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REVIEW

Chemogenetic Tools and their Use in Studies of Neuropsychiatric Disorders

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Summary

Chemogenetics is a newly developed set of tools that allow for selective manipulation of cell activity. They consist of a receptor mutated irresponsive to endogenous ligands and a synthetic ligand that does not interact with the wild-type receptors. Many different types of these receptors and their respective ligands for inhibiting or excitating neuronal subpopulations were designed in the past few decades. It has been mainly the G-protein coupled receptors (GPCRs) selectively responding to clozapine-*N*-oxide (CNO), namely Designer Receptors Exclusively Activated by Designer Drugs (DREADDs), that have been employed in research. Chemogenetics offers great possibilities since the activity of the receptors is reversible, inducible on demand by the ligand, and non-invasive. Also, specific groups or types of neurons can be selectively manipulated thanks to the delivery by viral vectors. The effect of the chemogenetic receptors on neurons lasts longer, and even chronic activation can be achieved. That can be useful for behavioral testing. The great advantage of chemogenetic tools is especially apparent in research on brain diseases since they can manipulate whole neuronal circuits and connections between different brain areas. Many psychiatric or other brain diseases revolve around the dysfunction of specific brain networks. Therefore, chemogenetics presents a powerful tool for investigating the underlying mechanisms causing the disease and revealing the link between the circuit dysfunction and the behavioral or cognitive symptoms observed in patients. It could also contribute to the development of more effective treatments.

Key words

Chemogenetics • DREADDs • PSAMs • Neuropsychiatric disorders

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Introduction

In neuroscience, especially brain research, the need for selective modulation of separate brain areas or neuronal subpopulations has become increasingly pressing. Especially in the case of brain diseases, understanding the brain's circuitry is crucial for finding the underlying causes and possible targets for medication. It has been shown that the dysfunction of specific networks or connections between different brain areas leads to the impairments and behavioral changes caused by the disease. It can be seen in Alzheimer's disease as the hyperactivity of the hippocampus preceding neurodegeneration [1], the hypofunction of the prefrontal cortex in schizophrenia [2], or the impairments in attention-deficit/hyperactivity disorder (ADHD) [3]. However, studying these circuits, their activity, and how they influence one another with the current methods is not always optimal. For example, lesions do not allow for investigation of different areas' interplay and functional connectivity [4]. Optogenetics is a very invasive method, only targeting a small spot for a short time [5]. That is why chemogenetics could lead to significant advances in this field.

How we got to chemogenetics

This paper is a part of the issue of the Physiological Research, which commemorates the 70th anniversary of the Institute of Physiology of the Czech Academy of Sciences. The Laboratory of Neurophysiology of Memory at this Institute has made significant advancements in the study of memory mechanisms, progressing from traditional techniques to the utilization of chemogenetics. This shift reflects a broader trend in neuroscience toward integrating molecular, cellular, and systemic approaches to understanding cognitive functions.

Initially, the department focused on traditional neurophysiological and behavioral studies. These included examining the hippocampus [6-8] and its roles in spatial navigation and memory, a classic model for studying the neurobiological mechanisms underlying learning, decision-making, and other higher cognitive processes. Over time, the department's research expanded to include various aspects of spatial orientation in laboratory rodents, the role of brain structures and neurotransmitter systems in spatial behavior [9-12], and the study of cognitive deficits associated with modeled neuropsychiatric diseases [13].

The transition to chemogenetics was a natural progression of the department's evolving research interests and capabilities. Chemogenetics offers a more precise way to manipulate and study specific neuronal populations and their functions. This method allows for the control of neuronal activity using engineered receptors and specific, pharmacologically inert ligands. By adopting this technique, the laboratory can investigate the neural basis of behavior with greater specificity and detail, contributing significantly to both fundamental knowledge and applied outcomes in neuroscience.

Through these developments, the department has advanced our understanding of memory and cognitive processes and made significant contributions to the study of neurodegenerative and neuropsychiatric disorders. The adoption of chemogenetics by the Laboratory of Neurophysiology of Memory exemplifies the ongoing evolution in neuroscience research, where cutting-edge techniques are increasingly employed to unravel the complexities of the brain and behavior.

What is chemogenetics

Chemogenetics is a field interconnecting biology and chemistry that seeks to engineer proteins (especially GPCRs) to show an altered interaction with endogenous and synthetic ligands for more precise and rapid control of cellular activity. These synthesized proteins retain all their functions except their ligand specificity, so they selectively respond only to specific small molecules, usually artificially synthesized [14,15]. These engineered proteins can then be used to investigate physiological mechanisms (on the level of cells, organs, or whole organisms and their behavior) or potentially for therapy. The creation of chemogenetic tools permitted the control of a specific type of GPCR (in a particular subpopulation of cells) by a single specific ligand. These tools allow for a more targeted control by eliminating the activation of other molecular targets. They also help identify the signalling pathways affected and the subsequent consequences for better analysis of cell functions [16,17]. Neuroscientists use these predominantly to non-invasively control cell signalling in freely moving animals (mainly rats and mice) to distinguish between such cells' normal and pathological function and their effects on certain behaviors [18].

The most commonly used chemogenetic tools are Receptors Activated Solely by Synthetic Ligands (RASSLs) [19] and DREADDs [20]. New tools have also emerged, like the engineered Ligand-Gated Ion Channels (eLGIC) [21].

RASSLs were first generated by modifying the extracellular loop of the G-protein coupled human κ-opioid receptor so it would not bind the endogenous protein ligands (like dynorphin). Because these receptors have a different binding site for the small molecules and the protein ligands, they remained capable of being activated by small synthetic molecules chosen for the experiment after the mutation. The limitations of this approach are the residual affinity of these small synthetic molecules for the wild-type receptors, which is primarily an issue for experiments *in vivo* [14], and the high constitutive activity of RASSLs [22].

DREADD technology is the one overcoming these shortcomings. These chemogenetic tools have low constitutive activity and suppressed affinity for any endogenous ligands, binding only biologically inert synthetic ligands. The muscarinic receptors are the GPCRs most often used for DREADDs, with the synthetic ligand being clozapine *N*-oxide, which is

biologically almost inert. They are usually created using a novel directed molecular evolution method [17].

Lastly, the eLGICs permit control of the ion conductance of neurons *via* a ligand-binding domain of a particular ion channel mutated into a Pharmacologically Selective Actuator Module (PSAM). PSAM responds only to a specific Pharmacologically Selective Effector Molecule (PSEM), which can activate or silence these neurons [21]. One of the first studies on LGICs used an invertebrate chloride channel activated by ivermectin to function as a neuron-silencing tool [23]. Later on, the effectiveness of this tool was improved by mutating a human α1 glycine receptor to respond to ivermectin because this receptor showed increased cell expression and better sensitivity [24].

The principles and mechanisms

Due to chemogenetics being a relatively new tool, this chapter goes over the different ways of generating the modified receptors, how they are delivered to the site of interest, and methods for activating them. Generally speaking, chemogenetics is based on orthogonal chemical genetics, where a particular protein is changed (mainly in its binding domain) to respond only to a specific molecule. This small molecule is artificially synthesized and usually silent in interactions with endogenous receptors [16,25]. Except for RASSLs, these small molecules can interact with wild-type receptors, making this tool unsuited for *in vivo* systems where their activity could interfere with that of the RASSLs [14].

Furthermore, the specific protein is mutated, so it would be activated by the orthogonal ligand (one that does not interact with the endogenous system) and retain all the other protein functions. It is usually also engineered not to accept the endogenous ligands, only the orthogonal ones, making it an orthogonal protein [26]. The directed molecular evolution approach usually executes this mutation and selection process in DREADDs (and eLGICs). This approach creates a library of mutants by random mutagenesis *via* error-prone polymerase chain reaction (PCR). These mutants are afterward screened for high affinity for the designer ligand while showing low to no affinity for the endogenous ligand. The selection is achieved by making the GPCR activity in the yeast population crucial for survival and placing them on a selective medium with the synthesized ligand. Like this, only the yeast cells with GPCRs activated by the synthetic ligand will survive and be used for the experiments [17].

On the other hand, RASSLs are created by rational design. This approach consists of modeling and evaluating a chosen protein's structure to mutate it by site-specific mutagenesis. The rational design method is faster and more directed, but structural data is needed compared to the directed molecular evolution approach [27].

