

**Exploring the Behavioural Effects of Compound-21 Using Novel Object Recognition and  
Object in Place Tests in Long Evans Rats**

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## ABSTRACT

Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) have gained popularity as a tool to investigate the neural substrates of behaviour in rodents. When used with spontaneous behavioural tests of memory in rodents, DREADDs allow for a unique opportunity to advance our understanding of how specific neuronal populations contribute to cognition.

While data on the use of DREADDs to study memory with spontaneous tasks in rats is somewhat limited, there is evidence to suggest that the canonical DREADD agonist, clozapine-*N*-oxide (CNO), exhibits off target effects on recognition memory assessed with the Novel Object Recognition (NOR) test. While newer DREADD agonists are available, an understanding of how these novel compounds impact rat behaviour unspecific to DREADD activation is lacking.

Therefore, I sought to test whether the DREADD agonist, Compound 21 (C21), affected recognition memory assessed by NOR or, associative memory assessed by the Object-in-Place (OiP) test. I also investigated whether DREADD-mediated inhibition of parvalbumin (PV+) gamma-amino butyric acid (GABA)ergic interneurons of the medial prefrontal cortex (mPFC) would impair associative memory as measured by OiP. I showed that C21 did not affect either sex in NOR, or females in OiP. Male rats failed to exhibit robust discrimination in OiP following either control or C21 treatment; however, total object exploration times of male rats were not altered by C21. Lastly, PV-Cre rats transfected with an inhibitory DREADD in the mPFC and treated with C21 showed normal exploration of objects in OiP. Poor discrimination in OiP and low vector co-expression of DREADD with mPFC parvalbumin-containing interneurons precluded conclusions about potential impacts of inhibiting these cells on associative memory. While C21 did not impair discrimination of objects in females tested in OiP, further work is needed to replicate this finding in males.

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This page was the last, and surprisingly most difficult section to write for this thesis. Not because I don't know who to thank, but because that list would far exceed anything I could write. So, I will try to execute one of the many skills I have attempted to learn during my training: conciseness (for reference, my first "20-minute" talk given in a lab meeting was actually 60+ slides and lasted an hour).

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As I conclude this chapter of my life, and this section, I can't help but to cap it all off with John's imaginary voice enthusiastically exclaiming, "onwards and upwards!!!".

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## LIST OF ABBREVIATIONS

AAV	Adeno-Associated Virus
ACh	Acetylcholine
AMPA	$\alpha$ -Amino-3-Hydroxy-5-Methyl-4-Isoxazolepropionic Acid
ANOVA	Analysis of Variance
BBB	Blood Brain Barrier
BLA	Basolateral Amygdala
C21	Compound-21
CA1	Cornu ammonis 1
CaMKII	Calcium/Calmodulin-Dependent Protein Kinase II
CAV2	Canine Adenovirus Type 2
CNO	Clozapine-N-Oxide
CNQX	6-Cyano-7-Nitroquinoxaline-2,3-Dione
CSF	Cerebrospinal Fluid
DIO	Double-Floxed Inverted Open reading frame
DMNS	Delay Non-Match to Sample
DNA	Deoxyribonucleic Acid
DR	Discrimination Ratio
DREADD	Designer Receptors Exclusively Activated by Designer Drugs
EC50	Half-Maximal Concentration
GABA	$\gamma$ -Aminobutyric Acid
GFP	Green Fluorescent Protein
GIRK	G Protein-Coupled Inwardly Rectifying Potassium Channel

GPCR	G Protein-Coupled Receptor
hM3Dq	Excitatory DREADD Receptor
hM4Di	Inhibitory DREADD Receptor
hSyn	Human Synapsin 1
Ki	Inhibitory Constant
KORD	DREADD based on kappa-opioid receptor
LC	Locus Coeruleus
mPFC	Medial Prefrontal Cortex
NMDA	<i>N</i> -Methyl- <i>D</i> -Aspartate
NOR	Novel Object Recognition
OiP	Object in Place
OST	Odour Span Task
P-gp	P-Glycoprotein
PLC	Phospholipase C
PRhC	Perirhinal Cortex
PV	Parvalbumin
RM	Recognition Memory
TH	Tyrosine Hydroxylase
WM	Working Memory
5-HT	5-Hydroxytryptamine

## 1. INTRODUCTION

This introduction will be broken down into 3 main sections and presented in the same logical order of ideas which ultimately lead to the experiments conducted for this project. I will begin by giving an overview of how behavioral pharmacology has been used to study the brain circuitry in rats involved in two different spontaneous task paradigms (Dere et al., 2007). Second, I will briefly discuss certain limitations with the traditional pharmacological lesion technique and use this as the foundation for a general discussion of Designer Receptors Exclusively Activated by Designer Drugs, or “DREADDs”. Neuroscience has seen an exponential increase in the use of DREADDs over the last decade, and as such the crux of my project seeks to elaborate on the technique and establish its utility with two spontaneous behavioural tests. I will discuss DREADDs in more detail later, however the general premise involves expressing a synthetic receptor *in vivo* that can only be activated by an otherwise ‘physiologically inert’ agonist in order to modulate cellular G-protein signalling (Armbruster et al., 2007). There are, however, numerous reports that DREADD agonists are not inert (Bonaventura et al., 2019; Gomez et al., 2017; Goutaudier et al., 2020; Jendryka et al., 2019). Clozapine-*N*-oxide, or “CNO”, is the prototypical DREADD agonist and has been more extensively characterized than newer, second-generation agonists such as Compound-21 (C21). My project seeks to expand on a limited literature base regarding off-target behavioral effects of C21. It is critical to assess these agonists prior to their use for DREADD manipulations to establish whether they cause any unspecific effects that may not be attributed to activation of the DREADD alone. C21 has some demonstrable advantages of CNO, however its behavioral properties still need to be validated. My experimental results will be used to bring together everything discussed in the introduction,

and my discussion will make a case for the relevance of my project in the expanding DREADD literature.

### **1.1 Spontaneous Recognition Tasks for Evaluating Recognition Memory in Rodents**

Behavioral tests of learning and memory can be broadly divided into two categories: those that use trained rules and some form of reinforcement/punishment to encourage behavior, and those which behavior is solely reliant on innate motivation. It is common to use a rewarding stimulus within behavioural paradigms as they are an effective motivator for training rodents to perform a given task, however these paradigms often require long, laborious training sessions that span months. For example, the odour span (pun intended) task (OST) is a type of delayed nonmatching to sample (DNMS) task in which rodents must select a novel odour from an increasing number of familiar odours over time to attain a food reward (Dudchenko et al., 2000). However, shaping and training rats to perform this task typically takes approximately 1-2 months before they can perform ‘test’ trials, not including any surgical procedures which add another 1-2 weeks before animals can be used for behavioral testing (Dudchenko et al., 2000). While the OST is considered a test of working memory (WM) capacity, recognition memory (RM) is also required for rats to identify whether a given stimulus is novel or has been previously encountered (Otto & Eichenbaum, 1992). Most, if not all mammals reliably display a preference for novelty, thus this innate behavior has been exploited in what are termed ‘spontaneous recognition tests’. In broad terms, spontaneous tests are used to evaluate RM, which falls under the branch of declarative or episodic memory (Squire et al., 2007). Intact RM is inferred when an animal spends more time interacting with ‘novel’, versus ‘familiar’ stimuli. While there are numerous versions of these spontaneous tests, the general testing scheme involves 1) a sample phase which animals can investigate and encode modality-specific characteristics of a given stimulus in

memory, 2) a delay phase in which animals are required to retain the memory, and 3) a test phase in which some aspect(s) of the presented stimuli are changed in a novel way. The specific *type* of memory being utilized may differ between tests, and thus care must be taken to ensure the validity of test parameters in testing one form over another. Various test variables are amenable to manipulation such as the number and modality presented (olfactory, visual, textile, spatial) of the stimuli, the length of the delay period, the orientation or position of the stimuli, or whether a novel stimulus is introduced to the subject during the test phase.

One of the simplest and most common tests of RM is the novel object recognition (NOR) test (for a comprehensive review of the NOR task see Antunes & Biala, 2012). In general, the test begins with a sample phase where rodents are allowed to explore 2 identical objects (A, A). The rodent is subsequently removed from the test arena for a defined delay phase that typically spans anywhere from minutes or hours (Barker et al., 2007; Barker & Warburton, 2011, 2013; Cross et al., 2013; Pinizzotto et al., 2022; Reger et al., 2009) up to as long as 1-2 days (Barker & Warburton, 2011; Reger et al., 2009). In the ensuing test phase, the rodent is reintroduced to the test arena where they then explore one copy of the original, familiar object and another novel object (A, B). In this example, rodents typically spend more time interacting with object B than A. Thus, the NOR paradigm can be thought of as a simple test of visual/tactile recognition memory.

Many studies have used physical or pharmacological lesioning to study the neuronal circuitry involved in this test, especially in rat. Deficits in RM of humans are observed in certain pathological conditions such as schizophrenia (Calkins et al., 2005; Danion et al., 1999), therefore rat models are often used to elucidate the neuronal substrates of this form of memory as a model of the human condition. To this end, the studies I highlight here primarily focus on rat

unless stated otherwise. Lesioning the perirhinal cortex (PRhC) alone *prior* to NOR appears to completely abolish recognition of a novel versus a familiar object in the standard paradigm already discussed (Albasser et al., 2011; Barker et al., 2007; Barker & Warburton, 2011; Ennaceur & Aggleton, 1997b; Olarte-Sánchez et al., 2015). While the mPFC is implicated in spatial WM tasks (Ennaceur & Aggleton, 1997a; Hannesson et al., 2004; Kesner & Holbrook, 1987; Yang et al., 2014), it is not required for strict RM using either object (Barker et al., 2007; Barker & Warburton, 2011; Ennaceur & Aggleton, 1997a), or spatial (Hannesson et al., 2004) tests. In contrast, studies regarding the role of the hippocampus are largely conflicting. In some instances, hippocampal lesions prior to NOR testing impaired RM (Broadbent et al., 2010; Clark et al., 2000; Gaskin et al., 2003; Stanley et al., 2012), while in others the manipulation did not cause any impairments (Barker & Warburton, 2011, 2013). Interestingly, another group argued that infusion of the muscimol, a GABA<sub>A</sub> agonist, between the sample and test phases enhances RM (Oliveira et al., 2010). The apparent discrepancies may be partially explained by the nature in which “NOR” was modified in studies of hippocampal involvement: 1) an alternative sampling format was used wherein animals were allowed to explore the initial objects over multiple days, 2) lesions of the hippocampus did not occur until after the conclusion of the sample phase(s), and 3) the actual test phase, while similar to canonical NOR paradigms was conducted several weeks or months after the sample phase (Broadbent et al., 2010; Gaskin et al., 2003; Stanley et al., 2012). It is therefore arguable that in those contexts, the hippocampus is clearly involved in the *retrieval* of a long-term memory of a previously investigated object, but perhaps not acute recognition which seems to rely more on the PRhC. Thus, as stated previously we must take care in explicitly identifying the specific forms of memory being recapitulated in these paradigms.

Another commonly used test for RM is the ‘Object-in-Place’ (OiP) test (Dix & Aggleton, 1999). This test uses a similar progression as NOR by still relying on the same order of sample, delay, and single test phase. Briefly, in the sample phase a subject can explore 4 unique objects positioned in the corners of a testing box. During the test phase, two of the objects will have swapped positions but no new objects are introduced to the arena. We consider the two displaced objects to be ‘novel’, while the stationary objects are ‘familiar’; animals will preferentially explore the displaced (novel) objects more than the stationary ones. Like NOR, the delay phase is subject to variation which can alter the difficulty of successful memory retention. Given that this test is a slight departure from strict visual RM as it also includes a spatial component, one would expect that additional circuits would be involved. In line with the structures discussed in NOR, studies have shown that OiP requires the PRhC, mPFC, and hippocampus, as well as their connections (Barker et al., 2007; Barker & Warburton, 2008, 2011, 2013).

## **1.2 Overview of DREADDs and Use for Behavioral Studies**

While studies employing pharmacological lesion have provided the basis of our understanding of the neural circuitry responsible for behavior, certain questions remain. First, a “lesion” in this context may refer to a number of manipulations. For example, the use of NMDA to cause excitotoxicity is a permanent abolishment of neuronal activity (due to neuronal cytotoxicity), whereas lidocaine as a general sodium channel blocker can temporarily block essentially all neuronal activity in an area. Other drugs that selectively block certain receptors are used to more closely investigate the types of signalling, rather than just the general cortical areas responsible for behavior. For example, successful discrimination in OiP depends specifically on AMPA receptor signalling between the PRhC and mPFC (Barker & Warburton, 2008). Secondly, while receptor selective drugs are incredibly useful for differentiating between signalling

pathways in certain brain circuits, single receptors are not necessarily reliable markers for specific neuronal subtypes. AMPA receptors are found in various cell types, including both glutamatergic and GABAergic populations (Isaac et al., 2007), thus AMPA receptor antagonists such as CNQX cannot necessarily be used to interrogate ‘excitatory’ or ‘inhibitory’ cells separately. To attain more cellular-specific pharmacological targeting, advances in genetics combined with advances in pharmacology have opened a new field of research known as ‘chemogenetics’. This field brought forth the idea that one can use the unique genetic (and more specifically, the transcriptomic) background of specific cell types to selectively target them with an engineered receptor and appropriate drug to activate that receptor (Alexander et al., 2009; Armbruster et al., 2007; Roth, 2016; Urban & Roth, 2015).

