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REVIEW ARTICLE

The use of chemogenetics in behavioural neuroscience: receptor variants, targeting approaches and caveats

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The last decade has seen major advances in neuroscience tools allowing us to selectively modulate cellular pathways in freely moving animals. Chemogenetic approaches such as designer receptors exclusively activated by designer drugs (DREADDs) permit the remote control of neuronal function by systemic drug administration. These approaches have dramatically advanced our understanding of the neural control of behaviour. Here, we review the different techniques and genetic approaches available for the restriction of chemogenetic receptors to defined neuronal populations. We highlight the use of a dual virus approach to target specific circuitries and the effectiveness of different routes of administration of designer drugs. Finally, we discuss the potential caveats associated with DREADDs including off-target effects of designer drugs, the effects of chronic chemogenetic receptor activation and the issue of collateral projections associated with DREADD activation and inhibition.

Abbreviations

AAVs, adeno-associated viral vectors; BBB, blood–brain barrier; CAMKII, calmodulin-dependent protein kinase II; CAVs, canine adenoviruses; CNO, clozapine *N*-oxide; DREADDs, designer receptors exclusively activated by designer drugs; hM3D, human M_3 muscarinic DREADD; hM4D, human M_4 muscarinic DREADD; HSV, herpes simplex viral; hSyn, human synapsin; KORD, κ-opioid-based receptor designer receptor exclusively activated by designer drugs; LGICs, ligand-gated ion channels; NAc, nucleus accumbens; RASSL, receptor activated solely by a synthetic ligand; SalB, salvinorin B; VTA, ventral tegmental area

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Introduction

During the last decade, there has been a revolution in neuroscience techniques that have resulted in increasingly precise methods to manipulate neural systems in awake, behaving animals. Understanding the relationship between brain function and behaviour is critical for the advancement of both neuroscience research and targeted medication development. Chemogenetics refers to the technique that allows for the reversible remote control of cell populations and neural circuitry *via* systemic injection or microinfusion of an activating ligand (Alexander *et al.*, 2009; Armbruster *et al.*, 2007). The chemogenetic technique uses engineered receptors and biologically inert ligands to achieve this aim. Unlike optogenetics, which has the ability to control cells and neural circuitry with light, the use of designer drugs makes chemogenetics simple to use, removing the need for optical fibre probes and tethers. While the temporal resolution of chemogenetics is lower than optogenetics, this relatively non-invasive technique is still effective for functional mapping, cell-type-specific manipulations and multiplexed control of neurons.

In this review, we will evaluate the different strategies that have been used to restrict chemogenetic receptors to defined neuronal populations. We will also highlight the use of a dual virus approach at targeting projection neurons and the effectiveness of different routes of administration of designer drugs. Finally, we discuss the potential caveats associated with chemogenetics including off-target effects of designer drugs as well as the issue of collateral projections associated with chemogenetic activation and inhibition.

Development of chemogenetic receptors

Necessary conditions for chemogenetic interrogation of brain function

Chemogenetic receptors are used to selectively modulate the activity of defined neuronal populations, primarily through a systemic drug injection. To be an effective behavioural neuroscience tool, a chemogenetic receptor must meet the following conditions: (i) the modified receptor must not be receptive to any endogenous ligand; (ii) the modified receptor needs to have minimal or no endogenous activity in the absence of ligand binding; and (iii) the modified receptor must have a high affinity for the ligand that has no pharmacological activity at other endogenous receptors (Urban and Roth, 2015).

Early chemogenetic receptors

GPCRs are at the forefront of current chemogenetic practice. The earliest evidence for the use of specifically engineered GPCRs was by Strader *et al*. (1991) who substituted a single amino acid residue to mutate the **β-adrenoceptor** to become activated by catechol-containing esters and ketones, compounds that do not activate endogenous β-adrenoceptors. This paper introduced the idea of modifying endogenous receptors, altering their specificity and binding properties, so that they can be activated at a time point chosen by the experimenter. Coward *et al*. (1998) subsequently developed GPCRs that responded solely to synthetic ligands and termed these receptors RASSLs (receptors activated solely by a synthetic ligand). One limitation of RASSLs was that the synthetic ligands often had high affinity at the native receptor (Coward *et al*., 1998). In addition, when RASSL expression was very high, they were found to have endogenous activity in the absence of ligand binding (Sweger *et al*., 2007), limiting their applicability *in vivo*.

