Pharmacosynthetics and the Cell-Type-Specific Control of Neuronal Signaling

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ABSTRACT

MARTILIAS STEPHEN FARRELL: Pharmacosynthetics and the Cell-Type-Specific Control of Neuronal Signaling
(Under the direction of Bryan L. Roth)

Pharmacology, in its broadest interpretation, is defined as the study of drug action. In modern neuropsychopharmacology, there is a conceptual boundary between the "drug" and the "action", with the drug itself on one side and signal transducer (receptor), the signal transduction cascade (effector proteins, second messengers), the cellular response (transcriptional regulation, activity modulation), the organ response (brain circuitry modulation), and, finally, the whole organism response (behavior) on the other. In other words, pharmacology has structured itself around the idea that the exogenous molecule (the drug) encodes a "signal" leading to everything on the other side including, in extreme instances, a physiological response. The inference is that engaging a particular signal transduction pathway in a defined cell type leads inexorably to a prototypic physiological response. Here, I suggest that the invention of synthetic ligand—GPCR pairs (aka DREADDs, RASSLS, 'pharmacogenetics') permits the study of pharmacology using a shifted equation: with the signal transduction elements moved to the left and, subsequently, under experimental control. For the purposes of disambiguation and to clarify this approach as a creation of pharmacological manipulation, I present the term pharmacosynthetics to

describe what has heretofore been called pharmacogenetics or chemicogenetics. In this document I will review previous work utilizing this technology, present my work validating a variation of this technology in a heretofore untested cellular context, and provide a perspective on how this technology can advance the field of pharmacology.

I dedicate this dissertation to my future wife Candace and my parents.

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LIST OF ABBREVIATIONS

A2A – Adenosine A2A receptor

AAV – adeno-associated virus

adora2A – Adenosine A2A receptor gene

AgRP – agouti-related protein

AKT, pAKT – Protein Kinase B (PKB), phosphorylated

AMPAR – alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

AMPH – Amphetamine

ANOVA – Analysis of Variance

ARC – arcuate nucleus

ATP – adenosine triphosphate

BAC – Bacterial Artificial Chromosome

BCA – Bicinchoninic acid assay

bp – base pair

BSA – Bovine Serum Albumin

cAMP: cyclic adenosine monophosphate

CNO – Clozapine N-oxide

D1-Dopamine receptor 1

D2 – dopamine receptor 2

DARPP-32: Dopamine and cAMP regulated neuronal phosphoprotein

dB – decibels

DMEM – Dulbecco's Modified Eagle Medium

DMSO – Dimethyl Sulfoxide

DNA – Deoxyribonucleic Acid

Drd1 – Doparmine Receptor D1

Drd2 – Doparmine receptor D2

DREADD – Designer Receptors Exclusively Activated by Designer Drug

EGFP – enhanced green fluorescent protein

EPSCs – Excitatory postsynaptic currents

ERK, pERK – Extracellular signal regulated kinase, phosphorylated

Flp – Flippase

FRT – Flippase recognition target

FW – Forward

GDP – guanosine diphosphate

GENSAT – Gene Expression Atlas of Mouse Central Nervous System

GPCR: G protein-coupled receptor

GTP – guanine triphosphate

HBSS – Hank's Buffered Salt Solution

HEK - human embryonic kidney cells

HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

hM3Dq – Human Muscarinic Receptor 3 Gq DREADD

hM4Di – Human Muscarinic Receptor 4 Gi DREADD

i.p. – intraperitoneally

IRES – Internal Ribosome Entry Site

kb: kilobase

mEPSC: miniature excitatory post synaptic current

MeSH – Medical Subject Headings

Min – minutes

MSN: medium spiny neuron

NAc – Nucleus accumbens

NIH – National Institutes of Health

NINDS – National Institute of Neurological Disorders and Stroke

NMDAR – N-methyl-D-aspartate receptor

NREM: Non Rapid Eye Movement

P23 – Post Natal Day 23

PAGE – Polyacrylamide Gel Electrophoresis

PBS – Phosphate Buffered saline

PCR – Polymerase Chain Reaction

PDZ – post synaptic density protein (PSD95), Drosophila disc large timor suppressor (Dlg1), and zonula occludens-1 protein(zo-1) domain

PKA – protein kinase A

PLSD – Fisher's protected least-significant difference

Rbp4: retinol binding protein 4

REV – Reverse

RFP – Red Fluorescent Protein

rM3Ds – Rat Muscarinic Receptor 3 Gs DREADD

Rpm – revolutions per minute

RT – Room temperature

SDS – Sodium Dodecyl Sulfate

TBST – Tris Buffered Saline with 0.05% Tween 20

TRE – Tetracycline Response Element

tTa – tetracycline transactivator

LIST OF SYMBOLS

Gα – G protein alpha.

Gβ/γ – G proteins beta and gamma

$G\alpha_s$ – Gs-type G protein alpha – stimulates adenylyl cyclase

$G\alpha_{olf}$ – G alpha olf, a Gs-type G protein

$G\alpha_i$ – Gi-type G protein alpha – inhibits adenylyl cyclase

$G\alpha_q$ – Gq type G protein alpha – stimulates phospholipase C

CHAPTER 1. BACKGROUND

1.1. INTRODUCTION

The gap between our understanding of receptor mediated signaling and the ultimate functional output of the brain is shrinking. The past decade has witnessed the advent of multiple technologies that allow the exquisite manipulation of neurons in an intact animal, providing the opportunity to definitively determine the neuronal correlates of complex brain function. The primary technologies are optogenetic – the modulation of transgenic receptors and channels via photons-- and pharmacogenetic – the modulation of transgenic receptors via pharmacologic agents. Here I will focus on pharmacogenetics.