DREADDs were borne out of the desire to modulate G protein-coupled receptor (GPCR) signalling in only a desired cell-type. Modulating cell activity in this way should theoretically allow interrogation of specific cells populations, rather than potentially diverse cell types that all express a given receptor. The initial DREADD was developed by mutating the human muscarinic acetylcholine subtype-3 (hM<sub>3</sub>) receptor so that it displayed minimal levels of endogenous activity by losing affinity for acetylcholine (ACh), and gaining affinity for clozapine-*N*-oxide (CNO), a purported physiologically “inert” compound (Armbruster et al., 2007). This hM<sub>3</sub> DREADD was found to couple with an excitatory Gq protein subunit (thus the receptor was named “hM<sub>3</sub>Dq”) upon binding with CNO, which subsequently led to the generation of phosphorylated extracellular signal-regulated kinase 1 & 2 which also occurs following activation of wild type hM<sub>3</sub> receptors by ACh (Armbruster et al., 2007). The original hM<sub>3</sub>Dq was also found to activate the phospholipase-C (PLC) pathway, as indicated by increased levels of inositol monophosphate in response to CNO (Armbruster et al., 2007). As a result of

activating the PLC pathway, one would suspect that in neurons specifically, this causes a calcium influx which should aid in depolarization. Indeed, CNO causes depolarization in mouse CA1 neurons in the presence of hM<sub>3</sub>Dq expression compared to non- hM<sub>3</sub>Dq controls (Alexander et al., 2009). In contrast, hM<sub>4</sub>Di (which was originally derived from hM<sub>4</sub> receptors) was shown to silence hippocampal neuronal firing by reducing inward current through G protein-coupled inwardly-rectifying potassium, or “GIRK” channels in response to CNO (Armbruster et al., 2007). Since then, various DREADD’s have been generated from the muscarinic receptor family which are coupled to various G-proteins (Gq, Gi, Gs,  $\beta$ -Arrestin), (Urban & Roth, 2015 for a review). Depending on how a researcher wants to manipulate neuronal activity, one simply needs to choose a DREADD which couples to the G-protein that will induce the desired signalling effect. It should be noted that there are now non-muscarinic synthetic receptors such as the kappa-opioid based DREADDs (Aldrin-Kirk et al., 2016), but here will primarily focus on the former as they are much more prevalent in the literature.

Viral vectors, and primarily adeno-associated viruses (AAVs) are the delivery system of choice for obtaining DREADD expression in cells (Urban & Roth, 2015). Inside the viral particle is a piece of DNA that encodes the DREADD receptor. The viral vector is generally delivered to neural tissue via micro infusion (Alexander et al., 2009; Roth, 2016), and expression can be observed throughout not only the soma, but also the projections emanating from the site of infusion (Kim et al., 2017). *In general*, DREADD vectors include 1) a promotor sequence that drives transgene expression targeted towards desired cell/tissue-type(s), 2) the sequence encoding the DREADD itself, and 3) a reporter sequence, usually encoding a fluorescent protein. In addition, some vectors are used with the Cre-loxP system (Kim et al., 2017; Roth, 2016; Urban & Roth, 2015). Cre-dependent vectors have the DREADD sequence inverted and flanked

by loxP sites on either side and often have the abbreviation “DIO”, which stands for “double-floxed inverted open reading frame” listed between the promoter and protein coding sequence. When Cre-dependent vectors are expressed in modified animals that contain the enzyme Cre recombinase in a particular cell population of interest, Cre recombinase binds the loxP sites, excises them and inverts the sequence *back to its original, translatable orientation*. In this way, transgenic rat and mouse lines expressing Cre recombinase in specific cells or tissues can be used in conjunction with Cre-dependent vectors to attain cellular specificity. Alternatively, a second viral construct which itself encodes for the Cre recombinase enzyme can be targeted to certain cells via a cell-specific promoter, along with a Cre-dependent vector that is controlled under a constitutive or ubiquitous promoter. For example, by either using Parvalbumin (PV)-Cre rats and a DIO DREADD construct (Binette et al., 2023), or using a single viral DREADD construct with the PV promoter sequence in typical rats (Chamberlin et al., 2023), DREADD expression will occur in PV+ cells. The human synapsin 1 (hSyn) promoter is a ubiquitous promoter sequence used in many DREADD constructs; therefore, expression is primarily limited by the volume distribution of virus during surgical infusion. In this way, the cells being targeted are not necessarily different than pharmacological lesion. However, hSyn is often the promoter of choice in Cre-dependent vectors (see Table 1 for example references) since the level of specificity in this case is determined by the localization of Cre recombinase and not the DREADD promoter. Other viruses besides AAVs have been used, such as the Canine adenovirus containing retrogradely-transported Cre (CAV2) which can be infused in one brain region, while simultaneously injecting a second, Cre-dependent DREADD vector (such as pAAV-hSyn-DIO-HM4Di-mCherry) into a connected brain region to achieve expression specifically between the

two regions (O’Neal 2019). Nonetheless, by and large, adeno-associated viruses (AAVs) are the most prominent viral carrier used for transfecting mammalian cells.

In-depth explanations of DREADD strategies can be found elsewhere (Chao et al., 2022; Roth, 2016); here I highlight that there are many ways to achieve pharmacological specificity using DREADDs in rats. Table 1 (see supplemental material) highlights some representative examples of the DREADD systems used in rats to transfect neurons. There are some differences noted in the rate and efficiency with which some AAV serotypes transfect cells. For example, neurons of the red nucleus in rat are more rapidly transfected by serotypes 1 and 6 by 1 week, whereas serotypes 5 and 8 allowed for much larger expression levels by 1 month (Blits et al., 2010). Indeed, among a select sample of behavioral DREADD studies in rat, serotypes 2, 5, and 8 have all been frequently used and as such are all likely sufficient for most behavioral studies. In addition to, and perhaps more critical than the chosen serotype of the AAV is the actual viral transgene itself. Muscarinic based DREADDs are the most frequently used receptor type. The newer kappa-opioid receptor-based DREADD (KORD) has been used in rat too (Aldrin-Kirk et al., 2016; Marchant et al., 2016; Vardy et al., 2015), although not as commonly.

DREADDs are being used as a technique for further investigation of the circuitry behind RM, albeit there are few studies currently published. For example, DREADD excitation of the PRhC using a DREADD vector with the ubiquitous hSyn promotor reverses methamphetamine-induced RM deficits in NOR, reaffirming the importance of this cortical region in novelty recognition (Peters et al., 2018). Interestingly, DREADD inhibition targeted to all hSyn<sup>+</sup> cells of the mPFC after an acute stressor impairs RM assessed by NOR (Jeon et al., 2022), in contrast to previously discussed evidence the mPFC is not involved in NOR under no stress conditions (Barker et al., 2007; Barker & Warburton, 2011; Ennaceur & Aggleton, 1997a). Given the

ubiquitous promotor sequences used in these studies, the DREADD-dependent effects would likely be similar to those observed following the infusion of a drug such as lidocaine into the same area. While lidocaine is a sodium channel blocker and should cause an almost complete reduction in action potential firing at sufficient doses, Gi DREADDs simply *modulate* neuronal firing by hyperpolarizing the cell. In other studies with more specific DREADD promoters, a CaMKII driven Gi DREADD was used to show that inactivation of excitatory ventral hippocampus projections rescued high fat-diet induced deficits in RM as assessed by NOR (Naneix et al., 2021). Behavioural studies employing traditional pharmacology and DREADDs have also been done in rat, such as using DREADDs that target orexin<sup>+</sup> cells of the of the hypothalamus, alongside orexin receptors antagonists (Eacret et al., 2019; Grafe et al., 2017). At the circuit level, a recent study using the NOR paradigm combined the use of an inhibitory DREADD in tyrosine hydroxylase (TH<sup>+</sup>) cells of the locus coeruleus (LC), with infusion of CNO into the basolateral amygdala (BLA) to specifically target the LC→BLA projections (Llorca-Torralba et al., 2019). Expression of excitatory DREADD in mPFC PV<sup>+</sup> interneurons using PV Cre rats and DIO constructs have been used to show that increases in PV<sup>+</sup> GABAergic interneurons firing following CNO administration impairs performance in the temporal order memory task (Armenta-Resendiz et al., 2022). Since the mPFC is also required for OiP (Barker et al., 2007), it is possible that PV-interneurons are important for associative memory too. Interestingly, it has been demonstrated that pathological reduction in PV<sup>+</sup> interneurons of the hippocampus and mPFC impair OiP performance in rat (Reichelt et al., 2015), indirectly confirming that these regions are neural substrates for the task (Barker et al., 2007; Barker & Warburton, 2011, 2013) and suggesting that these interneurons play a role.

### 1.3 DREADD Agonists

In this section, I will discuss and compare the pharmacological characteristics of DREADD agonists CNO, and C21. DREADDs were initially designed to lose affinity for endogenous ACh and gain affinity for the reportedly ‘physiologically inert’ CNO (Armbruster et al., 2007). However, a growing literature demonstrates off-target effects of almost all DREADD agonists, which brings their validity for certain paradigms into question. Other considerations that are particularly relevant to CNO include metabolism into other pharmacologically active compounds. Understanding how these agonists affect mammalian systems alone is critical, especially given the large number of DREADD studies employing CNO (see Table 1). I will now discuss the pharmacokinetics and pharmacodynamics of DREADD agonists beginning with CNO, with a stronger emphasis on endogenous effects. We will then compare those findings with similar data on newer agonists like C21. While C21 is a promising alternative to CNO, there is still a need for studies investigating its non-specific effects. I will also highlight studies that support the use of proper controls, such as agonist-only controls or even agonist-GFP vector controls. As has been emphasized elsewhere (Lawson et al., 2023), proper controls should always be required irrespective of the choice of DREADD agonist.

#### *Pharmacokinetics of CNO and Clozapine*

The original DREADD developed by Armbruster et al., (2007) was created and selected based around its ability to specifically bind CNO, which they purported was a “pharmacologically inert drug-like and bioavailable compound”. Interestingly, studies that preceded this study suggest that CNO has off-target effects, at least under some conditions. CNO is the major metabolic by-product of the atypical antipsychotic, clozapine, and as such has been the focus of biochemical characterization studies that date back to the 1990s in rat (Lin et al.,

1996), mice (Bender et al., 1994) and humans (Avenoso et al., 1998; W. H. Chang et al., 1998; Jann et al., 1994; Lin et al., 1994). Among these reports, it was initially noted that CNO and clozapine appeared to undergo interconversion reactions in both humans and guinea pigs (Jann et al., 1994), with additional confirmation of this in humans (W. H. Chang et al., 1998). A more recent report showed that this interconversion is also present in mice and rats following treatment with 10mg/kg CNO (Manvich et al., 2018) or 3.5mg/kg in mice (MacLaren et al., 2016). Notably, 1mg/kg CNO did not yield detectable plasma levels of clozapine (Manvich et al., 2018). It has been established with other antipsychotics such as chlorpromazine that N-oxide metabolites are readily converted back to their parent compound in rat (Jaworski et al., 1988) and even locusts (Hellman et al., 2016), lending more credibility to findings of interconversion of clozapine and CNO. In mice, systemic administration of clozapine and CNO both resulted in maximal brain penetration at  $\leq 7$  minutes, however CNO levels only peaked at half that of clozapine and rapidly dropped as early as 10 minutes (Bender et al., 1994). Further mouse studies demonstrated that while administration 3.5mg/kg of CNO yields high plasma levels for up to one-hour, cerebrospinal fluid (CSF) levels dropped below half of the half-maximal concentration (EC50) of hM4Di, and cortical levels were undetectable by this point (Jendryka et al., 2019). Importantly, Jendryka et al. (2019) also confirmed that CNO administration yielded low plasma, undetectable CSF, yet exceptionally high cortical levels of clozapine. These findings of low cortical CNO, and high clozapine levels after CNO administration were also demonstrated in mice elsewhere (Thompson et al., 2018). Furthermore, there is additional evidence showing that CNO is a substrate for the permeability glycoprotein (P-gp) transporter *in vitro* (Gomez et al., 2017; Raper et al., 2017), where clozapine is not (Bonaventura et al., 2019; Gomez et al., 2017). P-gp is an important efflux pump used to remove foreign substances from the across the

blood-brain barrier (BBB) from brain tissue back to the circulation (Wessler et al., 2013).

Collectively, these data show that CNO rather poorly crosses into the brain, and is converted into clozapine which may exert off-target effects on both endogenous receptors and other proteins in the brain.

