Chapter 6

The Use of DREADDs (Designer Receptors Exclusively Activated by Designer Receptors) in Transgenic Mouse Behavioral Models

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Abstract

Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) are increasingly used to manipulate activity in specific neuronal populations in the brains of awake, behaving mice. Here we review the pros and cons of DREADDs relative to other genetically encoded neuromodulation technologies and describe in detail methods for using DREADDs with transgenic mouse behavioral models. This approach can not only provide insight into the role of specific neural circuits in behavior but also identify potential neuromodulation targets for the treatment of neuropsychiatric disorders.

Key words DREADDs, CNO, Reverse pharmacogenetics, Addiction, Mouse behavior, Transgenic mice

1 Background and Introduction

Since the development of the Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) [1] and their first use in awake behaving mice [2], there have been an increasing number of studies using this technology to examine the behavioral relevance of specific neural circuits in mice. A number of these studies have focused on the circuitry of the striatum, examining the differential roles of the direct and indirect pathways in amphetamine sensitization [3, 4] or the effect of modulating ventral striatum in a preclinical model of alcohol abuse [5]. Others have taken advantage of the cell-type specificity of DREADDs to determine the differential roles of AgRP-expressing [6] and POMC-expressing [7] neurons of the hypothalamus in feeding behavior as well as orexin-expressing hypothalamic cells in sleep-related behavior [8].

Given the increasing availability of Cre recombinase mouse driver lines selective for different neuronal populations [9] and the growing relevance of mouse behavior in neuroscience in general,

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this trend will only continue. While DREADDs are a robust technology, there are nevertheless a number of potential pitfalls to be avoided when using them in mouse behavioral assays. In this chapter, we describe several of these pitfalls as well as the framework and protocols that we have found to be effective in avoiding them. We hope that this will facilitate the successful application of this technology to dissecting the functional roles of specific neural circuits as well as to identifying novel targets and strategies for the treatment of neuropsychiatric disorders.

2 Experiment Planning

2.1 DREADDs Versus the Alternatives

The first issue to address in the planning stage is to determine whether or not DREADDs are the optimal tool for the particular experimental question at hand. The primary features of DREADDs are that they enable (1) genetic targeting of specific neuronal subtypes, (2) reversibility on a timescale of hours, (3) focal neuromodulation without chronically implanted hardware, and (4) modulation of large brain structures that are difficult to illuminate. If any of these are not required for the experiment at hand, other techniques should also be considered. For example, if genetic encodability is not required, techniques such as lesioning, electrical stimulation, or focal drug infusion strategies may be preferable (Table 1). Similarly, if genetic targeting is required but reversibility is unnecessary or even undesirable, alternatives include genetically encoded tetanus toxin [10] or ion channels such as NaChBac [11] or Kir2.1 [12, 13], which depolarize or hyperpolarize the membrane, respectively.

If both genetic targeting and reversibility are required, then alternative genetically encoded neuromodulation technologies should still be considered. Two of the most widely used options include optogenetics [14, 15] and the PSAM/PSEM system [16]. The primary advantage of optogenetics is its fast reversibility, which can be advantageous in certain behavioral paradigms in which modulation and control trials can be interleaved on timescales of seconds. However, optogenetics requires indwelling optical fibers and lasers for illumination, severely restricting the throughput of behavioral assays. In contrast, DREADDs enable a large number of animals to be tested in parallel without the need for extensive equipment. Additionally, DREADDs can target multiple sites simultaneously, which is often necessary for large brain structures and can be difficult using optogenetics because it requires complex implantations of multiple optical fibers.

The PSAM/PSEM system is another reverse pharmacogenetic system in which engineered receptors are expressed ectopically in the neurons of interest and activated by administration of an otherwise-inert ligand. A key difference between PSAMs and

Table 1

Comparison of several common methods for modulating neural activity in behaving mice

Method	Reversible?	Cell-type specificity	Effect on membrane potential	Onset of effect	Duration of effect
DREADDs	Yes	Yes	Depolarize or hyperpolarize	~30 min with IP administration	2–3 h with IP administration
Microbial opsins (e.g., channelrhodopsin-2)	Yes	Yes	Depolarize or hyperpolarize	Milliseconds	Milliseconds to hours
PSAM/PSEMs	Yes	Yes	Depolarize or hyperpolarize	Minutes	~10 min with IP injection
Kir2.1	No	Yes	Hyperpolarize	Days	Permanent
NaChBac	No	Yes	Depolarize	Days	Permanent
AAV-Tetanus toxin	No	Yes	Blocks synaptic vesicle release	Days	Permanent
Lesioning	No	Variable	Cell death	Days	Permanent
Electrical stimulation	Yes	No	Depolarize	Milliseconds	Milliseconds to hours
Local drug infusion	Yes	No	Depolarize or hyperpolarize	Seconds	Minutes to hours