Directed molecular evolution

Approaches to protein engineering are critical for designer receptor development, involving gene shuffling, random mutagenesis and analysis of structure and sequence (see Steiner and Schwab, 2012). RASSLs were generated using a rational design approach whereby receptors were generated with deliberate mutations at key residues important for native ligand binding (Strader *et al*., 1991; Coward *et al*., 1998). Dong *et al*. (2010) used a novel approach to receptor engineering, termed directed molecular evolution, to generate the next generation of chemogenetic receptors. The directed evolution of GPCRs that are exclusively activated by certain ligands involved the generation of a random mutagenesis library of amino acid substitutions in the DNA for **muscarinic receptor**s, through error-prone PCR (Dong *et al*., 2010). This was followed by screening the resultant population of mutant receptors for the ability to be activated by a designer ligand. This selection step was done by hijacking the natural signalling pathway of endogenous GPCRs in yeast to make GPCR activity critical for survival of the yeast host cell. The biologically inert ligand clozapine *N*-oxide (CNO) was selected as the designer ligand to activate the modified GPCR, and thus, the yeasts were screened for growth in medium with CNO. Multiple rounds of mutagenesis and selection were employed until receptors were identified that exhibited high affinity for **clozapine** and CNO but not **ACh** (Armbruster *et al*., 2007). This novel approach to receptor engineering resulted in a new class of RASSLs termed DREADDs (designer receptors exclusively activated by designer drugs) (Armbruster *et al*., 2007).

Types of chemogenetic receptors

Human muscarinic DREADDs and κ-opioid receptor DREADD

The muscarinic-based DREADDs meet the necessary criteria to be effective tools for behavioural neuroscience research. They are insensitive to the endogenous ligand (ACh), they have low constitutive activity, and they have orders of magnitude greater sensitivity to the ligand CNO compared with the endogenous ligand (Armbruster *et al*., 2007). Different muscarinic-based DREADDs have been developed that can either increase neuronal activity (Alexander *et al*., 2009) or decrease neuronal activity (Armbruster *et al*., 2007), and the mechanism of action for both requires the action of associated G proteins. The three main types of signalling pathways for muscarinic-based DREADDS are G_q, G_i and G_s. The G_q DREADD increases neuronal firing by stimulating phospholipase C, releasing intracellular calcium stores (Conklin *et al*., 2008). G_s DREADDs are less commonly used, stimulating **cAMP** production (Conklin *et al*., 2008). G_i DREADDs inhibit

cAMP production (Urban and Roth, 2015). Electrophysiological *in vivo* recordings of DREADD-expressing neurons show an initial effect of systemic CNO administration on neuronal activity after 5–10 min, with peak activity demonstrated after 45–50 min, and effects can last up to 9 h (Alexander *et al.*, 2009; Guettier *et al.*, 2009). The solubility of CNO varies depending on concentration and source. For example, CNO obtained from the National Institutes of Health (NIH) appears to be less soluble, requiring DMSO concentrations of up to 15% in a 10 $mg{\cdot}mL^{-1}$ solution (Raper *et al.*, 2017). However, 2 $mg{\cdot}mL^{-1}$ CNO obtained from NIH has been dissolved in sterile PBS (Carvalho Poyraz *et al.*, 2016). CNO from Biomol International has been shown to dissolve in 0.9% saline at concentrations of up to 10 $mg{\cdot}mL^{-1}$ (Guettier *et al.*, 2009).

One limitation from an experimental design perspective is that both excitatory and inhibitory receptors are activated by the same ligand, and therefore, selective manipulation of neurons within the same animal is not possible. Recently, Vardy *et al.* (2015) addressed this limitation by developing an inhibitory κ-opioid-based receptor DREADD (KORD) activated by the ligand salvinorin B (SalB), a metabolite of the **κ-opioid receptor** agonist **salvinorin A**. SalB has limited solubility, dissolving in 100% DMSO, but is faster acting than CNO, affecting neuronal activity *in vivo* within a few minutes after systemic administration and lasting approximately 1 h (Vardy *et al.*, 2015). KORD permits multiplexed control of diverse neuronal populations within the same animal, expression of both the human M_3 DREADD (hM3D) and KORD in the same population of neurons is possible, and behaviour can be bidirectionally controlled by systemic application of each DREADD ligand (Vardy *et al.*, 2015). Marchant *et al.* (2016b) showed that systemic injection of SalB in rats with ventral tegmental area expression of KORD reduces locomotor behaviour, demonstrating its efficacy *in vivo* in rats.

