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The use of chemogenetic tools in the research of brain diseases
Využití chemogenetických nástrojů ve výzkumu onemocnění mozku

Bachelor's thesis

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Prohlášení

Prohlašuji, že jsem tuto závěrečnou bakalářskou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

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Podpis:

Abstract

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 inhibition or excitation of 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 offer 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. Furthermore, the effect of the chemogenetic receptors on neurons lasts longer, and even chronic activation can be achieved. That can be useful for behavioural testing. The great advantage of chemogenetic tools is especially apparent in the case of the research of 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 present a powerful tool for investigating the underlying mechanisms causing the disease and revealing the link between the circuit dysfunction and the behavioural or cognitive symptoms observed in patients. It could also contribute to the development of more effective treatment.

Keywords: chemogenetics, DREADDs, PSAMs, neuropsychiatric disorders

Abstrakt:

Chemogenetika je označení pro nově vytvořené nástroje pro manipulaci aktivity buněk. Skládají se z receptoru, který je mutovaný tak, aby neodpovídal na endogenní ligandy, a ze syntetického ligandu, který naopak neinteraguje s endogenními receptory. V průběhu posledních několika dekád bylo vytvořeno mnoho různých typů receptorů a k nim korespondujících ligandů jak pro inhibici, tak pro excitaci subpopulací nervových buněk. Přesto jsou to hlavně receptory spřažené s G-proteiny odpovídající na CNO, konkrétně DREADDs, které se používají ve výzkumu. Chemogenetika má velký potenciál, jelikož aktivita oněch receptorů je reverzibilní, spustitelná pouze příslušným ligandem, a neinvazivní. Také díky použití virových vektorů je možné selektivně ovládat určitou skupinu či typ nervových buněk. Navíc, efekt těchto receptorů na neurony je dlouhodobější, dokonce se dá docílit i chronické aktivace či inhibice, což může být užitečné zvláště u behaviorálních testů. Výhoda chemogenetických nástrojů je zejména patrná v případě výzkumu onemocnění mozku, jelikož dokážou ovlivňovat celé neuronální okruhy či propojení mezi různými oblastmi mozku. Mnohá psychiatrická a jiná onemocnění mozku se vyznačují právě dysfunkcemi v neuronálních okruzích, takže je chemogenetika silným nástrojem pro výzkum zásadních mechanismů způsobujících dané onemocnění a souvislostí mezi dysfunkcí neuronálních sítí a behaviorálními či kognitivními symptomy pozorovanými u pacientů. Také by mohla přispět i k vývoji efektivnější léčby.

Klíčová slova: chemogenetika, DREADDs, PSAMs, neuropsychiatrická onemocnění

Seznam použitých zkratek:

(r)AAV	(recombinant) adeno-associated virus
ACC	anterior cingulate cortex
A β	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	Ca ²⁺ /calmodulin-dependent protein kinase II
CDK2	cyclin-dependent kinase 2
CNO	clozapine- <i>N</i> -oxide
DGC	dentate granule cells
DMN	default mode network
DREADD	designer receptor exclusively activated by designer drugs
EEG	electroencephalography
eGluCl	engineered glutamate Cl ⁻ channel
eLGIC	engineered ligand-gated ion channel
EphB	ephrine 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	<i>N</i> -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 synthetic ligand
SSRI	selective serotonin reuptake inhibitors
SN	substantia 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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1 Introduction

In neuroscience and especially brain research, the need for selective modulation of separate brain areas or neuronal subpopulations has become increasingly pressing. Especially in the case of the diseases of the brain, 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 behavioural changes caused by the disease. It can be seen in Alzheimer's disease as the hyperactivity of the hippocampus preceding neurodegeneration (O'Brien *et al.*, 2010) or the hypofunction of the prefrontal cortex in schizophrenia (Fletcher *et al.*, 1998) or the impairments in attention-deficit/hyperactivity disorder (ADHD) (Flores-Dourojeanni *et al.*, 2021). But 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 the interplay and functional connectivity of different areas (Padmanabhan *et al.*, 2019). Optogenetics is a very invasive method, only targeting a small spot for a short period of time (Eban-Rothschild *et al.*, 2016). That is why chemogenetics could lead to significant advances in this field.

Chemogenetics are a novel tool in research, primarily used in neuroscience but applicable for general manipulation of cell activity (Jain *et al.*, 2013). The first attempts were made in the 1980s by altering GTPases or kinases to be activated only on demand by a synthetic ligand, mainly to discover their specific role in the cell (Hwang and Miller, 1987; Shah *et al.*, 1997). Later, the G-protein coupled receptors came into focus, and new tools for neural modulation were constructed. Their main advantages are the reversibility of their effects, low invasiveness of the method and the ability to modulate the chosen neuronal subpopulation selectively. They can efficiently inhibit or activate only the targeted neurons expressing the chemogenetic receptors by administering a biologically inert and highly selective ligand. The activation of the chemogenetic receptor has a long enough duration for testing the neuronal modulation's effects on behaviour (Foldi, Milton and Oldfield, 2017). Atop their low constitutive activity, the non-responsiveness to endogenous ligands eliminates off-target activity and allows for reliable recognition of the link between the neuronal subpopulation's activity and the behaviour (Armbruster *et al.*, 2007).

These novel tools allow for flexible control of neuronal populations to explore their function and effect on behaviour, which could help uncover the causes of many brain diseases. They are often diagnosed based solely on behavioural symptoms as there is a lack of visible brain insult. But tracing these behavioural symptoms back to the neuronal populations using chemogenetics could lead to significant improvements in treatment, along with the general understanding of the brain (Boekhoudt *et al.*, 2017; Donegan *et al.*, 2019). This thesis seeks to offer an overview of chemogenetics, its advantages and disadvantages, and how it can be used in neuroscientific research of a few exemplary brain diseases.

2 What is chemogenetics

Chemogenetics is a field interconnecting biology and chemistry which seeks to engineer proteins (especially GPCRs) to show an altered interaction with the endogenous and synthetic ligands for more precise and rapid control of the cellular activity. These synthesised proteins retain all their functions except for their ligand specificity, so they selectively respond only to specific small molecules, usually artificially synthesised (Coward *et al.*, 1998; Small *et al.*, 2001). These engineered proteins can then be used to investigate physiological mechanisms (on the level of cells, organs, or whole organisms and their behaviour) 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 (Shah *et al.*, 1997; Armbruster *et al.*, 2007). 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 behaviours (Nguyen *et al.*, 2014).

The most commonly used chemogenetic tools are Receptors Activated Solely by Synthetic Ligand (RASSLs) (Alvarez-Curto *et al.*, 2011) and DREADDs (Vardy *et al.*, 2015). There have also been new tools emerging like the engineered Ligand-Gated Ion Channels (eLGIC) (Magnus *et al.*, 2011).