DREADDs is that PSAM ligands (PSEMs) have significantly shorter half-lives than the DREADD ligand CNO and are also not available commercially. Another important difference is that PSAMs are ion channels that alter membrane potential directly, but DREADDs are G protein-coupled receptors that influence membrane potential indirectly by modulating endogenous ion channels. This means that the efficacy of DREADDs will differ between different cell types and developmental time points. For example, one important limitation of DREADD-hM4Di is that it is unlikely to be effective at early postnatal time points (e.g., before P10) because of insufficient expression of endogenous GIRK channels [17, 18]. Even in adult mice, DREADD-hM4Di may only be sufficient to partially silence some cell types. Additionally, DREADDs alter G-protein dependent cellular processes beyond membrane potential, which may complicate interpretation of results in some experiments. Nevertheless, DREADDs have proven to be a robust technology that works in a variety of cell types and brain regions.

2.2 Targeting the Cells of Interest

Having decided on the DREADD/CNO system, the next step is to determine how to express the chosen DREADD in the cells of interest. The two main methods are transgenic DREADD-expressing mouse

lines and viral vector approaches. Several DREADD mouse lines developed by the Dymecki and Roth laboratories are now available from the Jackson Laboratory [4, 19, 20]. The primary advantage of transgenic mouse lines over viral vectors is that with viral vectors only a subset of the cells are infected, while with transgenic animals virtually 100 % of the cells of interest will express the DREADD given an efficient driver line. This also leads to greater uniformity of expression levels across the cell population. However, this approach is frequently problematic if DREADDs are expressed in undesired brain regions or outside the brain, leading to nonspecific effects if CNO is administered systemically.

For this reason, we generally use adeno-associated viral (AAV) vectors to express DREADDs in the brain, which enables us to restrict expression to specific anatomical regions. In addition, AAV provides high transduction efficiencies (up to 80 %) and expression levels with low toxicity, and it is commercially available at relatively low cost. There are several possible methods for targeting AAV expression to particular cells of interest, but we will focus on three: using cell-type specific promoters, using Cre-dependent AAV to target specific anatomical projections, and using Cre-dependent AAV in Cre-expressing transgenic mice.

Cell type-specific promoters are of limited utility in AAV for several reasons. Most promoters are too large for the ~5 kb packaging limit, drive transgene expression too weakly, and/or exhibit leaky expression in undesired cell types, possibly due to the high number of viral genomes introduced to each cell. To date, the only well-validated cell-type specific promoter for use in AAV is a minimal CaMKIIa promoter that in cortex and hippocampus drives expression selectively in excitatory pyramidal cells. This promoter was first validated in lentiviral vectors and then in AAV [21, 22]. Other viral vectors with larger capacity can carry longer promoter sequences that in theory could provide higher specificity than AAV; nevertheless, this approach frequently leads to leaky nonspecific expression.

As a result, the more common approach is to use AAV containing Cre-dependent "FLEX" [23] or "DIO" [24] cassettes, in which the transgene of interest (e.g., a DREADD) is initially in the reverse orientation and hence inactive. The presence of Cre recombinase irreversibly inverts the orientation of the transgene, activating transcription (Fig. 1). Using this approach, it is possible to target cells based on projection pattern by injecting Cre-dependent AAV near the somata of the cells of interest and a retrograde Cre vector near the axon terminals [25–28]. However, a more common method is to use transgenic/knock-in mouse lines that express Cre in a subset of cells based on the expression profile of a given gene. A growing number of Cre driver lines are commercially available from vendors such as Jackson Laboratories, as well as from publicly funded projects such as the GENSAT project [9].