Ligand-gated G proteins: allatostatin neuropeptide receptor

Other methods for reversible inhibition of neural activity involve the use of the insect allatostatin neuropeptide receptor. The G-protein coupled *Drosophila* allatostatin receptor is activated by the peptide ligand allatostatin and has been used to silence neuronal activity (Lechner *et al.*, 2002). Allatostatin is soluble in saline and water and is fast acting (Haettig *et al.*, 2013). Electrophysiological recordings of allatostatin receptor-transduced neurons show neuronal silencing within minutes of bath application of allatostatin (Tan *et al.*, 2006), which is recovered within minutes following washout (Menuet *et al.*, 2017; Tan *et al.*, 2006). Haettig *et al.* (2013) used this technique to inactivate CA1 hippocampal interneurons and showed impairments in long-term memory for object location. Additionally, Menuet *et al.* (2017) used the allatostatin receptor to show a functional link between respiratory modulation of BP and hypertension *via* the inhibition of rostral ventrolateral medulla adrenergic (C1) neurons. Importantly though, allatostatin does not cross the blood–brain barrier (BBB), limiting its clinical potential.

Ligand-gated ion channels

Another method for controlling neuronal activity *in vivo* is with the use of **ligand-gated ion channels (LGICs)** (Magnus *et al.*, 2011). LGICs permit the control over ion conductance, allowing for the activation or inhibition of neurons (Magnus *et al.*, 2011). This strategy developed chimeric LGICs with unique conductance properties originating from combinations of ligand binding domains and ion pore domains (Magnus *et al.*, 2011). It requires intracranial injection of a virus encoding a pharmacologically selective actuator molecule (PSAM) element. An i.p. injection of the inert pharmacologically selective effector molecule binds to the LGIC causing activation or inhibition of PSAM-expressing neurons (Simonds *et al.*, 2014). Simonds *et al.* (2014) used this technique to demonstrate the involvement of leptin receptor-expressing neurons in the dorsomedial hypothalamus in increasing BP and heart rate in mice.

Modified receptors that are activated by **ivermectin** may be especially well suited for future clinical trials compared with muscarinic receptor-based DREADDs, because ivermectin is currently a FDA approved anti-parasitic drug. Lerchner *et al.* (2007) developed a glutamate and **ivermectin**-gated chloride channel that could be activated by systemic ivermectin administration *in vivo*. However, this channel had low expression levels. Lynagh *et al.* (2010) improved upon this design by identifying the A288G mutation of the human **α1 glycine receptor**, which had increased expression and ivermectin sensitivity. Islam *et al.* (2017) have also developed glycine receptor chloride channels, members of the LGIC family. Ivermectin has a $t_{1/2}$ of approximately 24 h in humans (Edwards *et al.*, 1988). However, neuronal silencing and reversal following ivermectin administration in animals is relatively slow with onset occurring within hours and lasting up to several days (Lerchner *et al.*, 2007). Additionally, ivermectin is insoluble in water but can be dissolved in methanol, high concentrations of ethanol, propylene glycol and DMSO (Lerchner *et al.*, 2007).

Viral methods for chemogenetic receptor expression

Types of viral vectors and promoters

The type of viral vector and promoter used may affect the neuronal transduction and expression of DREADDs. Typically, DREADD expression in behavioural neuroscience experiments is mediated by viral vector-induced neuronal transfection. The type of viral vector used depends on the experimental question. One of the most common methods is to use intracranial injections of recombinant adeno-associated viral vectors (AAVs)-encoding DREADDs for neuronal transfection. AAVs were developed to improve transduction capability and tropism by using capsid genes from other AAV serotypes (Gao *et al.*, 2002). AAVs are relatively non-toxic and achieve long-term (months to year) expression (Morsy *et al.*, 1998). There are several different serotypes of AAV, and each serotype has different transduction and retrograde transport efficiencies depending on the infected brain region (Aschauer *et al.*, 2013; Nair *et al.*, 2015). Lentiviral and herpes simplex viral (HSV) vectors are also used to transduce DREADD expression *in vivo* (Ferguson *et al.*, 2011; Mahler *et al.*, 2014). Lentiviral vectors appear to have greater transduction properties compared with AAV; however, they have

poor retrograde transport capabilities (Blomer *et al.*, 1997). HSV vectors provide specific neuronal transduction along with highly efficient retrograde transport. However, transduction using HSV vectors is typically lower than AAV or lentiviruses (Palella *et al.*, 1989; Soudais *et al.*, 2001).