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* (Coward *et al.*, 1998), and the high constitutive activity of RASSLs (Sweger *et al.*, 2007). 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 by a novel method called directed molecular evolution (Armbruster *et al.*, 2007). Lastly, the eLGICs permit control of ion conductance of neurons via a ligand-binding domain of a particular ion channel mutated into a Pharmacologically Selective Actuator Module (PSAM). PSAM then respond only to a specific Pharmacologically Selective Effector Molecule (PSEM) which can activate or silence these neurons (Magnus *et al.*, 2011). One of the first studies on LGICs concerned using an invertebrate chloride channel activated by ivermectin to function as a neuron silencing tool (Slimko *et al.*, 2002). Later on, the effectiveness of this tool was improved by mutating a human $\alpha 1$ glycine receptor to respond to ivermectin because this receptor showed an increased expression in the cells and better sensitivity (Lynagh and Lynch, 2010).

2.1 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 synthesised and usually silent in interactions with endogenous receptors (Shah *et al.*, 1997; Gillespie *et al.*, 1999). Except for RASSLs, in which case 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 (Coward *et al.*, 1998).

Furthermore, the specific protein is mutated, so it would be activated by the orthogonal ligand (one that is not interacting with the endogenous system) but 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 (Bishop *et al.*, 2000). This mutation and selection process in DREADDs (and eLGICs) is usually executed by the directed molecular evolution approach. This approach consists of first creating a library of mutants by random mutagenesis via error-prone polymerase chain reaction (PCR). These mutants are afterwards 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 synthesised ligand. Like this, only the yeast cells with GPCRs activated by the synthetic ligand will survive and be used for the experiments (Armbruster *et al.*, 2007). On the other hand, RASSLs are created by rational design. This approach consists of modelling and evaluating the structure of a chosen protein to be able to mutate it by site-specific mutagenesis. The rational design method is a more directed and faster approach, but structural data are needed compared to the directed molecular evolution approach (Steiner and Schwab, 2012).

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 useful and safer for studying behaviour (Nathwani *et al.*, 2011). Also, different serotypes of recombinant AAV allow for targeted gene transfer into specific tissues or organs. Some of these serotypes even permit labelling of neuronal populations that are harder to reach via retrograde transport (Aschauer, Kreuz and Rumpel, 2013). 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 (Tervo *et al.*, 2016).

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 (Blömer *et al.*, 1997). Some types of herpes simplex virus (HSV) move retrogradely and allow studying even the regions of the brain that are harder to reach (Foldi, Milton and Oldfield, 2017).

HSV is also useful for their larger capacity since they can carry longer gene sequences or multiple transgenes (Krisky *et al.*, 1998).

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 (Keaveney *et al.*, 2020; Maestas-Olguin, Fennelly and Pentkowski, 2021). 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. And 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 double-floxed inverted open reading frame), making it available for transcription (Atasoy *et al.*, 2008).

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

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 of this method is its invasiveness as the cannulas are surgically implanted, going through the skull into the brain region. (Ge *et al.*, 2017). 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. Afterwards, the expressed DREADDs can be activated by systematically injecting the drug, which is not invasive and allow for easier manipulation (Foldi, Milton and Oldfield, 2017).

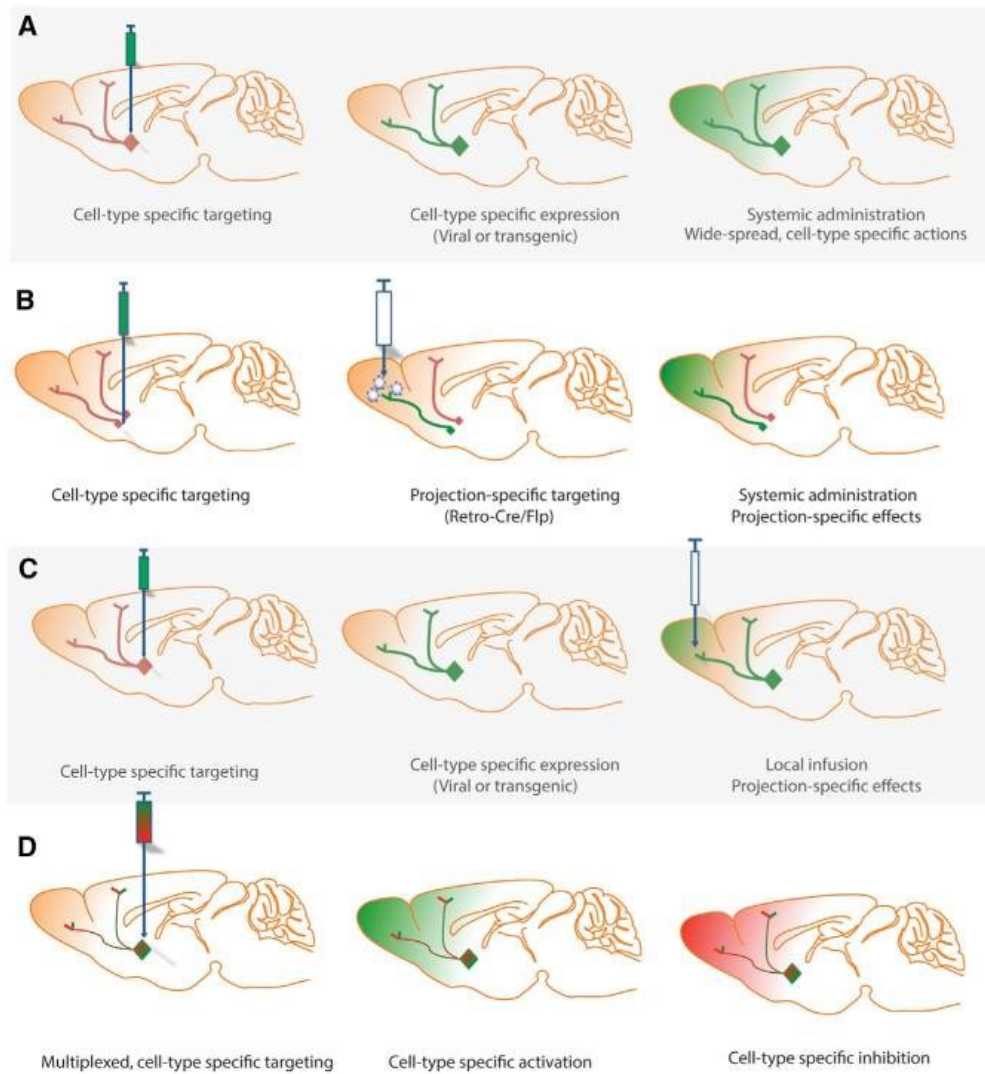


Fig. 1. Schematic representation of different approaches to DREADD expression and activation in the brain. (A) Expression of DREADDs in a specific cell type by viral vectors or transgenically (via an externally inducible transgene promoter) and their subsequent activation by systemic administration of 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 (adapted from Roth, 2016).