### *Receptor Binding Profiles of Clozapine, CNO, and N-desmethylozapine*

Since CNO is converted to both clozapine and *N*-desmethylozapine, it is important to consider the bioactivity of all compounds. Early reports in rat showed that clozapine and *N*-desmethylozapine (the second major metabolite of clozapine), but not CNO, strongly antagonize serotonin type 1C (5-HT<sub>1c</sub>) and 5-HT<sub>2</sub> and weakly antagonize dopamine type 1 (D1) and dopamine type 2 (D2) receptors (Kuoppamäki et al., 1993). However, more recent evidence suggests that CNO causes >50% inhibition of binding of test ligand at 5-HT<sub>1A</sub>, 5-HT<sub>1B</sub>, 5-HT<sub>2A</sub>, 5-HT<sub>2B</sub>, D<sub>2</sub>, and D<sub>2S</sub> receptors (Jendryka et al., 2019). Gomez et al., (2017) reported that CNO antagonizes histamine type 1 (H1), 5-HT<sub>2A</sub>, M1, M4, D1, and D2 receptors *in vitro*. Another report also showed that clozapine potently inhibits various 5-HT<sub>2</sub> receptors (Bymaster et al., 1997). Later, it was demonstrated in rat prefrontal cortex that CNO caused appreciable antagonism at D<sub>2</sub>, D<sub>3</sub>, and 5-HT<sub>2A</sub> receptors following systemically administered doses of 2mg/kg, but not at 0.5mg/kg, 5mg/kg or 8mg/kg (Bærentzen et al., 2019). Interestingly, CNO at 5mg/kg in rat attenuates amphetamine-induced increases in dopamine signalling, whereas CNO alone has no effect (MacLaren et al., 2016). It was also demonstrated that CNO and *N*-desmethylozapine lack appreciable antagonism compared to clozapine at H<sub>3</sub> receptors in rat (Alves-Rodrigues et al., 1996), although CNO may exhibit significant antagonism at H<sub>1</sub> receptors in mice (Jendryka et al., 2019). Clozapine and *N*-desmethylozapine, but not CNO also weakly antagonize GABA<sub>A</sub> receptors in rat hippocampal, cortical, and cerebellar tissue

(Schlicker & Marr, 1996; Wong et al., 1996). CNO antagonizes adrenergic (Jendryka et al., 2019) and muscarinic receptors (Jendryka et al., 2019; Thompson et al., 2018). Similar receptor binding profiles of clozapine and CNO led to further comparisons of these compounds at DREADDs themselves. Clozapine is demonstrably more potent than CNO *in vitro* at hM<sub>4</sub>Di (Gomez et al., 2017; Jendryka et al., 2019) and hM<sub>3</sub>Dq DREADDs (Gomez et al., 2017). Clozapine, not CNO, has also been shown to bind to AAV-DREADDs in mice using autoradiography (Gomez et al., 2017). Taken together, the pharmacokinetic and pharmacodynamic profiles suggest that CNO is readily transformed in the more bioactive clozapine, and both can activate both endogenous receptors and DREADDs. It is also likely that CNO-derived clozapine is at least partly responsible for DREADD activation in the brain, as opposed to CNO itself (Bonaventura et al., 2019). Thus, the lack of specificity of CNO *in vitro* warrants investigation of other DREADD agonists that may cause fewer unspecific effects.

### *Behavioral and Neurochemical Effects of CNO Administration*

CNO activity unspecific to DREADD activation represents a potential confound in behavioural experiments, which is especially concerning given that many DREADD experiments are conducted using CNO (Table 1). However, it has been noted elsewhere that the actual number of studies explicitly demonstrating unspecific effects of CNO is limited (Rodd et al., 2022). To supplement our discussion, both findings from studies strictly on the endogenous effects of CNO, and control data from DREADD studies on rodent behavior will be included here. We will explore the claims and cited literature of studies employing CNO to further answer questions about notable off-target effects. Currently, there is conflicting evidence on whether CNO alters locomotion in mice and rats. Some studies have shown that locomotion is unaffected by doses of up to 10mg/kg CNO in both non-DREADD mice (Gomez et al., 2017; Jendryka et

al., 2019; Kljakic et al., 2022) and non-DREADD rats (Gomez et al., 2017; Nentwig et al., 2022). Clozapine (0.1mg/kg and 1mg/kg significantly reduces locomotion in hM4Di expressing rats and mice while sparing control mice, however control rats display reduced locomotion at 1mg/kg (Gomez et al., 2017) highlighting potential differences between rodent species. While one study reported that 5mg/kg CNO did not alter cocaine-induced locomotion in non-DREADD rats (Wunsch et al., 2017), a different group showed that locomotion is reduced by 5mg/kg CNO if the animals have a prior history of cocaine-administration (Padovan-Hernandez & Knackstedt, 2018). Further investigations in mice revealed that even chronic CNO administration does not alter locomotion but might induce a mild anxiolytic-like phenotype (Tran et al., 2020). In rat, 1mg/kg CNO attenuates acoustic startle but not pre-pulse inhibition, whereas amphetamine-induced hyperlocomotion is only reduced at doses of 5mg/kg (MacLaren et al., 2016). In rats, CNO up to 5mg/kg, and 10mg/kg also do not affect cocaine-reinstatement (Mahler et al., 2019) and heroin-reinstatement (O'Neal et al., 2020) behaviours, respectively. CNO also alters the sleep quality and patterns in mice at doses of 5mg/kg (Traut et al., 2023).

Data related to the neurochemical effects of CNO is rather sparse. At 5mg/kg, CNO significantly blunts amphetamine-induced increases in neuronal dopamine release in the rat nucleus accumbens and locomotion (MacLaren et al., 2016). Similarly, dopamine and glutamate levels are greatly increased in rat mPFC at doses of 0.5mg/kg and 1mg/kg, respectively (Rodd et al., 2022). However, *in vitro* application of CNO to rat dorsal striatum did not induce increases in glutamate, where CNO activation of Gq-DREADD in the nucleus accumbens did (Scofield et al., 2015).

## *Importance of CNO-only, or CNO/Reporter Vector Control Groups for Behavioral Studies*

A significant portion of behavioral studies using DREADDs have included animals expressing a control vector that receive CNO injection showing that CNO alone does not affect the behaviours being investigated (Bull et al., 2014; Casado-Sainz et al., 2022; Chamberlin et al., 2023; S. E. Chang et al., 2015; Haaranen, Schäfer, et al., 2020; Haaranen, Scuppa, et al., 2020; Jeon et al., 2022; Kostin et al., 2022; Maestas-Olguin et al., 2021; Mahler et al., 2019; Marciante et al., 2019, 2020; Naneix et al., 2021; Nentwig et al., 2022; Schmidt et al., 2019; Tomek et al., 2020). Some other studies have employed non-transfected, CNO-only controls (Amer & Martin, 2022; Casado-Sainz et al., 2022; Chamberlin et al., 2023; S. E. Chang et al., 2015; Eacret et al., 2019; Lawson et al., 2023; Naneix et al., 2021; Nentwig et al., 2022; Schmidt et al., 2019). However, there are also studies that only employed combined DREADD expressing, vehicle injected controls (Binette et al., 2023; Panoz-Brown et al., 2018; Peters et al., 2018; Rorabaugh et al., 2017; Schmidt & Redish, 2021; Sharma et al., 2020). Panoz-Brown et al, (2018) used additional behavioral assays to argue that CNO likely did not mediate unspecific effects, but arguably both the viral transfection and CNO injection represent confounds. A recent report showed CNO significantly improved RM in NOR in non-DREADD rats fed a high fat diet relative to vehicle injected rats, which was improved even more in rats expressing hM<sub>4</sub>Di in CaMKIIa<sup>+</sup> cells of the ventral hippocampus (Naneix et al., 2021). This finding directly challenges the results of Peters et al, (2018) who showed CNO activation of hM<sub>3</sub>Dq in rat PRhC improves RM in NOR without CNO only, or CNO/control vector rats. Taken together, the risk of off-target effects of CNO has led many groups to search for additional agonists with better specificity.

### *C21: Is it Really a Better DREADD Agonist?*

To address the potential drawbacks of CNO, second-generation agonists were generated to similarly activate muscarinic-based DREADDs with more favorable pharmacokinetics and fewer unspecific effects, the most common of which is C21 (Chen et al., 2015). Here, I will carefully dissect the few large-scale studies which characterize C21 and facilitate a comparison with CNO. C21 is a more potent full agonist at hM3Dq with less agonism at 5-HT<sub>2A</sub>, 5-HT<sub>2C</sub>,  $\alpha_{1A}$  and H<sub>1</sub> receptors than clozapine *in vitro* (Chen et al., 2015). DREADD agonism of C21 at hM3Dq and hM4Di was later confirmed in an *in vivo* mouse model (Thompson et al., 2018). Like CNO, weak agonistic activity of C21 has been noted at hM3 (Chen et al., 2015), as well as hM1 and hM4 (Thompson et al., 2018) which is not entirely surprising as it is targeted for muscarinic DREADDs. In both rats and mice, hM3Dq was activated by C21 at 0.1mg/kg, whereas hM4Di required 1mg/kg (Bonaventura et al., 2019). Measurements of the *in vitro* potency of C21 at DREADDs have been somewhat inconsistent across studies. Interestingly, studies which argued that C21 was a “potent” DREADD actuator used EC<sub>50</sub> as their metric (Chen et al., 2015; Thompson et al., 2018), whereas studies that largely argued “weaker” potency had measured the inhibitory constant, “K<sub>i</sub>” from C21’s ability to displace tritiated compounds from DREADDs (Bonaventura et al., 2019; Nagai et al., 2020). Furthermore, unlike CNO C21 is not a P-gp substrate (Bonaventura et al., 2019) and is not back metabolized to clozapine (Thompson et al., 2018). A comparative study in mice employing either chronic CNO and C21 injections of 1mg/kg each over 16 weeks found that neither agonist caused any obvious behavioural effects on locomotion or anxiety-like behaviours in mice (Tran et al., 2020). A different study showed that at 1mg/kg, acute C21 does not alter locomotion in non-DREADD mice but can still mediate DREADD-specific changes in behavior in both mice and rats

(Bonaventura et al., 2019). One study raised concern over the use of C21 after showing it had differential sex-effects both specific and non-specific to DREADD activation (Goutaudier et al., 2020). These effects are especially important to consider when interpreting data collected in rats with hM4Di and C21 systems that lack C21-only controls (Grigsby et al., 2020). Data on whether C21 impacts sleep in mice is also conflicting (Ferrari et al., 2022; Traut et al., 2023). Like with CNO, many studies incorporate C21-alone or fluorophore vector + C21 controls (Botterill et al., 2021; Dean et al., 2022; Du et al., 2022; Miranda et al., 2022), although some studies have not (Roselli et al., 2020). Despite concerns of the non-specific effects of C21 (Goutaudier et al., 2020), proper controls still permit its use in some behavioural studies (Goutaudier et al., 2023).

### *Experimental Objectives and Hypotheses*

To the best of my knowledge, C21 has not been validated for use in spontaneous tasks such as NOR and OiP, and a behavioral phenotype after C21 administration in rat is largely lacking. Thus, my first objective was to determine whether C21 impaired RM in rats assessed with NOR at 0.5mg/kg (Goutaudier et al., 2020) and 1mg/kg (Bonaventura et al., 2019). I then investigated whether C21 would impair associative memory, as measured by the OiP test.

Lastly, I investigated whether DREADD inhibition of PV+ interneurons of the rat mPFC would impair associative memory in OiP. Previous studies have highlighted a relationship between a reduction in PV+ interneurons of the hippocampus and mPFC and impaired OiP performance (Reichelt et al., 2015). Thus, I hypothesized that inhibiting PV+ interneurons would impair associative memory in the OiP test.

## 2. METHODS

### *Animals*

Two separate cohorts of adult male and female Long Evans (LE) rats were used for testing the effects of C21 on RM in NOR (n=11 males and n=12 females) and OiP (n=8 males and n=8 females) (Charles River Laboratories, Kingston, NY, USA). An additional cohort of LE-transgenic (Tg) (Pvalb-iCre)<sup>2</sup>Otc (PV-Cre) rats were used for a DREADD manipulation in OiP (purchased from Rat Resource & Research Center, Missouri US). Long Evans rats were either double or triple housed for the C21 experiments, whereas the PV-Cre rats were either singly or doubly housed. The PV-Cre rats had previously been trained on OST, and were subsequently used in the present experiment with OiP. All rats were allowed to acclimatize undisturbed to our facilities for 2 weeks upon their arrival. All rats had water and food *ad libitum* except during testing, however the PV Cre rats had previously undergone food restriction during OST training and testing which ended ~1 month prior to OiP testing. Ventilated plastic home cages in a temperature- and humidity-controlled vivarium were used to house the rats, with a 12:12-h lighting cycle (lights on at 0700). Environmental enrichment was provided in the form of a plastic tube in each home cage. All experiments were conducted in accordance with the standards of the Canadian Council on Animal Care and the University of Saskatchewan Animal Research Ethics Board.

### *Experimental Design*

NOR testing took place between December 2022 and January of 2023. Rats for NOR were tested as 2 separate cohorts, with squad one consisting of n=6 females and n=5 males, and squad 2 consisting of n=6 females and n=6 males. NOR squad one was handled by two experimenters. The rats were either injected by just one of the two handlers, or a third

experimenter who was not conducting behavioral testing. NOR squad 2 was handled by a lone experimenter, and injected by a separate person who handled the rats to familiarize them with the injection procedures. C21/OiP testing took place between March 2023 and April 2023. Rats used to assess the effects of C21 on OiP were all handled and injected as a single squad by a lone experimenter. The PV-Cre rats for the DREADD OiP experiment were all handled and tested during April of 2023. Rats for testing the effects of C21 in either NOR or OiP received each of the 3 treatments in a pseudorandomized order across the 3 testing days, with the number of rats receiving a given treatment being counterbalanced against testing day, object set used, and which object in a set was assigned to be the familiar/novel stimulus. The PV-Cre rats received saline and 1mg/kg C21 in random order across 2 test days. Each rat was always tested at the same time of day, ranging from 9:00 AM to 5:00 PM.

#### *Testing Apparatus & Equipment*

For NOR, two identical boxes made of white corrugated plastic were cut to dimensions 60cm x 60cm x 60cm to serve as the testing arenas, however a given rat was only ever habituated and tested in the same testing arena. For OiP, two additional test boxes were used which were similar to the NOR boxes, except one of the walls was black and always oriented west. To hold the objects in place during testing, small pieces of female Velcro were stuck to the bottom of the box in the corners where the stimuli would be placed (2 adjacent corners for NOR boxes, all corners for OiP boxes). A Logitech camera was connected to a portable laptop computer and mounted to the ceiling above the testing box to record behavioral tests. Since the test boxes were frequently moved for cleaning and in between animals, a tape outline on the floor was used to ensure the box was always located in the same location relative to the camera. Logi Capture

recording software was used to record all test sessions. All videos were recorded at 30 frames per second with 1080 x 1080 resolution.

