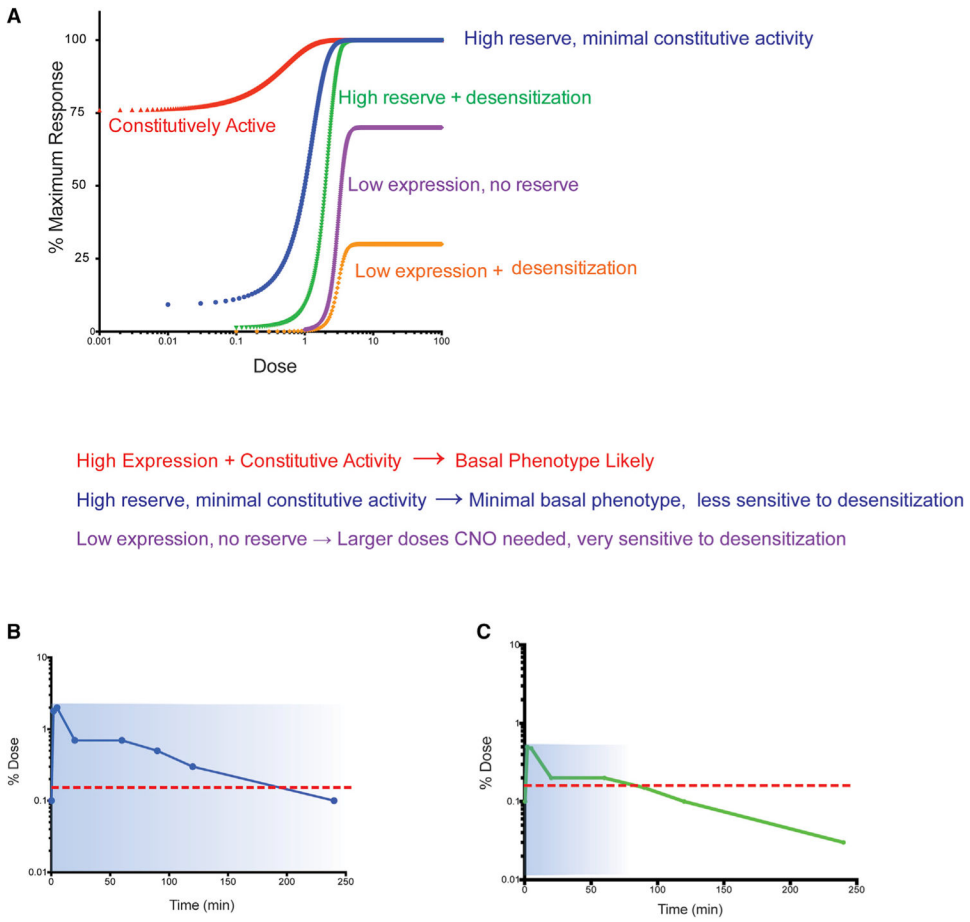

Figure 3. Available DREADDs and Chemical Actuators

(A) The available DREADDs, their current accepted nomenclature, and the potential downstream neuronal effects of activation.

(B and C) (B) Shows the structures of currently available chemical actuators for CNO-based DREADDs, while (C) shows the structure of the KORD ligand salvinorin B.