As was already partially discussed, there are different ways of designer drug administration. The administration choice is dependent on the type of the chemogenetic receptor 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, the most commonly utilised ligand for DREADDs (the prevalent chemogenetic tool used) is CNO (Manvich *et al.*, 2018). 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) (Michael *et al.*, 2015). However, the dose of the synthetic ligand can have varying effects based on the receptor used, the type of brain region, and subsequently, the behaviour targeted (Zhu *et al.*, 2016). Intracranial administration permits selective control of specific brain regions or even the subpopulations of neurons and their specific projections. But as already suggested, its invasiveness reduces the method's applicability (Mahler *et al.*, 2014). Last, oral administration is usually used for chronic activation of the chemogenetic receptor. It is added to either food or water, which is freely available to the tested animals and is less stressful for them than injections. Still, the presence of CNO in water could aggravate its taste and, therefore, lead to higher consumption of water when removed, which could interfere with the study results (Cassataro *et al.*, 2014).

2.1.1 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 and why they are an effective tool for cell activity modulation, as well as some of their 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 signalling pathways (Shukla *et al.*, 2014). 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 (DeWire *et al.*, 2007). Furthermore, the type of ligand or the type of receptor and the cellular context can bias the receptor toward one signalling pathway over others, also called functional selectivity (Wacker *et al.*, 2013).

Even though various ligands activate G-protein coupled receptors, they can also signal without them, with higher or lower probability based on the type of a receptor, which is 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 (Sweger *et al.*, 2007).

The signalling pathways activated by GPCRs 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 signalling pathways. However, it may be important information since the isoforms can lead to differing additional changes in cell activity (Marti-Solano *et al.*, 2020). Furthermore, some of them have separate extracellular binding sites for endogenous protein ligands 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 original mode of function of the receptor (Armbruster *et al.*, 2007).

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 a great tool to be used in a wide variety of scenarios. It permits studying a whole range of cellular activities and behaviours. So even despite some caveats, they have undeniable advantages for use as chemogenetics.

2.2 Types of chemogenetic tools

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

2.2.1 Kinases

One of the first genetically modified proteins to work as chemogenetic tools was kinases. They were generated by creating a functionally silent mutation in the ATP (Shah *et al.*, 1997) or GTP (Hwang and Miller, 1987) binding sites, respectively, and then an already existing corresponding kinase (or GTPase) inhibitor was synthesised to bind to the altered site. Thanks to this approach, the effect of its inhibition on the cell could be observed. Furthermore, the specific signalling pathway it engages in and the role of the kinase in the cell could be reconstructed. This approach is essential for treating the diseases since it can uncover the signalling 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 signalling 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 (Bishop *et al.*, 1998). A similar study was conveyed only with a different ATP-analogue for the v-Src kinase (Liu *et al.*, 1998). 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 the larger 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 signalling in many areas of neuronal development (Soskis *et al.*, 2012).

2.2.2 Enzymes

Another class of proteins grouped under the chemogenetic tools is enzymes. Here the studies focus on developing artificial enzymes which 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 together 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 (Gillespie *et al.*, 1999). On the other hand, in the study by Häring and Distefano (2001), the introduction of 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.

2.2.3 G-protein coupled receptors: RASSLs and DREADDs

The chemogenetic technology based on GPCRs is, to date, the most popular tool in many areas of science, especially in behavioural 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, it is crucial to study their affinity and selectivity for ligands. 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 (Strader *et al.*, 1991).

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.* (1998) created chimeric receptors with parts from μ and δ opioid receptors, which 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 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 (MacLaren *et al.*, 2016) and allows for multiplexed control of cell activity (Vardy *et al.*, 2015).

2.2.4 Ligand-gated ion channels

The last category of chemogenetic technologies, based on ion channels, is eLGIC, sometimes called PSAM. They serve as a rapid control of ion conductance on the cell membrane, 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 behaviour. 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 also in the possible use of multiple eLGICs within one cell population or together with different tools like optogenetics (Magnus *et al.*, 2011).

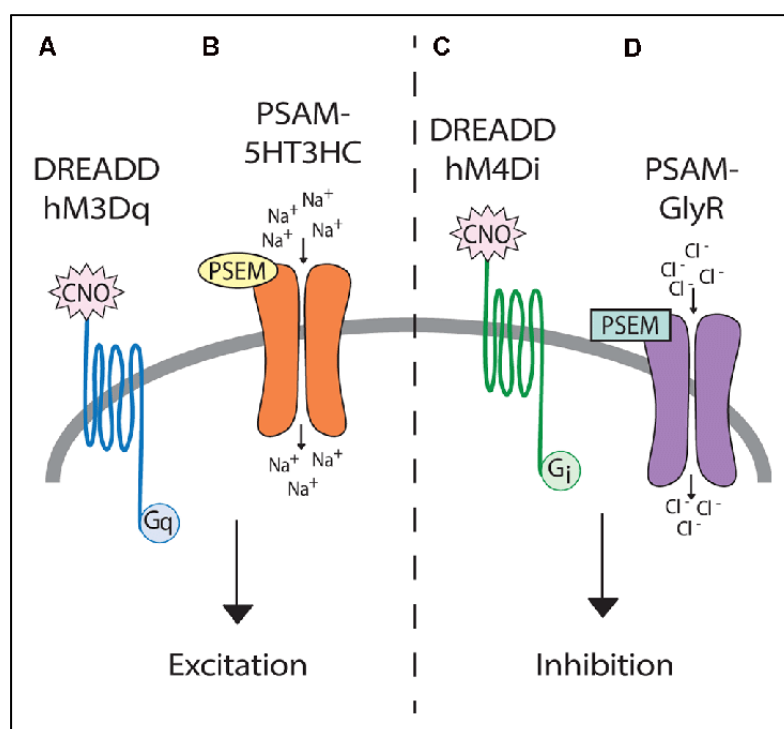


Fig. 2. Schematic representation of four different types of chemogenetic tools. On the left, (A) DREADD hM3Dq and (B) PSAM-5HT3HC act through depolarization of the neuronal membrane to produce excitation. On the right, (C) DREADD hM4Di and (D) PSAM-GlyR hyperpolarise the membrane to induce inhibition. The DREADDs activate the respective G-protein coupled signalling 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) (adapted from Eisdorfer *et al.*, 2020).

2.2.5 Synthetic receptors and ligands

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, there are multiple different classes of proteins 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 giving rise to DREADD technology. Due to that, this chapter will be focused mainly on the DREADDs. Multiple types of DREADDs have been engineered and coupled with different G proteins and, therefore, various signalling pathways, G_q , G_i or G_s , as specified further.

