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Abstract

Chemical genetics has played an important role in linking specific G protein-coupled receptor (GPCR) signalling to cellular processes involved in central nervous system (CNS) functions. Key to this approach has been the modification of receptor properties such that receptors no longer respond to endogenous ligands but rather can be activated selectively by synthetic ligands. Such modified receptors have been called Receptors Activated Solely by Synthetic Ligands (RASSLs) or Designer Receptors Exclusively Activated by Designer Drugs (DREADDs). Unlike knock-out animal models which allow detection of phenotypic changes caused by loss of receptor functions, RASSL and DREADD receptors offer the possibility of rescuing "knock-out" phenotypic deficits by administration of the synthetic ligands. Here we describe the use of these modified receptors in defining the physiological role of GPCRs and validation of receptors as drug targets.

The use of chemogenetic approaches to study the physiological roles of muscarinic acetylcholine receptors in the central nervous system.

Sophie J. Bradley, Andrew B. Tobin and Rudi Prihandoko*

Centre for Translational Pharmacology, Institute of Molecular, Cell and Systems Biology, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, G12 8QQ, UK

*to whom correspondence should be addressed: rudi.prihandoko@Glasgow.ac.uk

Corresponding author:

Dr. Rudi Prihandoko

Centre for Translational Pharmacology,

Institute of Molecular, Cell and Systems Biology,

College of Medical, Veterinary and Life Sciences,

University of Glasgow,

Glasgow, G12 8QQ, UK

Phone (+44) 01413306454

Email: Rudi.Prihandoko@glasgow.ac.uk

Abstract

Chemical genetic has played an important role in linking specific G protein-coupled receptor (GPCR) signalling to cellular processes involved in central nervous system (CNS) functions. Key to this approach has been the modification of receptor properties such that receptors no longer respond to endogenous ligands but rather can be activated selectively by synthetic ligands. Such modified receptors have been called Receptors Activated Solely by Synthetic Ligands (RASSLs) or Designer Receptors Exclusively Activated by Designer Drugs (DREADDs). Unlike knock-out animal models which allow detection of phenotypic changes caused by loss of receptor functions, RASSL and DREADD receptors offer the possibility of rescuing "knock-out" phenotypic deficits by administration of the synthetic ligands. Here we describe the use of these modified receptors in defining the physiological role of GPCRs and validation of receptors as drug targets.

Keywords

Abbreviations

mAChRs: muscarinic acetylcholine receptors, DREADDs: Designer Receptors Exclusively Activated by Designer Drugs, RASSLs: Receptors Activated Solely by Synthetic Ligands, CNO: clozapine-N-oxide, ACh: acetylcholine.