The type of promoter chosen depends on the type of cell the experimenter is trying to target. In regard to cell-type-specific promoters, the size of the genetic material required to target these cells is important. This is also dependent on the type of viral vector chosen, with HSV vectors having the greatest capacity for multiple gene cassettes (Nair *et al.*, 2015). Several commercially available promoters are commonly used to examine the behavioural response to DREADD manipulations. For example, the human synapsin (hSyn) promoter is pan-neuronal, whereas **calmodulin-dependent protein kinase II** (CAMKII) predominately targets excitatory neurons, although not always (Jennings *et al.*, 2013; Yizhar *et al.*, 2011; Yau and Mcnally, 2015). There are also some specific promoters such as human glial fibrillary acidic protein, which is expressed in astrocytes (Yizhar *et al.*, 2011). However, it is common to find that the *in vitro* specificity of expression based on gene promoters do not always translate faithfully to the *in vivo* models.

Strategies to restrict chemogenetic receptor expression to neuronal subtypes

A key advantage of using viral vectors to mediate DREADD expression occurs when it is combined with Cre systems to restrict DREADD expression in genetically defined neuronal populations. Cre-dependent viral vectors permit restriction of DREADDs in neurons defined by the expression of specific genetic markers. Atasoy *et al.* (2008) designed an AAV system that uses a FLip and EXcise approach (Schnutgen *et al.*, 2003), similar to double-floxed inverted open reading frame, to restrict expression of viral-transduced DNA in Cre-expressing neurons. This technique has been widely adopted because of its efficiency in restricting expression of DREADD receptors to cells that express Cre. However, an important caveat with Cre systems that should be taken into account is the risk of 'tumour-causing' off-target effects *in vivo* (Janbandhu *et al.*, 2014). Another method for expressing DREADDs in specific neuronal subtypes is with the use of transgenic mice expressing DREADD receptors. Farrell *et al.* (2013) developed this model expressing the G_s DREADD specifically in striatopallidal neurons.

Circuit-specific uses of chemogenetics

Like optogenetics, chemogenetics can be used for selective interrogation of neuronal circuitry and manipulation of behavioural output. Two different strategies have been developed that allow experimenters to achieve this. One way is through local intracranial administration of the activating ligand (Figure 1). Typically, DREADD expression is induced through non-selective expression of DREADDs in the projection region, using AAV with promoters such as hSyn or CAMKII. In addition, intracranial cannulas are implanted above the projection target region. To date, several studies have used local infusions of CNO into the projection target region to cause selective manipulation of only the DREADD-expressing terminals in the target region (Lichtenberg *et al.*, 2017; Mahler *et al.*, 2014; McGlinchey and Aston-Jones, 2017; Stachniak *et al.*, 2014; Venniro *et al.*, 2017). For example, Ge *et al.* (2017) used this approach to show that inactivation of entorhinal cortex terminals in the dorsal dentate gyrus significantly decreased context-induced reinstatement of heroin seeking. While this technique has significant advantages to examine the neural circuitry of complex behaviours, the invasive methodology limits its clinical applicability. The use of high (1 mM) concentrations of CNO for microinfusions may lead to off-target effects (Gomez *et al.*, 2017). However, there have been no reports of general locomotor deficits with this intracranial dose of CNO (Ge *et al.*, 2017; Mahler *et al.*, 2014; McGlinchey and Aston-Jones, 2017; Venniro *et al.*, 2017).

Another approach for circuit-specific modulation of neuronal activity with chemogenetics uses a dual viral–vector approach (Figure 2). In these experiments, the Cre vector is a

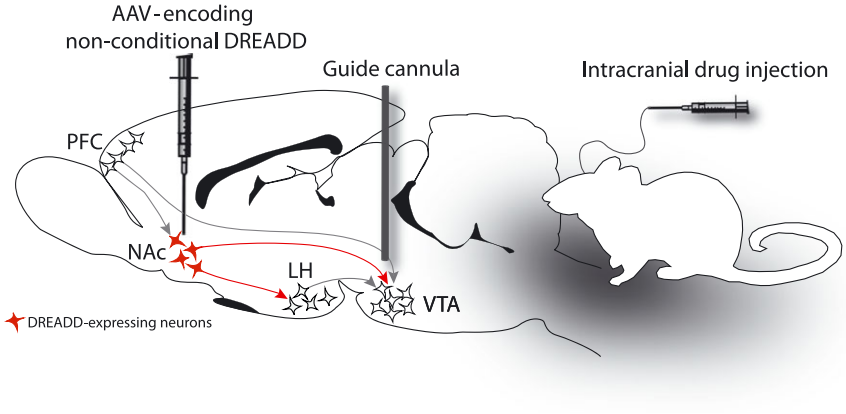

Figure 1

Interrogation of neuronal circuitry using chemogenetics: local intracranial administration of the activating ligand. Intracranial injection of DREADD ligands can be used to selectively manipulate neuronal circuits and behavioural output. In this sagittal rat brain schematic, an AAV-encoding non-conditional DREADD is injected into the NAcs, and intracranial guide cannulas are implanted above the VTA. Intracranial infusion of the DREADD ligand into VTA will change the activity of DREADD-expressing terminals in VTA.