### *Objects*

Objects used were small household items such as small Lego shapes, ceramic garden decorations, and 3D-printed plastic shapes (**Figure 1**). The height of each object was approximately at eye-level with the rats. Each object had male Velcro attached to the bottom, which was used to secure it in place where the female Velcro was placed in the testing arena. For NOR, there were three object sets that were each comprised of 2 unique objects with 3 copies of each, so that a rat never saw the exact same copy in the test phase as in the sample phase. For OiP, there were three object sets that were each comprised of 4 objects with 2 copies of each. All objects were verified by our lab to yield satisfactory levels of animal exploration from a previous OiP experiment in our lab (data unpublished). Furthermore, object sets/pairs were chosen based on previous data comparing levels of exploration at particular objects used in various combinations.

### *Drug Preparation and Injections*

Water soluble C21 dihydrochloride was purchased from Hello Bio (Princeton, NJ, USA). C21 was dissolved in 0.9% saline at concentrations of 1mg C21/ mL saline, and 0.5mg C21/ mL saline before being frozen in -20 degrees Celsius 1-day prior to each test. All rats were weighed in the morning before the beginning of behavioural experiments to determine the required drug dosages. The drug solutions were thawed the morning of each test day and were always thoroughly vortexed prior to drawing up solution with a syringe for subsequent injections. C21 was administered via intraperitoneal injection at dosages of 0.5mg/kg or 1mg C21/ kg rat body

weight. All saline injections were administered via intraperitoneal injection as 1mL saline/ kg bodyweight. The specific time of treatment administration for each experiment *always* occurred 30 minutes prior to the test phase, however more details can be found under the testing procedures for each test. The experimenter injecting the rats was blinded to the treatment on that particular day.

### *Viral Vector Infusion Surgeries*

All surgeries were done at the University of Saskatchewan by an experienced graduate student (Dan McElroy) who did not conduct behavioural testing of the PV-Cre rats. Rats were anesthetized with a combination of isoflurane and subcutaneous 0.6mg/kg buprenorphine for surgery and mounted on stereotactic apparatus. During all procedures rats' vitals were monitored with a pulse oximeter placed on the hind paw, and rectal temperature probe. An incision in the scalp was then made to expose the dorsal surface of the skull. A hand-operated drill was used to drill two holes in the skull, one over the mPFC in each hemisphere (AP + 3.5-3.8 mm and ML +/- 0.5 mm *from bregma*, DV -3.5 mm from the *dorsal surface of the brain*). A Cre-dependent Inhibitory DREADD construct (pAAV-hSyn-DIO-hM<sub>4</sub>Di-mCherry) was packaged into serotype 5 AAV (Addgene: 44362-AAV5) for viral infusions. 0.65µL of vector per hemisphere was infused into mPFC using 1.0µL Hamilton Neuros 32-gauge syringes, which were left in place for 5 min to ensure that all vector was expelled from the needle. The surgeries took place February 15<sup>th</sup> and 16<sup>th</sup> of 2023, and the animals were allowed to rest until February 27<sup>th</sup> before being tested on a different experiment. For this study, the animals were tested in OiP on April 5<sup>th</sup> and 11<sup>th</sup>.

### *Handling and Habituations*

After the initial 2-week acclimatization period in the vivarium, rats were handled for three days by the same experimenter that would conduct behavioural testing. Next, rats underwent 3 days of habituation to both the testing box and testing room. For habituation days, each rat underwent the i.p. injection procedures except that an empty syringe with no needle was used, before being subsequently carted to the testing room and spending 10 min in the test box with no objects. Test day 1 occurred 24h after the 3<sup>rd</sup> habituation day. Rats would also undergo an identical habituation 24h prior to test days 2, and 3 to ensure that their familiarity with the testing conditions and procedures was maintained.

### *NOR Testing Procedures*

The first cohort of rats were used to assess behavioural effects of C21 in NOR. The same testing protocol was conducted on 3 separate test days separated by 1 week each, with each rat receiving a different given treatment each day. Males and females were tested in a quasi-alternating fashion to control for any potential sex by time-of-day effects. Furthermore, experimenters would switch lab coats before handling the other sex, and sex-specific transfer cages were used to transport animals to the injection table and testing room. Rats were first placed on a tower rack outside the testing room for 20 min. Next, rats were removed from their home cage and transported via transfer cage to a table in the same hallway for injection. After receiving an injection, animals were returned to their home cages on the tower rack for 20 minutes before being transported to the testing box to begin the sample phase. During the sample phase, rats could explore 2 identical copies of a particular object ( $A_1$ ,  $A_2$ ) for 5 minutes. Next, rats were removed from the box for a 5-minute delay phase. During this time, the test box was thoroughly cleaned with 70% ethanol to remove any scent markings. Additionally, the sampled

objects were replaced by one identical copy of the now ‘familiar’ stimulus, and one copy of a novel stimulus ( $A_3$  and  $B_1$ , respectively) to ensure that any attempt to scent mark the objects would not confound subsequent exploration. After the delay, the rats were returned to the test box for a 5-min test phase where they could explore the familiar and novel objects. All sample/test phases were recorded, and the time spent interacting with each object was subsequently hand scored by a researcher using a stopwatch.

### *OiP Testing Procedures*

A second cohort of rats were used to assess the effects of C21 in OiP. The same testing protocol was conducted on 3 separate test days separated by 1 week each, with each rat receiving a different given treatment each day. Males and females were tested in a quasi-alternating fashion to control for any potential time of day effects. Furthermore, the experimenter would switch lab coats before handling the other sex, and sex-specific transfer cages were used to transport animals to the injection table and testing room. Animals were first placed on a tower rack outside the testing room for 20 minutes. For the 1-hour delay OiP, animals would begin the experiment by going to the testing room for a 5-min sample phase where they could explore 4 unique objects in the arena placed in each corner. Next, rats would be returned to the tower rack for a 1h delay phase. After a 30 min delay, rats were carted to a table down the hall to receive their respective treatments, and then returned to the tower rack for the remaining half of the delay phase. For 5-min OiP, rats were injected 20 min prior to the sample phase. During the delay phase, the 4 objects in the testing box were all swapped for identical copies, except that 2 of the objects had exchanged positions. Importantly, there were only ever two “variations” in which objects could be swapped- If the rat was facing the black wall, then either the two objects on the “left” or the two on the “right” were switched. This was done to give the rat a consistent reference point with

which to remember the objects against. After the delay, the rat was placed in the testing arena for a 4-min test phase to explore the objects.

### *Scoring and Data Analysis*

All sample/test phases were recorded, and the time spent interacting with each object was subsequently hand scored by a researcher using a stopwatch. For the test phases in each experiment, the following formula: (time exploring novel – time exploring familiar)/ total test exploration, was used to calculate a discrimination ratio (DR) representing the proportion of time a rat spends with either object. A positive DR is indicative of a novelty preference, 0 no preference, and negative familiarity preference. Scores were taken at both 2 and 5 min of NOR testing and at 2 and 4 min for OiP to monitor any effects of C21 across time in the test phase. Statistical analysis and creation of graphs were conducted in GraphPad Prism version 9.4 (GraphPad Software, San Diego, USA). Critical p-value for all statistical tests was set to  $p < 0.05$  to determine statistical significance. For assessing the effects of C21 on non-DREADD rats in NOR, all data was analyzed by repeated measures two-way ANOVA. For assessing the effects of C21 on non-DREADD rats in OiP, data was first analyzed by factors Sex and Treatment using repeated measures two-way ANOVA. Next, factors Object Set and Sex for OiP DRs was analyzed by fitting a mixed-effects model to the data. For assessing the effects of DREADD inhibition of PV+ cells of the mPFC in OiP, paired t-tests were used to compare rat behaviours when injected with saline versus C21.

### *Histology*

All rats were euthanized with isoflurane and transcardially perfused with 1x PBS, followed by extraction of whole brains which were frozen prior to sectioning. Sections were

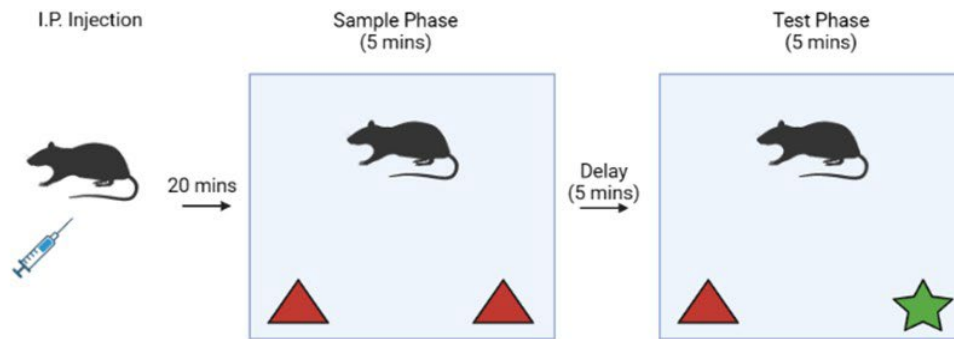
rinsed with PBS buffer and PBSx (PBS buffer + 0.25% (volume per volume) Triton X). Blocking was achieved using 5% normal goat serum in PBSx. Primary antibodies used were 1:1000 mouse anti-parvalbumin (#MAB1572) and 1:3000 rabbit anti-mCherry (#abcam 167453) to stain PV+ cells and cells expressing the DREADD mCherry reporter, respectively. Secondary antibodies used were Alexa 568 (Red, 1:500; Invitrogen #A11011) and Alexa 488 (Green, 1:500; Invitrogen #A11011) to detect DREADD reporter+ and PV+ cells, respectively. Sections were again rinsed in PBS and counterstained with Hoescht (1:2000 from stock), followed by additional rinses with PBS. The stained sections were then mounted onto gelatin-coated microscope slides, airdried, and cover slipped prior to being imaged with a LSM 700 laser scanning confocal microscope (Carl Zeiss).

### 3. RESULTS

#### *C21 Did Not Impact RM as Measured by NOR*

Rats were injected with either C21 (0.5mg/kg or 1mg/kg) or saline 20 min prior to the beginning of NOR testing with a 5-minute delay (experimental timeline depicted in Figure 1). Mean exploration times during the sample phase (Figure 2A) were not statistically different across Treatment ( $F_{(2, 42)} = 1.47, p = 0.24$ ) or Sex ( $F_{(1, 21)} = 0.15, p = 0.70$ ), with no significant interaction between factors (Treatment by Sex,  $F_{(2, 21)} = 1.93, p = 0.16$ ). Similarly, the test phase exploration times (Figure 2B) were not different by Treatment ( $F_{(2, 42)} = 0.74, p = 0.48$ ) or Sex ( $F_{(1, 21)} = 1.19, p = 0.29$ ) with no interaction between factors ( $F_{(2, 42)} = 1.23, p = 0.30$ ). In the test phase, rat DRs were determined using both 2 (Figure 2C) and 5 min (Figure 2D) of the trial to investigate potential changes over time. For 2 min, mean DRs were not statistically significant when analyzing the effects of Sex ( $F_{(1, 21)} = 3.64, p = 0.070$ ) or Treatment ( $F_{(2, 42)} = 0.90, p = 0.41$ ), and no interaction between factors was observed ( $F_{(2, 42)} = 0.45, p = 0.64$ ). Similarly, I saw no differences in group DRs after the full 5-min test phase of any our groups (Treatment:  $F_{(2, 42)} = 1.49, p=0.24$ ; Sex:  $F_{(1, 21)} = 0.99, p=0.33$ ; Treatment by Sex:  $F_{(2, 42)} = 0.43, p = 0.65$ ).

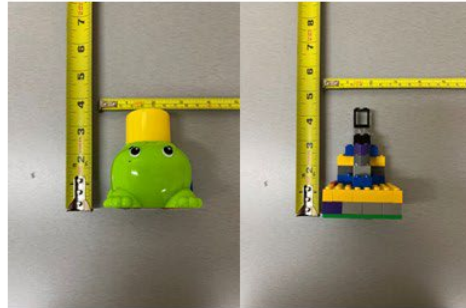
### C21 Novel Object Recognition Experimental Paradigm



#### Object Set “A”



#### Object Set “B”



#### Object Set “C”



Figure 1: NOR testing paradigm (top panel) and object sets used (bottom panel). Rats were injected with either 1) saline, 2) 0.5mg/kg C21, or 3) 1mg/kg C21. 20 min post injection, rats began the sample phase where they could explore 2 identical objects. Rats were then removed from the test box for a 5-min delay. Lastly, rats returned to the test box and could explore an identical copy of the now “familiar” object, as well as a “novel” object.