Name	Protein(s)	Ligand	Reference
Representative kinases			

Allele-specific kinase inhibitors	v-I388G	Compound 3g	(Liu <i>et al.</i> , 1998)
Analogue-sensitive kinases	v-Src (I388G, v-Src-as1), c-Fyn (T339G, c-Fyn-as1), c-Abl (T315A, c-Abl-as2), CAMK II α (F89G, CAMK II α -as1) and CDK2 (F80G, CDK2-as1)	K252a and PPI analogues	(Bishop <i>et al.</i> , 1998)
Rapamycin-insensitive TOR complex	TORC2 V2227L	BEZ235	(Bishop <i>et al.</i> , 2000)
ATP-binding pocket mutations in EphB1/2/3	<i>Ephb1</i> ^{T697G} , <i>Ephb2</i> ^{T699A} , and <i>Ephb3</i> ^{T706}	PP1 analogues	(Soskis <i>et al.</i> , 2012)
ATP-binding pocket mutations of TrkA/B/C	<i>TrkA</i> ^{F592A} , <i>TrkB</i> ^{F616A} , and <i>TrkC</i> ^{F617A}	1NMPP1 and 1NaPP1	(Chen <i>et al.</i> , 2005)
Representative enzymes			
Metalloenzymes	Achiral biotinylated rhodiumdiphosphine complexes		(Collot <i>et al.</i> , 2003)
Engineered transaminases	Chemically conjugating a pyridoxamine moiety within the large cavity of intestinal fatty acid binding protei	Enhanced activity	(Häring and Distefano, 2001)
Representative GPCRs			
Allele-especific GPCRs	β 2-adrenergic receptor, D113S	1-(3',4'-dihydroxyphenyl)-3-methyl-L-butanone (L-185,870)	(Strader <i>et al.</i> , 1991)
RASSL-G _i	κ -opioid chimeric receptor	Spiradoline	(Coward <i>et al.</i> , 1998)
Engineered GPCRs	5-HT _{2A} serotonin receptor F340→L340	Ketanserin analogues	(Westkaemper <i>et al.</i> , 1999)
G _i -DREADD	M2- and M4 mutant muscarinic receptors	Clozapine-N-Oxide	(Armbruster <i>et al.</i> , 2007)

G _q -DREADD	M1, M3, and M5-mutant muscarinic receptors	Clozapine-N-Oxide	(Armbruster <i>et al.</i> , 2007)
G _s -DREADD	Chimeric M3-frog Adrenergic receptor	Clozapine-N-Oxide	(Guettier <i>et al.</i> , 2009)
Arrestin-DREADD	M3Dq R165L	Clozapine-N-Oxide	(Nakajima and Wess, 2012)
Axonally-targeted silencing	hM4D-neurexin variant	Clozapine-N-Oxide	(Stachniak, Ghosh and Sternson, 2014)
KORD	κ-opioid receptor D138N mutant	Salvinorin B	(Vardy <i>et al.</i> , 2015)
Representative channels			
GluCl	Insect Glutamate chloride channel; Y182F mutation	Ivermectin	(Lerchner <i>et al.</i> , 2007)
TrpV1	TrpV1 in TrpV1 knock-out mice	capsaicin	(Arenkiel <i>et al.</i> , 2008)
PSAM	Chimeric channels PSAMQ79G,L141S PSAM-GlyR fusions	PSEM9S PSEM89S; PSSEM22S	(Magnus <i>et al.</i> , 2011)

Tab. 1. Table illustrating the different types of chemogenetic tools used. 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 Ca²⁺/calmodulin-dependent protein kinase II (CAMKII) or cyclin-dependent kinase 2, target of rapamycin complex 2 (TORC2), EphB2 or TrkA) and their respective ligands. Then it is the groups of enzymes 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. Each example also has a corresponding study in which it has been used in Reference section (adapted from Roth, 2016).

For DREADDs based on G_q signalling, human M1, M3 and M5 muscarinic receptors are mutated to obtain selective activation by CNO. From these, the human muscarinic M3 DREADD (hM3Dq) is most prevalent. Their activation leads to phosphatidylinositol hydrolysis, which stimulates the release of intracellular Ca²⁺, resulting in increased neuronal firing (Armbruster *et al.*, 2007; Alexander *et al.*, 2009). Because the release of Ca²⁺ ions plays an essential role in many cell types, it

has been used more widely. For example, in the research of glial cells (Michael *et al.*, 2015) or pancreatic β cells, where the activation of pathways including insulin receptor substrate 2 (IRS2) by these DREADDs was crucial (Jain *et al.*, 2013).

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 $G\beta/\gamma$, which subsequently leads to hyperpolarization of the neuron, therefore silencing the neuron's firing (Armbruster *et al.*, 2007). 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 (Vardy *et al.*, 2015).

Last, in what concerns the types of DREADDs, G_s -coupled ones have been developed and used *in vivo*, to study medium spiny neurons (Farrell *et al.*, 2013) or β -cells in the pancreas (Guettier *et al.*, 2009). 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 behaviours. In the case of striatopallidal medium spiny neurons activation, it decreases the locomotion of tested rats (Farrell *et al.*, 2013).

For PSAMs, many different receptors have been engineered both for silencing and enhancing neuronal firing. The $\alpha 7$ -5HT3 channels have been developed as the activators while eLGICs based on the glycine and γ -Aminobutyric acid (GABA) receptors act as inhibitors (Magnus *et al.*, 2011).

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 (Armbruster *et al.*, 2007). 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*-desmethylozapine (NDMC) in both rats and mice (Manvich *et al.*, 2018). And these molecules are not biologically inert (McQueen *et al.*, 2021). Gomez *et al.* (2017) 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, Jendryka *et al.* (2019) 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 an accord on

whether these off-target effects cause some behavioural changes or not. A study using the five choice-serial-reaction task did not observe any (Jendryka *et al.*, 2019), while another one reported changes in amphetamine-induced locomotion and startle response following even low doses of CNO (MacLaren *et al.*, 2016).

One possible alternative to CNO use is low doses of clozapine which do not seem to cause behavioural 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. But there would remain the uncertainty about the off-target effects of clozapine (Jendryka *et al.*, 2019). 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. But no behavioural consequences of this effect have been reported (Jendryka *et al.*, 2019). 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 (Goutaudier *et al.*, 2020). 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 so it would not activate the endogenous receptor or other receptors (like the muscarinic ones). It is successfully used as the KORD activator resulting in significant behavioural changes through the silencing of specific neuronal populations (Vardy *et al.*, 2015). Last, perlapine could serve as another valuable ligand to substitute CNO in activation of 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 (Chen *et al.*, 2015).

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 (Thompson *et al.*, 2018) 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 also binds to the native dopamine D2 receptors and causes treatment side effects (Weston *et al.*, 2019).