First and foremost, I present an alternative name for this technology to disambiguate the topic from other uses of the word pharmacogenetic. The term "pharmacogenetic" is already an established MeSH (Medical Subject Headings) term, defined as "a branch of genetics which deals with the genetic variability in individual responses to drugs and drug metabolism". This word has been well adopted, retrieving 3417 results from PubMed as of October 10th, 2012, has been in use for an extended period of time (Gonzalez-Vacarezza *et al*, 2012; La Du, 1972; Weinshilboum *et al*, 1999) and is cited as the foundation of personalized medicine (Cohen, 1997; Kohane, 2012). For similar reasons, the term "chemical genetics" (or its portmanteau chemicogenetic), while not being assigned its own MeSH term, has been defined as "the study of gene-product function in a cellular or organismal context using exogenous ligands" (Stockwell, 2000). Here I present an alternative terminology and

reimagination of pharmacogenetics (the modulation of transgenic receptors via pharmacologic agents) as *pharmacosynthetics*. This term integrates the true meaning and functional mechanisms of the technology: *pharmaco-* meaning drug and *-synthetic* meaning the combination of two or more parts in an artificial manner. *Pharmacosynthetics* provides a clear distinction from both pharmacogenetics and chemicogenetics and, to date, has not been used to describe any phenomenon or identify any technology.

I present the formal definition of pharmacosynthetics as "a branch of biology which deals with the creation of pharmacological modulation using artificial components". While it is possible to equate conventional drugs with pharmacosynthetics (or having been developed through pharmacosynthesis), there are distinctions within the semantics that should be explored to provide clarification. A chemical is synthesized to have a particular pharmacology, and this pharmacology is based on the system with which that chemical interacts. On the other hand, a pharmacosynthetic approach creates pharmacological modulation within a system using artificial components. While a pharmacological agent may be synthesized, at no point in this effort is the pharmacology of the agent created – instead, it is measured. In one way of thinking about it, a pharmacology (as defined as the study of drug action) is engineered for an otherwise inert chemical by engineering a receptor and inserting the receptor into a living system. On the other hand, when a novel chemical is synthesized, its pharmacology in a living system is studied to determine whether or not it is a drug or has drug-like properties.

The pharmacosynthetic tools currently utilized include the Designer Receptors Exclusively Activated by Designer Drug (DREADDs), the latest iteration of a long-standing concept of creating orthologous ligand-receptor pairs to remotely control cellular GPCR

signaling (Conklin *et al*, 2008). The original DREADDs were human muscarinic acetylcholine receptors engineered to be activated by clozapine N-oxide (CNO), an otherwise inert pharmacological agent. Additionally, DREADDs are insensitive to the endogenous ligand, acetylcholine. There are currently three DREADDs in common use – the hM3Dq that activates $G\alpha_q$ signaling, the hM4Di that activates Gαi signaling, and the rM3Ds that activates $G\alpha_s$ signaling. These three DREADDs share the same point mutations (Figure 1) that simultaneously engender CNO efficacy and acetylcholine inefficacy (Armbruster *et al*, 2007). The rM3Ds was engineered to couple $G\alpha_s$ by replacing intracellular loops 2 and 3 of the hM3Dq with those from the turkey β1-adrenergic receptor (Guettier *et al*, 2009). With these three DREADDs, it is possible to control 3 of the G protein signaling cascades found in the mammalian brain.

Other pharmacosynthetic tools have been developed in addition to DREADDs. The precursor to DREADDs, the Receptors Activated Solely by Synthetic Ligand (RASSL), were also G protein-coupled receptors that had been engineered to respond to synthetic ligands instead of their cognate ligand (Conklin *et al*, 2008). These receptors required the use of a knockout background of the parent receptor because the synthetic ligand still activates the native receptor. Additionally, these receptors often exhibited constitutive activity, rendering temporal control of signaling intractable. Another GPCR system is the allotstatin receptor (AlstR) and ligand, consisting of the *D. melanogaster* neuropeptide allostatin and its cognate receptor (Gosgnach *et al*, 2006). The system confers Gi/o signaling and can silence neurons through GIRK channel modulation. This system provides temporal and spatial control similar to DREADDs, but is more invasive due to the requirements of local microinjection of a neuropeptide. In addition to G protein modulation, pharmacosynthetic tools exist that

modulate ion conductance via ligand gated ion channels. Namely, the pharmacologically selective actuatory modules (PSAMs) and their cognate pharmacologically selective effector molecule (PSEM) agonists (Magnus *et al*, 2011) have been shown to be effective neuronal modulators. This system permits for the direct modulation of ion conductance via pharmacological means using chimeras of ligand-binding domains of the alpha7 nicotinic acetylcholine receptor and ion pore domains of other Cys-loop receptors. Similar to DREADDs, PSAMs were engineered to respond to PSEMs in a two-way selective manner, providing for exclusive control of neuronal signaling. Additionally, ivermectin-gated ion channels provide similar modulation of membrane ion conductance using glutamate-gated chlorid channel receptor (GluClR) activated by ivermectin (Lerchner *et al*, 2007), though this technology has not been extensively utilized.