The application of chemogenetics requires gene transfer, facilitated mainly by viral vectors (more precisely, retroviruses). Adeno-associated viruses (AAV) are the most commonly used as they do not produce high immune response cytotoxicity. Therefore, their expression lasts longer, making them practical and safer for studying behavior [28]. Also, different serotypes of recombinant AAV allow for targeted gene transfer into specific tissues or organs. Some of these serotypes even permit labeling neuronal populations that are harder to reach *via* retrograde transport [28]. Moreover, AAV (more precisely recombinant rAAV) can be altered through direct evolution to increase their efficiency of retrograde transport and expression, for example, by changing capsid variants. Such alterations can be helpful in research and clinical gene therapy [30].

Other types of vectors can also be used for gene transfer in chemogenetics. Lentiviruses (based on HIV) were shown to have more efficient transduction, meaning that the vector spreads into more cells [31]. Some types of herpes simplex virus (HSV) move retrogradely and allow studying even the regions of the brain that are harder to reach [32]. HSV is also useful for their larger capacity since they can carry longer gene sequences or multiple transgenes [33].

The choice of the promoter is also crucial as it enables the targeting of specific cell types based on the nuanced expression levels of certain genes [34,35]. To further specify the location of the transcript, transgenic mice expressing the enzyme Cre-recombinase in a specific cell type are used. In that case, the DREADD coding gene is inverted. It can be transcribed only in the cells with Cre-recombinase because it can excise the gene based on loxP sites (in the case of a double-floxed inverted open reading frame), making it available for transcription [36].

DREADDs can be effectively used for circuit-specific interrogation, particularly useful for studying brain diseases (schematically shown in Fig. 1). First, the designer receptors are applied to the area by injecting the virus vector, then activated on demand by a specific ligand. This targeting and activation of a particular neuronal circuitry can be accomplished in multiple ways.

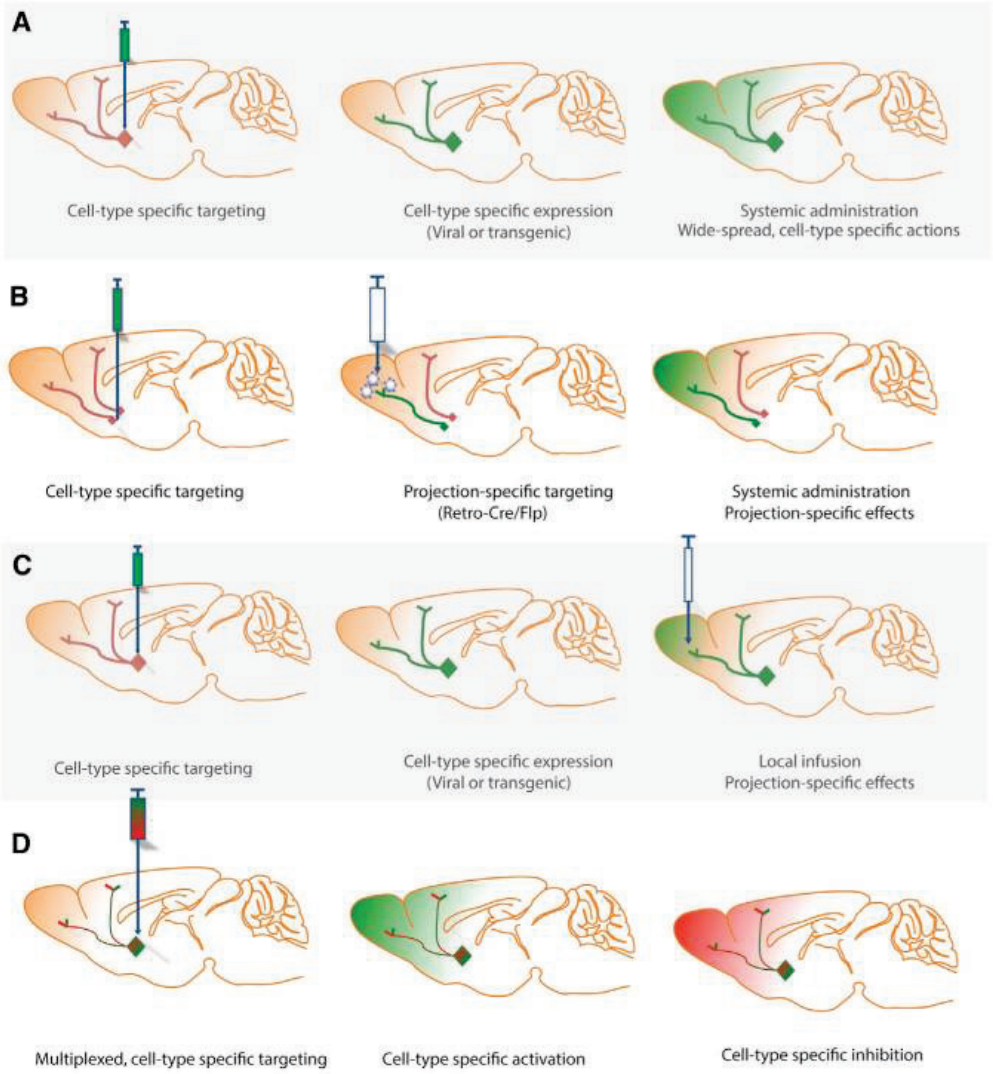

Fig 1. Schematic representation of different approaches to DREADD expression and activation in the brain. (**A**) Expression of DREADDs in a specific cell type by viral vectors or transgenically (*via* an externally inducible transgene promoter) and their subsequent activation by systemic administration of the appropriate ligand. (**B**) Injection of cell-type-specific viral vector and then of the Cre-recombinase carrying viral vector transported retrogradely from the neuron's projection site to activate the transcription of the sequence from the first virus. The expressed DREADD is then activated by systemic administration of the ligand. (**C**) Cell-type-specific expression of DREADDs as in (A) but with the selective activation of chosen projections by local (intracranial) ligand injection. (**D**) Expression of different DREADDs in specific cell types for multiplexed control of their activity (source: from [38], copyright purchased).

It can be done by targeting a specific cell type thanks to a corresponding promoter and then selectively activating the subpopulation by local intracranial administration of the ligand. Usually, it is CNO microinjected through the intracranial cannula(s) into a particular brain subregion of interest. Like this, DREADDs can be activated solely in this region, such as the dorsal dentate gyrus terminals of the entorhinal cortex. The issue with this method is its invasiveness as the cannulas are surgically implanted, going through the skull into the brain region [37]. Another possibility for modulating the activity of specific brain circuits is the dual viral-vector methodology. For example, Cre-dependent DREADD is injected into a region with cell bodies of the studied neurons. The retrograde vector with Cre-recombinase is injected into the area where the studied neurons are projecting. Therefore, the expression of DREADDs will be limited only to the neurons projecting from the first region to the second one injected. Afterward, the expressed DREADDs can be activated by systematically injecting the drug, which is not invasive and allows for easier manipulation [32].

As was already partially discussed, there are different ways of designer drug administration. The administration choice depends on the chemogenetic receptor type and the experimental design. It can be

delivered systemically, intracranially, or orally. Also, there are multiple different ligands from which to choose (this will be discussed in greater detail further). Nonetheless, CNO is the most commonly utilized ligand for DREADDs (the prevalent chemogenetic tool used) [39]. Systemic drug injection is advantageous due to its easy manipulation and non-invasiveness while keeping an effective and rapid control of targeted neurons (within 10 minutes after systemic CNO application, the response is activated, with a peak after 20 minutes) [40]. However, the dose of the synthetic ligand can have varying effects based on the receptor used, the type of brain region, and the behavior targeted [41]. Intracranial administration permits selective control of specific brain regions or even the subpopulations of neurons and their specific projections. However, as suggested, its invasiveness reduces the method's applicability [42]. Last, oral administration is usually used for chronic activation of the chemogenetic receptor. It is added to food or water, freely available to the tested animals, and is less stressful than injections. Still, the presence of CNO in water could aggravate its taste, leading to higher water consumption when removed, which could interfere with the study results [43].