Introduction

Muscarinic acetylcholine receptors (mAChRs) play key roles in many physiological functions in both the central and peripheral nervous system (Hulme et al., 1990). Furthermore, aberrations in muscarinic expression and/or signalling have been implicated in the pathophysiology of numerous disease states, and as such mAChRs represent therapeutic targets for an array of human diseases (Kruse et al., 2014b; Wess, 2004). Of the five mAChR subtypes (M_1-M_5), four crystal structures have been solved revealing a high degree of similarity within the acetylcholine binding pocket and, therefore, design of subtype selective pharmacological entities that target the same binding site as the endogenous ligand has proven challenging (Kruse et al., 2014a; Kruse et al., 2012; Kruse et al., 2013; Thal et al., 2016). Hence, much effort has been directed toward developing ligands that target alternative (allosteric) binding sites on the receptor. Such allosteric modulators have the potential to be more subtype selective as the allosteric binding sites on GPCRs are less evolutionarily conserved (Conn et al., 2014). A number of positive allosteric modulators have indeed been successfully developed for the M_1 and M_4 muscarinic receptors including BQCA (M_1 positive allosteric modulator) (Bradley et al., 2017; Ma et al., 2009; Shirey et al., 2009) and LY2033295 (M_4 positive allosteric modulator) (Chan et al., 2008), which showed superior subtype selectivity and usefulness in animal studies. Despite these successes, allosteric modulators have their drawbacks, most notably, the ligands require the presence of the endogenous neurotransmitter to function. Therefore, in disease situations where ACh levels are depleted, the effects of allosteric modulators on receptor function might not be detected. Over the past fifteen years, knock-out animals lacking each of the five muscarinic receptor subtypes have been developed, which provided a wealth of information on the role of these receptors in the CNS (Wess, 2004). For example, M_1 mAChR deficient mice show deficits in learning and memory tasks (Bradley et al., 2017) indicating a clear role for this muscarinic receptor subtype in cognitive function. Furthermore, knockout of M_1 in parvalbumin-containing neurons in hippocampal CA1 region also leads to impairment in working memory and cognition (Yi et al., 2014). Taken together, these data highlight CNS M_1 mAChRs as therapeutic targets in diseases associated with impaired cognitive function, such as Alzheimer's Disease and schizophrenia. However, such knockout models are limited to the detection of biological responses caused by a loss of receptor function and are unsuitable for identifying the consequences of or biological responses associated with receptor activation. Additional potential drawbacks associated with knockout models include cellular compensation and disruption of the basal 'tonic' neurotransmitter release, which may subtly

affect cellular/synaptic excitability. To overcome these limitations, researchers have developed more sophisticated approaches, which involve the use of chemical genetics to enable receptor activation *in vivo* with exquisite selectivity and precise spatiotemporal control. The premise of the chemical genetic approach is to modify the receptor such that the receptor no longer responds to the endogenous ligand but can be activated by inert drug like synthetic ligands. Such mutant receptors have been termed Receptors Activated Solely by Synthetic Ligands (RASSLs) or Designer Receptors Exclusively Activated by Designer Drugs (DREADDs). Unlike the mode(s) of action of allosteric modulators, which depends on the presence of the endogenous neurotransmitter, RASSL/DREADD receptor functions independently of the endogenous ligand. Expression of these modified receptors in place of the wild type muscarinic receptor theoretically should result in a phenotype similar to that of the receptor knockout. Administration of the surrogate "synthetic" ligand activates the RASSL/DREADD receptor allowing for the identification of cellular and physiological responses associated with activation of the mutant receptor. This approach can not only be used to establish the physiological responses and signalling pathways that lie downstream of a GPCR but importantly, this approach mimics the responses that might be expected if a receptor subtype selective drug were to be developed. Hence, this approach would enable assessment of both the potential clinically beneficial and adverse effects of receptor activation.

Chemical genetic approach to generate muscarinic DREADDs

The premise of chemical genetic is to engineer cellular signalling systems that can be employed to alter *in vivo* physiological and behavioural responses in a highly specific manner. Key to this approach is the availability of surrogate ligands that have favourable pharmacokinetic properties and are otherwise pharmacologically inert in normal physiological conditions. In addition, the genetic modification introduced to the receptor should render the receptor unresponsive to the endogenous ligand and does not produce unintended response in the absence of the surrogate ligands. Classically, chemical genetic approach employed rationally designed mutation of residues known to interact with endogenous ligands, such as the highly conserved aspartate residue in the biogenic amine receptors ($D^{3.32}A$ in the β_2-adrenoceptor and $D^{3.32}A$ in 5-HT$_4$ receptor) (Claeysen et al., 2003; Strader et al., 1991). Although, these mutations reduced the affinity and efficacy of the endogenous ligands, the affinity and efficacy afforded to the synthetic ligands were insufficient to warrant *in vivo* study (Strader et al., 1991), and in the case of the 5-HT$_4$