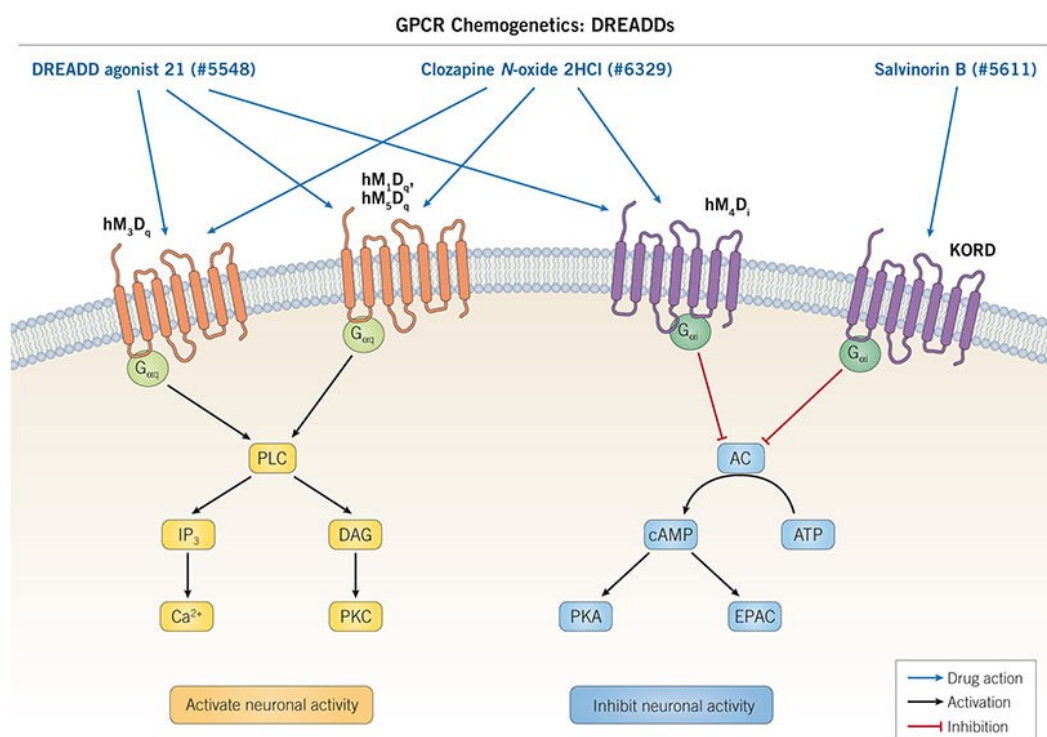


Fig. 3. The schematic illustration of the effect of different DREADD agonists. Compound 21 and CNO bind to G_q-DREADDs (hM3Dq, hM4Dq, hM5Dq), leading to neuronal activation through the action of phospholipase C (PLC), and the inhibitory G_i-DREADD (hM4Di) acting via inhibition of adenylyl cyclase (AD). The salvinorin B is a selective agonist for KORD, which affects the same inhibitory pathway as hM4Di (adapted from <https://www.tocris.com/product-type/dreadd-ligands>).

3 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.

3.1 Alzheimer's disease

3.1.1 General characteristic

Alzheimer's disease (AD) is a highly prevalent neurodegenerative disease, mainly manifesting at an older age. This chapter focuses primarily on the impairment of functional networks in the brain disease. But the molecular mechanisms like the accumulation of β -amyloid (A β) plaques and neurofibrillary tangles of tau protein must be mentioned here as they are directly tied to the dysfunction or dysregulation of the brain networks (Rodriguez *et al.*, 2020).

An underlying issue occurring in AD patients is the hypometabolism of many brain areas, especially the parietal and temporal lobes (Kennedy *et al.*, 1995). This deficit is also prominent in the hippocampus. It could then lead to a compensatory increase of generalised activity in different

temporal lobe areas, like the entorhinal complex or even the neocortex in more severe AD cases (Desgranges *et al.*, 2002). It could have a similar influence on the default mode network (DMN). This network is deactivated in healthy adults when focusing on an external task. However, in patients with AD, this inactivation is not present. Furthermore, the activity of DMN, particularly in the posterior cingulate cortex and precuneus, is even increased compared to young, healthy adults. Since this network is connected to the entorhinal cortex (and by extension to the hippocampus), which shows reductions in metabolic activity and consequent atrophy, the DMN could be compensating for that by the increased activity (Lustig *et al.*, 2003). Other findings suggest that this observed DMN hyperactivity could result from a loss of regulation due to the reduced connectivity between the hippocampus and DMN areas like the precuneus (Wang *et al.*, 2006). Another critical aspect of the impairment of DMN in AD is A β plaques accumulation. It is primarily the posterior parietal cortex, precuneus, and some parts of the prefrontal cortex where the deposition of A β plaques is prominent and could result in the observed disruption of the functionality of DMN (Palmqvist *et al.*, 2017). But there is some discussion on whether the accumulation of A β plaques leads to the change in DMN activity or the hyperactivity of DMN neurons makes them more prone to A β plaques acquisition (Rodriguez *et al.*, 2020).

Another significant impairment in AD concerns the functionality of the hippocampus. Interestingly, it seems that the increased activity of hippocampal neurons, as detected by functional magnetic resonance imaging (fMRI), is a good predictor of subsequent clinical decline and reduction in activity leading to memory deficits, which is one of the predominant symptoms of AD (O'Brien *et al.*, 2010).

3.1.2 Application of chemogenetics in Alzheimer's disease

Chemogenetics have been used in the research to determine the underlying causes of AD impairments and possible targets for early diagnosis or even for treatment. Rodriguez *et al.* (2020) showed that when the entorhinal cortex activity was reduced, the accumulation of A β 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)/A β 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 A β plaques accumulation.

A similar study by Peng and Grutzendler (2016) further supports these findings. They used the hM3Dq for neuronal activation and hM4Di for neuronal inhibition to evaluate their effects on A β plaques. CNO was administered daily for 30 or 60 days. They also found that the attenuation of neuronal activity reduced the accumulation of A β plaques, as well as the diameter of the halo of oligomeric A β (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 A β 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 A β 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 minutes 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 of the significant AD's symptoms: spatial memory and learning deficits (Rorabaugh *et al.*, 2017). To sum up, chemogenetics prove to be a handy set of tools for researching the causes of AD and possibly finding new targets for treating the debilitating symptoms.

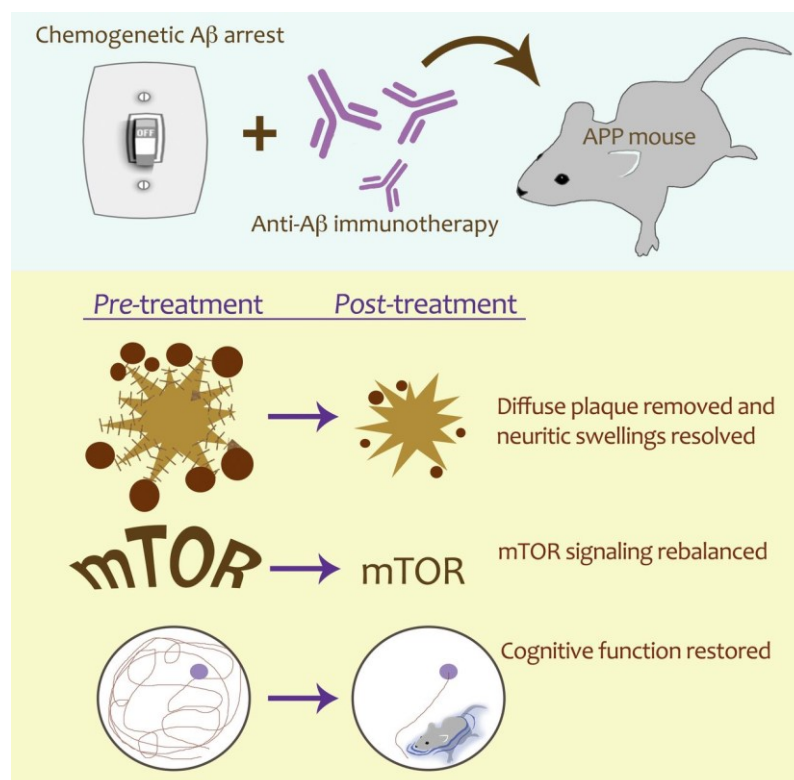


Fig. 4. Image illustrating the possibility of improving cognitive impairments in an AD mouse model by employing chemogenetic and immunotherapeutic approaches to reduce A β plaques accumulation and restore normal mammalian TOR signalling (adapted from Chiang *et al.*, 2018).