G-protein coupled receptors

Since the most prevalently used chemogenetic tools are DREADDs (and RASSLs) based on GPCRs, it is necessary to discuss their mode of function, why they are an effective tool for cell activity modulation, and some shortcomings. GPCRs are seven-transmembrane receptors forming complexes with G-proteins or β-Arrestins. These two compete for the receptor as the complex with β-Arrestins blocks the binding of G-proteins on the receptor, desensitizing it and impeding the G-protein coupled signaling pathways [44]. Therefore, the activated receptor can bind only to one of the two at a time, creating agonist-induced selectivity. Atop the desensitization effect, β-Arrestins can also lead to the internalization of the GPCR and, therefore, cause issues concerning the dosing of the ligand in experiments using GPCR-based chemogenetics [45]. Furthermore, the type of ligand, receptor type, and cellular context can bias the receptor toward one signaling pathway over others, also called functional selectivity [46].

Even though various ligands activate G-protein coupled receptors, they can also signal without them, with higher or lower probability based on the receptor type, called constitutive activity. That is especially crucial to account for when using chemogenetics. If the expression of the receptor in the target cell is high, it can generate a phenotype even in the absence of a ligand due to its constitutive activity. Even though DREADDs generally have low constitutive activity, it is essential to consider this factor [22].

The signaling pathways GPCRs activate are numerous, and their effects can be more far-reaching than just silencing or enhancing neuronal activity. Unfortunately, it is not always sure whether the silencing or enhancing of neuronal firing happens through a canonical or non-canonical pathway and how the different receptor isoforms may influence the signaling pathways. However, it may be important information since the isoforms can lead to additional changes in cell activity [47]. Furthermore, some have separate extracellular binding sites for endogenous protein and small molecule ligands such as CNO. Therefore, the binding site for an endogenous ligand can be altered to be dysfunctional while keeping a high affinity for the small molecule and the original mode of function of the receptor [17].

All the above and the fact that GPCRs are one of the most prevalent receptors in cells, therefore playing a part in many cell functions, make them an excellent tool for use in various scenarios. It permits studying a whole range of cellular activities and behaviors. So even, despite some caveats, they have undeniable advantages for use as chemogenetics.

Types of chemogenetic tools

Chemogenetics is a superordinate term for multiple tools (as outlined in previous chapters) based on engineering proteins to alter their ligand specificity for rapid and precise control of cell signaling *in vivo*. Therefore, specifying these different types and how they function would be appropriate (schematically shown in Fig. 2).

Kinases

Kinases were among the first genetically modified proteins to work as chemogenetic tools. They were generated by creating a functionally silent mutation in the ATP [16] or GTP [48] binding sites, respectively, and then an already existing corresponding kinase (or GTPase) inhibitor was synthesized to attach to the altered site. Thanks to this approach, the effect of its inhibition on the cell could be observed. Furthermore, the specific signaling pathway it engages in and the role

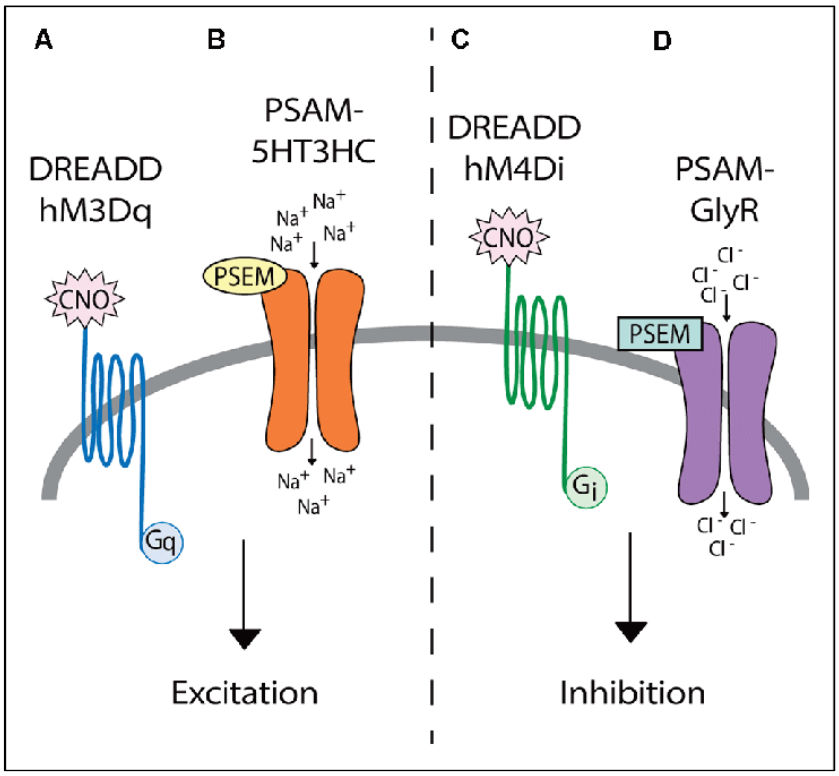

Fig. 2. Schematic representation of four different types of chemogenetic tools. On the left, (**A**) DREADD hM3Dq and (**B**) PSAM-5HT3HC act through depolarization of the neuronal membrane to produce excitation. On the right, (**C**) DREADD hM4Di and (**D**) PSAM-GlyR hyperpolarize the membrane to induce inhibition. The DREADDs activate the respective G-protein coupled signaling cascades after CNO binds. The PSAMs, on the other hand, are activated by PSEMs and directly open ion channels, causing the appropriate effect by selective ion influx (sodium for excitation and chloride for inhibition) [55].

of the kinase in the cell could be reconstructed. This approach is essential for treating diseases since it can uncover the signaling cascade and specific targets for drugs in therapy. For example, the research on the v-Src and Fyn kinases, which play a significant role in oncogenesis, focused on uncovering their signaling pathways. These kinases were mutated in their binding site (point mutation of Ile338 to Gly) to only respond to a synthetically altered inhibitor, ATP-analogue, called compound 3g [49]. A similar study was conducted only with a different ATP-analogue for the v-Src kinase [50]. The paper on ephrine B-type (EphB) tyrosine kinase, which plays a significant role in brain development, employed a similar approach. A targeted mutation of Ile amino acid residue to Gly permitted the activation of such kinases by a synthetic molecule (like PP1) inert with the wild-type kinase. This study revealed the importance of EphB tyrosine kinase signaling in many areas of neuronal development [51].

Enzymes

Another class of proteins grouped under the chemogenetic tools is enzymes. Here, the studies focus on developing artificial enzymes that mimic the naturally occurring enzymatic processes to study their natural function or, for example, to improve their efficiency. A protein scaffold and a catalytic group can be linked to create a new enzyme. On top of that, a change in the position of functional groups in the binding site can play a significant role in ligand selectivity, affecting the efficiency of the enzymatic reaction. This use of chemogenetically modified enzymes in research was demonstrated in a study on the myosin isoenzyme. Its binding site was changed to selectively bind an ATP-analogue that does not activate other isoenzymes to determine the enzyme's specific function [25], introducing Lys residues into the active site of artificial transaminase was conducted to improve its selectivity for the substrate. The improved selectivity led to an increase in the kinetic rate of the reaction.

G-protein coupled receptors: RASSLs and DREADDs

To date, the chemogenetic technology based on GPCRs is the most popular tool in many areas of science, especially in behavioral studies. These tools include the RASSL and DREADDs. Similar attempts as with the kinases have been introduced for GPCRs. Furthermore, GPCRs are also often targets of pharmacological remedies; therefore, studying their affinity and selectivity for ligands is crucial. It was first explored on the β-adrenergic receptor by embedding a point mutation of Asp113 in the binding site to change the receptor's specificity for a ligand, creating the first GPCR-based chemogenetic tool [53].

The RASSLs were created to study the downstream effects of G-protein-coupled receptors because it is hard to separate the function of one from the other due to their abundance in the organism. The RASSLs were first based on the κ-opioid receptor coupled with the G_i protein. Coward *et al*. [14] created chimeric receptors with parts from μ and δ opioid receptors. This resulted in decreased affinity for the peptide ligands but retained it for the small molecules, as these have different binding sites on the receptor. Like this, it is possible to study and control the activity of GPCRs and their physiological effects selectively by small molecule drugs.