receptor, the mutation produced an unwanted constitutive receptor activity (Claeysen et al., 2003). A second method, which involves domain swapping, was introduced. Here, the second extracellular loop of the κ-opioid receptor was replaced with that of the δ-opioid receptor to produce a chimeric receptor that displayed 200-fold reduction in the binding of the endogenous ligand, dynorphin, but maintained normal binding and activation by small molecule ligand, spiradoline (Coward et al., 1998). Conditional expression of this chimeric receptor, termed Ro1 in tissues such as the heart and salivary glands resulted in physiological responses that are consistent with G_i signalling through the κ-opioid receptor, such as reduction in heart rate and salivary secretion (Redfern et al., 1999). Despite these successes, Ro1 is not an ideal system as the surrogate ligand, spiradoline is pharmacologically active at the native κ-opioid receptor, which necessitates expression of Ro1 in a knockout background (Sweger et al., 2007). To circumvent these issues, Roth and colleagues employed directed molecular evolution and a yeast based growth assay on the M_3 mAChR with the aim of evolving the receptor to respond to clozapine-N-oxide (CNO) and concomitantly eliminate the binding and signalling efficacy of acetylcholine (ACh) (Armbruster et al., 2007). CNO was chosen as a surrogate ligand due to its drug likeness and favourable pharmacokinetic properties. The ligand is also reported to be pharmacologically inert and hence less likely to produce off target effects (Weiner et al., 2004). This strategy uncovered mutations of two residues, one on TM3 ($Y^{3.33}C$) and the other on TM5 ($A^{5.46}G$) which significantly reduced the activity of acetylcholine but instead generated responsiveness to CNO (Armbruster et al., 2007). Moreover, these mutations did not produce constitutive receptor activity, making muscarinic DREADDs an ideal tool for *in vivo* studies. The residues that make up the DREADD mutations are also conserved among the five members of the muscarinic receptor family and analogous mutations in these receptors have resulted in the generation of DREADDs for all five (M_1-M_5) muscarinic receptor subtypes (Armbruster et al., 2007).

Pharmacology of muscarinic DREADDs

Early *in vitro* characterisation of the M_3 DREADD showed that in response to CNO, the engineered receptor was able to stimulate G protein-mediated signalling pathways such as phosphorylation of ERK1/2, inositol phosphate accumulation and calcium mobilisation (Armbruster et al., 2007). In contrast, acetylcholine, the natural ligand for the M_3 mAChR, showed significantly reduced activity at the M_3 DREADD receptor. Further work on the M_4 DREADD showed that when expressed in neurons, the receptor was able to cause

hyperpolarisation and neuronal silencing, indicating that the DREADD is able to replicate the M_4 receptor function in a native cellular environment.

It is now widely accepted that different ligands binding to the same GPCR can differentially direct receptor signalling via one pathway in preference to another, a concept referred to as "stimulus bias" or "functional selectivity" (Galandrin et al., 2007; Stallaert et al., 2011). Given this, it is possible that DREADD receptors, activated by CNO may signal differently to the wild-type acetylcholine-stimulated receptor. It has therefore been imperative to thoroughly examine the signaling properties of the DREADD receptors in particular to determine if the DREADD ligand shows functional bias. In the case of the M_3 DREADD expressed in cell lines, it was shown that no functional bias was observed in a range of cellular signalling assays including ERK1/2 phosphorylation, calcium mobilization, receptor phosphorylation, β-arrestin recruitment and receptor internalisation (Alvarez-Curto et al., 2011). M_3 DREADD expressed in neurons also did not display functional bias as similar afterdepolarisation and action potential frequency were observed in the M_3 DREADD-expressing neurons treated with CNO compared to wild-type neurons stimulated with muscarine (Yi et al., 2014). Hence, activation of the M_3 DREADD receptor with CNO closely reflects that of the acetylcholine-stimulated wild-type receptor.