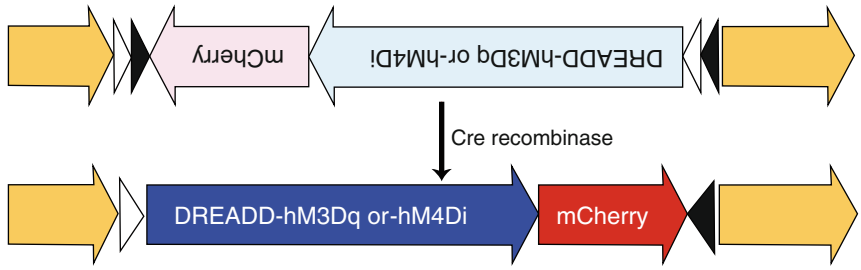

Fig. 1 The flip-excision ("FLEX")/double-inverted open reading frame ("DIO") method restricts expression of DREADDs to Cre-positive cells

2.3 Mouse Breeding

Another important consideration that should be planned as far in advance as possible is the breeding scheme for the experiments. Power calculations should be performed to estimate the number of animals needed, and the breeding scheme should be based on this estimate. Relevant considerations also include whether males, females, or both will be used; the proportion of mice expected to carry the required allele/alleles; and the number of mice required for control groups including Cre-negative littermates and Cre-positive littermates injected with a control AAV not encoding a DREADD. The background strain of the mice is also important, and transgenic mouse lines should generally be backcrossed onto an inbred background such as C57BL/6 in order to minimize variability in anatomical targeting and behavioral performance. While C57BL/6 is the most common inbred strain used for behavior assays, we generally prefer to use C57BL/6×FVB/NJ F1 hybrids because FVB females generate larger litter sizes, and hybrid mice are larger, healthier, and, at least in our hands, more reliable overall.

2.4 Incorporating DREADDs into the Behavioral Paradigm of Interest

One of the key features of DREADDs is that the modulatory effect is reversible, which enables partial within-subjects experimental designs in which the behavioral effects of DREADDs are ascertained by comparing the difference between the CNO and CNO-negative control condition within a single animal. Although this may slightly complicate analysis, we strive to use within-subjects designs whenever possible because using each animal as its own control helps to minimize variability and reduce the number of animals required. Nevertheless, a number of control conditions are usually necessary. Because a number of Cre lines exhibit abnormal behavior, it is important to perform control experiments in which Cre-positive animals are injected with an AAV that does not encode a DREADD. An equally important but lesser-recognized control involves injecting Cre-dependent AAV into Cre-negative littermates, which we have found for some AAVs can have a surprisingly large effect on behavior. Depending on the behavior being tested,

it is frequently necessary to perform experiments controlling for nonspecific effects such as impaired locomotion or motor coordination. Although the need for this type of control experiment applies to any intervention, DREADDs offer the advantage of a within-subjects design for these experiments as well (e.g., comparing open field locomotion in saline vs. CNO conditions).

Finally, the CNO delivery method, delivery time, and dose must also be determined. The most common delivery methods are oral administration and IP injection, though focal infusions directly into the brain are also effective [29]. For long-term administration (e.g., overnight), we generally use CNO in drinking water at 0.1 mg/mL. Since a 30 g mouse generally drinks about 3 mL/day, this amounts to an approximate dose of 10 mg/mL of CNO daily. For shorter-term administration, we dissolve CNO in sterile PBS and inject intraperitoneally at a dose of 1–5 mg/kg. Under these conditions, DREADD activation generally takes ~30 min and lasts about 2–4 h. However, Alexander et al. [2] have reported a longer duration of effects.

3 Preexperiment Optimization

The single most important factor in the success of behavioral experiments using DREADDs is obtaining high levels of DREADD expression selectively in the cells and target region of interest. Careful preexperiment optimization is crucial.

3.1 Optimizing Anatomical Targeting

Before starting experiments, it is important to test viral injection conditions and optimize targeting of the brain structure of interest. Stereotactic coordinates from atlases are a good starting point, but in our experience these coordinates typically require refinement. When targeting a new brain structure, especially structures that are small and/or ventral, we first start by injecting small volumes (e.g., 50 nL) of Evans Blue followed immediately by sectioning the brain to verify targeting accuracy. Alternatively, one can also place a small electrolytic lesion (100 μA cathodal current for two seconds from a 50 μm tungsten electrode) and section the brain 24–48 h later. The use of dye or lesions rather than AAV-EGFP in pilot injections provides rapid feedback and a more precise estimate of the true location than EGFP expression, which fills the processes of the cells and thus extends to a larger spatial area. It is important to use mice of the same background strain, age, and sex that will be used for the experiments. If possible, we use inbred mice or F1 hybrids so that all mice are genetically identical, minimizing anatomical variability. After refining and validating coordinates, we inject commercially available AAV-EGFP to verify the amount of spread and optimal volume of virus to inject. The degree of spread is dependent on serotypes and titer, so it is best to use the same serotype that will be used for experiments.