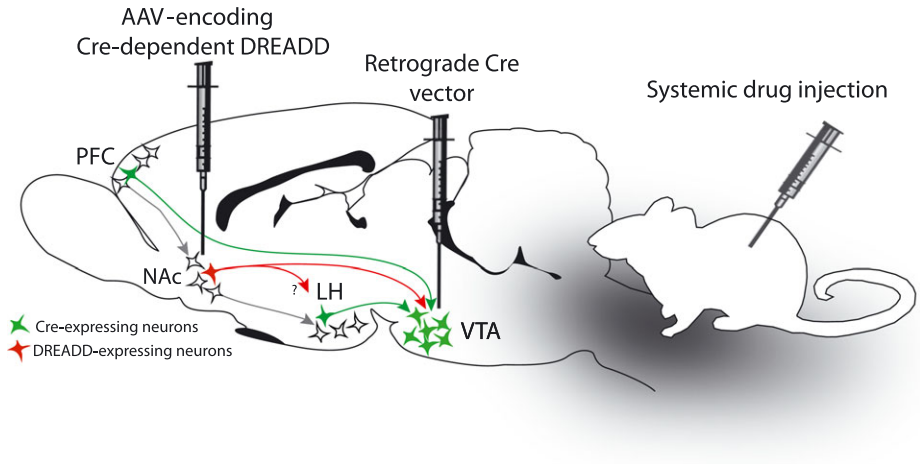

Figure 2

Interrogation of neuronal circuitry using chemogenetics: dual viral vector approach. A dual-virus approach may be used to manipulate neurons defined by their anatomical projections. In this sagittal rat brain schematic, an AAV-encoding Cre-dependent DREADD is injected into the NAcs, and a retrograde vector-encoding Cre is injected into the VTA. With this arrangement, Cre expression can be expected in the inputs to VTA, such as NAc, prefrontal cortex (PFC) and lateral hypothalamus (LH). However, chemogenetic receptor expression will be confined to NAc → VTA neurons. Systemic injection of the DREADD ligand will affect the activity of NAc → VTA neurons. However, this manipulation will also affect collateral projections of NAc → VTA neurons, such as to LH, if they exist.

retrograde transport type and is injected into a brain region that has anatomical connectivity with the brain region that receives the Cre-dependent vector. One advantage of this approach is that selective manipulation of neurons defined by their anatomical projection is possible through a systemic drug injection. The extent of retrograde labelling varies across brain regions depending on the serotype that is used (Aschauer *et al.*, 2013). Canine adenoviruses (CAVs) were pioneered by Kremer *et al.* (2000) as an alternative to human adenoviruses, because they display effective retrograde transport properties (Soudais *et al.*, 2001). Boender *et al.* (2014) used CAV to show that this approach is feasible for DREADD control over nucleus accumbens (NAc) projecting VTA neurons. More recently, Foldi *et al.* (2017) also used the CAV approach in VTA → NAc projection neurons and showed that excitation of this projection increased food intake, ameliorating activity-based anorexia-induced weight loss. This CAV–DREADD technique has also been used in other neural pathways. For example, Burgos-Robles *et al.* (2017) inhibited the projection from the basolateral amygdala to the prelimbic cortex using a combination of CAV and the human M_4 (hM4Di) DREADD to demonstrate a functional role for this pathway in fear-associated memories. The AAV serotype 2/5 has also been shown to have retrograde tropism (Aschauer *et al.*, 2013). Marchant *et al.* (2016a,b) used a similar approach to restrict KORD expression in ventral subiculum neurons that project to the NAc shell. They found that KORD-mediated inhibition of these projection neurons decreased context-induced relapse to alcohol seeking after punishment-imposed abstinence (Marchant *et al.*, 2016a).

Recently, Tervo *et al.* (2016) used a directed evolution approach to develop an AAV serotype specifically selected for retrograde transport. The serotype they developed (rAAV2-retro) was shown to label projection neurons at least as effectively as the traditional retrograde tracer FluoroGold. The application of this retrograde AAV serotype is likely to increase the effectiveness of circuit-specific manipulations using chemogenetics. The efficiency of DREADD expression within a specific pathway is likely to determine the magnitude of the behavioural effect that can be observed by chemogenetic manipulations. It is interesting to note that the dual-virus approach has also been tested in non-human primates. Oguchi *et al.* (2015) used *Macaca fuscata* monkeys and showed that Cre-dependent expression of a reporter protein (mCherry) is found in prefrontal cortex neurons that project to the caudate nucleus in monkeys with a highly efficient retrograde gene transfer (HiRet) lentivirus injected into the caudate nucleus.