3.2 Epilepsy

3.2.1 General characteristic

Epilepsy is a heterogeneous brain disease characterised by recurrent seizures, which are uncontrolled synchronous outbursts of the electrical activity of neurons. They may either start in a localized area and then spread into other parts of the brain (focal seizures) or affect larger areas of the brain from the start (generalised seizures). The exact causes of epilepsy are diverse and sometimes unknown, ranging from immunological and molecular dysfunction through genetic predisposition and a brain insult (like stroke) to the cell-signalling pathway and whole circuit disruptions. Further confusion arises when epileptic seizures are part of or a result of a different disease. Also, epilepsy can manifest in very differing ways: from somatosensory to cognitive impairments (Fisher *et al.*, 2005, 2014).

Treatment consists of a myriad of different drugs like lamotrigine or levetiracetam, anticonvulsants like carbamazepine or oxacarbazepine, valproate sodium and others (Canevini *et al.*, 2010) or a surgical disconnection of some regions of the brain. Yet, the drugs are not effective for all types of epilepsy. Around 30 % of patients are drug-resistant (Kwan and Brodie, 2000). And surgery is not advised (especially in adults) due to the possibility of causing long-term cognitive impairments, especially in the areas of language or motor and sensory functions (d’Orio *et al.*, 2019). These impairments occur probably because the most common epilepsy resistant to medical treatment is the temporal lobe epilepsy which usually influences the hippocampus, amygdala and temporal cortex, further propagating to the midline thalamus (Wicker and Forcelli, 2016). Moreover, the mediodorsal thalamus is an important brain region in propagating excitatory signals in general, causing limbic motor seizures (Cassidy and Gale, 1998). It is often the redundant connections (to the excitatory neurons) and their density that leads to excessive stimulation resulting in epilepsy, as shown on dentate granule cells (DGCs) (Zhou *et al.*, 2019).

3.2.2 Application of chemogenetics in 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 the research of different types of epilepsy or their treatment. Especially the temporarily controlled silencing of seizures makes it more advantageous than permanent genetic approaches. The issues of gene therapy concerning viral vector targeting or potential oncogenesis could have serious adverse effects (Watakabe *et al.*, 2015; Howe *et al.*, 2008).

First, DREADDs are immensely useful for the research of epilepsy 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 the 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 to be true. Like this, the pro-epileptic neural circuits involving hippocampal DGCs, and their ability to provoke seizures in temporal lobe epilepsy, were identified (Zhou *et al.*, 2019).

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 threshold for seizures and, therefore, facilitated the onset of the seizure (Drexel *et al.*, 2017).

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. The inhibitory hM4Di was activated by CNO after the injection of pilocarpine or picrotoxin to induce a motor or a behavioural seizure. Surprisingly rapidly, it significantly attenuated the epileptic seizure (already within 10 minutes of the CNO injection) (Kätzel *et al.*, 2017). Other studies like that by Wicker and Forcelli (2016) 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 minutes before seizure stimulation. The impact on cognitive abilities was not thoroughly explored, even though it could be significant as this region encompasses many neuronal fibres and interconnections to other brain regions (Wicker and Forcelli, 2016).

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 (Lieb *et al.*, 2018).

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, it would be favourable to test other ligands and their effectivity (like KORD). Even though some studies found no off-target effects of CNO and that even low doses of CNO might still be effective and selective enough (Wicker and Forcelli, 2016). 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 certain neuronal populations may prove quite challenging and possibly even cause off-target signalling, for example, in the midline thalamus (Wicker and Forcelli, 2016). 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 (Kätzel *et al.*, 2017).

3.3 Depression and anxiety

3.3.1 General characteristic

Depression is a highly prevalent disease occurring in around 16 % of the adult population (in the United States) (Kessler *et al.*, 2003). It is commonly treated with Selective Serotonin Reuptake Inhibitors (SSRI), but unfortunately, only 63 % of patients respond to the treatment, and only 47 % achieve remission (Gartlehner *et al.*, 2011). That is probably because depression and anxiety disorders are very heterogeneous and target many different brain regions and circuits, which may or may not include the drug's target monoamine oxidase, as is discussed below.

First, DMN is one of the networks significantly dysregulated in depression. DMN is a large-scale brain network, reducing its activity when focusing on a cognitive task (usually aimed externally or engaging the working memory) in healthy subjects. But it failed to deactivate in patients with depression. The anterior-medial prefrontal cortex and the posterior cingulate cortex from the DMN remained significantly active even during the cognitive task (Bartova *et al.*, 2015). Also, the connectivity between DMN and other brain regions like the precuneus, thalamus, and subgenual cingulate (subregion of anterior cingulate cortex) is higher compared to healthy subjects, possibly contributing to the dysregulation (Greicius *et al.*, 2007). Furthermore, the increased activity was correlated with higher levels of maladaptive and negative rumination in patients with depression (Hamilton *et al.*, 2011). These differences in brain activity in patients with depression occurred even in those with complete remission. They were more pronounced in adults with adolescent-onset depression, pointing to the effect of depression on the development of the brain (Bartova *et al.*, 2015). Similarly, in social anxiety disorder, the medial prefrontal cortex and angular gyrus showed lowered connectivity (synchronous activation) during a resting state than healthy controls. This suggests there is some dysregulation of the DMN in anxiety disorders as well (Qiu *et al.*, 2011).

Second, a crucial network impaired in depression is the reward circuit which most likely leads to the common symptom of anhedonia. People with major depressive disorder showed decreased

activation mainly in the caudate, thalamus, anterior cingulate cortex, insula and the orbitofrontal cortex. They showed increased activation in the dorsolateral prefrontal cortex during reward processing (Dichter *et al.*, 2012; Zhang *et al.*, 2013). Also, another dopaminergic structure, the nucleus accumbens (NAc), seems to react less to rewards in patients than in healthy subjects (Pizzagalli *et al.*, 2009). It would be intriguing to investigate the effect of dopamine-based stimulants in depression treatments more closely. Interestingly, there seems to be a significant difference in depressive patients compared to controls during the reward anticipation where the anterior cingulate gyrus and the midfrontal gyrus are over-activated, possibly creating an inadequate reaction to the stimuli. These differences in activation of the reward circuit could also serve as a diagnostic parameter for susceptibility to major depressive disorder (Dichter *et al.*, 2012).