Nonetheless, these small molecules can still activate wild-type receptors, so DREADDs were developed for use *in vivo*. They are also based on the GPCRs but are created so only a synthetic naturally inert ligand activates them, eliminating the simultaneous activation of wild-type GPCRs. DREADDs are now the most used chemogenetic tools with various types of receptors and ligands (more specified in the Synthetic receptors and ligands chapter). A particular one is the κ-opioid DREADD (KORD), which is based on the human κ-opioid receptor and is used to inhibit neuronal activity. It is different since it responds to Salvinorin B instead of CNO, unlike the other DREADDs (human muscarinic receptor-based). Thanks to that, it deals with the issues arising from the back-metabolization of CNO to clozapine [54]. It allows for multiplexed control of cell activity [20].

Ligand-gated ion channels

The last category of chemogenetic technologies, based on ion channels, is eLGIC, sometimes called PSAM. They rapidly control ion conductance on the cell membrane and, therefore, neuron activity. Again, that makes them valuable in studying the effects of these ions on the molecular level and their far-reaching consequences on behavior. A chimeric LGIC has a mutated ligand-binding domain called PSAM, which can interact only with pharmacologically selective effector molecules (PSEMs). These are small molecules that do not bind to the native receptor. Like this, effective neuron-activating or -inhibiting systems can be created, depending on the ion channels chosen and altered. Their advantage is using multiple eLGICs within one cell population or with different tools like optogenetics [21].

Recent advances in ligand-gated ion channel (LGIC) research have introduced the eLGIC type, a novel system based on acetylcholine receptors called BARNI (Biologically Advanced Receptor for Novel Interactions). This innovative type of LGIC is noteworthy due to its activation through a clinically approved ligand, which underscores its potential for simplified clinical translation and application. The ability of BARNI to be activated by an existing, clinically approved compound significantly reduces the regulatory and developmental hurdles typically associated with new therapeutic interventions, making it an attractive candidate for rapid integration into clinical practice [56].

The BARNI system leverages acetylcholine receptors' structural and functional properties, which play critical roles in various physiological processes, including muscle activation and cognitive function [57]. The BARNI system circumvents the often lengthy and costly process of novel ligand development and approval by utilizing a clinically approved ligand. This strategy aligns with recent trends in drug repurposing, where existing drugs are adapted for new therapeutic applications, thus speeding up the transition from bench to bedside [58].

Moreover, the clinical applicability of the BARNI system is further enhanced by its potential to be used in a range of therapeutic contexts. For instance, acetylcholine receptor modulation has been implicated in treating neurological disorders such as Alzheimer's disease and myasthenia gravis [59]. The eLGIC type's ability to precisely target these receptors could lead to more effective therapies with fewer side effects than traditional treatments [60]. Furthermore, using a clinically approved ligand means that safety profiles are already well-established, thereby reducing the risk of adverse effects in patients [56].

Synthetic receptors and ligands

Receptors

Over the years of using chemogenetic tools in science, many receptors and ligands have been created to suit the needs of the experimental design or improve the required characteristics of chemogenetic tools, like selectivity or off-target activity.

As seen in Table 1, multiple classes of proteins are used in chemogenetics: kinases, enzymes, channels, and GPCRs, which are the most explored and widely applied. From GPCRs, the most employed receptors are those based on the human muscarinic receptor, which has given rise to DREADD technology. Due to that, this chapter will be focused mainly on the DREADDs. Multiple types of DREADDs have been engineered and

Table 1. Table illustrating the different types of chemogenetic tools used.

Name	Protein(s)	Ligand	Reference
Representative kinases			
Allele-specific kinase inhibitors	v-I388G	Compound 3g	[50]
Analogue-sensitive kinases	v-Src (I338G, v-Src-as1), c-Fyn (T339G, c-Fyn-as1), c-Abl (T315A, c-Abl-as2), CAMK IIα (F89G, CAMK IIα-as1) and CDK2 (F80G, CDK2-as1)	K252a and PPI analogs	[49]
Rapamycin-insensitive TOR complex	TORC2 V2227L	BEZ235	[26]
ATP-binding pocket mutations in EphB1/2/3	*Ephb*1^{T697G}, *Ephb*2^{T699A}, and *Ephb*3^{T706}	PP1 analogs	[51]
ATP-binding pocket mutations of TrkA/B/C	*TrkA*^{F592A}, *TrkB*^{F616A}, and *TrkC*^{F617A}	1NMPP1 and 1NaPP1	[61]
Representative enzymes			
Metalloenzymes	Achiral biotinylated rhodium diphosphine complexes		[62]
Engineered transaminases	Chemically conjugating a pyridoxamine moiety within the large cavity of intestinal fatty acid binding protein	Enhanced activity	[52]
Representative GPCRs			
Allele-specific GPCRs	β2-adrenergic receptor, D113S	1-(3′,4′-dihydroxy phenyl)-3-methyl-L-butanone (L-185,870)	[53]
RASSL-G_i	κ-opioid chimeric receptor	Spiradoline	[14]
Engineered GPCRs	5-HT2A serotonin receptor F340→L340	Ketanserin analogues	[63]
G_i-DREADD	M2- and M4 mutant muscarinic receptors	Clozapine-N-Oxide	[17]
G_q-DREADD	M1, M3, and M5- mutant muscarinic receptors	Clozapine-N-Oxide	[17]
G_s-DREADD	Chimeric M3-frog Adrenergic receptor	Clozapine-N-Oxide	[64]
Arrestin-DREADD	M3Dq R165L	Clozapine-N-Oxide	[65]
Axonally-targeted silencing	hM4D-neurexin variant	Clozapine-N-Oxide	[66]
KORD	κ-opioid receptor D138N mutant	Salvinorin B	[20]
Representative channels			
GluCl	Insect Glutmate chloride channel; Y182F mutation	Ivermectin	[67]
TrpV1	TrpV1 in TrpV1 knock-out mice	capsaicin	[68]
PSAM	Chimeric channels PSAMQ79G,L141S PSAM-GlyR fusions	PSEM9S PSEM89S; PSSEM22S	[21]

The kinases as the first chemogenetics developed at the top, including the allele-specific inhibitors, analogue-sensitive kinases, the rapamycin-insensitive target of rapamycin (TOR) complex, ATP-binding pocket mutations in ephrin B-type receptor (EphB) 1/2/3 and ATP-binding pocket mutations of tropomyosin receptor kinase (Trk) A/B/C. Each subtype of kinases has examples of proteins used as chemogenetic tools (like Ca^{2+}/calmodulin-dependent protein kinase II (CAMKII) or cyclin-dependent kinase 2, the target of rapamycin complex 2 (TORC2), EphB2 or TrkA) and their respective ligands. Then, the groups of enzymes are modified to classify as chemogenetics: metalloenzymes and engineered transaminases and their effect, such as enhanced activity. The third group is the largest, containing the GPCRs used as chemogenetics with allele-specific and engineered GPCRs, RASSLs, and DREADDs, again with the specific receptors (like the muscarinic, adrenergic, serotonin, or opioid receptors) and their ligands, which have been used in research. The last group includes the channels: glutamate Cl^- channel (GluCl), transient receptor potential vanilloid 1 (TrpV1) channel, and PSAM like the PSAM-Glycine receptor (GlyR) fusion.

coupled with different G proteins and, therefore, various signaling pathways, G_q, $G_{i,}$ or G_s, as specified further.

For DREADDs based on G_q signaling, human M1, M3, and M5 muscarinic receptors are mutated to obtain selective activation by CNO. The human muscarinic M3 DREADD (hM3Dq) is most prevalent from these. Their activation leads to phosphatidylinositol hydrolysis, which stimulates the release of intracellular Ca^{2+}, resulting in increased neuronal firing [17,69]. Because the release of Ca^{2+} ions plays an essential role in many cell types, it has been used more widely. For example, in the research of glial cells [40] or pancreatic β cells, where the activation of pathways, including insulin receptor substrate 2 (IRS2), by these DREADDs was crucial [70].