Despite the equivalence in the pharmacology of the acetylcholine-bound wild-type receptor versus a CNO-bound DREADD receptor, the allosteric interactions at these receptors might not be the same as allosteric ligands have distinct binding pockets with complex pharmacology. That this is the case is evident at the M_4 DREADD. While acetylcholine and LY2033298 (an allosteric potentiator) display positive co-operativity with respect to binding at the wild-type receptor, the interaction between clozapine-like compounds and LY2033298 at the DREADD receptor was neutral. Furthermore, LY2033298 could rescue acetylcholine responses at the M_4 DREADD receptor a result which indicates for the M_4 DREADD at least, that this chemical genetic approach could extend to the examination of physiological responses modulated by both allosteric and orthosteric synthetic ligands (Nawaratne et al., 2008). Further differences in allosteric action at DREADD receptors was revealed by an in-depth study investigating the action of the M_1 mAChR positive allosteric modulator, BQCA, at wild-type and M_1 DREADD receptors (Abdul-Ridha et al., 2013). BQCA is a positive modulator of ACh binding and function at wild-type M_1 mAChRs, and does not engender stimulus bias (Canals et al., 2012). However, BQCA engenders stimulus bias at the

DREADD receptor, with the degree of co-operativity differing depending on the signalling pathway being probed. These studies indicate that clozapine derivatives can stabilize unique receptor conformations with BQCA at the M_1 DREADD receptor to reveal significant differences in downstream signalling pathways. Hence, it might be possible to employ DREADDs to investigate signalling bias in vivo.

In vivo **applications of muscarinic DREADDs**

An alternative approach to delineating the physiological roles of GPCRs is to pharmacologically manipulate the activity of a given receptor using synthetic agonists or antagonists. However, development of subtype selective orthosteric ligands for GPCRs has proven challenging owing to the highly conserved binding pockets for endogenous ligands across GPCR subtypes. The DREADD approach provides a novel and powerful tool to overcome these barriers. As described above, the residues mutated to generate the M_3 DREADD are conserved across all members of the muscarinic acetylcholine receptor family, and as such a battery of muscarinic DREADDs have been created which have been used in numerous transgenic studies to control $G_{q/11}$, $G_{i/o}$ or G_s signalling.

Conditional expression of the $G_{q/11}$-coupled M_3 DREADD in pancreatic β-cells using insulin promoter revealed a role for $G_{q/11}$ signalling in the acute- and second-phase insulin release, whereby CNO co-administration with glucose led to dramatic increases in *in vivo* insulin release (Guettier et al., 2009). Furthermore, in mice expressing M_3 DREADD selectively in β-cells and maintained on a high-fat diet, glucose and CNO co-administration was found to improve glucose tolerance and *in vivo* insulin release compared to mice treated with glucose alone (Guettier et al., 2009; Jain et al., 2013).

In addition to exploring a role for $G_{q/11}$ signalling in glucose homeostasis by driving expression in pancreatic β-cells, the M_3 DREADD has been used to probe the function of $G_{q/11}$ signalling in specific CNS pathways. Targeting the M_3 DREADD to agouti-related protein (AgRP) neurons showed that stimulation of AgRP activity can induce feeding and reduce energy expenditure in mice (Krashes et al., 2011). Furthermore, the M_3 DREADD under the control of a tetracycline CAMKII promoter has also been selectively targeted to forebrain pyramidal neurons; in the absence of the exogenous ligand, these mice displayed normal physical appearance and behaviour. However, mice that were treated with CNO showed significant enhancement in locomotion and limbic seizures, unveiling a role for $G_{q/11}$

signalling in mediating locomotion and seizures (Alexander et al., 2009). The usefulness of M_3 DREADD in providing mechanisms of cholinergic driven seizures was further demonstrated by studies on M_3 DREADD expressed in parvalbumin-positive (PV) interneurons, a CA1 region of the hippocampus that also express the M_1 muscarinic receptor (Yi et al., 2015). Chronic or continued administration of pilocarpine to PV neurons of wild-type mice resulted in increased action potential, which developed into a state of depolarisation block and a deficit in GABAergic inhibition (Yi et al., 2015). This state leads to the onset of epileptic seizure-like activity. Interestingly these processes were mimicked by the M_3 DREADD upon stimulation with CNO and was absent in the PV neurons devoid of M_1 muscarinic receptor (Yi et al., 2015). Together, these data indicate that chronic activation of pyramidal forebrain neurons and parvalbumin-positive (PV) interneurons of the hippocampus plays an important role in the induction of epileptic seizures.