Connecting receptor-mediated signaling to overt brain function is the defining challenge of neuropsychopharmacology research. The hypothesis that aberrant neuronal activity underlies neuropsychiatric disease combined with the knowledge that drugs modulate neuronal activity via receptors has fueled the persistence of this challenge. To date, small molecule therapeutics are the first line treatments for debilitating mental illness including schizophrenia, Parkinson's disease, and depression, to name a few. DREADDs offer a unique opportunity to study the neurophysiological correlates of therapeutic efficacy due to the nature of the DREADD technology and the therapeutic mechanisms of efficacy. First and foremost, DREADDs are G-protein coupled receptors – a drug target class of which 36% of all currently approved drugs either directly or indirectly modulate (Klabunde and Hessler, 2002). Furthermore, DREADDs are modulated in a drug-like fashion since the small-molecule ligand exhibits drug-like properties (Bender *et al*, 1994; Guettier *et al*, 2009).

Finally, therapeutic efficacy is most often obtained through modulation of diffusely expressed albeit specific drug targets (Roth *et al*, 2004). These three characteristics can only be mimicked via the systemic injection of drug and the dispersed expression of the DREADD. This similarity to conventional therapeutics may thus facilitate an immediate crossover of insights gleaned from research utilizing DREADDs to the physiological phenomena responsible and necessary for therapeutic efficacy.

DREADDs are capable of providing non-invasive temporal control of neuronal signaling for three important reasons that clearly distinguish DREADDs from previous pharmacosynthetic technology. The first is the two-way selectivity of the receptor-ligand pair, in that CNO does not modulate other known effectors in a biological system and that the engineered receptor is not activated by effectors present in the biological system. Secondly, DREADDs do not exhibit constitutive activity – i.e., in the absence of CNO, the DREADDs do not modulate neuronal signaling. The variant rM3Ds has been shown to exhibit constitutive activity in pancreatic beta cells (Guettier *et al*, 2009), though constitutive activity has not been observed in striatal neurons (See Section 2.4.1). Finally, the drug used to activate the DREADD is bio-available and drug-like, meaning that a simple administration method (injection, drinking water, food, etc.) can be used to modulate DREADD activity (Bender *et al*, 1994; Guettier *et al*, 2009). These advancements are perhaps the most important in terms of the ultimate goal of neuropsychopharmacology, as it permits the investigation of specific signaling states on changes in overt animal behavior with minimal invasiveness.

1.2. APPLICATION OF PHARMACOSYNTHETICS

1.2.1. Selective mimicry of endogenous receptors

One application is to use pharmacosynthetic constructs as very selective pharmacological agents. In this manner, DREADDs can be expressed in a neuronal population that matches a pharmacologically intractable endogenous receptor. The Gs-DREADD validation work in this thesis can be viewed as an example of this type of application, in which the DREADD enables selective modulation of a distinct population of neurons residing in a nucleus of heterogenous neuronal composition. In this study, the Gs-DREADD was driven by the adora2A gene in a bacterial artificial chromosome. In the mouse genome, the native adora2A gene drives the expression of the adenosine A2A receptor, which itself is a Gs-coupled receptor. To date, the availability of selective adenosine A2A agonists is limited, and CGS 21680 is the most commonly used. Thus, by mimicking the expression of the adenosine A2A receptor with the Gs-DREADD, we essentially created a highly selective, spatially restricted adenosine A2A receptor ligand-pair. Utilizing the DREADD for selective mimicry is limited, though, in that the DREADD must be of the same coupling-type as the endogenous receptor for which it is mimicking. In the instance above, the Gs-DREADD and the adenosine A2A receptor both couple to Gs signaling pathways. Indeed, the advent of functional selectivity (a.k.a agonist-directed trafficking, biased agonism) introduces complications (or potential benefits) that will be discussed in section 3.3). One can imagine applying pharmacosynthetics in this manner to mimic the effects of orphan receptor modulation, where receptors with unique neuronal expression patterns have no known modulators.

1.2.2. Creating drug-like modulation where none exists

Additionally, the DREADD system can be conceptualized of as a way to pharmacologically modulate spatially defined neuronal populations for which no pharmacological modulatory agents exist. Krashes et al. (2011) used DREADDs in this fashion to study the arcuate nucleus (ARC) of the hypothalamus. This nucleus has been implicated in regulating energy homeostasis and has therefore been a focus of intensive study for the understanding and treatment of obesity, with particular focus on agouti-related protein (AgRP) neurons expressed in this area. To date, investigative efforts into the function of these neurons have been limited to conventional genetic, invasive, and ablative approaches: overexpression of AgRP in transgenic mice, central administration of peptides, and ablation of AgRP neurons. The first approach removes the temporal specificity required for definitive experimentation, whereas the latter approaches introduce confounds associated with non-reversible and invasive administration techniques. Krashes et al. (2011) utilized DREADD technology to study the acute effects of AgRP neuronal activity. The hM3Dq DREADD was targeted to AgRP neurons using a Cre-recombinase dependent adeno-associated virus (AAV) injected into AgRP-Ires-cre mice. Following i.p. administration of CNO (0.3 mg/kg), hM3Dq-expressing mice began feeding and consumed almost four times as much food than control mice in the first half hour. Additionally, Krashes et al. infected the same neurons with the hM4Di to induce neuronal silencing and observed a decrease in food intake. This study demonstrates that DREADDs can be used to introduce pharmacological modulation to nuclei for which drug-like compounds do not exist.

