How structure informs and transforms chemogenetics

Bryan L Roth

Chemogenetic technologies such as Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) are widely used to remotely control neuronal and non-neuronal signaling. DREADDs exist for most of the canonical G protein-coupled receptor signaling pathways, and provide a synthetic biology platform useful for elucidating the role of neuronal signaling for brain function. Here, a focused review is provided that shows how recent insights obtained from GPCR structural studies inform our understanding of these chemogenetic tools from a structural perspective.

Address

Department of Pharmacology and Division of Medicinal Chemistry and Chemical Biology, University of North Carolina, Chapel Hill School of Medicine, Chapel Hill, NC 27514, United States

Corresponding author: Roth, Bryan L (bryan_roth@med.unc.edu)

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Introduction

G protein-coupled receptors (GPCRs) — with more than 800 members — represent the largest family of membrane proteins in the human genome [1]. GPCRs also represent the single largest family of therapeutic drug targets in the druggable genome [2^{••}], with 20–30% of approved drugs having GPCRs as their major molecular target [3,4]. GPCRs are expressed in essentially every major organ in the body, with approximately 80% being expressed in the brain [5[•]]; many GPCRs have their highest level of expression in nervous tissue [5[•]]. GPCRs transduce their signals via binding to and activating heterotrimeric G proteins; G proteins couple to various downstream effectors to modulate second messengers including cAMP, inositol trisphosphate (IP3), calcium (Ca²⁺) and others [3]. GPCRs also can signal via arrestin proteins [6] which bind to activated GPCRs following phosphorylation by G protein receptor kinases (GRKs) [7]. Arrestins also arrest G-protein signaling and interact with clathrin to promote endocytosis [8].

Within neurons, GPCRs, via G protein activation, regulate neuronal excitability [9], vesicle release [10], ion channel activity [11,12], and a variety of intracellular second messengers [see Ref. [13] for review]. How these alterations in neuronal signaling and firing ultimately result in various brain processes such as perception, cognition, emotion, motivation, and so on is yet unclear. Some years ago, Francis Crick proposed that in order to ultimately understand the neuronal basis of these brain activities [14,15^{••}] we would need:

“ . . . to identify the many different types of neurons in the cerebral cortex and other parts of the brain. One of the next requirements . . . is to be able to turn the firing of one or more types of neuron on and off in the alert animal . . . One way-out suggestion is to engineer these neurons so that when one of them fires it would emit a flash of light of a particular wavelength . . . ” [15^{••}]

The past decade has seen remarkable progress on this front and essentially all of the items in Crick’s ‘wish list’ are in place. First, RNA-seq based technologies have begun cataloging the many different types of neurons [16] and large numbers of engineered mouse lines are available to ultimately provide genetic access to individual types of neurons [17,18]. Second, optogenetic [19^{••},20] and chemogenetic [21,22^{••}] technologies have provided tools ‘to turn the firing of one or more types of neuron on and off in the alert animal’. Finally, genetically encoded calcium [23] and voltage [24] sensors are available to image neurons that ‘emit a flash of light of a particular wavelength’ [25] and be visualized with fiber optics [26] and miniature microscopes [27].

It still remains unclear; however, how GPCR signaling is ultimately harnessed by neurons to modulate various brain functions. Given the vast number of neuronal phenomena that can be modulated, engineering synthetic ligands and GPCRs that can be used to remotely control neuronal could provide a technology useful for elucidating the role of GPCRs in brain function. For decades, it has been appreciated that if one could engineer GPCRs so that they could be activated by an inert drug but were insensitive to their endogenous ligand, they could be used to illuminate the role of GPCR signaling in these processes [28–30]. The notion was that once such ‘designer receptor/ligand pairs’ were created, they could be expressed in defined cell types via transgenic or viral methods. Behavioral and physiological monitoring before and after activation by the designed ligand would then illuminate the role of a particular GPCR

Figure 1

DREADD-based chemogenetic tools modulate cellular function.

Shown is a hypothetical pancreatic beta cell wherein a Gq-coupled DREADD can be activated by CNO to induce insulin release (orange circles). Red stars show the approximate locations of the Y3.33C and A3.56G mutations which render the M3-muscarinic receptor insensitive to acetylcholine and potentially activated by clozapine-*N*-oxide shown above.

signaling cascade for a particular process (Figure 1). Although these pioneering chemogenetic technologies were useful for archetypical proof-of-concept studies [31,32], they were not widely adopted. To circumvent problems inherent with the first-generation chemogenetic tools (lack of inert ligands and high basal activity of receptors), my lab developed DREADDs (Designer Receptors Exclusively Activated by Designer Drugs) [21], which are now routinely used by neuroscientists and others to modulate neuronal activity and cellular signaling [22]. DREADDs are also used to clarify how GPCR signaling processes are important for mediating normal and abnormal physiology [33,34,35,36].