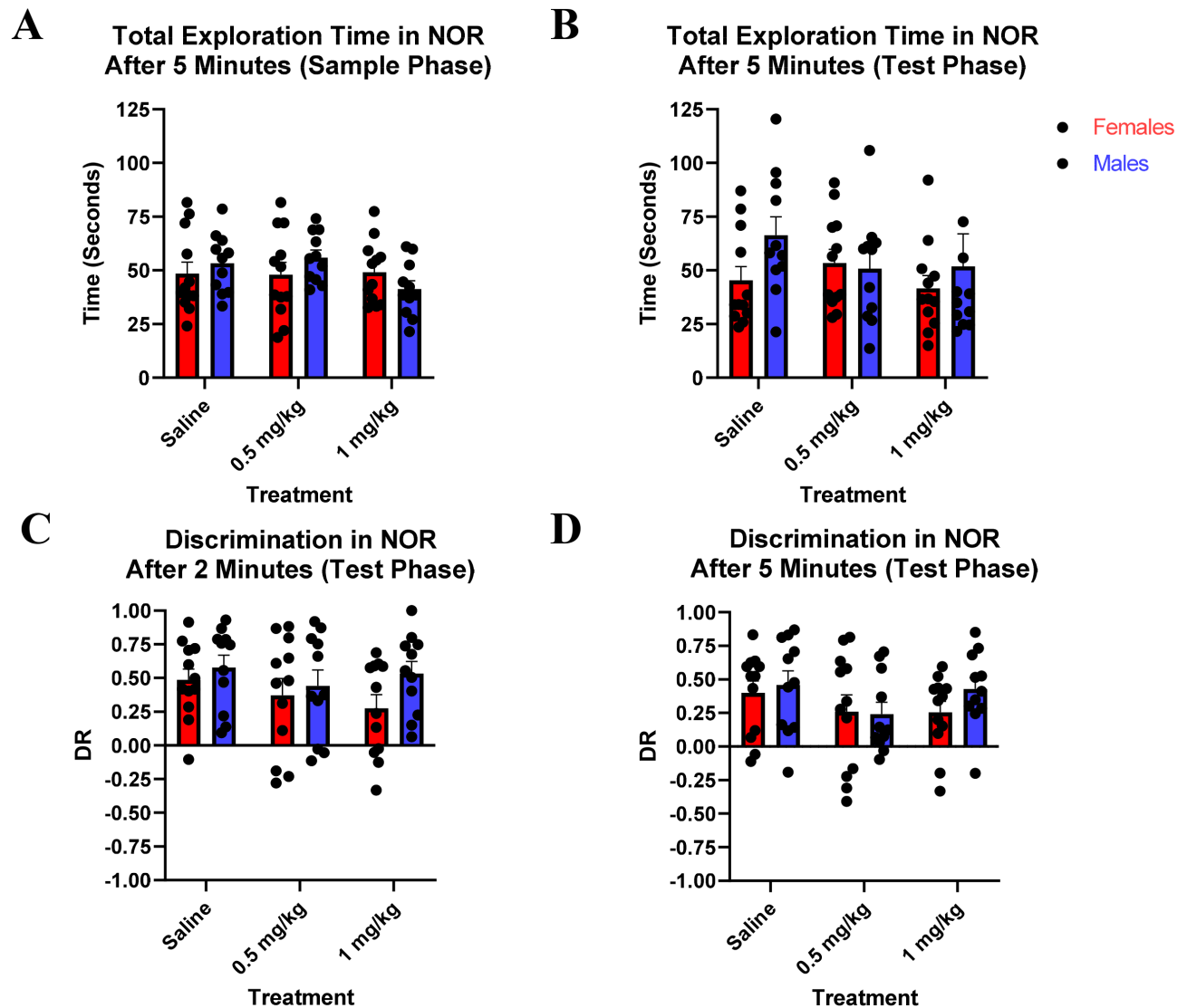


Figure 2: Effects of C21 in NOR as assessed with male and female Long Evans rats. N=24 rats (12 male, and 12 female) rats were administered 0.5mg/kg C21, 1mg/kg C21, and saline 20 min prior to the sample phase. Data were analyzed by repeated measures two-way ANOVA. [A] There was no difference between treatment groups in the amount of total exploration during the sample phase. [B] There was no difference between treatment groups in the amount of total exploration during the test phase. [C-D] Time spent exploring the novel and the familiar stimulus was used to calculate a discrimination ratio (DR, y-axis). There was no difference between treatment groups in the proportion of time spent exploring novel versus familiar stimuli.

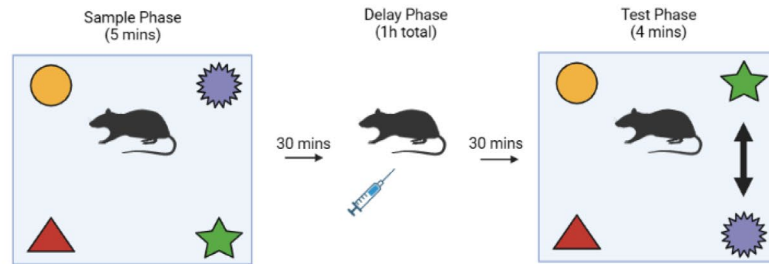
### *C21 Did Not Impact Associative Recognition Memory as Measured by OiP*

A second cohort of N=16 rats (8 males and 8 females) were injected with C21 at 0.5 mg/kg, 1mg/kg, or 1mL/kg of saline and tested in OiP across three separate days using a within subject's design (Figure 3). We were interested in whether C21 would impair memory recall as opposed to acquisition, so injections took place after the sample phase. Prior to treatment, each group of rats explored the objects for equal amounts of time during the sample phase (Figure 4A: Treatment [ $F_{(2, 28)} = 0.46, p = 0.63$ ], Sex [ $F_{(1, 14)} = 0.57, p = 0.46$ ], and no interaction of factors [ $F_{(2, 28)} = 1.10, p = 0.35$ ]). During the test phase, exploration times (Figure 4B) were significantly lower in females (Sex,  $F_{(1, 14)} = 4.69, p = 0.048$ ), however exploration was not different across Treatment ( $F_{(2, 28)} = 1.43, p = 0.26$ ) and there was no interaction of factors ( $F_{(2, 28)} = 0.23, p = 0.80$ ). Analysis of the 2-min DRs during the test phase (Figure 4C) revealed no main effects of either Treatment ( $F_{(2, 28)} = 0.05, p = 0.96$ ) or Sex ( $F_{(1, 14)} = 1.39, p = 0.26$ ) and no interaction of factors ( $F_{(1, 14)} = 3.18, p = 0.057$ ). After the full 4 min of the test phase, mean male DRs were lower than females (Figure 4D: Significant effect of Sex [ $F_{(1, 14)} = 5.60, p = 0.03$ ]), but there was no effect of Treatment ( $F_{(2, 28)} = 0.93, p = 0.40$ ) or interaction between factors ( $F_{(2, 28)} = 0.39, p = 0.68$ ).

To investigate the reason for low male DRs across all treatment groups, rat DRs were analysed again according to which object set was used (Figure 3). All except one rat had used each set once, the exception being a female that was tested on one of the object sets twice (object set B was used on test 1 and 3, and the results of both tests were comparable so data from the second test was included as a separate value for the purposes of this analysis). Figure 4E shows that after 2 min, rats preferentially explored the novel object over the familiar when they were tested with object set's A and B, however with object set C the average DRs were +0.16 and -

0.15 for females and males, respectively. No differences were found after performing mixed-effects analysis on the factors Object Set and Sex among 2-min DRs (Object Set:  $F_{(2, 27)} = 1.64$ ,  $p = 0.21$ ; Sex:  $F_{(1, 15)} = 1.01$ ,  $p = 0.33$ ; Object Set by Sex:  $F_{(2, 27)} = 0.60$ ,  $p = 0.56$ ). Among 4-min DRs (Figure 4F), there was a significant main effect of Sex ( $F_{(1, 15)} = 5.25$ ,  $p = 0.037$ ), but not Object Set ( $F_{(2, 27)} = 0.25$ ,  $p = 0.78$ ) or interaction between factors ( $F_{(2, 27)} = 1.16$ ,  $p = 0.33$ ). Next, DRs were analyzed according to test day to check whether performance in OiP changed across time. Two-way repeated measures ANOVA did not reveal any significant differences in 2-min DRs (Figure 5A) across Sex ( $F_{(1, 14)} = 1.03$ ,  $p = 0.33$ ) or Time ( $F_{(2, 28)} = 0.80$ ,  $p = 0.46$ ) with no interaction of factors ( $F_{(2, 28)} = 1.25$ ,  $p = 0.30$ ), although females had an average DR of 0 on test day 3, while males had an average DR of 0 on day 2. Furthermore, there were no significant differences in the full 4-min DRs (Figure 5B) for across Sex ( $F_{(1, 14)} = 4.06$ ,  $p = 0.06$ ) or Time ( $F_{(2, 28)} = 3.07$ ,  $p = 0.06$ ) or interaction of factors ( $F_{(2, 28)} = 1.91$ ,  $p = 0.17$ ).

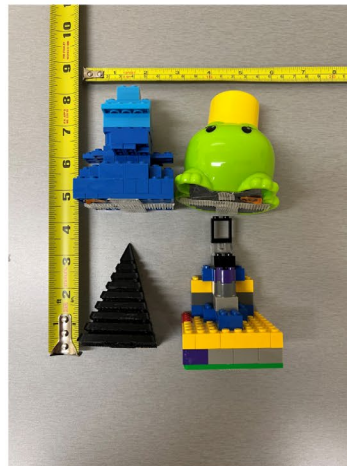
### C21 Object in Place (1 Hour Delay) Experimental Paradigm



### Object Set “A”



### Object Set “B”



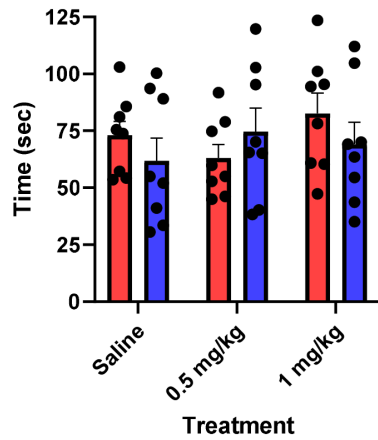
### Object Set “C”



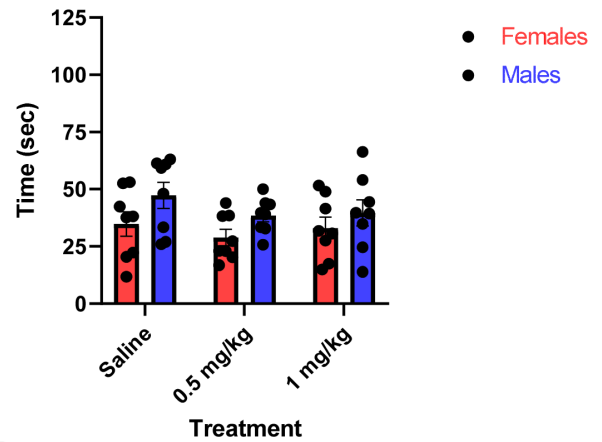
Figure 3: OiP experimental paradigm (top panel) and object sets used (bottom panel). During the sample phase, rats explored 4 unique objects placed in the corners of the testing box for 5 min. Next, the rats underwent a total 1-hour delay. 30 min into the delay, rats were injected with saline, 0.5mg/kg C21, or 1mg/kg C21. Lastly, rats returned to the testing box where they could explore 2 displaced (novel), or two stationary (familiar) objects. Objects were all 3-5 inches tall to be approximately at eye-level with the rats.

**A**

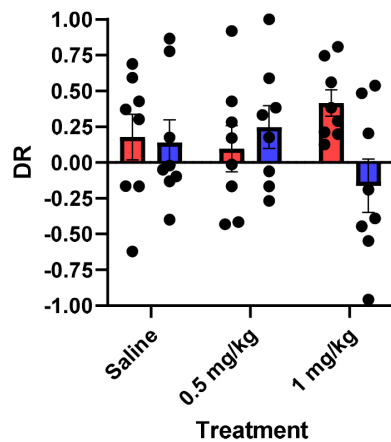
Total Exploration Time in OiP  
After 5 Minutes (Sample Phase)

**B**

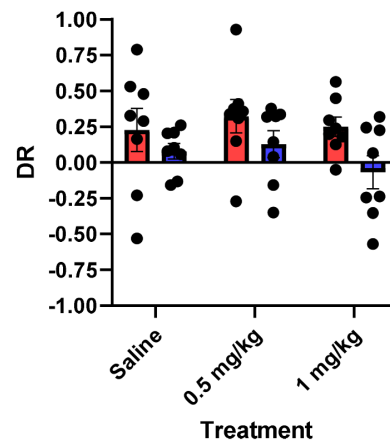
Total Exploration Time in OiP  
After 4 Minutes (Test Phase)

**C**

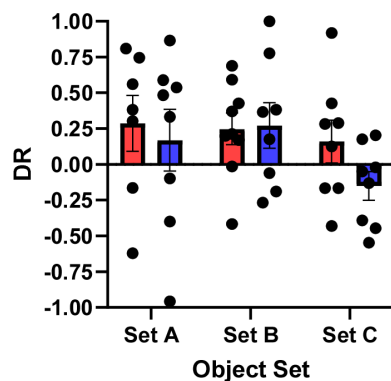
Discrimination in OiP  
After 2 Minutes (Test Phase)

**D**

Discrimination in OiP  
After 4 Minutes (Test Phase)

**E**

2-Minute Discrimination Across  
Object Sets Used in OiP (Test Phase)

**F**

4-Minute Discrimination Across  
Object Sets Used in OiP (Test Phase)

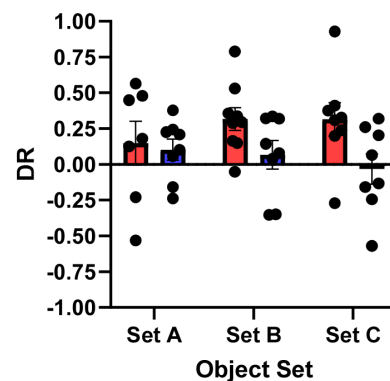


Figure 4: Effects of C21 in OiP assessed with Long Evans rats. N=16 rats (8 male, and 8 female) were administered 0.5mg/kg C21, 1mg/kg C21, and saline 30 min prior to the test phase. Data was analyzed by two-way repeated measures ANOVA, except for DRs by object set which were assessed using a mixed-effects model. [A-B] All treatment groups explored the objects for equal amounts of time in the sample phase, however females explored less than males during the test phase. [C-D] Time spent exploring the displaced/novel and the stationary/familiar stimuli were used to calculate a discrimination ratio (DR, y-axis). A positive DR is indicative of novelty preference, whereas a 0 or negative DR is indicative of no preference, or a familiarity preference, respectively. DRs after 2 minutes were not different between groups. After the full 4 minutes of the test phase, males had lower DRs than females. [E] Average male DR with object set C was negative, however there were no significant differences between groups. [F] After 4 minutes, males had small average DRs with all object sets, and these were significantly lower than females.