This neuronal circuitry was further explored using virally mediated delivery of DREADDS by Atasoy et al. (2012). Although the above study demonstrated that AGRP

modulation modulated food intake, the fact that the AGRP neurons project to disparate brain nuclei raises the question of which of these downstream nuclei is integral for the ultimate behavioral effect of AGRP activation. This was determined through functional-connectivity mapping utilizing a combination of optogenetics and phamacosynthetic tools. In this study, the hM4Di was used to silence pro-opiomelanocortin (POMC) expressing neurons in the arcuate nucleus, a population of neurons modulated by AGRP neurons from within the same nucleus, to determine whether inhibition of these neurons modulated food intake. Atasoy et al. found that CNO (5.0 mg/kg) administration did not significantly alter food intake over 1 hour, but repeated treatment (5.0 mg/kg, 3 injections every 8 hours) increased food intake over a 24 hour period. The paraventricular hypothalamic (PVH) nucleus also receives AGRP innervation, and administration of CNO to mice expressing the hM4Di in these neurons caused an increase in food intake. Furthermore, these mice displayed an increase in break point during a progressive ratio operant task for food reinforcement, indicating that PVH suppression itself can mimic the food seeking and food consumption effects of whole-circuit AGRP activation. These experiments were complemented by activating all of the AGRP neurons via systemic CNO injection and then selectively silencing the projections to the PVH using GABA or NPY antagonists. Even with activation of brain-wide AGRP neural circuits, selective inactivation of these projections caused signifantly reduced food intake. The use of pharmacological agents provides a physiological context for the circuitry described in this report.

A similar approach was used to modulate orexin neurons in the lateral hypothalamic area. These neurons have been implicated in sleep and wakefulness and produce the neuropeptides orexin A and orexin B. As mentioned above, the pharmacological modulation

of neuropeptide systems is historically difficult due to issues of invasiveness (local microinjection of purified peptide), lack of temporal control (genetic modulation) or off-target effects (physical ablation). Using virally mediated gene transfer, Sasaki et al. (2011) was able to express the hM3Dq and hM4Di in the orexin neurons of the lateral hypothalamic area. Following intraperitoneal administration of CNO (5.0 mg/kg) during the light phase (when mice typically sleep), the percent of wakefulness during the following hour was significantly greater and the NREM time was significantly shorter. Similarly, administration of CNO during the dark phase (when mice are typically awake), caused a significant increase in wakefulness. Conversely, administration of CNO to mice expressing the hM4Di (the inhibitory DREADD) in the orexin neurons decreased wakefulness during the dark phase and the light phase.

Introducing pharmacological modulation to non-druggable neuronal populations was also demonstrated by Ray et al. (2011). Here, the hM4Di (referred to as Di in the report) was expressed in serotonergic neurons of the brainstem using conditional intersectional genetics. In normal mice, an increase in the amount of carbon dioxide inhaled causes an increase in breathing. Upon administration of CNO (10 mg/kg), the hM4Di transgenic mice had a blunted response to increased CO_2 concentrations. These data indicate that the serotonergic neurons of the brainstem are involved in the modulation of respiratory activity. Additionally, they found that CNO injection decreased oxygen consumption in room air independent of ventilation modulation, indicating that these neurons can modulate metabolic rate independent of respiration. Furthermore, this group found that modulation of these neurons caused long-term changes in body temperature (up to 10 hours in duration), and that this effect became desensitized following repeated CNO treatment.

1.2.3. Encoding and modulating diffuse neuronal ensembles

A very elaborate application of pharmacosynthetics was performed by Garner et al. (2012) in which the neuronal ensemble encoding a memory trace was captured and recreated by DREADDs. To achieve this engram capturing ability, a transgenic mouse was designed to express the hM3Dq in active neurons during a conditioning task. This was achieved using a c-fos promoter-driven tTa transgenic mouse crossed with the TRE-hM3Dq mouse. The protein c-fos is a transcription factor that is upregulated in response to increases in neuronal activity. Thus, by using the c-fos promoter sequence, the active neurons of transgenic mice are labeled with hM3Dq receptor. These labeled neurons can then be activated via CNO administration to re-create the neuronal activity pattern responsible for the initial neuronal labeling. Garner et al. used these mice to determine whether a synthetic memory trace could be formed and re-created using pharmacosynthetics. To do this, they used a fear conditioning protocol in which a mouse is electrically shocked in a novel environment. In this paradigm, a mouse learns very quickly that particular environments are associated with an electric shock, and when later placed in the shock-paired environment will "freeze" – a well characterized behavior associated with learned fear. During these sessions, the neurons involved in the formation of these memories will become more active and thus express the hM3Dq. Interestingly, they found that the synthetic memory trace encoded by the DREADD-tagging could not produce a fear response on its own. The DREADD-encoded memory trace, however, was powerful enough to interfere with a naturally encoded memory trace. This study exemplifies the ability of pharmacosynthetics to modulate a diffuse but specific population of neurons.