DREADDs [22] are GPCRs that have been engineered to: (a) lack appreciable responses to their endogenous ligand; (b) have minimal basal (constitutive) activity and (c) be activated by a pharmacologically inert ligand. Many types of DREADDs currently exist, including those based on muscarinic [22], k-opioid [37] and the FFA2 fatty acid GPCRs [38]. All of these DREADDs activate the canonical G proteins to which they are coupled after stimulation by their otherwise inert cognate ligand. Thus, the M2 and M4-DREADDs (hM2Di and hM4Di, respective) activate Gi-family proteins, M1-DREADDs, M3-DREADDs and M5-DREADDs (hM1Dq, hM3Dq and hM5Dq respectively)

activate Gq/11. The k-opioid DREADD (KORD) activates Gi-family receptors, while the FFA2 DREADD activates Gi and Gq family G proteins. A particularly useful DREADD has been developed, in which the highly conserved arginine in the conserved 'DRY' motif essential for receptor activation [3,39,40] is mutated. This particular mutation—R3.50L—yields an M3-(R3.50L)-DREADD that is able to recruit arrestin, but can no longer activate G protein signaling [41]. This M3-(R3.50L)-DREADD can be used to determine the role of arrestin signaling in cellular processes.

DREADDs can be expressed via virally mediated transduction [42] or can be genetically encoded in essentially any cell type [43,44] and activated non-invasively via drug-like small molecules [45] to reveal the physiological consequences of GPCR signaling in defined cells. Accordingly, DREADDs provide a synthetic biological platform for selectively and non-invasively modulating GPCR signaling. Here, I will summarize emerging structural insights into the actions of DREADDs that promise to transform and extend the usefulness of this chemogenetic platform.

Structural insights into muscarinic DREADD actions

The first DREADDs were based on human muscarinic acetylcholine receptors. These muscarinic DREADDs

(hM1Dq, hM2Di, hM3Dq, hM4Di, and hM5Dq) were created by directed molecular evolution in yeast [22[•],46] to be activated by the inert clozapine metabolite clozapine-*N*-oxide (CNO). To date, muscarinic DREADDs are the most widely used chemogenetic tools in the neurosciences. Two of these — hM2Di and hM4Di — inhibit neuronal activity [22[•]] via Gβ/γ-mediated activation of hyperpolarizing GIRK channels and inhibition of synaptic release of neurotransmitters [47]. The Gq-coupled DREADD (hM3Dq) is most frequently used to enhance neuronal activity [48], via Gq-mediated depolarization and modulation of ion channel activity [44]. When combined with Cre-responsive viruses and genetically encoded Cre-recombinase expressed in discrete neurons, DREADDs can be used to remotely control neuronal activity in defined neurons and, consequently, modulate a number of behaviors and physiological responses [49,44].

Although widely used, these DREADDs are not without liabilities. Thus, for instance, CNO may be metabolized to clozapine at low levels in mice [50] and rats [51] and to a greater extent in guinea pigs and humans [52]. As clozapine is an antipsychotic drug with high affinity for a large number of neurotransmitter receptors [53], such a conversion may be problematic and various controls are now recommended when using CNO [see Ref. [44] for review]. Alternatively, CNO analogs with improved bioavailability and without metabolic liabilities such as C21 and perlapine can also be used [45,54[•]]. Going forward, novel DREADD chemogenetic actuators having different chemotypes with enhanced bioavailability will be useful.

In discussing the structural features of DREADDs, I use the numbering system developed by Ballesteros and Weinstein [55]. Thus, for the muscarinic DREADDs the conserved amino acids of interest are Y3.33 in TM3 and A5.46 in TM5 (numbered according to Ballesteros and Weinstein [55]). When these are mutated to Y3.33C and A5.46G they yield muscarinic receptors insensitive to the endogenous ligand acetylcholine and potentially activated by CNO [22[•]] (Figure 2a and c). As these particular mutations were arrived at via several cycles of directed evolution and employed unusual [non-conservative] substitutions (e.g. Y → C and A → G) it was not immediately clear why these particular mutations were essential for the desired chemical and biological phenotype.

Several years after the invention of the muscarinic DREADDs, both inactive [56] and active-state structures of the M2 muscarinic receptor were published [57]. Figure 2 also shows an active state structure of the wild-type M2 muscarinic receptor (2D [57]), a model of an active state hM2Di-DREADD (Figure 2b) and a summary of key transitions (Figure 2c). Although it is not entirely evident from the structure why these mutations transform CNO from a weak antagonist into an agonist, our recent studies provide a clue. Thus, we found that a similar A5.46G mutation of the 5-HT_{2B} serotonin receptor transforms the antagonist methysergide into a potent agonist [58]. This is accomplished by providing bulk-tolerance for the *N*-methyl group of methysergide, which allows for the inward movement of TM5 required for stabilizing the active state. Presumably, a similar transition in the hM2Di-DREADD (Figure 2b–d) is key for

Figure 2

Structural insights into DREADD actions.

Shown are snake plots of the M2 (a) and M3 (e) [72] muscarinic receptors with the locations of the Y3.33C and A3.56G mutations highlighted in red. Panel (d) shows the active state structure of the M2 muscarinic receptor while (b) and (c) show a model of the M2-DREADD and the transition from inactive to active state DREADD.