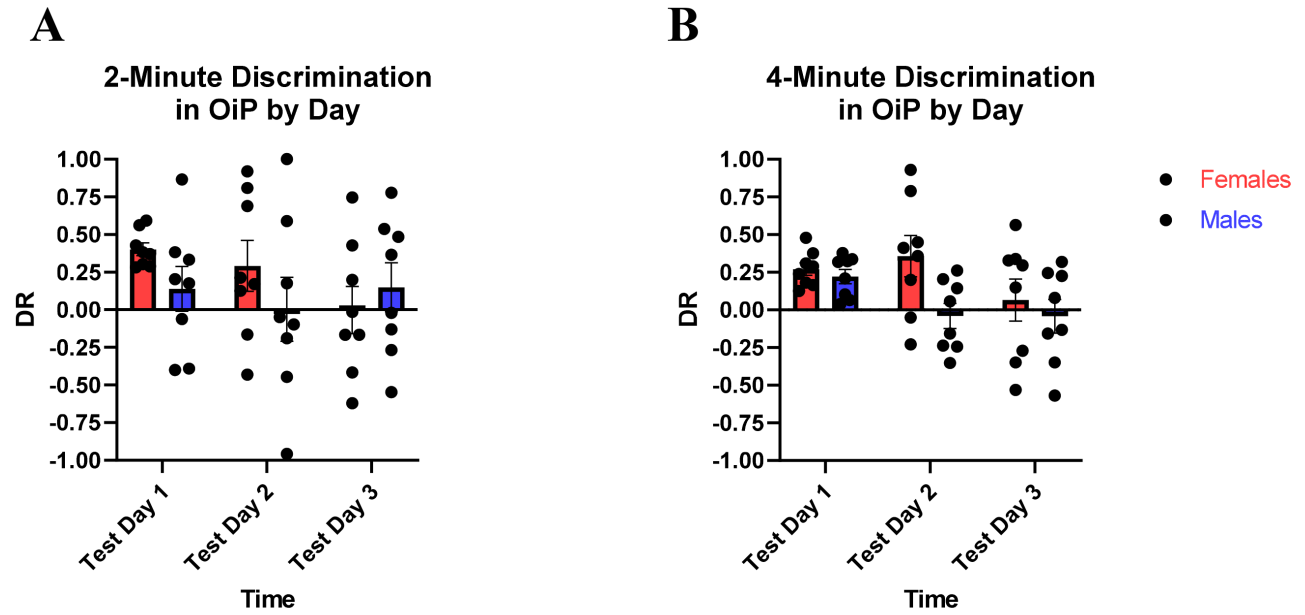


Figure 5: OiP performance across test days. [A-B] OiP DRs assessed at 2 and 4 min into the test phase were not significantly different across test days.

*Associative Memory was not Impacted in Rats Transfected with hM4Di in mPFC*

Our lab obtained PV-Cre rats (2 males, 3 females) which were transfected with an inhibitory (hM4Di) DREADD in the mPFC and tested on the OST by another researcher in our lab. Given that the mPFC is a neural substrate for OiP (Barker et al., 2007; Barker & Warburton, 2013), I wanted to test whether inhibition of PV-interneurons in mPFC would impair associative RM. We employed a version of OiP that was similar to experiment 2 reported here, except a 5-min, rather than 1-hour, delay was used (Figure 6i). Our reasoning for this change was due to concerns that the PV-Cre rats relatively older age could negatively impact memory capabilities. Thus, the shorter delay would make the task easier. Importantly, this meant that injections took place 20 minutes prior to the sample phase, similar to our NOR paradigm. We used a repeated measures design with saline, and 1mg/kg C21 across two test days, with treatment order being counterbalanced. Furthermore, since each rat would only be tested twice, we only used object sets “A” and “B” out of concerns that set “C” may have yielded poor discrimination. Due to the small sample size, the sexes were pooled and analyzed by two-tailed paired t-tests to compare DRs after saline, and 1mg/kg C21 injection. As shown in Figure 6A, there was no significant difference between the mean total exploration times during the sample phase ( $t_{(4)} = 0.30$ ,  $p = 0.78$ ). Furthermore, test phase exploration (Figure 6B) was not different by treatment ( $t_{(4)} = 0.96$ ,  $p = 0.39$ ). DRs after 2 min in the test phase (Figure 6C) were low, and not significantly different between treatments ( $t_{(4)} = 0.41$ ,  $p = 0.70$ ). DRs after the full 4 min (Figure 6D) were slightly higher than after 2 min, but still not different between treatments ( $t_{(4)} = 0.09$ ,  $p = 0.93$ ).

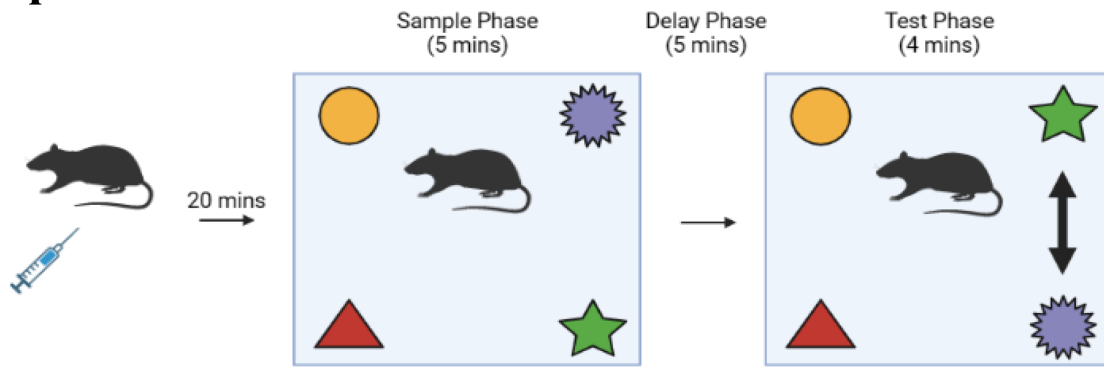
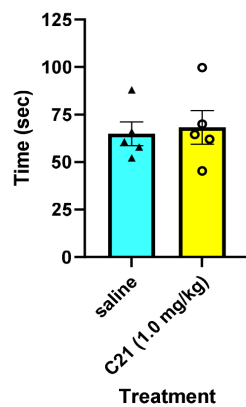
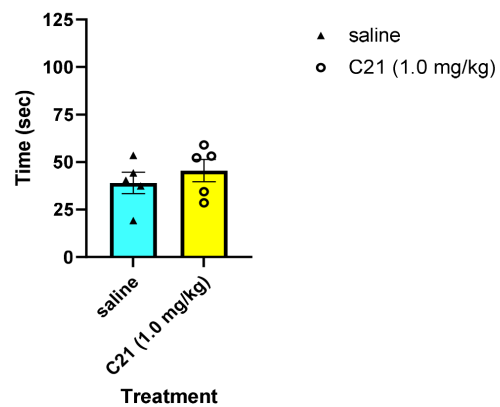
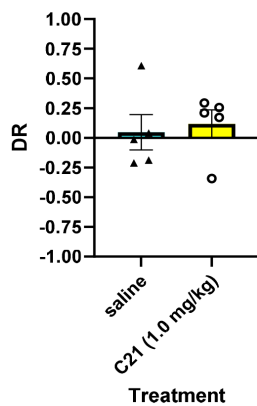
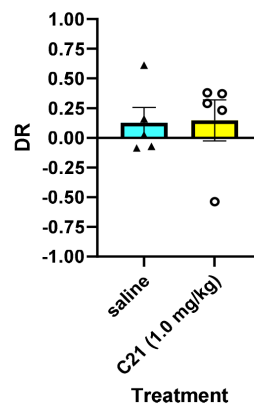
**i****C21 Object in Place (5 Minute Delay) Experimental Paradigm****A****Total Exploration Time in OiP After 5 Minutes (Sample Phase)****B****Total Exploration Time in OiP After 4 Minutes (Test Phase)****C****Discrimination in OiP After 2 Minutes (Test Phase)****D****Discrimination in OiP After 4 Minutes (Test Phase)**

Figure 6: Effect of DREADD inhibition of mPFC in the OiP test. PV Cre rats (n=5) which had been transfected to selectively express hM4Di DREADD in the mPFC were tested on OiP. [i] This version of OiP utilized a 5-min delay phase, so rats were injected 20 min prior to the sample phase. Rats were each tested twice, once with 1mg/kg C21 and once with saline in a pseudorandom order across two test days. [A] Exploration times during the sample phase were not different when rats received either saline or C21. [B] Exploration times during the full 4-min test phase were not different when rats received either saline or C21. [C] DRs after 2 min in the test phase were not different when rats received either saline or C21. [D] DRs after 4 min in the test phase were not different when rats received either saline or C21.

### *hM4Di Expression in mPFC Interneurons*

Following completion of OiP testing, PV-Cre rat brains were sectioned and co-localization of hM4Di transgene expression with PV interneurons in the mPFC were determined using immunohistochemistry. Representative sections from the mPFC of each rat are shown in Figure 7. Upon visual inspection of the stained slices, only one rat (male 1) displayed obvious bilateral colocalization of GFP and mCherry in the mPFC with GFP-alone and mCherry-alone stained cells accounting for 63% and 31% of cells, respectively, while dual-stained GFP/mCherry cells only accounted for 6% of cells (Figure 7A). Male 2 (Figure 7B) and female 1 (Figure 7C) displayed small levels of colocalization in only one hemisphere, while females 2 (Figure 7D) and female 3 (Figure 7E) displayed sparse levels of colocalization. Since our transfection protocol did not yield significant levels of hM4Di expression in PV interneurons, C21 likely was not able to exert sufficient DREADD inhibition of these neurons in the mPFC.

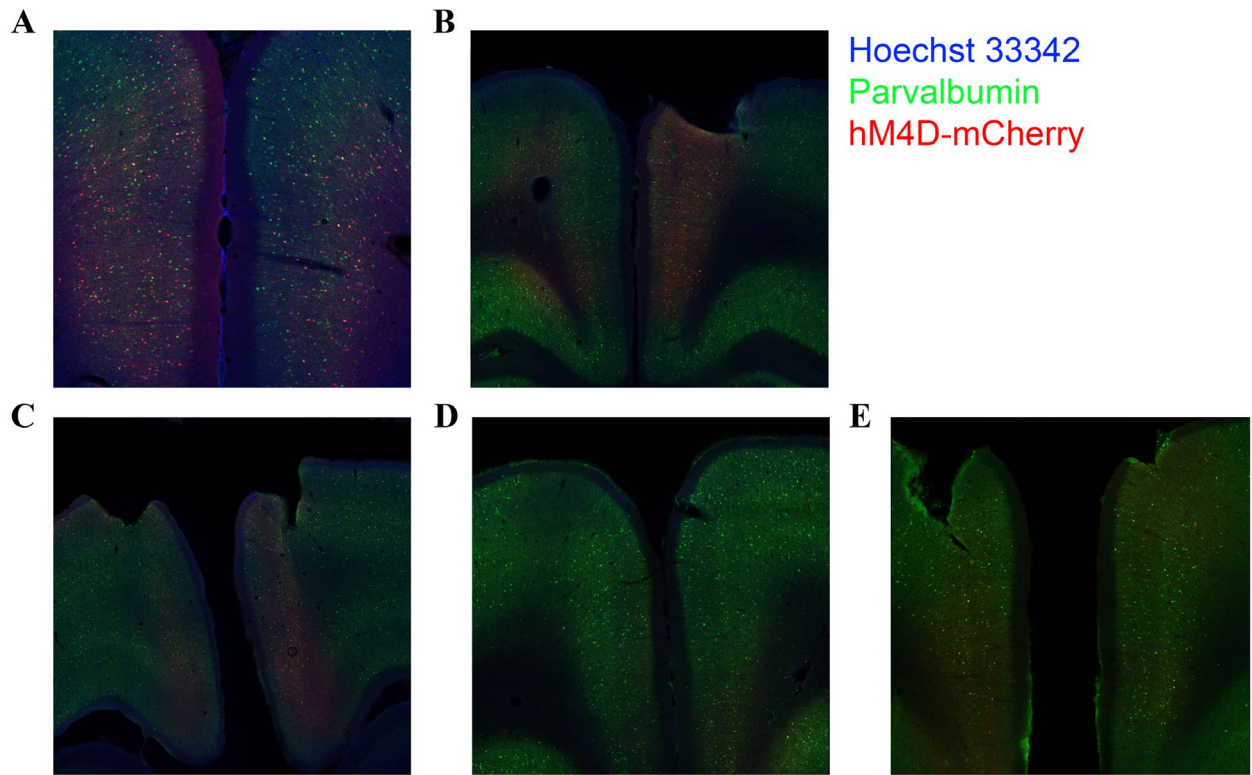
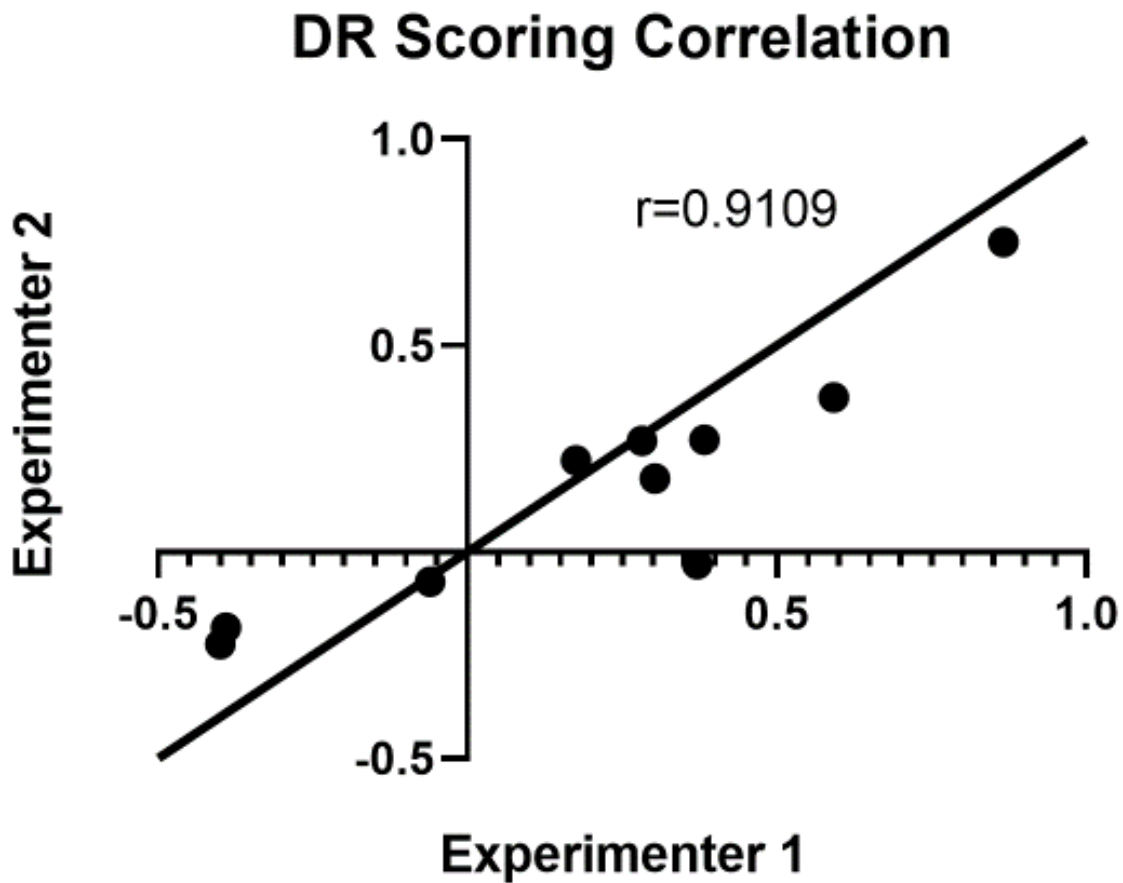