3.2 Optimizing Cellular Targeting

To target a molecularly defined cell population with Cre driver lines and Cre-dependent AAV, both the mouse line and the viral vector must be validated and optimized if this has not been done in the past. An underappreciated aspect of using AAV is that even among serotypes with known tropism for neurons, different serotypes infect various neuronal subtypes with different degrees of efficiency. When targeting a novel cell type, we first test a panel of several AAV serotypes (e.g., AAV1, AAV5, and AAV9) to see which is the most efficient for the cell type of interest. For our studies in striatum, we generally use AAV5, which infects most striatal neurons efficiently with relatively low levels of retrograde infection. AAV-DREADDs are now commercially available in AAV2, 5, and 8 from the University of North Carolina Vector Core, providing several options.

When using an untested Cre driver line, it is important to verify that expression is actually restricted to the cells of interest. This is particularly true for transgenic Cre lines, in which the expression pattern is less likely to mimic the endogenous expression pattern than with knock-in lines. The simplest technique is to inject a Cre-dependent AAV encoding a fluorescent protein such as GFP, then use immunohistochemistry to verify that all of the green cells also express the marker gene of interest (Fig. 2). For marker genes where no suitable antibody is available, a logical approach would be to cross the Cre driver with a Cre-dependent fluorescent protein reporter line, then inject the Cre-dependent AAV and quantify colocalization of the two fluorescent proteins. However, we and others [30] have found that this approach is not reliable because most Cre-dependent AAVs will suppress expression of Cre-dependent genomic reporters. Additionally, expression patterns in reporter lines may be broader than with Cre-dependent AAV due to recombination at early developmental time points. As a result, the best approach in this case is to find alternative markers for immunohistochemistry or to perform in situ hybridizations.

4 Performing the Experiment

4.1 Stereotactic Injection Protocol

A protocol for stereotactic injections will vary depending on the specific hardware and has been covered in detail elsewhere [31]. Nevertheless, given the importance of proper injection technique, we will review the factors that we consider to be critical.

- Fill the injector pipet/syringe with AAV before anesthetizing the first animal. We typically fill the pipet with enough AAV to inject several animals in a row. Upon receiving a new AAV stock, we make 8 μL aliquots in PCR tubes and store at −80 °C. Only thaw each aliquot once—after thawing, an aliquot is stable

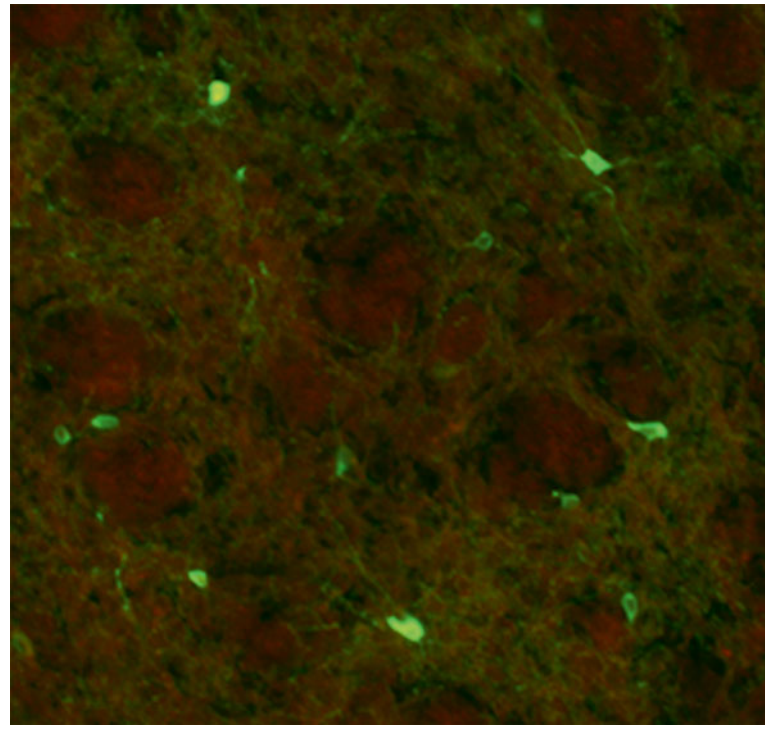

Fig. 2 Validation of cre driver line by immunohistochemical colocalization of a cellular marker (*green*) and DREADD-mCherry (*red*). Since the AAV does not infect all neurons, some green neurons do not express mCherry, but all mCherry-expressing neurons are positive for the immunohistochemical marker, consistent with the cre driver line restricting DREADD-mCherry expression to the cells of interest

at 4 °C for up to a month. Prepare all other instruments and supplies prior to starting anesthesia to minimize the duration of anesthesia.