Figure 3

Structural features of the k-opioid DREADD.

Shown in (a) is the inactive state structure of the k-opioid receptor indicating the location of D3.32 shown in sphere representation. (b) shows a close-up of the binding pocket of the active state k-opioid receptor highlighting the change in rotamer of D3.32 from inactive (purple) to active (green). (c) shows a model of the k-opioid DREADD wherein the D3.32N mutated residue is pushed out of the binding pocket, freeing up energy of desolvation for the DREADD-specific ligand salvinorin B.

accommodating bulky constituents of CNO, although direct structural studies will ultimately be essential to determining this for certain.

Structure-inspired design of a κ -opioid DREADD

The first DREADDs were developed using an unbiased, directed evolution approach and, although successful for many applications, they suffered from the inability to ‘multiplex’. To be able to use synthetic biological approaches to separately activate distinct GPCR signaling cascades sequentially or simultaneously in the same neurons would solve the problem of multiplexing. With the explosion in structural information related to GPCRs [59] we set out to use this information to design a new DREADD. We chose as our template the k-opioid receptor (KOR) for which a high resolution structure was available [60] and for which NMR-based structural information was available for the active state [61]. We had previously discovered that the non-nitrogenous natural product salvinorin A was a potent and selective KOR agonist [62] and that it did not rely on the conserved Asp3.32 found in the binding pocket of many biogenic amine receptors [63] for binding or activation. Indeed, Salvinorin A binding and functional potency were enhanced by a D3.38N mutation while binding of the native peptide ligand dynorphin A (1–17) was abolished [63].

Accordingly, we created a D3.38N mutant and found that not only was Salvinorin A’s potency enhanced, but that the relatively inert metabolite Salvinorin B (SALB) was transformed into a potent agonist [37]. In a series of studies, we were able to show that SALB was inert at the tested doses *in vivo* but was able to afford potent and

efficacious neuronal silencing *in vivo* in genetically defined neurons [37]. We dubbed this the k-opioid receptor DREADD (KORD) and note that it has been used widely in the neurosciences to silence genetically identified neurons [64–66]. Recent structural studies of the active KOR have revealed a potential mechanism for SALB’s potency at KOR [40].

In accordance with a new active state structure of KOR [40], we found that the epoxymorphinan-derivative MP1104 bound to KOR at some distance (2.6 Å) from the highly conserved D3.38 (Figure 3b). The D3.38A mutation of this residue had a modest effect on binding of MP1104, although it abolishes binding of all known endogenous KOR ligands [37]. The D3.38N mutation is predicted to result in a movement of ~ 3 Å outward from the binding pocket due to repulsive forces with the positively charged groups of morphinans and opioid peptides. Such a movement is predicted, based on simulations, to enhance SALB binding by desolvation [37] thereby providing a molecular mechanism for SALB’s remarkable efficacy at KORD. The D3.38N mutation also provides repulsive energy to hinder the binding of endogenous opioid peptides, which are known to interact with this residue in opioid receptors for high affinity binding [61,67] in the active state.

Future potential developments

DREADDs and other chemogenetic tools [68] along with optogenetic technologies [69] have transformed neuroscience and other disciplines. The currently available chemogenetic and optogenetic technologies provide many orthogonal approaches to manipulate cellular signaling and neuronal firing. With the current revolution occurring in membrane protein structural determination via

Figure 4

Computational approaches for structure-guided discovery of novel chemogenetic actuators.

In the top panel is shown a comparison of the D3- (purple) [73] and D4- (green) [71] dopamine receptor structures highlighting the selectivity filter which provides a template for large-scale docking campaigns [71]. The lower panel illustrates how a similar process could be used on a mutant receptor engineered from a template of the D4 to identify potential novel chemotypes as chemogenetic actuators.

crystallography and cryo-electron microscopy, the fields are poised to deliver a new generation of tools inspired and designed with high resolution structural insights (see Refs. [37,70] for examples). Combining high resolution structures with automated docking, for instance, could provide novel chemogenetic actuators for engineered receptors, as has been done for native GPCRs [71] (Figure 4).

In addition to developing novel chemogenetic actuators and new DREADDs, it continues to be useful to engineer GPCRs to activate designed signaling networks. Thus, for instance, DREADDs are available for Gi [22,37], Gq

[22], Gs [33] and arrestin [41] signaling, although none are available that are selective for G12/13, gustducin, transducin or Golf. As well, although the Gi and Gs-selective DREADDs are currently available, they also mobilize arrestin to at least some extent, and to have those that do not interact with arrestin at all (as has been done for a Gq/11-DREADD [36]) will be useful for interrogating arrestin-dependent and independent pathways in cells and intact animals.

Conclusion

From the preceding examples, it should be clear that the field of chemogenetics is maturing, and that, when

combined with detailed structural studies, tools will continue to be developed that will allow a much more sophisticated understanding of intracellular, cellular, and especially neuronal signaling *in vitro* and *in vivo*. The ability to multiplex these tools will allow a more nuanced view of such signaling in the future, and thus will allow us to extend Crick's vision yet further.

Conflict of interest statement

Nothing declared.

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