Mood disorders like depression and anxiety disorders are tightly connected to emotion regulation. Therefore, it is no surprise that some significant differences have been found in the function of the regions like the amygdala, insula, prefrontal cortex (PFC), and anterior cingulate cortex (ACC) compared to healthy adults. In the anxiety disorders (like social anxiety and specific phobia), the insular and amygdala hyperactivity seems crucial (Etkin and Wager, 2007). When analyzing facial expressions, the insula showed increased activity in patients with generalised social anxiety disorder. Furthermore, the ACC showed lower activation when extensive attention was required for non-emotional cues contrary to controls. This led the researchers to conclude that this hypoactivity of ACC resulted from the dysregulated and hyperactive insula causing more vigorous attention to emotional stimuli, leading to anxiety (Klumpp *et al.*, 2013). Patients suffering from anxiety also showed decreased recruitment of dorsomedial-PFC in case of emotional conflict together with increased activation of the amygdala, again implicating the impairment of top-down control on emotion processing centres (Etkin *et al.*, 2010). In patients with depression, a bias towards negative faces was apparent. It was achieved by increased amygdala activity when presented with sad faces, even though the showing time was under the conscious awareness threshold (Victor *et al.*, 2010). The top-down control of emotions is also impaired in depression as the dorsolateral PFC showed decreased activity both with and without a negative emotional stimulus. The negative stimulus was then over-activating the amygdala in patients with depression. That is probably due to the dysfunction of the emotion regulation system between the dorsolateral PFC and the amygdala (Siegle *et al.*, 2007).

3.3.2 Application of chemogenetics in 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, therefore less of a 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 as the most likely target of the SN neuronal projections since they showed higher expression of c-fos when CNO activated the hM3Dq in SN. The activation of 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 (Bouchet *et al.*, 2018).

Another study focused on the impairment of regulatory circuits and centres 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. The tetanus toxin induced chronic presynaptic inhibition, impacting the proportion of excitatory and inhibitory neurons active in medial PFC by increasing the activity of inhibitory interneurons. While 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 the depressive symptoms. But 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 (Kato *et al.*, 2019). 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 by 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 towards potential threats, not the immediate ones (Maestas-Olguin, Fennelly and Pentkowski, 2021).

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.

Afterwards, 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 the SSRI antidepressants (Han *et al.*, 2017).

In the case of depression and anxiety, chemogenetic tools could be very useful 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 different brain areas.

3.4 Schizophrenia

3.4.1 General characteristic

Schizophrenia is a psychiatric disorder characterised by a wide range of symptoms, usually classified into the positive ones like hallucinations and delusions, negative ones including apathy or inappropriate emotional responses and cognitive symptoms like impairments in working memory or speech (World Health Organization, 1993; Suttkus *et al.*, 2021). It usually manifests in early adulthood. But studies suggest that it is a neurodevelopmental disease caused by brain defects stemming either from perinatal age (for example, from maternal infection) (Brown *et al.*, 2000) or from adolescence. In those later diagnosed with schizophrenia, the neural pruning (reduction in grey matter in youth) was significantly greater and faster (Thompson *et al.*, 2001). Especially in the parietal and superior frontal cortices, the motor cortex and dorsolateral PFC, and the temporal cortices. The worsening of positive symptoms was correlated with a higher rate of decline of the temporal cortices. In contrast, the negative symptoms seemed to be associated with the accelerated grey matter loss in frontal cortices (Thompson *et al.*, 2001).

Regarding the circuits and brain areas impaired in schizophrenia, the frontal cortices were identified as majorly impacted, the patients showing so-called hypofrontality. Primarily the function of PFC in working memory was explored, showing that patients with schizophrenia fail to activate enough the PFC as the working memory task gets more demanding. Also, a reduced activity in the posterior parietal region, implicated in long-term memory retrieval, was observed during this task. However, the interpretations of whether it is due to the impairment of working memory, long-term memory and retrieval, or general cognitive decline are unclear (Fletcher *et al.*, 1998). A different study focused on context processing and showed that schizophrenic patients performed worse on such tasks due to impaired ability to activate the dorsolateral PFC. Therefore, this region could potentially be behind the inadequate responses in people with schizophrenia (Barch *et al.*, 2001).

Another critical network affected in schizophrenia is the DMN, which is characterised by higher activity at rest. In schizophrenia patients, the DMN is impaired as it remains activated even during a working memory task. That is especially apparent in the medial PFC and posterior cingulate cortex. Furthermore, there was greater connectivity between these two regions and significantly lower connectivity of DMN to the dorsolateral PFC in schizophrenia patients. This altered activity was also linked to the severity of symptoms (Whitfield-Gabrieli *et al.*, 2009).

Hippocampus is also majorly impaired in schizophrenia patients. It manifests by the reduction in volume, especially in the dentate gyrus and the CA3, CA4 regions (Nelson *et al.*, 1998; Kraguljac *et al.*, 2013). Furthermore, the baseline hyperactivity of the anterior hippocampus in patients also plays an important role, leading to its reduced recruitment during a task (McHugo *et al.*, 2019). The anterior hippocampus has reduced connectivity to other brain regions, especially between the anterior subiculum and the limbic striatum (Gangadin *et al.*, 2021). In general, the different connectivity patterns in the brain, but especially between the hippocampus and DMN or the other resting-state networks, seems to be an essential driver in schizophrenia (Uscătescu *et al.*, 2021).

Concerning the dysregulations of the neurotransmitter-specific neuronal populations, the dopaminergic activity in the associative striatum is elevated in patients and even those with prodromal symptoms, suggesting it could be a good predictor of an impending schizophrenia diagnosis (Howes *et al.*, 2009). There is also evidence for increased glutamate levels in the hippocampus of schizophrenia patients (Kraguljac *et al.*, 2013). In what also concerns the hippocampus, the increased GABA_A tonic activation seems to normalise the function of the ventral hippocampus in the methylazoxymethanol acetate (MAM) rodent model of schizophrenia (Donegan *et al.*, 2019)

3.4.2 Application of chemogenetics in 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 the 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 the manipulation of 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 behaviour 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 (Nguyen *et al.*, 2014).

The connection between the GABAergic structures of the hippocampus and the dopamine system of the brain was explored in a study using the overexpression of the $\alpha 5$ subunit of the GABA_A receptor in pyramidal cells and inhibition of the ventral hippocampus by hM4Di. The overexpression of the $\alpha 5$ subunit reduced the dopaminergic neuron's activity in the VTA in the MAM model of schizophrenia. That was probably mediated by the ventral hippocampus – NAc pathway since reducing this pathway's activity by hM4Di resulted in 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 $\alpha 5$ 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 (Donegan *et al.*, 2019).

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 were unable to modulate their behaviour 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 (Parnaudeau *et al.*, 2015).