- Anesthetize the animal. We prefer the use of isoflurane to injectable anesthetics such as ketamine/xylazine because the animals recover much more rapidly after surgery. When using isoflurane, be sure to use a heating pad and monitor the animal's body temperature and respiratory rate every 15 min. If the rate drops below 20 respirations in 15 s, lower the isoflurane concentration. For complex surgeries requiring more than three hours of anesthesia, we administer atropine 1 mg/kg intraperitoneally at the start of the procedure to reduce secretions that can lead to respiratory compromise.
- Fix the animal's head in the stereotactic frame securely. This may sound trivial, but doing this properly is the most technically difficult aspect of the procedure and is a key step in achieving accurate, reproducible results. First, coat the tips of the ear

bars with topical lidocaine cream, which decreases anesthesia requirements. When using isoflurane anesthesia, place the animal in the nosepiece first and stabilize the level of anesthesia at a higher isoflurane concentration than used for maintenance anesthesia (e.g., 2 %). Next, loosen the nosepiece so the skull can tilt up and down freely, and lift the animal by the tail until the skull is level before securing the ear bars. Note that ear bars should not be placed in the ear canals but on the skull anterior to the ear opening, which provides greater stability. After tightening the ear bars and nosepiece, push on the head to verify that the skull does not move. Next, cover the eyes with eye ointment or a mix of petroleum jelly and mineral oil, and reduce the isoflurane concentration to a maintenance level (e.g., 0.8–1 %).

- After removing the hair and prepping the skin with betadine, inject bupivacaine subcutaneously, and open the scalp with sharp scissors cutting from posterior to anterior along the midline. Bupivacaine decreases anesthesia requirements, but it may still be necessary to increase the isoflurane concentration for this step. Clear away the connective tissue covering the skull, and hold the incision open with small retractors or hooks fashioned from bent needles. Push the skull with blunt forceps to verify that the head is fixed securely, and if necessary, reposition or tighten.
- Under maximum magnification, mark the calibration points bregma and lambda on the skull with a scalpel or sterile razor blade. Mark these locations with an "X" by interpolating along the skull sutures—do not use the actual intersections of the skull sutures, which vary considerably from animal to animal. Touch the corners of the "X" with a new fine-tip permanent marker—the ink will flow into the "X," marking it for easy visualization.
- Ensure that the skull is level, first by visual inspection and next by measuring the *Z*-axis (vertical) position of points 2.5 mm to the left and right of bregma. For small and/or ventral targets, our rule is that the difference in *Z*-axis measurements must be 80 μm or less. Next, ensure that the difference in *Z*-axis positions of lambda and bregma is also 80 μm or less. For this step, it is highly advantageous to have a stereotactic frame that allows adjustment of head angle without loosening the ear bars, such as the Kopf Instruments 1900.
- Mark the desired injection sites and drill craniotomies. Stop drilling before puncturing the dura and clear skull fragments from the craniotomy using a small (~28–30 gauge) needle with the tip bent away from the opening to form a hook. The dura is difficult to visualize in mice, so the best way to determine

if it is intact is to poke the brain surface lightly with a sharp needle. If the dura is intact, the brain surface will indent visibly around the needle. After cutting craniotomies, keep them from drying out using sterile saline and saline-soaked gel foam.

- Fit the injector onto the frame and re-calibrate coordinates to bregma and lambda. Lower the pipet slowly, punching through the dura, to reach the target coordinates.
- Infuse AAV at a rate of 50–100 nL/min, then wait 5 min after the injection to remove the needle/pipet. Withdraw the pipet slowly to minimize backflow of virus along the injection tract.
- Close the incision, stop anesthesia, and monitor the animal until it has recovered.

4.2 Post-surgery Recovery

For the first 3–4 days after injection, house animals individually to prevent other mice from opening the wound. After this period, we recommend group housing animals if possible to minimize the behavioral effects of social isolation. Although fluorescent protein expression is detectable as early as 3 days after AAV injection, we recommend waiting at least 2 weeks before starting experiments. Expression typically peaks around 3–4 weeks after injection and generally persists for months.