G_i-coupled DREADDs are used for neuronal silencing, with the human muscarinic M4 DREADD (hM4Di) being a popular inhibitor for experiments. This DREADD derived from the human M4 muscarinic receptor activates the G-protein coupled inwardly rectifying potassium channel (GIRK) pathway by Gβ/γ, which subsequently leads to hyperpolarization of the neuron, therefore silencing the neuron's firing [17]. Furthermore, relatively recently, a new inhibitory DREADD called KORD was created. It exploits a mutated version of the κ-opioid receptor, which answers to the binding of Salvinorin B instead of CNO. Due to the activation by different ligands, KORD, and muscarinic-based receptors can allow for more diverse, bi-directional (when G_q-based DREADDs are used) control when expressed in the same neuronal population [20].

Last, concerning the types of DREADDs, G_s-coupled ones have been developed and used *in vivo* to study medium spiny neurons [71] or β-cells in the pancreas [64]. These are based on the human M3 muscarinic receptor and signal either by $G\alpha_s$ or the closely related subunit $G\alpha_{olf}$. The activation of these G-proteins results in increased cyclic adenosine monophosphate (cAMP) concentration. This activation of neurons expressing the corresponding DREADD manifests as certain associated behaviors. In the case of striatopallidal medium spiny neuron activation, it decreases the locomotion of tested rats [71].

For PSAMs, many different receptors have been engineered for silencing and enhancing neuronal firing. The α7-5HT3 channels have been developed as the activators, while eLGICs based on the glycine and γ-Aminobutyric acid (GABA) receptors act as inhibitors [21].

Ligands

Regarding the types of ligands, the choice depends on the specific chemogenetic tool, but since DREADDs are the most explored ones, this paragraph will focus on ligands used for DREADD activation. CNO is the most popular and first paired with DREADDs because it has a high affinity for human muscarinic receptors. Also, it is biologically inert in the presence of only the wild-type receptors [17]. Unfortunately, this was later found to be only partially true since CNO does exhibit some off-target activity and is also back-metabolized in the brain to clozapine and *N*-desmethyl clozapine (NDMC) in both rats and mice [39]. And these molecules are not biologically inert [72]. Gomez *et al.* [73] suggested that it might be the back-metabolized clozapine that crosses the blood-brain barrier and possibly activates the DREADDs instead of CNO (which has low permeability of the blood-brain barrier) since it also shows a great affinity for DREADDs.

On the other hand, [74] showed that the CNO crosses the blood-brain barrier (BBB) to an extent and could be more readily available to bind to the DREADDs instead of clozapine which binds unspecifically in the brain tissue. Moreover, it was shown that CNO, even in the micromolar doses, causes off-target effects on many endogenous receptors (dopaminergic, serotonergic, muscarinic, or even adrenergic), which again hinders its suitability for use as a DREADD ligand. Nonetheless, there has not been agreement on whether these off-target effects cause some behavioral changes or not. A study using the five-choice-serial-reaction task did not observe any [74], while another one reported changes in amphetamine-induced locomotion and startle response following even low doses of CNO [54].

One possible alternative to CNO use is low doses of clozapine, which do not seem to cause behavioral effects, instead of high concentrations of CNO (needed for it to cross the BBB). This approach could eliminate the back-conversion that is highly variable between species or even sexes and can confound the experimental results. However, uncertainty about the off-target effects of clozapine remains [74]. Another ligand that could substitute CNO is compound 21 (C21). It does not back-metabolize to clozapine, and it readily crosses the BBB. Unfortunately, it exhibits off-target competitive inhibition of many endogenous GPCRs. However, no behavioral consequences of this effect have been reported [74].

Moreover, in low doses (of 0.5 mg/kg), the off-target effects can be eliminated while keeping sufficient activation of the DREADD. Nonetheless, the optimal dose may differ between males and females, complicating the experimental design [75]. The next ligand used for DREADDs is salvinorin B. It is a derivate of salvinorin A, a specific ligand of the κ-opioid receptor, mutated not to activate the endogenous receptor or other receptors (like the muscarinic ones). It is successfully used as the KORD activator, resulting in significant behavioral changes by silencing specific neuronal populations [20]. Last, perlapine could serve as another valuable ligand to substitute CNO in activating hM3Dq, but no other types of DREADDs as it does not have a strong enough affinity for those. Its affinity for the hM3Dq is even higher than that of CNO [76].

Unfortunately, not many of these ligands can potentially be used as therapeutics. CNO is back-metabolized to clozapine with off-target effects, and perlapine does not have an affinity high enough for hM4Dq (the DREADDs most prospective for the treatment of brain diseases like schizophrenia). The only ligands with possible applications are C21 [77] or an already approved drug for schizophrenia, olanzapine, which was newly discovered to have an affinity for hM4Dq receptors and, therefore, could be used as a therapeutic ligand in humans. Unfortunately, in the doses needed for this activation, it binds to the native dopamine D2 receptors and causes side effects of treatment [78].

Deschloroclozapine is a potent, selective, and metabolically stable agonist used in chemogenetics to activate designer receptors exclusively activated by designer drugs (DREADDs). This compound is particularly valuable due to its high affinity and efficacy in binding to the engineered G protein-coupled receptors (GPCRs) without interacting with endogenous receptors, thus minimizing off-target effects [79]. In experimental settings, deschloroclozapine can selectively modulate neuronal activity, enabling precise control over specific neural circuits [38]. This facilitates the study of complex brain functions and behaviors and the investigation of disease mechanisms and potential therapeutic interventions in neurological and psychiatric disorders [73]. Its usage represents a significant advancement in chemogenetic methodologies, providing a powerful tool for dissecting cellular and molecular functions *in vivo* with high specificity and temporal precision.

Selected brain diseases studied using chemogenetics

Since DREADDs are the most used chemogenetic tools, this work will mainly focus on the application of DREADDs in the research of brain diseases, with the occasional mention of other chemogenetic technologies. It will focus on the neuronal circuit's dysfunctions since chemogenetics are most apt to regulate these as they can be expressed in larger areas of the brain or the downstream projections of specific neuronal populations and for more extended periods.

Alzheimer's disease

Chemogenetics has been used in the research to determine the underlying causes of AD impairments and possible early diagnosis or treatment targets (schematically shown in Fig. 3). Rodriguez *et al.* [80] showed that when the entorhinal cortex activity was reduced, the accumulation of Aβ plaques and tau neurofibrils decreased, too. They used the hM4Di and its synthetic ligand CNO to produce chronic attenuation of neuronal activity for six weeks in human amyloid protein precursor (hAPP)/Aβ overproducing 16-month-old transgenic mice. They detected mainly diffuse, small-sized plaques affected by the change in firing activity, pointing to its effect on new plaque accumulation but not clearance. Furthermore, it impacted the phosphorylated tau protein aggregation in neurons downstream from the entorhinal cortex in the hippocampus. Still, the effects were not distinct compared to the Aβ plaques accumulation.

A similar study by Peng and Grutzendler [81] further supports these findings. They used the hM3Dq for neuronal activation and hM4Di for neuronal inhibition to evaluate their effects on Aβ plaques. CNO was administered daily for 30 or 60 days. They also found that the attenuation of neuronal activity reduced the accumu-

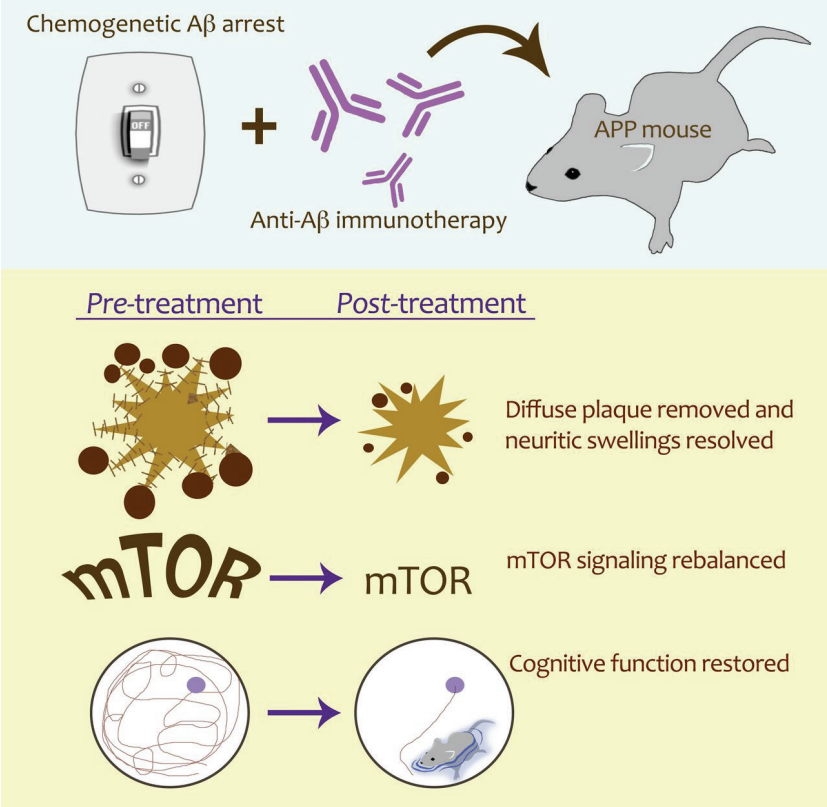

Fig. 3. This picture illustrates the possibility of improving cognitive impairments in an AD mouse model by employing chemogenetic and immunotherapeutic approaches to reduce Aβ plaque accumulation and restore normal mammalian TOR signaling (adapted from [83]).