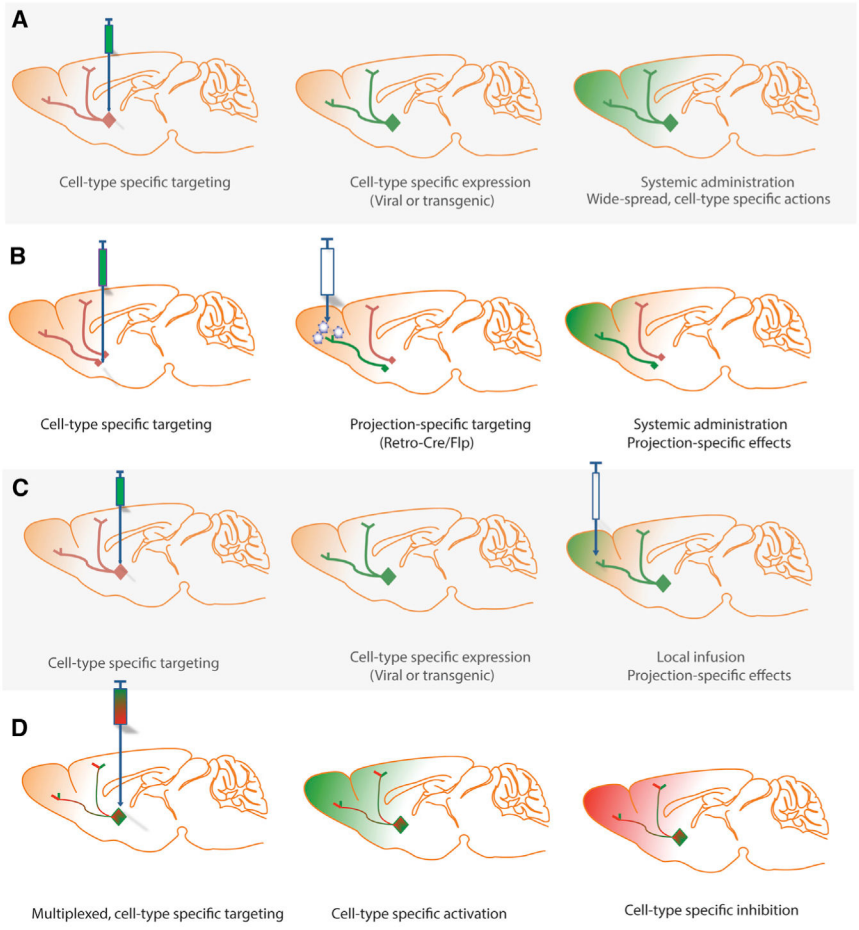

Figure 4. Potential Approaches for Cell- and Projection-Specific Modulation of Neuronal Activity Using DREADDs

(A) The standard approach whereby DREADDs are expressed in a cell-type-specific manner (either virally or transgenically) and then activated by systemic administration of chemical actuator.

(B) How a combination of cell-type-specific expression (e.g., localized injection of AAV-FLEX-hSyn-DREADD) and projection-specific infusion of CAV-Cre allows for the projection-specific expression and activation of DREADDs.

(C) How local infusion of a chemical actuator provides for projection-specific effects with a limited area of activation.

(D) How distinct DREADDs may be expressed in a cell-type-specific fashion to afford multiplexed chemogenetic modulation of neural activity and physiology.

Table 1

Representative Chemogenetic Technologies

Name	Protein(s)	Ligand	Reference
Representative kinases			
Allele-specific kinase inhibitors	v-I388G	Compound 3g	Liu et al., 1998
Analogue-sensitive kinases	v-Src (I338G, v-Src-as1), c-Fyn (T339G, c-Fyn-as1), c-Abl (T315A, c-Abl-as2), CAMK IIα (F89G, CAMK IIα-as1) and CDK2 (F80G, CDK2-as1)	K252a and PPI analogues	Bishop et al., 1998
Rapamycin-insensitive TOR complex 2	TORC2 V2227L	BEZ235	Bishop et al., 2000
ATP-binding pocket mutations in EphB1, EphB2 and EphB3	$Ephb1^{T697G}$, $Ephb2^{T699A}$, and $Ephb3^{T706A}$	PP1 analogues	Soskis et al., 2012
ATP-binding pocket mutations of TrkA, TrkB and TrkC	$TrkA^{F592A}$, $TrkB^{F616A}$, and $TrkC^{F617A}$	1NMPP1 and 1NaPP1	Chen et al., 2005
Representative Enzymes			
Metalloenzymes	Achiral biotinylated rhodium-diphosphine complexes		Collot et al., 2003
Engineered transaminases	Chemically conjugating a pyridoxamine moiety within the large cavity of intestinal fatty acid binding protein	Enhanced activity	Häring and Distefano, 2001
Representative GPCRs			
Allele-specific GPCRs	β2-adrenergic receptor, D113S	1-(3′,4′-dihydroxyphenyl)-3-methyl-L-butanone (L-185,870)	Strader et al., 1991
RASSL-Gi (receptors activated solely by synthetic ligands)	κ-opioid chimeric receptor	Spiradoline	Coward et al., 1998
Engineered GPCRs	5-HT2A serotonin receptor F340→L340	Ketanserin analogues	Westkaemper et al., 1999
Gi-DREADD	M2- and M4 mutant muscarinic receptors	Clozapine-N-Oxide	Armbruster and Roth, 2005; Armbruster et al., 2007
Gq-DREADD	M1, M3, and M5- mutant muscarinic receptors	Clozapine-N-oxide	Armbruster and Roth, 2005; Armbruster et al., 2007
Gs-DREADD	Chimeric M3-frog Adrenergic receptor	Clozapine-N-oxide	Guettier et al., 2009
Arrestin-DREADD	M3Dq R165L	Clozapine-N-oxide	Nakajima and Wess, 2012
Axonally-targeted silencing	hM4D-neurexin variant	Clozapine-N-oxide	Stachniak et al., 2014
KORD	κ-opioid receptor D138N mutant	Salvinorin B	Vardy et al., 2015
Representative Channels			
GluCl	Insect Glutmate chloride channel; Y182F mutation	Ivermectin	Lerchner et al., 2007