Figure 7: Colocalization of PV+ cells and DREADD reporter expression in rat mPFC. PV+ cells are labelled with GFP (green), while DREADD reporter expression was determined using an mCherry reporter (red) and colocalization being indicated in yellow/orange. Representative images are shown for [A] male 1 (10x magnification), [B] male 2 (5x magnification), [C] female 1 (5x magnification), [D] female 2 (5x magnification), and [E] female 3 (5x magnification).

### *Inter-scorer Reliability*

To ensure that scoring of exploration times and DRs was consistent, a second researcher with experience conducting and scoring these spontaneous tasks analyzed 10 random videos of OiP test phases to compare with that of the primary experimenter. Since scoring is identical in NOR and OiP, this was considered an appropriate proxy to check reliability in all experiments. We chose to compare DRs because they represent more standardized values than do raw times. DRs scored by the two experimenters were highly correlated with each other (Figure 8:  $r = 0.91$ ,  $p = 0.0002$ ).



**Figure**

8: Correlation of DRs for inter-scorer reliability. Ten OiP videos were scored by a second experimenter and compared against data of the primary experimenter. DRs were highly correlated when scored by the primary (x-axis) and secondary experimenter (y-axis).

#### 4. DISCUSSION

The primary objective of this study was to determine whether systemic C21 treatment alone affects NOR or OiP tests in rats. Our secondary objective was to demonstrate whether DREADD inhibition of PV interneurons of the mPFC impaired RM in the OiP test. I will discuss the relevance of my data to the existing body of literature, as well as potential shortcomings of my work.

##### *C21 Did Not Impair NOR in Male or Female Rats*

Our first cohort of animals were used to test the effects of C21 on NOR, given that the only other similar experiment to my knowledge used CNO (Jeon et al., 2022). To the best of my knowledge, C21 has not been used for DREADD manipulations with spontaneous RM tests of rats, and as such, the present data are the first of its kind to evaluate its effects on rat RM. I first demonstrated that C21 caused no impact on exploration during either sample or test phases, in either NOR or OiP tests. Total test phase exploration times in my experiments were consistent with previous reports in control rats (Mathiasen & Dicamillo, 2010; Sutcliffe et al., 2007). These results are a good indication that C21 did not impact other behaviours such as motivation, or perhaps (indirectly) locomotion. My data also support the conclusion that C21 did not impair RM in the NOR test, which is strengthened by the fact that the DRs reported here are comparable control rats elsewhere (Brymer et al., 2021). These data are reassuring given evidence that CNO is not inert in the NOR paradigm with rats (Naneix et al., 2021), therefore C21 may be an attractive alternative DREADD agonist for the NOR test using rats.

### *Effects of C21 in OiP in Male and Female Rats*

Unlike NOR, interpretation of the experimental results of C21 on associative memory assessed in OiP is more difficult. After 2 min, the saline groups had average DRs of 0.17 and 0.14 for females and males, respectively, which is slightly lower than expected for control rats (Barker & Warburton, 2008, 2011; Cost et al., 2012; Dix & Aggleton, 1999; Howland et al., 2012; Saucier et al., 2008). Female DRs assessed at 2 min were inconsistent with averages of 0.18, 0.10, and 0.42 in saline, 0.5mg/kg C21, and 1mg/kg C21 groups, respectively. Additionally, males averaged 0.14, 0.24, and -0.163 for saline, 0.5mg/kg C21, and 1mg/kg C21 groups, respectively. When evaluating DRs for the full 4 min, the females were more consistent with averages of 0.23, 0.32, and 0.25 for saline, 0.5mg/kg, and 1mg/kg doses. In contrast, males after 4 minutes had low DRs in all groups (saline= 0.08, 0.5mg/kg C21= 0.13, 1mg/kg C21= -0.07), albeit the saline group had minimal variability. Since these differences were not statistically different, but the male DRs are consistently lower, I conclude that females' associative memory was not impaired by C21 in OiP when assessed across the full 4-min test phase. On the other hand, since male DRs were quite low, I conclude that male rats simply did not display robust discrimination in OiP. That said, C21 did not alter total sample or test exploration times in either sex.

It is not clear why some mean group DRs were so low, but I will propose a few possible explanations. Females 2-minute DRs were not consistent across treatment. Male saline DRs were much lower than anticipated, but with a larger SEM it is possible that our sample mean was simply lower than the population mean.

Second, there was a notable ventilation issue that occurred during the second test day. Due to maintenance, the ventilation system was forcing increased volumes of air into the test room such

that the door could not close entirely, and this may have distracted or stressed the rats. The timing of this issue was especially apparent during the testing of the first 4 females and 4 males that day. Another issue that was particularly troublesome for the females arose when on the third test day the fire alarm was sounded just prior to four of the female rat's tests that spanned an hour afterward. These animals had been undergone the sample phase and been injected, so I opted to continue with testing. Two of those females were responsible for the lowest 2-min DRs in the 0.5mg/kg and saline groups, and the other two rats (also split between 0.5mg/kg and saline groups) were negative too. These data points resulted in a lower average DR for these two groups after 2 min, but interestingly three out of four of those females had positive DRs after 4 min (the remaining rat still had the lowest DR of the saline females). There were also other minor audible disturbances throughout testing as a result of directly adjacent rooms to testing being occupied by other researchers on the floor.

Thirdly, injections themselves may have been disproportionately stressful for our males specifically despite being habituated to the process beforehand. Males were noted to be increasingly vocal during every single injection, whereas females were almost never vocal. Two of the males from the 1mg/kg group were noted to have been squirming significantly during the injection, requiring additional attempts and or further restraint with a towel to carry out the injection. One of these rats scored a DR of -0.44 after 2 min and -0.24 at 4 min, however the other rat had high DRs of 0.48 and 0.24 at 2 and 4 min, respectively. Throughout tests one and two, one of the rats unfortunately had bloody urine and was seemingly very uncomfortable with being handled, perhaps due to bladder pain. During each injection he was particularly vocal, although he hadn't been noted as squirmy. Nonetheless, this rat consistently scored negative DRs across all 3 tests.

Fourth, I considered whether the objects used need to be further optimized. To check this, I looked at the exploration of the individual object sets by comparing the DRs across each of the 3 objects sets (Figure 5). Females had averaged 2-min DRs of 0.29, 0.25, and 0.16 for object sets A, B, and C, respectively. At 4 min, females still showed robust discrimination across sets with averages of 0.16, 0.32, and 0.32 for sets A, B, and C, respectively. Males had average 2-minute DRs of 0.16, 0.27, and -0.15 for sets A, B, and C, respectively. At 4 minutes, males had exceedingly low averages of 0.10, 0.07, and -0.03 for sets A, B, and C, respectively. Upon closer inspection of the male data, 4 of the 8 rats were tested on object set C when treated with 1mg/kg C21, and had scored 2-min DRs of -0.39, 0.20, -0.55, and -0.45. After 4 min, those same rats scored DRs of 0.32, 0.07, -0.56, and -0.24. Nonetheless, if we remove those rats tested on object set C, the 1mg/kg group still has an average DR of virtually 0 (-0.03) while the 4 remaining male saline rats still only average 0.11 (data not shown). While it is difficult to make statistical comparisons, visual inspection of these numbers hint at the fact that object set C was not to blame. Thus, it is difficult to parse out any indication that the object sets are to blame for males' poor performance. Regarding counterbalancing, the exact same treatment assignments have been used in other rat cohorts for previous experiments in our lab by another experimenter, and no issues were noted with resultant control DRs. My data is also not consistent with prior data from our lab which shows that object set C yields DRs of about 0.2 in male Long Evans rats (data not shown).

Lastly, I searched the current literature to determine if sex differences have been previously noted in OiP among studies of Long Evans rats. It was shown elsewhere that both control female and male Long Evans rats explore the novel objects in OiP at for least 70-80% of the total exploration time after a 90 min delay (Reichel et al., 2012), which should correspond to

a DR of about 0.2-0.3 for both sexes. It's also been shown that female associative memory in OiP depended on levels of sex hormones, and that females can't discriminate objects in OiP with 30 min or 1h delays (Cost et al., 2012). However, male rats show robust discrimination with delay periods of 30 min (Cost et al., 2012) and 1h (Howland et al., 2012). More studies support male, not female discrimination abilities in OiP, which contrasts with data shown in this thesis demonstrating that female, and not male rats exhibit robust discrimination in OiP after 1h. Interestingly, female Long Evans are superior performers over males in OiP if there are 4, consecutive sample phases followed by the test phase 24h later (Saucier et al., 2008). It is interesting that our group previously showed that males, not females display robust discrimination in OiP after 1h (Howland et al., 2012). Studies employing OiP testing of rats typically reported DRs equivalent to 0.2-0.3 in successful groups (Cost et al., 2012; Dix & Aggleton, 1999; Howland et al., 2012; Reichel et al., 2012), however, it has also been reported that rats with intact associative memory can display DRs of upwards of 0.4-0.5 (Barker et al., 2007; Barker & Warburton, 2008, 2011, 2013; Pinizzotto et al., 2022). We hypothesized that a 1h delay would yield robust discrimination in both sexes, however only females, not males, exhibited a preference for novelty in OiP in the current study. Furthermore, females mainly scored mean DRs of 0.2-0.3. It is not yet clear why this discrepancy exists in the OiP literature. Importantly, OiP experiments conducted by our group in the past with a 1h delay (Howland et al., 2012) were carried out by different researchers than those involved here. Inconsistent findings of discrimination capabilities across studies suggest that performance of rats in OiP may depend on certain laboratory or handler conditions. If OiP performance primarily depends on extraneous factors outside of rat's innate behaviour or experimental manipulations, any results gleaned from OiP tests need be interpreted with caution, and a given handler may need to carry

out multiple experiments to determine what “successful” discrimination would look like in a particular setting.