lation of Aβ plaques and the diameter of the halo of oligomeric Aβ (which plays a vital role in axonal dystrophy) in the controlled neurons of cortical layer V. The exact mode of action leading to the decreased deposition of Aβ plaques was not found. The examination of thalamic nuclei, which receive the projections from DREADD-controlled neurons, showed that the neuronal activity could influence the production and axonal or somatodendritic release of Aβ plaques, leading to its accumulation and the subsequent neuronal dystrophy instead of changes in transcription or translation of APP or associated proteins. This could eventually lead to discovering potential treatment targets to reduce β-amyloid deposition and pathological neuronal dystrophy.

Another study focused on the locus coeruleus, an important noradrenergic region that plays a role in spatial memory and is impaired in AD. Here, the aggregation of the hyperphosphorylated tau protein over time in the transgenic TgF344-AD rats has been shown to lead to a loss of noradrenergic neurons in locus coeruleus. This most likely also impacted the stability of axons in projection areas like the entorhinal cortex. The DREADD technology was used to examine the effects of locus coeruleus on spatial memory and learning mediated by its noradrenergic neurons and the hippocampus. It was tested in the Morris water maze. When the hM3Dq was activated by CNO 30 min before each trial to stimulate the locus coeruleus, the memory, and spatial reversal learning improved (the rats managed to find the platform faster than those treated with the vehicle instead of CNO) in the transgenic rats. These findings suggest that locus coeruleus could be an intriguing target for treating some significant AD symptoms: spatial memory and learning deficits [82]. To sum up, chemogenetics proves to be a handy set of tools for researching the causes of AD and possibly finding new targets for treating the debilitating symptoms.

Epilepsy

Chemogenetic tools could offer the needed non-invasive controlled switching-on and -off of the neuronal activity in a specific area, therefore keeping it reversible and very customizable for researching different types of epilepsy or their treatment. Especially the temporarily controlled silencing of seizures makes it more advantageous than permanent genetic approaches. Gene therapy issues concerning viral vector targeting or potential oncogenesis could have serious adverse effects [84,85].

First, DREADDs are immensely useful for epilepsy research by allowing the identification of the crucial brain regions involved and the change in their activity. The study on hippocampal DGCs used hM4Di, which, upon activation by CNO, reduced the frequency of

epileptic spikes and spontaneous recurrent seizures. The connectivity of DGCs in the hippocampus was first identified using retrograde tracing by the modified *rabies* virus. They discovered that the DGCs that were newly formed around the time of pilocarpine-induced status epilepticus (seven days prior or three days after) had higher connectivity to many different areas (the entorhinal cortex, the forebrain, the hippocampus – especially reciprocal connections with cornu Ammonis (CA) 3. Moreover, it was primarily excitatory input connections. Such higher connectivity could impair the DGCs' ability to act as commutators between the entorhinal cortex and CA3. The hM4Di was used to examine whether the new DGCs were involved in generating a seizure. The inhibitory effect of these DREADD receptors expressed in DGCs led to a temporary decrease in spontaneous seizures after the injection of CNO. Furthermore, the hM3Dq was used to test whether the DGCs in an epileptic mouse were enough to generate an episode, which proved true. Like this, the pro-epileptic neural circuits involving hippocampal DGCs and their ability to provoke seizures in temporal lobe epilepsy were identified [86].

Another research focused on parvalbumin-expressing neurons in the CA1/subiculum in epilepsy. These neurons produce the GABA neurotransmitter and, therefore, have inhibitory activity. This study showed that the deactivation of these neurons contributed to the hyperexcitability of hippocampal neurons and the onset of an epileptic seizure. The hM4Di activated by CNO in the parvalbumin-containing interneurons proved that inhibiting the GABA release from these neurons lowered the seizure threshold and facilitated the seizure's onset [87].

Second, DREADDs have the potential to be used in clinical settings to treat epilepsy in humans. One study focused on its applicability in focal epilepsy in neocortical areas. CNO activated the inhibitory hM4Di after administering pilocarpine or picrotoxin to induce a motor or behavioral seizure. Surprisingly rapidly, it significantly attenuated the epileptic seizure (already within 10 min of the CNO injection) [88]. Other studies like that by Wicker and Forcelli [89] focused on proving the ability of DREADDs to silence an epileptic seizure on demand even further from the site of the outburst. That could be used to treat drug-resistant types of epilepsy like temporal lobe epilepsy. They used the hM4Di and CNO to reduce seizures from the amygdala in the mediodorsal thalamus.

Moreover, the dosing of CNO and timing were examined, showing that it significantly reduced seizure severity when treated with 2.5 mg/kg of CNO (or more). And that the effect of CNO was evident only when administered 30 min before seizure stimulation. The impact on cognitive abilities was not thoroughly explored, even though it could be significant as this region encompasses many neuronal fibers and interconnections to other brain regions [89].

Last, eLGIC technology exploiting the non-human (from *C. elegans*) engineered glutamate-gated Cl^- channel (eGluCl) with enhanced sensitivity was explored as a possible tool in focal epilepsy treatment. It was designed to answer to higher glutamate concentration in the extracellular space naturally resulting from an epileptic seizure. The application of eGluCl reduced the number of seizures in both models, with pilocarpine-induced and tetanus toxin-induced seizures, by hyperpolarizing the neuronal membrane. The great advantage of this approach is that it doesn't need any exogenous agonist [90].

The issue concerning the use of DREADDs for epilepsy treatment is undoubtedly the back-metabolization of CNO into clozapine, which could have other interfering effects on the brain. Therefore, testing other ligands and their effectivity (like KORD) would be favorable. Even though some studies found no off-target effects of CNO, even low doses of CNO might still be effective and selective enough [89]. Also, it might be difficult to activate the DREADDs before the full onset of the seizure *in vivo* in patients (without premonitory auras or other symptoms) since it takes tens of minutes for the DREADDs to be activated by a systematically administered ligand. Moreover, the specificity of viral targeting of specific neuronal populations may prove quite challenging and possibly even cause off-target signaling, for example, in the midline thalamus [89]. Nonetheless, chemogenetic tools are an essential part of epilepsy research and have a great potential to be used as a clinical treatment. This is especially true in drug-resistant patients and those with premonitory auras or other signs of an impending seizure [88].

Depression and anxiety

Chemogenetic tools have been used mainly in research to uncover the many pathways underlying depression and anxiety disorders. One of the studies used excitatory DREADD to study fear renewal after extinction to find new circuits implicated in this phenomenon in anxiety and post-traumatic stress disorder. The mice have been conditioned for auditory

fear, followed by a successful extinction of this fear (exposing them to conditioned stimuli without the unconditioned foot shock). In one group, neurons of substantia nigra (SN) were activated by hM3Dq during the fear extinction sessions. These mice then displayed lower freezing and, therefore, less fear renewal in the next session, especially in the new context (compared to the vehicle group). The activation of SN during fear extinction led to protection from fear renewal.

Furthermore, the activation of dopamine D1 receptors in the dorsal striatum was proved to be the most likely target of the SN neuronal projections since they showed higher c-fos expression when CNO activated the hM3Dq in SN. Activating dopamine D1 receptors in the dorsal striatum prevented fear renewal in a new environment (just like the activation of SN). The researchers then suggested that the SN and dopamine D1 receptors of the dorsal striatum might present a new target for eliminating relapse after successful fear extinction in mood disorders like anxiety or post-traumatic stress disorder [91].