Name	Protein(s)	Ligand	Reference
TrpV1	TrpV1 in TrpV1 KO mice	capsaicin	Arenkiel et al., 2008
PSAM	Chimeric channels PSAM^{Q79G,L141S}	PSEM^{9S}	Magnus et al., 2011
PSEM	PSAM-GlyR fusions	PSEM^{89S}; PSSEM^{22S}	Magnus et al., 2011

Table 2

Examples for Apparent Equivalency of Chemo- and Optogenetic Modulation

Cell Type	DREADD	Opsin	Electrophysiology	Behavior
AgRP Neurons	hM3Dq (Krashes et al., 2011)	ChR2 (Aponte et al., 2011)	Increased firing	Enhanced feeding
ETV-1 subfornical area neurons	hM3Dq (Betley et al., 2015)	ChR2 (Betley et al., 2015)	Increased firing	Enhanced drinking
Medial entorhinal cortex cells	hM4Di (Miao et al., 2015)	Arch (Miao et al., 2015)	Decreased firing	Remapping place cells
PBN CGRP Projection neurons	hM3Dq (Cai et al., 2014)	ChR2 (Cai et al., 2014)	Increased firing	Diminished feeding
Orexin neurons	hM4Di (Sasaki et al., 2011)	Halorhodopsin (Tsunematsu et al., 2011)	Decreased firing	Decreased wakefulness
Hippocampus	hM4Di (Zhu et al., 2014)	Arch (Sakaguchi et al., 2015)	Decreased firing	Suppression of contextual fear conditioning
Raphe serotonergic neurons	hM3Dq (Urban et al., 2015)	ChR2 (Ohmura et al., 2014)	Increased firing	Anxiogenic

Table 3

Mice Engineered to Express DREADDs

Mouse Name Genotype	Expression Type	First Publication	Availability
TRE-hM3Dq Tg(tetO-CHRM3*)1Blr/J	Conditional Tet-off/ Tet-on driven expression of hM3Dq	Alexander et al., 2009	JAX Mice; https://www.jax.org/strain/014093
TRE-hM4Di B6.Cg-Tg(tetO-CHRM4*)2Blr/J	Conditional Tet-off/ Tet-on driven expression of hM4Di	Zhu et al., 2014	JAX Mice; https://www.jax.org/strain/024114
Adora2A-rM3Ds B6.Cg-Tg(Adora2a-Chrm3*, -mCherry)AD6Blr/j	Selective expression in Adora2a-expressing D2 neurons in striatum	Farrell et al., 2013	JAX Mice; https://www.jax.org/strain/017863
FLOXED-Gi-DREADD mice B6N.129-*Gt(ROSA) 26Sor*$^{tm1(CAG-CHRM4*,-mCitrine)Ute}$/J	Conditional Cre-mediated expression of hM4Di	Unpublished resource	JAX Mice; https://www.jax.org/strain/026219
FLOXED-Gq-DREADD mice *Gt(ROSA) 26Sor*$^{tm2(CAG-CHRM3*,-mCitrine)Ute}$/J	Conditional Cre-mediated expression of hM3Dq	Unpublished resource	JAX Mice; https://www.jax.org/strain/026220
FLOXED-Gs-DREADD mice B6N;129-*Gt(ROSA) 26Sor*$^{tm3(CAG-Chrm3*,-mCitrine)Ute}$/J	Conditional Cre-mediated expression of GsDREADD	Unpublished resource	JAX Mice; https://www.jax.org/strain/026261
FLOXED/FLP conditional Gi-DREADD mice; RC::FPDi; RC::PDi; RC::FDi	Conditional and intersectional Cre- and FLX-mediated expression of Gi-DREADD	Ray et al., 2011; Brust et al., 2014	Dymecki Lab; http://genepath.med.harvard.edu/~dymecki/requests.html
β-cell-specific GqDRADD; β-R-q	Pancreatic β –cell-specific M3Gq DREADD	Guettier et al., 2009	Jurgen Wess lab; jurgenw@helx.nih.gov
β-cell-specific GsDREADD; β-R-S	Pancreatic β –cell-specific Gs DREADD	Guettier et al., 2009	Jurgen Wess lab; jurgenw@helx.nih.gov