4.3 Preexperiment Conditioning and Behavioral Data Collection

Perform any necessary preexperiment conditioning (e.g., habituation to a test chamber and IP injections), and collect the behavioral data. The actual procedure will vary depending on the assay, but the procedural aspects of CNO administration are the same as any other drug delivered through the same route. As with any behavioral assay, uniform handling is critical to minimize variability.

5 Post-experiment Verification and Data Analysis

After the experiment, harvest and process brains for histology according to any standard protocol. We sacrifice animals with an overdose of sodium pentobarbital, followed immediately by transcardial perfusion with a fresh solution of 4 % paraformaldehyde in ice-cold PBS. After perfusion, we harvest the brain and postfix in 4 % PFA for 1 h, then cryoprotect the tissue with 30 % sucrose in PBS overnight. For sensitive detection of fluorescent proteins, we cut 20 μm cryosections and perform immunohistochemistry against the fluorescent reporter (in our case, usually mCherry). However, in cases where the density of mCherry-positive cells is high, it is possible to save time by cutting thick (100–150 μm) sections, mounting them, and visualizing native mCherry fluorescence (Fig. 3). When rating the accuracy of anatomical targeting, it is crucial that the person rating the brains is blinded to the experimental group of each sample to prevent bias.

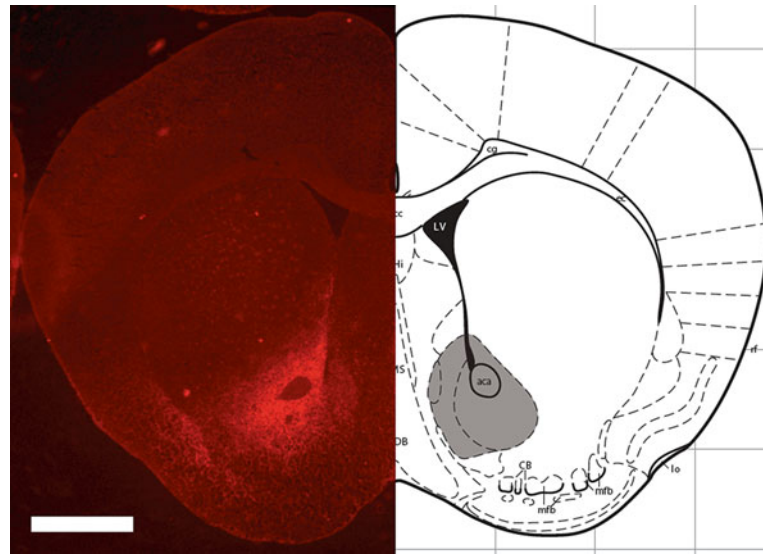

Fig. 3 Visualization of DREADD-mCherry expression after stereotactic AAV injection in the nucleus accumbens

6 Conclusion

The DREADD-CNO system enables targeted modulation of specific neuronal populations in vivo and possesses numerous features making it well suited for behavioral assays in transgenic mice. DREADDs provide genetic specificity and reversibility, allowing partial within-subjects study designs. Additionally, unlike optogenetics, DREADDs enable large numbers of animals to be tested in parallel without the need for lasers or indwelling implants and tethers that can restrict free movement of the animal. DREADDs can also affect cells over large anatomical regions that would be difficult to illuminate.

In this chapter, we focus on expression of DREADDs using Cre-dependent AAV with Cre driver lines that restrict expression to subsets of cells that normally express a given gene. This powerful approach provides two layers of specificity: cell-type specificity from restricted Cre expression and anatomical specificity due to precisely localized injections. For most applications, both of these are essential for minimizing off-target effects. Nevertheless, the genetic specificity of the DREADDs provides flexibility that extends well beyond this approach to include expression of DREADDs from genomic loci [4, 19], based on projection patterns [25–28], or even based on recent activity patterns [20, 32].

Using DREADDs to manipulate a specific cell population in a specific area of the brain not only adds to our understanding of the

roles of those cells in their circuit—it also clears a path toward potential clinical applications. A primary limitation of current treatments for neuropsychiatric disorders is insufficient specificity for affecting specific components of dysfunctional circuits. Information gained using DREADDs in transgenic mouse models may thereby help identify targets and methods for the eventual treatment of neuropsychiatric disorders in humans.

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