Another study focused on the impairment of regulatory circuits and centers in depression and anxiety, especially the medial PFC, exploring its connection to the paraventricular thalamus (PVT). They used tetanus toxin for presynaptic inhibition and hM3Dq activated by CNO for acute activation in PVT and then measured activity in medial PFC. Tetanus toxin-induced chronic presynaptic inhibition impacts the proportion of excitatory and inhibitory neurons active in the medial PFC by increasing the activity of inhibitory interneurons. At the same time, the DREADD-mediated acute activation (without the tetanus toxin effect) changed the firing rate in the pyramidal neurons. Furthermore, the DREADD-induced acute hyperactivity of PVT neurons led to more periods of hypoactivity in the long term (tested by wheel running). In the forced swimming test, the mice with chronic presynaptic inhibition seemed to have shorter immobility times. This suggests that the presynaptic inhibition (which increased the proportion of firing interneurons) could improve depressive symptoms. However, the differences between groups were not very pronounced. Therefore, it is not reliable to extrapolate from these results. On the other hand, the long-term activation of PVT influencing the mPFC pyramidal neurons seemed to worsen the depressive symptoms, increasing the number of depressive episodes [92]. Unfortunately, this study had a low number of subjects because 11 mice were excluded as they had not learned the standard wheel running used for the activity analysis, which could impair the strength of the statistical results.

The importance of the ventral hippocampus in anxiety was demonstrated in a study where they expressed the hM4Di in the glutamatergic cells of this brain region and activated them with CNO or clozapine. That led to the inhibition of those neurons. This inhibition then increased the exploratory time in the open arm in the elevated plus-maze and the illuminated part in the light/dark test. At the same time, it did not differ from controls in freezing after the foot shock. These observations suggest that the ventral hippocampus mediates anxiety toward potential threats, not immediate ones [35].

In what concerns the anhedonia symptoms connected to the reward network, there was a study on the role of medial habenula in depression. Among others, they examined the effect of medial habenula neurons on the activity of the ventral tegmental area (VTA) and the dorsal raphe nucleus. They expressed hM3Dq in the medial habenula and then peritoneally administered the CNO. Afterward, they tested for the tyrosine hydroxylase-immunopositive and tyrosine hydroxylase-immunonegative cells in both regions to uncover the activity patterns. It was discovered that the activation of medial habenula increased the activity of VTA dopaminergic neurons but reduced the number of serotonergic immunoreactive neurons in the dorsal raphe nucleus. This use of chemogenetics allowed researchers to test the influence of the cholinergic habenula on the areas implicated in reward and motivation and the associated anhedonia in depression. It could lead to better treatment of these symptoms irresponsive to SSRI antidepressants [93].

In the case of depression and anxiety, chemogenetic tools could be handy for finding the underlying causes and impairments since the circuits affected are very diverse, with many different neurotransmitters playing a role. It also shows excellent promises in detangling the regulatory aspects and hierarchy of influence between other brain areas.

Schizophrenia

In this disease, chemogenetics could help determine the dysfunctional networks and manipulate specific projections, like the dopaminergic ones, to assess their effect. Parvalbumin-positive (PV) interneurons have been of great interest in studies on schizophrenia since these GABAergic interneurons are prevalent in the anterior hippocampus and have an inhibitory function. Therefore, their dysfunctionality could be behind the

observed hippocampal hyperactivity. In one study, they focused on the effects of manipulating PV interneurons and GAD65 expressing interneurons by the inhibitory hM4Di. They tested it on pre-pulse inhibition (PPI), spontaneous alteration in T-maze, locomotor activity, and social interaction, looking for schizophrenia-like symptoms. They discovered that when CNO activated the hM4Di in PV interneurons during the trials (to silence the inhibitory action of the interneurons), the mice showed reduced PPI and impaired spatial memory. At the same time, GAD65 inhibition increased locomotor activity and produced even more stereotyped behavior on the spontaneous alternation task on spatial memory than the PV interneurons.

Furthermore, they proved that the locomotor activity induced by GABAergic interneurons was executed *via* the dopaminergic system. The haloperidol, an antagonist of the dopamine D2 receptor, reduced this hyperactivity when GAD65 were chemogenetically inhibited. This suggests that the disruption of hippocampal interneuron inhibition might considerably influence the dopaminergic system [18].

The connection between the hippocampus's GABAergic structures and the brain's dopamine system was explored in a study using the overexpression of the α5 subunit of the $GABA_A$ receptor in pyramidal cells and inhibition of the ventral hippocampus by hM4Di. The overexpression of the α5 subunit reduced the dopaminergic neuron's activity in the VTA in the MAM model of schizophrenia. The ventral hippocampus probably mediated that – NAc pathway since reducing this pathway's activity by hM4Di decreased dopaminergic cell activity. On the other hand, the same experimental design did not affect the VTA in the case of the ventral hippocampus – medial PFC pathway. Furthermore, they tested the cognitive deficits induced by the MAM model through attentional set-shifting experiments. The extradimensional set-shifting deficit was attenuated by the overexpression of α5 and entirely rescued by the inhibition of ventral hippocampus-medial PFC connection, while neither of those affected the reversal learning. Reversal learning was improved by inhibiting the ventral hippocampus – NAc pathway. These findings could lead to better treatment of the positive and cognitive symptoms of schizophrenia, which seem to be linked to the dopaminergic circuits [94].

The deficit in cognitive flexibility was observed in reversal learning tests during the chemogenetic inhibition of the mediodorsal thalamus by hM4Di. This silencing impaired the ability to adapt to the outcome's altered contingency, suggesting limiting cognitive flexibility. Furthermore, in the Pavlovian-to-instrumental task, the mice with mediodorsal thalamus inhibited during the Pavlovian task could not modulate their behavior to fit the new context of the instrumental task. Again, it could suggest some deficits in information integration and cognitive inflexibility. Unfortunately, the extent to which it is possible to extrapolate the findings to humans is debatable because the mediodorsal thalamus is not as prominent in mice [95].

ADHD

Studies concerning ADHD and chemogenetics mainly focus on discovering the functions and connections between certain brain areas or circuits and the behavioral symptoms linked to hyperactivity and attention or impulsivity. Hyperactivity is often tied to dopaminergic neurons, prevalent in the VTA and the SN projections into the ventral (with the NAc) and dorsal striatum. The increased activity of the neurons connecting VTA to the NAc seems to be mainly responsible for locomotor hyperactivity, such as in ADHD. Their locomotor activity changed when the transgenic mice expressing hM3Dq in either SN or VTA were injected with CNO. However, in those with DREADDs in VTA, the increase in home cage locomotor activity was much more pronounced. The same showed true for the VTA – NAc pathway expressing hM3Dq thanks to Cre recombinase and the canine-adenovirus (for retrograde transport to the projection neurons). The VTA – NAc pathway activation resulted in higher locomotor hyperactivity. The nigrostriatal pathway could be more involved in movement coordination than general locomotor hyperactivity since it produced only a mild increase in locomotor activity after stimulation. These findings offer a better understanding of the circuits and could lead to more targeted treatment of hyperactivity, not only in patients with ADHD [96]. Unfortunately, the activation was not strictly restricted to separate areas, as shown by the immunohistochemistry in this study, which could confound the results. Also, it does not clarify the underlying molecular functions, such as which dopamine receptors could be responsible for the observed hyperactivity and how.

A different study focused on the distinction between attention and impulsivity. They used a five-choice serial reaction time task in rats while chemogenetically activating either the VTA or SN,

projecting into the striatum – the hM3Dq and CNO as the specific ligands were used for this activation. The number of omissions in the task increased in the case of activation of both VTA and SN. Only in the case of SN did it also lead to latency in responding (as well as latency in collecting a reward) and a higher number of incorrect responses. VTA activity only lowered latency to collect the reward atop the increased omissions. Furthermore, there was no effect on the number of premature responses (impulsivity). VTA solely reduces the latency to initiate behavior, leading to higher distractibility. This points in the direction that neither VTA nor SN play a significant role in impulsivity but significantly impair attention in different ways.