Furthermore, the muscarinic receptor DREADD mice have been employed to demonstrate roles for $G_{q/11}$ signalling in learning and memory. Using a c-fos based transgenic approach the M_3 DREADD was expressed in an activity-dependent manner in neurons following exposure to an environmental stimulus (Garner et al., 2012). This allowed for the subsequent re-activation of a particular subset of neurons following administration of CNO, which formed a hybrid fear memory incorporating elements of an artificially-induced neural network. Taken together, these studies highlight the potential of these chemogenetic tools to remotely control the activity of $G_{q/11}$ signalling *in vivo*, in a manner that is selective and reversible.

Muscarinic DREADDs have also been used as an approach to suppress neuronal signalling; these studies employed an M_4 DREADD, which is coupled to $G_{i/o}$ proteins and induces membrane hyperpolarization by activation of G protein inward-rectifying potassium channels (GIRK) (Armbruster et al., 2007). In these transgenic mice where the M_4 DREADD is under control of a CAMKII promoter, CNO significantly impaired consolidation of contextual memory in the hippocampus by inactivating hippocampal neurons (Zhu et al., 2014). Furthermore, the use of viral vectors allowed for brain-region specific control of neuronal signalling, and showed that inactivation of ventral but not dorsal hippocampal neurons suppressed contextual memory consolidation, highlighting the use of DREADD technology to anatomically define sub regions of the brain, which are central to the behavioural effects (Zhu et al., 2014). This is further evidenced by studies in which expression of the M_4 DREADD receptor is driven selectively in the medio-dorsal thalamus (MD) revealing that

subtle decreases in activity in the MD was sufficient to induce profound impairments in cognitive tasks associated with the prefrontal cortex (Parnaudeau et al., 2013). These studies provided direct evidence linking reduced MD activity and prefrontal cortex-dependent learning and memory processes, a mechanism postulated to underlie cognitive impairment in schizophrenia. Neuronal silencing through expression M_4 DREADD in PV interneurons in the medial prefrontal cortex has also been associated with helplessness and enhanced susceptibility to stress (Perova et al., 2015), highlighting a role for this region in mood and depressive disorder. Furthermore, targeting of M_4 DREADD into motor cortex using a CAMKIIa promoter and adeno-associated delivery system, has also uncovered a role for the inhibition of seizures (Katzel et al., 2014; Krook-Magnuson and Soltesz, 2015). Acute administration of pilocarpine and epileptic promoting toxins (picrotoxin and tetanus toxin) locally into the cortex resulted in increased spiking frequency of Morlet-wavelet EEG spectra and the development of acute seizures. M4 DREADD activation in this brain region by CNO immediately after pilocarpine injection reduced the spiking frequency and seizure severity (Katzel et al., 2014).

Taken together, these approaches highlight the use of chemogenetic technology to reversibly and non-invasively control G protein signalling to probe physiological functions of specific G protein pathways and the contribution of certain brain regions in disease states. Despite this, muscarinic DREADDs are yet to be fully exploited for the physiological functions of specific muscarinic receptors. At present there is only one publication relating to the use of DREADDs to probe the muscarinic receptor activation *in vivo* (Butcher et al., 2016). In this study, a novel mouse model was generated in which the gene coding for the M_1 mAChR was replaced with an M_1 DREADD C-terminally tagged with a human influenza hemagglutinin (HA) epitope (Figure 1). Thus, expression of the M_1 DREADD is under the control of the endogenous M_1 mAChR promoter and, therefore, the DREADD receptor will only be expressed in the cell types in which the native receptor is expressed and at physiological expression levels. Administration of CNO to these mice resulted in an increase in phosphorylation of serine 228 of the M_1 DREADD receptor, a phosphorylation event that is used as a biomarker for receptor activation (Butcher et al., 2016). Further behavioural and electrophysiological work on this mouse model together with the M_4 DREADD mice which are already generated (see Figure 2 for pharmacological characterisation) will provide a very powerful tool to selectively and reversibly manipulate specific muscarinic receptor subtypes *in vivo* to define their physiological and pathophysiological functions.