1.2.4. Long-lasting specific neuronal modulation

A useful aspect of pharmacological modulation is the duration of activity inherent in the pharmacological approach. In addition to the pharmacokinetics of CNO activation, the hM3Dq, hM4Di, and rM3Ds have been shown to produce long-lasting neuronal modulation exceeding what can be explained by pharmacokinetics alone (see table 1 for summary). This longevity of action can be utilized in studies requiring chronic modulation of neuronal activity. For example, to determine the influence of neuronal circuit activity on excitatory synaptogenesis in medium spiny neurons, Kozorovitskiy et. al (2012) administered a chronic regimen of CNO (1.0 mg/kg, twice-daily, from post-natal day 8-15) to mice pups that had been infected at post-natal day 0-1 with an AAV carrying a cre-dependent hM4D. In this study, D1 and D2-Cre mice were used to target expression of the hM4Di in the two primary neuron populations of the striatum. The chronic regimen of CNO administration, theorized to produce long-lasting neuronal activity modulation, dampened the synaptogenesis of direct or indirect pathway MSNs as measured by mEPSC frequency and spine density on day 15 in mice expressing hM4Di throughout the striatum. In mice expressing the hM4Di unilaterally in a more dispersed manner (theorized to not effect circuitry activity), there was no effect of CNO on synaptogenesis. To determine whether the corticostriatal neurons (those that project to the striatum from the cortex) could influence synaptogenesis, the hM4D AAV was injected to the Rbp4-cre mouse, a mouse expressing Cre in corticostriatal neurons. These mice were treated with the same chronic regimen of CNO. On day 15, decreased mEPSC frequency and spine density was observed, indicating a decrease in synaptogenesis. Similar results were also observed when measured at postnatal day 25-28, indicating these changes persist into

adulthood. This study demonstrates the ability of the pharmacosynthetic approach to provide chronic neuronal modulation.

1.3. CONSIDERATIONS AND IMPLICATIONS OF THE PHARMACOSYNTHETIC APPROACH

Pharmacosynthesis requires a consideration of many factors to be effectively utilized. The key elements to be considered are the expression of DREADD and the dose of CNO required for experimental manipulation. Here I provide a primer on the consideration of these elements.

1.3.1. Expression systems

The primary challenge in pharmacosynthetics is inserting the DREADD receptor into the desired tissue of the model organism. To date, this has been achieved using virally mediated gene transfer and the genomic insertion of a transgene. Although a full review of each approach is beyond the scope of this dissertation, the benefits and complications of each approach will be briefly discussed.

1.3.1.1. Virally Mediated Gene Transfer

Virally mediated gene transfer utilizes viral mechanisms to insert the DREADD DNA into a neuronal population. There are many different types of viruses available, each having their own benefits and drawbacks. The reader is directed to reviews and applications of viral vectors and their utility (de Silva and Bowers, 2011; Foust *et al*, 2009; Teschemacher *et al*, 2005; Weinberg *et al*, 2012; Wu *et al*, 2006). The primary benefit of the viral approach is the relatively quick turnaround between project conception and functional animal model expressing the DREADD. In addition to the expediency provided by this approach, the local microinjection of viral particles enhances the spatial specificity of DREADD expression.

Finally, the viral approach permits utilization of the DREADD in model organisms for which transgenic approaches are not available or widespread (e.g., rats, monkeys). The drawbacks of the viral approach arise from the nature of local microinjections and the size limitations of viral packaging. This vector delivery method is invasive, potentially inducing an immune response and causing damage to tissue, including cell populations either directly or indirectly involved in the scrutinized output. Additionally, the spatial resolution provided can also be a limitation, in that DREADD expression is limited to the number of microinjection sites and the spread of viral particles. This latter point, however, can be a benefit depending on the goals of the study. Furthermore, the expression pattern of DREADDs between animal subjects will not be precisely identical due to differential stereotactic coordinate alignment, inconsistent viral diffusion, and experimental variation. Finally, viruses are only capable of carrying a certain quantity of DNA, potentially limiting the addition of desirable vector traits including targeting information (promoter sequences) and cell-type markers (fluorescent proteins).

1.3.1.2. Genomic Insertion of Transgene

Genomic insertion of a DREADD-encoding transgene involves the creation of a new line of genetically modified mice or the selective breeding of extant transgenic lines. The benefits of the transgenic approach are inherent to genome-level insertion of a transgene. First and foremost, the transgenic line of mice carries the transgene and expresses the DREADD in accordance to the genetic information in the transgene. Thus, no invasive interventions are necessary to obtain DREADD expression, providing a truly non-invasive means of cell-type specific signaling. Secondly, DREADDs can be expressed in a more dispersed manner using a transgenic approach. Whereas virally mediated gene transfer can

only provide expression of the DREADD in the area of viral diffusion, the transgene inserted into the genome is present in all cells. Expression of the DREADD is dependent upon the information contained in the transgene, and while the genetic sequences that confer cell-type specificity of expression are still a matter of research, certain promoter sequences have been determined. Thus, while the noninvasive component is definitively beneficial, the utility of dispersed expression patterns is dependent upon the research goals.

One type of transgenic approach is to create a new transgenic line to suit the needs of the intended research. When creating a new line of DREADD transgenic mice, the DREADD-encoding construct is inserted downstream of a promoter that is useful for the research goal. For instance, the Gs-DREADD mice described above utilized the adenosine A2A receptor bacterial artificial chromosome (BAC) to drive DREADD expression in striatopallidal medium spiny neurons, an area where the adenosine A2A receptor is naturally enriched. This permitted the selective activation of Gs signaling in those neurons in mice carrying a single transgene. One benefit of the single transgene approach is breeding efficiency: hemizygous breeding strategies can produce 50% transmission with the end result being 50% of the litter can be used for study. Additionally, because cell-type specificity is transmitted in one transgene, these mice can be easily crossbred with other types of engineered mice. The downside of this approach is that these single-transgene mice can only be used to study the cell population originally intended. An additional downside is that efforts to identify the genetic information responsible for tissue targeting have been, for the most part, unsuccessful, though a select few promoters have been commonly used in neuroscience research. One way to overcome this lack of knowledge is to knock-in the transgene, such that the endogenous promoter for a given neuronal subtype carries the