On the other hand, SN activation (and therefore activation of the dorsomedial striatum) leads to the overall impairment of attention and the appropriate response to stimuli, suggesting that the striatum plays a role in proper response regulation [97]. In the present study, it was also found that SN needed lower doses of CNO. Therefore, it had a lower threshold for stimulation to produce a significant effect (the number of omissions). This suggests that SN is more prone to getting dysregulated than VTA [97]. This study contributes to a better understanding of these dopaminergic circuits' function and possibly a better-targeted treatment for different types of ADHD.

There is not a large body of research on ADHD using chemogenetics, even though it could be beneficial. DREADD technology acts through G-coupled proteins, the same mode of action as dopamine or norepinephrine receptors effectively targeted by medication for ADHD like methylphenidate [98]. Therefore, it could help enlighten the precise effects of these receptors, as attempted in a study by Fitzpatrick *et al.* [99]. It showed that inhibiting dopaminergic neurons in VTA by hM4Di contributes to hyperactivity and attention impairment (reduced vigor and response speed). Boekhoudt *et al.* [97] study on VTA activation reveals that both insufficient and excessive activity of dopaminergic neurons causes symptoms associated with ADHD. Therefore, the treatment should aim to find this neurotransmitter's optimal concentration. It also touched on the effects of inhibition of norepinephrine neurons in locus coeruleus in attention (during more demanding tasks) and impulsivity [97].

Furthermore, it would be beneficial to employ chemogenetics in the research of the PFC, which is also impaired in ADHD. Studying its hypoactivity is crucial for understanding the cause of executive function impairments and the possible regulatory effects (and their dysregulation in ADHD) on other areas. It is mainly the hypofunction of the right dorsolateral PFC that seems to be important in ADHD [100]. Also, since the symptoms of ADHD are very heterogeneous, it is indeed essential to study other brain areas possibly involved (like the cerebellum, the cingulate cortex, or parietal regions) to map out the circuits impaired in or causing ADHD (like the DMN) [101]. Regarding the shortcomings of many studies on ADHD, there is a lack of comparison and acknowledgment of differences in symptoms and impairments between children and adult patients, even though they could be significant and cause confusion in the interpretation of results [100]. The same concerns the equal representation of male and female patients in ADHD studies, often focusing on boys exclusively [101].

Discussion

Chemogenetics is becoming more commonly employed in investigating various neuronal circuits, their connectivity, and their influence on behavior. Yet, there are still some shortcomings concerning the application of DREADDs and the effects of their ligands. The predominantly used CNO is usually considered a biologically inert molecule. Unfortunately, recent studies suggest that this might not be the case. CNO has been shown to have multiple off-target effects [74] and even an impact on behavior in the absence of DREADDs, which could impair the potential experimental results of DREADD employing studies [54].

Furthermore, it has been shown that CNO back-metabolizes into clozapine in the brain [39], and clozapine is not biologically inert [72]. That could again cause off-target effects after CNO administration. It has even been suggested that low doses of back-metabolised clozapine could mediate the effect of CNO as it binds effectively to DREADD receptors [54,73].

Moreover, the dosing of CNO in DREADD studies must be carefully assessed since it has been shown to exert different effects on neurotransmission depending on the dose, such as long-term potentiation or calcium concentration [102]. All this together asks for great precaution when using CNO as the DREADD activating ligand, especially in dosing and experimental control for CNO/clozapine off-target effects. It could be beneficial to thoroughly test and use other possible ligands like salvinorin B more routinely.

Another concern arises with the expression

levels of DREADDs because it has been shown that despite the low constitutive activity of GPCRs, they can produce some effects even in the absence of a ligand when they are overexpressed in the neuronal population [22]. In the case of chemogenetic tools other than DREADDs, there seems to be a lack of exploitation of the eLGIC technology, which offers exciting possibilities, for example, an effective therapeutic tool for epilepsy [23]. Different types of eLGICs or DREADDs can also be combined, which could be helpful for multiplexed modulation of brain networks [21,20].

There have also been some studies on optimizing the application of DREADDs for better targeting and more uniform vector distribution, which could be especially useful in laboratory animals with bigger brains, like rhesus monkeys. Fredericks *et al.* [103] suggested the co-infusion of magnesium ions to visualize and verify whether the vector was successfully injected into the desired brain area right after the surgery. Still, the virus vectors alone may differ in their ability to spread and infect the target cells, which should be considered when designing the experiment [84].

Lastly, there is the disadvantage of using rodents to study psychiatric diseases. Some of the symptoms prominent in human patients cannot be reliably replicated in mice or rats, such as hallucinations or delusions in schizophrenia. Furthermore, some critical brain areas are underdeveloped in mice, limiting the interpretation of the results of such studies [95]. It would be interesting to include more studies on non-human primates to simulate the human brain's functionality more closely [103]. Nonetheless, rodent models still offer a great resource and can work well for other symptoms for which the treatment is often ineffective [93,94].

Concluding remarks

This work demonstrates that chemogenetics, especially DREADDs, are powerful tools for researching brain diseases. That is mainly due to the reversibility of the neuron activity modulation and the minimal invasiveness of the method. The flexibility of activation and deactivation of neuronal populations and the longer duration of its effects than, for example, in optogenetics offers unprecedented possibilities in neuroscience research, emphasizing behavioral effects [5]. The application of viral vectors allows for exploring the function of whole neuronal populations or networks in the brain, which seems especially crucial for researching psychiatric diseases. The connectivity between brain areas like the hippocampus and PFC [94] or the dysfunction of whole networks like the DMN (default mode network) [104] plays an essential role. Their investigation could lead to a better understanding of the underlying issues and better treatment options. Since many patients often do not respond to classical therapeutics, like SSRI for depression [105], or there is no effective treatment, as in the case of Alzheimer's disease [82], chemogenetic tools may facilitate decisive discoveries. These could significantly improve the quality of life of the patients. Even DREADDs themselves might be used as therapeutics, for example, for drug-resistant epilepsy [89]. It can be used for general research on the brain, such as the function of different parts of a specific region, their connectivity to other areas, and the subsequent effect on behavior. Unfortunately, chemogenetics is still underused in the research. Even though they have some shortcomings that need to be addressed, like the effects of their ligands, they have great potential and, together with imaging technologies, could lead to significant discoveries in neuroscience.

Conflict of Interest

There is no conflict of interest.

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Abbreviations

(r)AAV, (recombinant) adeno-associated virus; ACC, anterior cingulate cortex; Aβ, β-amyloid; AD, Alzheimer's disease; ADHD, attention-deficit/hyperactive disorder; hAPP, human amyloid precursor protein; ATP, adenosine triphosphate; BBB, blood-brain barrier; C21, compound 21; CA, Cornu Ammonis; cAMP, cyclic adenosine monophosphate; CAMKII, Ca2+/calmodulin-dependent protein kinase II; CDK2, cyclin-dependent kinase 2; CNO, clozapine-N-oxide; DGC, dentate granule cells; DMN, default mode network; DREADD, designer receptor exclusively activated by designer drugs; EEG, electroence-phalography; eGluCl, engineered glutamate Cl^- channel; eLGIC, engineered ligand-gated ion channel; EphB,

ephrin type-B; fMRI, functional magnetic resonance imaging; GABA, gamma-aminobutyric acid; GIRK, G-protein coupled inwardly rectifying potassium channel; GPCR, G-protein coupled receptor; GTP, guanosine trisphosphate; HIV, human immunodeficiency virus; hMXD, human muscarinic MX DREADD; HSV, herpes simplex virus; IRS2, insulin receptor substrate 2; KORD, κ-opioid DREADD; MAM, methylazoxymethanol acetate; NAc, nucleus accumbens; NMDC, N-desmethyl clozapine; PCR, polymerase chain reaction; PFC, prefrontal cortex; PPI, pre-pulse inhibition; PSAM, pharmacologically selective actuator module; PSEM, pharmacologically selective effector molecule; PV, parvalbumin-positive; PVT, paraventricular thalamus; RASSL, receptor activated solely by a synthetic ligand; SSRI, selective serotonin reuptake inhibitors; SN, substatia nigra; TORC2, target of rapamycin complex-2; TrkA, tropomyosin receptor kinase A; TRPV1, transient receptor potential vanilloid 1 channel; VTA, ventral tegmental area

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