Caveats of chemical genetic approaches

While chemogenetic approach has afforded the ability to selectively target and activate particular signalling pathways and mAChR subtypes *in vivo* which would otherwise be challenging to achieve using directly acting pharmacological agents, there are several disadvantages associated with using this approach. One such disadvantage is that the temporal pattern of activation of the DREADD receptor might not be the same as the native receptor. For example, acetylcholine, the endogenous ligand for mAChRs is rapidly degraded by acetylcholinesterase after being released into the synaptic cleft, hence the ligand has a short duration of action. Furthermore, the release of acetylcholine is intimately linked with membrane potential and physiologically released in short bursts and pulsatile manner. However, CNO, the surrogate ligand for the muscarinic DREADDs is metabolically more stable relative to acetylcholine and hence likely to have a longer lasting effect on receptor activation. Indeed it has been shown that despite the fact that CNO levels in the plasma peak 30 min intraperitoneal administration, the physiological effect of the compound was still observed 6 hours later (Alexander et al., 2009; Guettier et al., 2009). This prolonged duration of action of CNO might not be useful for studying short-term effects of neuronal activity on behaviour. For better temporal control of neuronal responses, it may be necessary to complement DREADDs with optogenetics. Optogenetics utilise light to activate photosensitive receptor proteins and GPCR ligands, and the pattern of activation can be controlled such that it mimics action potential. Examples of optogenetic approaches include photoswitchable tethered ligands, which are otherwise inert and can only bind to the modified receptor when illuminated and optoXRs, optically sensitive chimeric proteins consisting of rhodopsin and the intracellular loops and C-terminal domain of GPCRs (Spangler and Bruchas, 2017). Although optoXRs for muscarinic receptors are currently unavailable, a photosensitive dualsteric ligand has recently been developed for M_1 muscarinic receptor which may be useful for probing the *in vivo* physiology of this receptor in the brain (Agnetta et al., 2017).

Several studies have also indicated that in some species, including non-human primates, guinea pigs and certain strain of rats, CNO is reverse metabolised to the active parent compound, clozapine (Chang et al., 1998; Jann et al., 1994; MacLaren et al., 2016). This may limit the application of CNO-dependent DREADDs to only certain species and the translational potential of the technology. This has facilitated the development of novel

ligands with lower risks of being transformed into active metabolites such as compound 21 and perlapine (Chen et al., 2015).

The majority of studies employing chemical genetic approaches were performed with DREADD receptors that have been overexpressed in tissues and not under the control of the endogenous promoters for muscarinic receptors. In this context, the physiological effects observed in these studies reflect the outcomes of the signalling pathways activated or inhibited by the DREADDs, but not necessarily related to the physiological roles of the endogenous muscarinic receptors. To utilise the DREADD-based chemo-genetic approaches for elucidating the physiological roles of muscarinic receptors *in vivo*, these DREADD receptors must be expressed in tissues at physiologically relevant levels under the control of the endogenous muscarinic receptor promoters. Indeed, studies are beginning to emerge that use just this approach (Butcher et al., 2016) and although still in their infancy, these muscarinic DREADDs will provide a very powerful tool to selectively and reversibly manipulate the activity of specific muscarinic receptor subtypes *in vivo* thereby defining their physiological and pathophysiological functions.