DREADD sequence in addition to the endogenous gene product. To avoid interfering with a particular genomic locus, an alternative transgenic strategy is to create a transgene containing the entirety of the genetic information associated with a particular cell-type specific protein. This approach can be achieved by using bacterial artificial chromosomes, which are capable of carrying 200-300 kb of genetic information, a drastically larger amount than other transgenic approaches utilize (Heintz, 2001). For instance, the camKII-alpha promoter sequence is 8.5 kb (Tsien *et al*, 1996), whereas the adora2A BAC is 175 kb. Finally, creating a transgenic mouse is both resource intensive and the effort has no guaranteed yield, depending on the strategy.

Alternatively, targeting DREADD expression to a cell type of interest can be achieved using conditional intersectional genetics. This approach involves the selective breeding of extant transgenic lines to produce mice carrying multiple transgenes; i.e., polytransgenic mice. In these mice, the expression specificity of the DREADD depends on the design of the transgenes, an approach called "intersectional genetics". A handful of technologies are available for this approach though the number of transgenic mice carrying these technologies is ever expanding. Prominently in use are the Cre, Flp, and Tet technologies, all of which are based on proteins and DNA sequences exogenous to the targeted model organism (in this case, the mouse) (Mallo, 2006). The Cre and Flp systems are based on the recombinases and their associated DNA targeting sequences. In these systems, one mouse carries a transgene for the Cre or FLP protein in which the protein sequence is downstream of a promoter that drives expression in a particular cell population (cell population A). A second mouse has a separate transgene, in which the DREADD sequence is inserted downstream of a ubiquitous promoter followed by a stop cassette that is flanked by either the Cre excision sites (LoxP) or

the Flp excision site (Flpe). Thus, in the cell population that expresses Cre or Flp, the stop cassette is removed from the genetic sequence. The nuclear expression machinery can then translate the DREADD sequence into protein in that cell population. In the cells that do not express Cre or Flp, the DREADD transgene remains silent due to the presence of the stop cassette. The Cre / Flp systems can also take advantage of a second phenomenon of the recombinatorial proteins in that they can reverse the direction of the sequence between the excision sequences. Dependent upon the orientation of the excision sequences, the Cre and Flp can either excise the bookended DNA or flip the direction (Atasoy *et al*, 2008). The "reversal" approach is less leaky than the excision method; i.e., the intended specificity of expression is more likely to occur.

In the Tet system (Kistner *et al*, 1996), one mouse is designed with a transgene containing the tet trans-activator (Tta) sequence downstream of a cell-type specific promoter. A second mouse is designed with a transgene containing the DREADD sequence downstream of the tet-response element (TRE) sequence. When the two mice are interbred to create double-transgenic offspring, the tet trans activator binds to the tet response element and permits DREADD expression. This system also permits for temporal control by the administration of doxycycline to the organism. Doxycycline binds to the trans activator and blocks its interaction with the tet response element, ultimately inhibiting expression of the DREADD. Alternatively, the reverse trans activator (rTta) can be used, in which doxycycline administration is necessary for the interaction of the trans activator and the response element. In this version of the system, doxycycline can be administered to induce the expression of the DREADD. The tet system has been enhanced recently with the tet-on 3G system (Fan *et al*, 2012), but the underlying principles remain.

1.3.1.3. Combinatorial approaches

Already the world of neuroscience research is seeing the full implementation of these technologies and the benefits of combining them. For example, the Krashes et al. (2011) and Sasaki et al. (2011) studies combined the specificity of expression provided by the genomic transgene approach with the spatial resolution and quick turnaround of the viral approach to achieve cell-type specific neuronal modulation. The Ray et al. (2011) study used intersectional genetics, combining Cre and Flp recombination to increase the specificity of DREADD expression with minimal invasiveness. At this point, the ability to target DREADD expression to specific tissue populations depends on the transgenic state of the art.

1.3.2. CNO Doses and Routes of Administration

In the articles mentioned above, a range of CNO doses is used to obtain effects at the whole-organism level, and these doses are summarized in Table 1. The dose of CNO required varies depending on the expression system, localization of expression and type of DREADD. For example, the hM3Dq DREADD is very effective at depolarizing neurons, and thus, relatively low doses can be used to elicit an effective neuronal response (Alexander *et al*, 2009) . On the other hand, the hM4Di is reportedly less effective at inhibiting neuronal firing, so higher doses of CNO are occasionally used (Ray *et al*, 2011; Sasaki *et al*, 2011). Beyond the inherent differences between Gq and Gi modulation of neuronal excitability, the effects of the CNO-DREADD mediated manipulation depend on the tissue distribution of the DREADD. For example, the hM3Dq mice originally characterized express the Gq DREADD in all neurons of the cerebrum using the calmodulin II kinase alpha promoter, creating a condition in which low doses of CNO could influence neuronal activity on a large scale (Alexander *et al*, 2009). Conversely, the transgenic mice used in Garner (2012) used the c-

fos promoter to drive expression of the hM3Dq, so the DREADD was expressed at lower levels and higher doses of CNO were apparently necessary. Furthermore, Krashes (2011) used a viral approach to express the hM3Dq in a small nucleus and administered 5.0 mg/kg CNO to elicit a response in these mice. From the body of work performed with DREADDs to date, it can be seen that the dose of CNO is variable and dependent on the type of DREADD and the expression system used.