### *Role of mPFC PV Interneurons in OiP?*

PV Cre rats did not display significant discrimination of novel over familiar objects, even with a 5-minute delay as has been successfully used elsewhere with non-transgenic rats (Cost et al., 2012). As noted already in this thesis, pathological reduction of PV+ GABAergic interneurons have been observed to accompany impaired OiP performance (Reichelt et al., 2015). Furthermore, DREADD expression targeted to PV+ interneurons have been shown elsewhere to yield high levels of colocalization (Armenta-Resendiz et al., 2022; Binette et al., 2023). Unfortunately, our PV Cre animals did not exhibit robust discrimination of objects in OiP when injected with either C21 or saline. It is possible that the older age of our rats impaired their ability to discriminate the objects on OiP, as age related deficits in rat RM have been noted using NOR (de Lima et al., 2005; Scali et al., 1997). Histological examination of rat mPFC transfected with DREADD vector revealed overall poor expression, with only one rat showing readily detectable levels of colocalization of PV+ cells with our DREADD reporter. It is unlikely that the construct used in the present study was the sole culprit behind poor expression given that it has been used successfully elsewhere (Armenta-Resendiz et al., 2022). It is also unlikely that the PV-Cre rats obtained for this study did not have sufficient, and/or specific expression of Cre recombinase in PV+ cells given that other animals from the same supplier have shown robust DREADD expression in PV+ cells elsewhere (Binette et al., 2023). To ensure sufficient Cre-recombinase expression in PV+ cells of rats, future experiments may need to employ polymerase chain reaction assays on each rat. Transduction of hippocampal neurons with hM4Di using AAV 2/7 can cause neuronal toxicity in mice (Goossens et al., 2021), however it is not clear at which

levels neuronal toxicity may or may not occur using AAV 5 in the mPFC of the rat. On the other hand, it is possible that our transfection protocol wouldn't yield robust DREADD expression at any time point. To rule this possibility out, future experiments should determine how expression of the construct used in this study with AAV 5 changes over time after surgery. Thus, we failed to show whether there is a direct role of PV interneurons in OiP, and further optimization of our transfection protocol is required before subsequent studies can be conducted in our lab to make more direct conclusions regarding the role of these interneurons in memory.

### *Future Directions*

Since males did not show robust discrimination in the OiP test, further work is needed to show whether C21 impairs male RM assessed in OiP. The first step to address this would be to establish a protocol that reliably produces male discrimination in OiP within our lab. Modifications to the object sets used could also help boost discrimination to values produced elsewhere (Barker & Warburton, 2008, 2011, 2013). Furthermore, our PV-DREADD experiment suffered from certain flaws. Future experiments will need to test more animals transfected with a control vector to assess impacts of the surgery and viral infection. As evident from our histology, further optimization of our surgical transfection protocol will be required to improve expression levels in rat brain. Furthermore, given evidence to suggest that hM4Di is not as readily activated by C21 (Bonaventura et al., 2019), other experiments that could serve as a positive control should be conducted to ensure the manipulation modulates PV interneuron firing. Another possibility is to employ CaMKII promoted inhibitory DREADDs to down-regulate all excitatory activity in the mPFC (Jeon et al., 2022) for a more robust response, and to test these animals in OiP where the lesion has reliably impaired performance before (Barker & Warburton, 2013).

Future experiments investigating the role of PV interneurons in OiP should also ensure that rats will not be old enough where age-related impairments are of concern.

## 5. CONCLUSIONS

Here, I show that C21 did not impair either male or female Long Evans rats in the NOR test. Furthermore, I also demonstrated that C21 did not impair exploration of objects in either male and female rats, or associative memory in female Long Evans rats. Since our males failed to show robust discrimination in OiP, we cannot conclude whether C21 impaired associative memory in male Long Evans rats. To the best of my knowledge, the data presented here are among the first to show the effects of C21 in spontaneous tasks in rat. Due to more favorable pharmacokinetics, C21 should be seriously considered as an alternative DREADD agonist to CNO for future studies. However, the effects of C21 on male rats in OiP need be evaluated further before employing DREADDs in both sexes within this spontaneous paradigm.

## 6. APPENDIX

**Table 1: Use of Viral DREADD Constructs in Rat Nervous System**

AAV/Construct	Rat/Genotype	Agonist Used	Primary Findings	Reference
1. AAV9-hSyn-DIO-hM4Di-mCherry	PV-Cre	CNO	mPFC PV interneurons regulate extinction learning	(Binette et al., 2023)
2. AAV9-CD68-hM4Di-mCherry	Sprague-Dawley	CNO	DREADD stimulation of spinal microglia induces and is required for allodynia following peripheral nerve injury	(Grace et al., 2018)
3. AAV9-CD68-hM4Dq-mCherry				
4. AAV9-CD68-hM4Di-mCherry	F344	CNO	Inhibition of microglia chronically prevents morphine sensitization	(Grace et al., 2016)
5. AAV8-fPV-hM3Dq-GFP	Unspecified	CNO	Inhibition of PV cells disrupts GABA transmission	(Chamberlin et al., 2023)
6. AAV8-hSyn1-hM4Di-mCherry	Alko Alcohol	CNO	Inhibition of anterior insula does not alter appetitive behaviour in alcohol-preferring rats, Activation of anterior insula attenuates alcohol and sucrose preference	(Haaranen, Scuppa, et al., 2020)
7. AAV8-hSyn1-hM3Dq-mCherry				
8. AAV8-hSyn-DIO-hM3Dq-mCherry (and CAV2-Cre)	Sprague-Dawley	CNO	Activation of indirect pathway medium spiny neurons mediate cue-induced reinstatement of heroin seeking,	(O'Neal et al., 2020)

9. AAV8-hSyn-DIO-hM4Di-mCherry (and CAV2-Cre)	Sprague-Dawley	CNO	inactivation of direct pathway medium spiny neurons mediate cue-induced reinstatement of heroin seeking	
10. AAV8-hSyn-DIO-hM3Dq-HA	Sprague-Dawley*	CNO, Salvinorin B	Elevated cAMP and 5-HT6 receptor activity on dopamine neuron grafts induces dyskinesia	(Aldrin-Kirk et al., 2016)
11. AAV8-hSyn-DIO-rM3Ds-mCherry	Sprague-Dawley*			
12. AAV8-hSyn-DIO-KORD-IRES-mCitrine	Sprague-Dawley*			
13. AAV8-hSyn-Gi-hM4Di-mCitrine	Long Evans	CNO	Inhibition of ventral pallidum impairs learning of sign-tracking	(Chang et al., 2015)
14. AAV8-hSyn-hM4Di-mCherry	Sprague-Dawley	CNO	Inhibition of hippocampus impairs episodic memory	(Panoz-Brown et al., 2018)
15. AAV8-hSyn-hM4Di-mCherry	Long Evans	CNO	Inhibition of dorsal anterior cingulate cortex alters default mode network	(Tu et al., 2021)
16. AAV8-CamKIIa-hM4Di-mCherry	Brown-Norway	CNO	mPFC is important for deliberate decision making and memory	(Schmidt et al., 2019)
17. AAV8-CamKIIa-hM4Di-mCherry	Brown-Norway	CNO		
18. AAV8-CaMKIIa-hM4Di-mCherry	Long Evans	CNO & Clozapine	Inhibition of excitatory cells in the ventral hippocampus reduces anxiety	(Maestas-Olguin et al., 2021)
19. AAV8-CamKIIa-hM4Di-mCherry	Sprague-Dawley	CNO	DREADD activation of anterior cingulate cortex rescues behavioural deficits induced by heroin	(Tomek et al., 2020)

20. AAV8-CaMKIIa-hM4Di-mCherry	Sprague-Dawley		DREADD activation of anterior cingulate cortex rescues behavioural deficits induced by heroin	
21. AAV8-CaMKIIa-hM4Di-mCherry	Brown-Norway	CNO	Inhibition of mPFC disrupts hippocampal firing and cognition	(Schmidt & Redish, 2021)
22. ssAAV-8/2-hSyn1-hM3Dq-mCherry-WPRE-hGHp(A)	Alko Alcohol	CNO	Demonstrated role of insula, amygdala, and nucleus accumbens alters alcohol-seeking behaviour	(Haaranen, Schäfer, et al., 2020)
23. ssAAV-8/2-hSyn1-hM4Di-mCherry-WPRE-hGHp(A)				
24. ssAAV-8/2-hSyn1-dlox-hM3Dq-mCherry(rev)-dlox-WPRE-hGHp(A)				
25. ssAAV-8/2-hSyn1-dlox-hM4Di-mCherry(rev)-dlox-WPRE-hGHp(A)				
26. AAV6-hSyn-DIO-mCherry-hM3Dq-WPRE	TH-Cre	CNO	Activation of dopaminergic neurons in the substantia nigra reduces anxiety and compulsive behaviour	(Casado-Sainz et al., 2022)
27. AAV5-GFAP-HA-hM3Dq-IRES-mCitrine	Long Evans	CNO	Nucleus accumbens core astrocytes mediate motivation to self-administer ethanol	(Bull et al., 2014)
28. AAV5-CaMKIIa-HM3Dq-mCherry	Sprague-Dawley	CNO	Excitatory median preoptic nucleus neurons cause increased nitric oxide	(Marciante et al., 2020)
29. AAV5-CaMKIIa-HM4Di-mCherry	Sprague-Dawley	CNO	Prefrontal cortex and basolateral amygdala contribute to anxiety during alcohol withdrawal	(McGinnis et al., 2019)

30. AAV5-CaMKIIa-HM3Dq-mCherry	Sprague-Dawley	CNO		
31. AAV5-hSyn-hM3Di-mCherry	Sprague-Dawley	CNO	mPFC mediates novel object recognition after acute stress	(Jeon et al., 2022)
32. AAV5-hSyn-DIO-hM4Di-mCherry	TH-Cre	C21	0.5mg/kg C21 activates hM4Di in substantia nigra without offtarget effects	(Goutaudier et al., 2020)
33. AAV5-hSyn-DIO-hM3Dq-mCherry & CAV2-CRE	Wistar	CNO	Mesolimbic pathway neurons amenable to DREADD manipulation and magnetic resonance imaging	(Roelofs et al., 2017)
34. AAV5-GFAP-HA-hM3D-IRES-mCitrine	Sprague-Dawley	CNO	Glial glutamate inhibits cue-induced cocaine seeking	(Scofield et al., 2015)
35. AAV5-hSyn-hM3Di-mCherry	Sprague-Dawley	CNO	Subthalamic nucleus mediates amphetamine sensitization and conditioned responding	(Nakata et al., 2022)
36. AAV5-hSyn-hM3Dq-mCherry	Sprague-Dawley	CNO		
37. AAV5-CaMKIIa-hM4Di-mCherry	Sprague-Dawley	CNO	Extracellular and cellular dehydration affect CaMKIIa neurons in the median preoptic nucleus and its hypothalamic projections	(Marciante et al., 2019)
38. AAV2-hSyn-DIO-hM3Dq-mCherry	Fischer-344	CNO	Activation of ventrolateral preoptic area projections to the perifornical-hypothalamic area induces sleep	(Kostin et al., 2022)
39. AAV2-hSyn-DIO-hM3Dq-mCherry	ChAT-Cre	CNO	DREADD activation of pedunculopontine cholinergic neurons rescues motor deficits in parkinsonian rats	(Sharma et al., 2020)
40. AAV2-hSyn-DIO-hM3Dq-mCherry	TH-Cre	Clozapine, C21, J52, J60	J52 and J60 are more potent DREADD agonists than C21 with better pharmacokinetics in rats, mice, and monkeys	(Bonaventura et al., 2019)
41. AAV8-hSyn-hM4Di-mCherry	Sprague-Dawley	J07		
42. AAV8-hSyn-hM3Dq-mCherry	Sprague-Dawley			
43. AAV2-CaMKIIa-HM4Di-mCherry	Sprague-Dawley	CNO	DREADD activation of motor cortex results in strengthening of motor cortex outputs	(Amer & Martin, 2022)

44. AAV2-CaMKIIa-HM4Dq-mCherry	Sprague-Dawley	CNO		
45. AAV2-hSyn-HA-hM3Dq-IRES-mCitrine	Sprague-Dawley	CNO	Activation of PRhC neurons rescues meth-induced NOR deficits	(Peters et al., 2018)
46. AAV2-hSyn-DIO-hM4Di-mCherry	TH-Cre	CNO	Increased Gq signalling in dopamine neurons of the ventral tegmental area enhances cocaine-seeking	(Mahler et al., 2019)
47. 37. AAV2-hSyn-DIO-hM4Dq-mCherry				
48. AAV2-hSyn-DIO-rM3Ds-mCherry				
49. AAV2-CaMKIIa-HM4Di-mCherry	Sprague-Dawley	CNO	DREADD activation of corticospinal tract neurons enhances axon length and branching	(L. Yang & Martin, 2023)
50. AAV2-CaMKIIa-HM4Dq-mCherry	Sprague-Dawley	CNO		
51. AAV2/7-CaMKIIa-hM4Di-mCherry	Sprague-Dawley	Clozapine	DREADD inhibition of excitatory neurons in the dentate gyrus reduces seizures	(Goossens, Boon, et al., 2021)
52. AAV2/7-CaMKIIa-hM4Di	Sprague-Dawley	Clozapine	High-titre hM4Di vector causes neurotoxic effects in the hippocampus	(Goossens, Boon, et al., 2021)
53. AAV2/7-CaMKIIa-hM4Di-mCherry	Sprague-Dawley	Clozapine		
54. AAV2/9-PRs8-HA-hM3Dq	TgF344-AD	CNO	Reversal learning in Morris Water maze in rat models of alzheimers is rescued by DREADD activation of the locus coeruleus	(Rorabaugh et al., 2017)
55. AAV1/2-hSyn-hM3Dq-mCherry	Sprague-Dawley	CNO	DREADD inhibition of mPFC after acute stress rescues RM assessed by NOR	(Jeon et al., 2022)
56. (?)AAV-hSyn-hM4Di-mCherry	Sprague-Dawley	CNO, Clozapine	CNO does not readily bind DREADDs or cross the blood brain barrier	(Gomez et al., 2017)

57. Lentiviral hM3Dq-IRES-mCherry	6-OHDA Lesioned	CNO	Induced dopaminergic neurons implanted into rat brain have therapeutic effects in parkinsons model	(Dell'Anno et al., 2014)
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