3.5 ADHD

3.5.1 General characteristic

Attention-deficit/hyperactivity disorder (ADHD) is a neurological developmental disorder with a very heterogeneous set of symptoms and, therefore, a complicated diagnosis. It starts to manifest in early childhood but persists into adulthood, often undiagnosed. The diagnosis is usually based on a report evaluating behavioural impairments instead of biochemical markers or technologies like fMRI or electroencephalography (EEG) (Ustun *et al.*, 2017). That is mainly because the link between behavioural deficits and neurological impairments is unclear since the breadth of symptoms in ADHD is grand and differs significantly between individuals. One of the popular approaches (out of many) states that ADHD stems from the dysfunction of two separate circuits (Sonuga-Barke, 2002), one concerning the executive functions with impairments in the prefrontal cortex (PFC), anterior cingulate cortex, and caudate nucleus, causing issues in planning or working memory. The other circuit is focused more on emotional response regulation connected to the nucleus accumbens, amygdala, and hippocampus, together resulting in dysregulation of

behavioural inhibition and attention (Posner *et al.*, 2013). Chemogenetic tools could be used precisely to determine the underlying circuits' impairments causing the behavioural symptoms of ADHD and possibly leading to the development of effective medication.

As mentioned above, a critical circuit affected in ADHD links the prefrontal cortex and basal ganglia, especially the striatum. It has been shown that ADHD is linked to dopamine insufficiency affecting the striatum (but also the PFC), as it has some essential dopaminergic projections like those in the nucleus accumbens or caudate nucleus. These are implicated in attention and response regulation (impulsivity) (Schneider, Sun and Roeltgen, 1994; Flores-Dourojeanni *et al.*, 2021). There is still a debate over the exact mechanisms causing this dopamine dysregulation; one possible explanation is a higher density of dopamine transporter, leading to decreased concentration of available dopamine (Dougherty *et al.*, 1999). But there are also studies finding different causes, like the reduced release of dopamine into the synaptic cleft (Volkow *et al.*, 2007) or even the hyperactivity of the dopaminergic system, that causes some of the symptoms (Boekhoudt *et al.*, 2016). In this case, chemogenetic tools could also help discover these specific mechanisms since they act through the G-protein coupled receptors, just like the dopamine receptors.

3.5.2 Application of chemogenetics in ADHD

Studies concerning ADHD and chemogenetics mainly focus on discovering the functions and connections between certain brain areas or circuits and the behavioural 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. It seems that mainly the increased activity of the neurons connecting VTA to the NAc is responsible for locomotor hyperactivity, such as seen in ADHD. When the transgenic mice expressing hM3Dq in either SN or VTA were injected with CNO, their locomotor activity changed. But 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. It seems that the nigrostriatal pathway could be more involved in movement coordination than in 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 (Boekhoudt *et al.*, 2016). Unfortunately, the activation was not strictly restricted to the separate areas, as showed 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 attention and impulsivity distinction. 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 ligand were used for this activation. The number

of omissions in the task increased in case of activation of both VTA and SN. Only in the case of SN it also led to latency to respond (as well as the latency to collect 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 behaviour, leading to higher distractibility. This points in the direction that neither VTA nor SN play a significant role in impulsivity but significantly impair attention, just 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 (Boekhoudt *et al.*, 2017). 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 the VTA (Boekhoudt *et al.*, 2017). 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 for 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 (Volkow *et al.*, 2001). Therefore, it could help enlighten the precise effects of these receptors, as attempted in a study by Fitzpatrick *et al.* (2019). It showed that the inhibition of dopaminergic neurons in VTA by hM4Di contributes to hyperactivity and attention impairment (reduced vigour and speed to respond). Boekhoudt *et al.* (2017) study on VTA activation reveals that both insufficient and excessive activity of dopaminergic neurons causes symptoms associated with ADHD. Therefore, the treatment should aim at finding the optimal concentration of this neurotransmitter. It also touched on the effects of inhibition of norepinephrine neurons in locus coeruleus in attention (during more demanding tasks) and impulsivity (Fitzpatrick *et al.*, 2019).

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 (Fernandez-Ruiz *et al.*, 2020). 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) (Yu-Feng *et al.*, 2007). Regarding the shortcomings of many studies on ADHD, there is a lack of comparison and acknowledgement 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 (Fernandez-Ruiz *et al.*, 2020). The same concerns the equal representation of male and female patients in ADHD studies often focusing on boys exclusively (Yu-Feng *et al.*, 2007).

4 Discussion

Chemogenetics are becoming more commonly employed in investigating various neuronal circuits, connectivity between those, and their influence on behaviour. Yet, there are still some shortcomings concerning the application of DREADDs and their ligands' effects. The predominantly used CNO is usually considered a biologically inert molecule. Unfortunately, recent studies suggest that it might not be the case. CNO has been shown to have multiple off-target effects (Jendryka *et al.*, 2019) and even an impact on behaviour in the absence of DREADDs, which could impair the potential experimental results of DREADD employing studies (MacLaren *et al.*, 2016). Furthermore, it has been shown that CNO back-metabolizes to clozapine in the brain (Manvich *et al.*, 2018), and clozapine is not biologically inert (McQueen *et al.*, 2021). 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 (MacLaren *et al.*, 2016; Gomez *et al.*, 2017).

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 (Pati *et al.*, 2019). 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 more routinely use other possible ligands like salvinorin B.

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're overexpressed in the neuronal population (Sweger *et al.*, 2007). 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 (Slimko *et al.*, 2002). Different types of eLGICs or DREADDs can also be combined, which could be useful for multiplexed modulation of brain networks (Magnus *et al.*, 2011; Vardy *et al.*, 2015).

There have also been some studies on optimizing the application of DREADDs for better targeting and more uniform distribution of the vector, which could be especially useful in laboratory animals with bigger brains like rhesus monkeys. Fredericks *et al.* (2020) suggested the co-infusion of magnesium ions for visualisation and verification of 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 (Watakabe *et al.*, 2015).

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 (Parnaudeau *et al.*, 2015). It would be interesting to include more studies on non-human primates to simulate the

human brain's functionality more closely (Fredericks *et al.*, 2020). Nonetheless, rodent models still offer a great resource, and they can work well for other symptoms for which the treatment often is not effective (Han *et al.*, 2017; Donegan *et al.*, 2019).

5 Conclusion

This work demonstrates that chemogenetics, especially DREADDs, are powerful tools for research of brain diseases. That is so 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 with the emphasis on behavioural effects (Eban-Rothschild *et al.*, 2016). The application of viral vectors allows for exploration of the function of whole neuronal populations or networks in the brain, which seems to be especially crucial for the research of psychiatric diseases. There, it is often the connectivity between different brain areas like the hippocampus and PFC (Donegan *et al.*, 2019) or the dysfunction of whole networks like the DMN (Hamilton *et al.*, 2011) which play 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 (Trivedi *et al.*, 2006), or there is no effective treatment, as in the case of Alzheimer's disease (Rorabaugh *et al.*, 2017), 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 (Wicker and Forcelli, 2016). It can be, of course, used for the general research of the brain, like the function of different parts of a specific region, their connectivity to the other areas, and the subsequent effect on behaviour.

Unfortunately, chemogenetics are still underused in the research. Even though they have some shortcomings which 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 the field of neuroscience.

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