To date, a majority of studies performed have used the intraperitoneal route of administration, though other routes of administration are possible. Our lab has demonstrated that CNO can be administered through the drinking water to create chronic administration conditions (10 mg/kg/day, unpublished observations).

1.4. LIMITATIONS OF CURRENT PHARMACOSYNTHETIC TECHNOLOGY

1.4.1. Pharmacological vs. Physiological Manipulation

Pharmacosynthetic modulation of neuronal signaling differs from physiological modulation of neuronal signaling that exists within the brain. Firstly, the drug-like modulation of receptor-mediated signaling is qualitatively different than the endogenous neurotransmitter modulation of receptor-mediated signaling. Whereas administration of a drug causes a uniform modulation of all available receptors simultaneously and with relatively similar concentrations, neurotransmitter modulation is dependent on the activity of neurons. These different types of modulation can be discribed as wiring transmission and volume transmission (Agnati *et al*, 2010). Pharmacological, and therefore pharmacosynthetic, modulation is akin to volume transmission. Neurons release neurotransmitter in a phasic, bursting nature, creating waves of neurotransmitters in the synaptic cleft to influence wiring transmission while simultaneously creating a tonic level of

modulation due to synaptic leakage to potentiall influence volume transmission (Goto *et al*, 2007). This phasic nature of ligand-induced signaling can not be replicated using pharmacosynthetics due to the uniform distribution of a pharmacological agent. Furthermore, the site of action of a neurotransmitter is mostly restricted to the synapse, though extra-synaptic receptors are present. This differential localization of endogenous receptor creates the possibility for differential response to neurotransmitter presence, based on the "leakage" from the synapse. This differential response will not be observed in a pharmacosynthetic system due to the uniform distribution of ligand. For these reasons, pharmacosynthetic tools are best utilized to study a pharmacological response of a system as opposed to the physiology of the system itself.

1.4.2. Limitations of Technology

To date, the primary consideration in the design of pharmacosynthetic tools has been the control of specific signaling types: G protein signaling pathways or ion conductances. Beyond the signaling types afforded by these receptors, there are other aspects of receptor proteins that ultimately influence the effects of receptor signaling. Firstly, the differential localization of a receptor can influence the effect of drug modulation. For instance, synaptic GPCR signaling can have differential effects than extrasynaptic GPCR signaling (Fuxe *et al*, 2012). To date, the targeting of DREADDs to specific cellular compartments is dependent on the targeting and trafficking information contained within the protein sequence of the muscarinic receptor, the endogenous receptor from which the DREADD was engineered. Studies into the localization of the muscarinic receptors are still underway, though it is known that the muscarinic M3 receptor is found in spiny dendrites and axon terminals (Nathanson, 2008). Furthermore, the targeting of the receptor construct validated in my

research (rM3Ds) is influenced by the presence of the turkey beta1 adrenergic receptor loops. Therefore, the targeting of the rM3Ds could be influenced by the trafficking information of both the muscarinic M3 receptor and the beta1 adrenergic receptor. The current targeting and trafficking information encoded by the available DREADDs represents the state-of-the art and is an area requiring further development to enhance the capabilities of pharmacosynthetic technology.

1.5. CONCLUSIONS

1.5.1. Key Differences Between Optogenetics and Pharmacosynthetics

It is important to note the differences between the pharmacosynthetic approach and the optogenetic approach. Using optogenetics, one can gain precise spatio-temporal control of neuronal firing using a combination of light and transgenic expression of engineered receptors. Among the optogenetic tools available, one difference is the level of invasiveness required for experimental manipulation when compared to pharmacosynthetics. Using optogenetics, one must deliver light to neurons- a process that currently requires implantation of fiber optics into the brain. Notwithstanding the proper implementation of control conditions, experiments can be imagined for which survival surgeries (and the subsequent hardware attachment) present insurmountable confounds. In its most noninvasive form - genomic transgene – pharmacosynthetic manipulation can be achieved with minimal invasiveness to the organism using peripheral administration of CNO (or even via food or drinking water). However, the prevalent use of virally mediated gene transfer in pharmacosynthetics (Table 1) would indicate that the level of initial invasiveness between approaches is similar. A second difference is the type of neuronal modulation afforded. Currently, the most widespread variation of optogenetics utilizes the channelrhodopsins, a

family of light activated ion channels that directly modulate the ion conductance of neuronal membranes and either hyperpolarize or depolarize neurons. In contrast, DREADDs modulate G-protein mediated signaling – signaling cascades for which neuronal hyperpolarization or depolarization are only one outcome. However, there are optogenetic tools available – the OptoXRs - that modulate G-protein mediated signaling using light, though these have not gained widespread use.