Chemogenetic control in the spinal cord and periphery

Chemogenetics may also be used to manipulate cells (neurons and other cells) outside the brain. Karadimas *et al.* (2016) demonstrated that chemogenetic (hM3Dq) activation of lumbar glutamatergic cells in mice resulted in greater locomotor ability following cervical spinal cord injury compared with controls. Additionally, Miller *et al.* (2017) showed that chemogenetic (hM4Di) inhibition of sensory cells expressing $Na_v1.8$ channels, in the dorsal root ganglion, reduced early stage osteoarthritis-associated pain in mice. DREADDs have also been used in pancreatic cells, chemogenetic (hM3Dq/Ds) stimulation of pancreatic beta-cell signalling has been shown to increase insulin release (Guettier *et al.*, 2009; Nakajima and Wess, 2012). Finally, Jaiswal and English (2017) showed successful chemogenetic transduction of motoneurons in the lateral gastrocnemius muscle following an i.m. injection of hM3Dq DREADD. These studies together highlight the potential for the use of DREADDs in many types of cells, beyond the CNS.

Effective designer drug administration

Systemic CNO administration

One of the key advantages of chemogenetics is that remote control of defined neuronal populations is possible with a systemic drug injection. However, there is substantial variability in the literature in terms of the effective dose of CNO used to achieve a behavioural effect. The type of DREADD used (G_q, G_i or G_s), as well as the size of the target brain region, can influence the required ligand dose. For example, hM4D is often found to be less effective at inhibiting neuronal activity compared with the ability of hM3Dq to activate neurons. As a consequence, experiments with hM4D often require greater doses of CNO to induce behavioural effects (Farrell and Roth, 2013; Yau and Mcnally, 2015; Mahler *et al.*, 2014). One possible reason for this is that it may be easier to cause a behavioural effect than to interfere with its expression.

Intracranial and intracerebroventricular CNO administration

Circuit-specific manipulation is also possible *via* intracranial injections of CNO. Chemogenetic receptors are expressed throughout the neuronal cell body, axons and terminals, and local application of CNO in the terminal projection regions enables researchers to isolate chemogenetic manipulation to pathways. Mahler *et al.* (2014) used this approach to selectively modulate the activity of ventral pallidum output pathways in animal models of relapse to **cocaine**-seeking. They infused a synapsin-driven lentivirus-encoding non-conditional hM4Di DREADD into different ventral pallidum subregions (rostral or caudal), and cannulas were implanted above the VTA. On test for reinstatement of extinguished cocaine seeking, CNO was infused into the VTA (1 mM), selectively inhibiting these pathways. They found that cue-induced reinstatement, but not cocaine-priming-induced reinstatement, was decreased by inhibition of the rostral ventral pallidum → VTA pathway but not the caudal ventral pallidum → VTA pathway. This result further exemplifies the specificity of intracranial injection of CNO (see Non-specific effects of the ligands section). Mahler *et al.* (2014) show that the actions of CNO are specific to DREADD-expressing neurons with intra-VTA injections of CNO in the caudal ventral pallidum of rats having no effect on reinstatement.

Finally, i.c.v. injections of CNO may have more applicability for experiments examining the effect of chronic chemogenetic activation of neuronal populations. Nakajima *et al.* (2016) have performed i.c.v. injections of CNO (1 μg) into mice with hM3Ds expression in **agouti-related peptide** neurons and examined food intake. They found that i.c.v. CNO caused a long-lasting effect on food intake and have argued that i.c.v. administration of CNO yielded more consistent results compared with systemic CNO administration.

Oral administration of CNO

CNO can also be administered p.o., *via* food or water, for studies examining chronic activation of DREADD-expressing neuronal populations. Using this method of voluntary CNO administration, saccharin or other sweeteners are often added to the CNO water to make it more palatable, introducing potential confounding factors in some studies. In a study examining the role of the NAc core in binge alcohol drinking in mice, Cassataro *et al.* (2014) used a dose of 0.1 $mg \cdot mL^{-1}$ CNO in tap water. They found that the mice consumed approximately 3 $mL \cdot day^{-1}$, resulting in a dose approximating 10 $mg \cdot kg^{-1}$ over 24 h. This dose was found to be sufficient to decrease ethanol consumption in mice expressing hM4Di in the NAc and to increase ethanol consumption in mice expressing hM3Dq in the NAc. This method is particularly attractive because of the non-invasive test method, which reduces stress particularly in cases of chronic or repeated testing with CNO (Jain *et al.*, 2013). Furthermore, CNO has been reported to retain effectiveness 5–10 h after a systemic injection (Alexander *et al.*, 2009). Thus, the time course of administration in drinking water might be considered comparable with systemic injections.