Conclusion

There is now a very large body of data that have used chemical genetic approaches to define the signalling pathways that drive physiological responses. It is now also possible to replace wild type receptors with receptors that respond only to synthetic ligands. These engineered mice will not only allow for an understanding of the physiological pathways driven by individual receptors, but importantly allow for the first time, investigation of the clinically important outcomes of ligands that selectively activate a given GPCR subtype. In addition, this approach will also inform us as to the adverse "on-target" effects of receptor activation. Only time will tell if these DREADD receptors will impact significantly to validating GPCR targets but there is no doubt that this technology will increase in use to reveal the fundamental biology of GPCRs.

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Figure legends

Figure 1: Targeting construct for the generation muscarinic receptor (M$_1$ subtype) DREADD. The gene encoding the native M$_1$ muscarinic receptor was replaced by the DREADD construct using homologous recombination. Under this scheme, expression of the DREADD receptor is controlled by the endogenous muscarinic receptor promoter (taken from Butcher *et al* 2016).

Figure 2: Pharmacology of M$_4$ DREADD receptor. Wild-type muscarinic M$_4$ receptor activates ERK1/2 phosphorylation in response to the endogenous ligand ACh but does not respond to the synthetic ligand clozapine-N-oxide (CNO) (A). In contrast, the M$_4$ DREADD gains responsiveness to CNO but loses ability to respond to ACh (unpublished data).
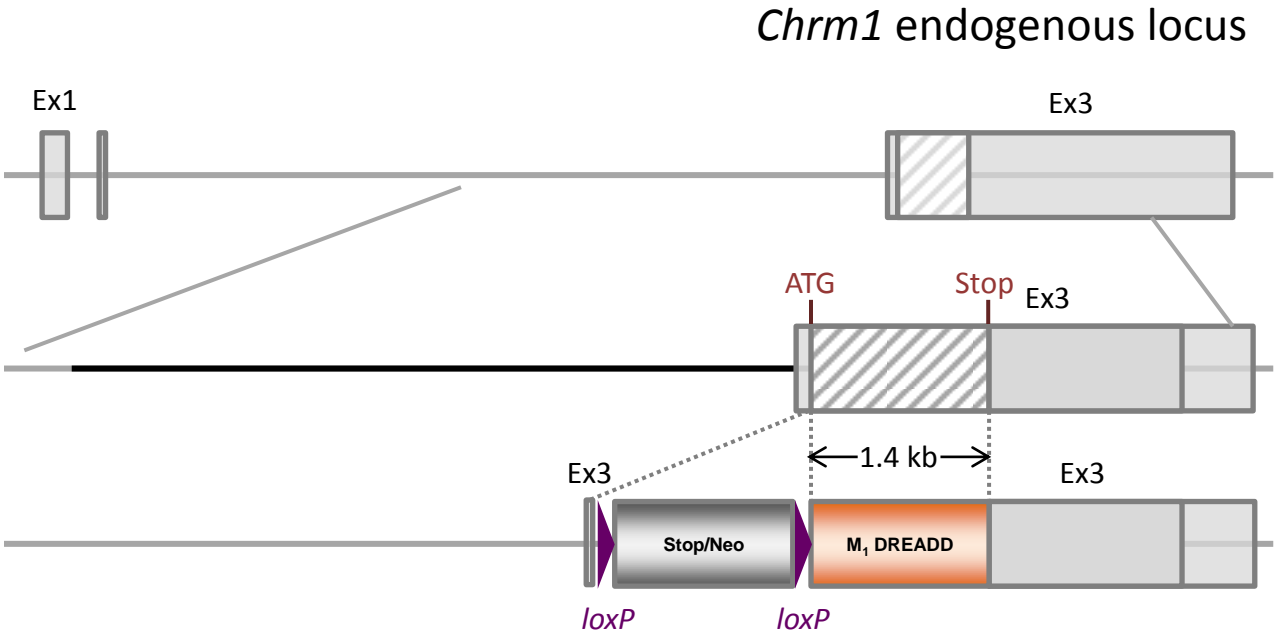
Figure 1

Chrm1 endogenous locus

Figure 2

A. M$_4$ wild-type

B. M$_4$ DREADD