Use of chemogenetics in non-human primates

The translational nature of DREADDS and its therapeutic potential in humans is highlighted by several non-human primate studies. Importantly, these studies have shown repeatable changes in reward-related behaviours following repeated DREADD-induced inactivation of several brain regions including the orbitofrontal cortex and rostromedial caudate (Eldridge *et al.*, 2016; Nagai *et al.*, 2016). These primate studies have also demonstrated the importance of anatomical connectivity and functional interactions using the hM4Di DREADD in combination with MRI scans (Grayson *et al.*, 2016). A recent study by Raper *et al.* (2017) examined the pharmacokinetics of s.c. CNO administration in rhesus monkeys. They found that CNO readily metabolizes to clozapine in monkeys, which may interfere with the behavioural interpretation of DREADD-based experiments in both humans and non-human primates. In both humans and rodents, CNO is also metabolized to clozapine (Chang *et al.*, 1998; Gomez *et al.*, 2017). It may be that effective DREADD manipulation in non-human primates and rodents will require low doses of clozapine. Given that muscarinic-based DREADDs have 100-fold greater sensitivity to clozapine than CNO (Armbruster *et al.*, 2007), it remains possible that there is a therapeutic window for selective manipulation of DREADDs with a dose of clozapine that has minimal off-target effects on the many receptors that clozapine acts on. Finally, it is important to note that other DREADD ligands exist. For example, Chen *et al.* (2015) suggest the use of perlapine, a hypnotic agent, or compound 21, which both have greater selectivity for hM3Dq over the native hM_3 receptor. However, *in vivo* testing for off-target effects of perlapine is yet to be conducted.

Potential caveats associated with chemogenetics

Collateral projections in circuit-specific DREADD experiments

The dual-virus approach has the advantage that manipulation of neurons defined by their anatomical projections is

possible with a systemic drug injection. However, one limitation of this approach is that collateral projections are also included in the manipulation. For example, using the dual-virus approach, while the DREADD-expressing neurons do by definition project to the target region where the retrograde Cre virus was injected, any collateral projections would also contain DREADDs (Figure 2). Thus, systemic drug injection of the chemogenetic ligand has the potential to alter the activity of more than one projection. This factor will vary depending on the circuit that is being interrogated and whether the transduced neurons have extensive collateral projections. For example, in the case of ventral subiculum projections to the NAc shell, Marchant *et al*. (2016a) used immunolabelling for the hemagglutinin tag for KORD to show that terminal expression was highest in the NAc shell, the site of retrograde Cre injection, with minimal observation of terminals in other known outputs of the ventral subiculum. Studies in other pathways, such as output pathways of the basolateral amygdala (Beyeler *et al*., 2016), have been similarly analysed and extensive collateralization has been found in some pathways (e.g. basolateral amygdala → ventral hippocampus), but not others (e.g. basolateral amygdala → central amygdala).

The extent to which this is a limiting factor for this approach remains to be shown. It raises interesting questions about whether information routing in the brain does occur within single circuits or whether activation of the collateral output targets are also necessary to mediate function. *In vivo*, neurons that have collateral projections do not discriminate between these output projections. Therefore, collateral projections are a critical part of normal brain function, where output projections exert their function through modulation of activity in all downstream nuclei, rather than in just one output target. Nevertheless, the use of intracranial ligand injections to selectively isolate circuit projections addresses the limitation of collateral projections.

Non-specific effects of the ligands

Chemogenetic receptors are sensitive to otherwise pharmacologically inert ligands. Recent debate has surrounded the potential for non-DREADD mediated effects of CNO or other ligands. In the example of human muscarinic DREADDs, clozapine is a major metabolite of CNO, a prototype atypical antipsychotic drug frequently used for the treatment of schizophrenia and other psychotic-related disorders (Geddes *et al*., 2000). A recent study by MacLaren *et al*. (2016) showed that small doses of CNO (1 mg·kg^{-1}) altered the startle response to a loud acoustic stimulus, and larger doses (5 mg·kg^{-1}) reduced **amphetamine**-induced hyperlocomotion in rats that do not express any DREADD receptors. However, it should be noted that while CNO altered these two behaviours in the absence of DREADD receptors, several other behaviours, including spontaneous locomotion and prepulse inhibition, were not affected. These data show that CNO is not entirely pharmacologically inert and that an effective dose of CNO must be established where off-target effects are minimalized. MacLaren *et al*. (2016) suggest that the inclusion of behavioural control groups, specifically a CNO-treated group without DREADD virus, will go a long way to address this limitation.

Until recently, the precise *in vivo* action of CNO had not been fully investigated. Gomez *et al*. (2017) recently reported no evidence that CNO crosses the BBB, in contrast to the findings of Ji *et al*. (2016). These data suggest that activation of DREADDs *in vivo* is likely to be mediated by metabolism of CNO to clozapine, which readily crosses the BBB. Furthermore, Gomez *et al*. (2017) showed that clozapine has a much higher affinity for the hM4D than CNO, which was demonstrated initially by Armbruster *et al*. (2007). One interpretation of these data is that a major premise of the muscarinic-based chemogenetic approach is compromised, because it is in fact clozapine that is causing activation of the muscarinic-based DREADDs. Based on this, Gomez *et al*. (2017) suggest that subthreshold doses of clozapine may be suitable for *in vivo* DREADD experiments, rather than CNO itself. This may result in confounding behavioural effects given clozapine has affinity for several serotonergic and dopaminergic receptors (Meltzer, 1994). However, concerns regarding this caveat should be tempered because the affinity of clozapine for muscarinic-based DREADDs is substantially higher than for native receptors, and an effective dose-window is achievable because of this. However, the inclusion of a control group without DREADD expression is a critical for interpretation of chemogenetic experiments. One potentially relevant consequence of this finding is that intracranial injections (see Intracranial and intracerebroventricular CNO administration section) avoid this complication. Because CNO is injected directly into the brain, the actions on DREADDs are more likely to be mediated by CNO, rather than clozapine.

Effects of chronic drug administration and DREADD activation

The most common use of chemogenetics is for acute manipulation of neuronal function to identify a critical role of the neuronal population expressing chemogenetic receptors, in a specific behaviour. This approach has provided, and will continue to provide, important findings in terms of basic neuroscience. However, any potential clinical applications will rely on chronic or repeated drug administration and receptor activation. With this in mind, the effect of chronic activation of muscarinic-based DREADDs, which exert their actions through G-protein coupled signalling cascades, is a factor that can be given greater focus. While chronic (daily for 4 weeks) administration of CNO (i.p.; 1 mg·kg^{-1}), stimulating beta cells expressing hM3Dq, has been used to examine high-fat-diet-induced diabetes in mice (Jain *et al*., 2013), the off-target behavioural effects of chronic CNO were not examined. Urban *et al*. (2016) sought to examine the long-term effects of serotonergic neuron stimulation assessing the effect of both acute (one 2 mg·kg^{-1} i.p. injection) and chronic (5 mg·kg^{-1}·day^{-1} for 3 weeks in drinking water) activation of these neurons in **5HT transporter**-Cre mice. Interestingly, they found that chronic administration effectively reduced anxiety-like behaviours. These clinically relevant results highlight the different behavioural effects that follow acute or chronic stimulation of a population of neurons. Furthermore, they suggest that chronic administration of CNO can induce important neural adaptations that acute experiments are not able to detect. In summary, the effects of chronic DREADD ligand administration, as well as chronic receptor activation, are critical factors that need to be extensively studied before the translational potential of chemogenetics is realized.

Conclusion and future directions

The chemogenetic technique has allowed for significant progress in the basic neuroscience mechanisms of animal behaviour. Because this approach is relatively less invasive than optogenetics, it may be favoured for clinical application. The use of AAV in clinical studies has precedent, with studies involving transgene expression in patients with Parkinson's disease (Christine *et al*., 2009). Thus, at the very least, viral vector transfection is not a limiting factor for the translatability of this technique. Indeed, recent advances have been made, which enable efficient transduction and non-invasive gene delivery throughout the central and peripheral nervous systems *via* i.v. AAV injection (Chan *et al*., 2017). However, important caveats regarding the specificity of existing DREADD ligands, as well as the lack of evidence that chronic DREADD activation will yield the same effects as acute activation, limit immediate translatability. Nevertheless, this technique has significant future potential for basic neuroscience discoveries into the neural control of animal behaviour.

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding *et al*., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander *et al*., 2017a,b,c,d).

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Conflict of interest

The authors declare no conflicts of interest.

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