Whereas the *type* of signaling afforded by these two approaches is not considerably different, nor the *level of invasiveness* considering the use of viral-mediated gene transfer, it can be argued that the *nature* of the neuronal modulation provided by these two technologies is what clearly separates them as distinct experimental tools with considerably different utility. The nature of the neuronal modulation created using pharmacosynthetics more closely resembles the hormonal signaling mechanisms found in the brain, in that the modulatory agent (CNO) "lingers" in the extraneuronal space and activates signal transduction mechanisms (GPCRs) dependent on diffusion, clearance, and receptor regulatory mechanisms (internalization, desensitization). This nature of activity more closely resembles the nature of metabotropic neurotransmitter or drug activity than any other process. On the other hand, optogenetics more closely resembles the electrochemical functionality of neurons. That is, the manipulation of membrane conductance more closely resembles the end result of factor-induced neuronal changes. In other words, pharmacosynthetics permits for the study of neuronal modulation itself, whereas optogenetics permits for the study of what a modulated neuron actually does in the brain. While these differences should fundamentally affect the type of experimentation performed with the respective technologies, pharmacosynthetics has yet to be fully implemented in this fashion.

1.5.2. Concluding remarks

Pharmacosynthetics provides an effective means to study the physiological effects of artificial, drug-like modulation of distinct neuronal populations. The specificity and exclusivity of GPCR modulation afforded by the technology can be utilized to explore mechanisms of therapeutic efficacy to further the development of small-molecule therapeutics. Further development of pharmacosynthetics will create levels of experimental manipulation that will challenge our conventional understanding of signal transduction and pharmacology. With this reimagination of this technology, it can be seen that the pharmacosynthetic approach provides unprecedented ability to truly answer the defining question of neuropharmacology – *how do drugs modulate brain activity?*

CHAPTER 2. NEURONAL VALIDATION OF GS COUPLED DREADD (RM3DS)

2.1. INTRODUCTION

Circuits of the basal ganglia, a brain region responsible for voluntary behavior and reward, are implicated in a variety of neuropsychiatric disorders including Parkinson's disease, Huntington's disease, Obsessive Compulsive Disorder, and addiction. A more thorough understanding of the signaling circuits that modulate basal ganglia function could lead to the development of better therapeutics for these disorders. The ability to exclusively modulate the intracellular signaling pathways of distinct circuits *in vivo* would be an extremely useful tool to facilitate studies investigating how circuits function in the brain. To this end, the Roth laboratory has recently created and validated a family of designer G protein-coupled receptors (GPCRs) that facilitate exclusive modulation of G protein signaling pathways. A family of Designer Receptors Exclusively Activated by Designer Drug (DREADDs) was developed such that the receptors (1) are not activated by their native ligand, acetylcholine; (2) lack any detectable intrinsic activity; and (3) are selectively activated by the bioavailable, inert synthetic ligand, clozapine-N-oxide (CNO), a metabolite of clozapine. By coupling DREADD technology with a transgenic approach to target DREADD expression to a specific subpopulation of neurons, it may be possible to achieve exclusive modulation of intracellular signaling pathways in distinct circuits *in vivo*. This proposal investigates the hypothesis that the $G\alpha_s$ DREADD, expressed in a specific cell type, allows for the spatiotemporal control of $G\alpha_s$ signaling in distinct circuits in vivo, and further

tests the utility of DREADDs in interrogating the relationship between intracellular signaling in specific neuronal circuits and behavior. This enabling technology has the potential to provide neuroscientists an innovative research strategy (pharmacological control of cell type-specific signaling with unprecedented precision) to advance towards Strategic Objective 1 of the National Institute of Mental Health (promote discovery in the brain and behavioral sciences to fuel research on the causes of mental disorders). Ultimately, the knowledge obtained could yield new insights into basal ganglia function.

2.2. THE STRIATUM AS AN IDEAL REGION FOR TESTING SELECTIVE CONTROL OF NEURONAL SIGNALING IN DEFINED NEURONAL POPULATIONS.

Cell-type specific control of neuronal signaling can provide valuable insight into the neuronal correlates of behavior, disease, and mechanisms of therapeutic efficacy. Medium spiny neurons (MSNs) are the projection neurons of the striatum and are segregated into two populations defined by their efferent projections and their neuropeptide and receptor expression profiles. Striatonigral MSNs project to the substantia nigra and express dynorphin and substance P neuropeptides, in addition to being enriched in D1-dopamine receptors. Striatopallidal MSNs project to the globus pallidus, express enkephalin and are enriched in D2-dopamine and A2A-adenosine receptors (Ferre *et al*, 1992; Gerfen *et al*, 1990; Svenningsson *et al*, 1998). The activation of striatopallidal neurons is thought to produce an inhibitory effect on motor behavior (DeLong, 1990; Kravitz *et al*, 2010), and these D2-dopamine receptor containing neurons have been implicated in both the etiology and potential therapy of many neuropsychiatric diseases (Beaulieu and Gainetdinov, 2011;

Emilien *et al*, 1999). The distinctive functional and biochemical composition of these neurons present a suitable region to validate the ability of the DREADDs to selectively control cell type specific neuronal signaling.

The $G\alpha_s$- and $G\alpha_i$- G protein signaling cascades, modulated by D1- and D2-receptors, respectively, are implicated in both the short-term excitability and the long-term plasticity of MSNs (Centonze *et al*, 2001; Surmeier *et al*, 2007). Striatal G protein signaling cascades have primarily been studied as a consequence of activating dopamine receptors, but activation of $G\alpha_s$ signaling downstream of other GPCRs also has significant effects. For example, striatopallidal $G\alpha_s$ signaling modulated by the A2A-adenosine receptor influences psychostimulant activity (Brown and Short, 2008). To create a mouse model in which the cellular and behavioral consequences of striatopallidal-specific Gs-type signaling (G protein signaling that increases cAMP production) can be studied, we took advantage of technology we developed whereby evolved GPCRs (DREADDs, or *D*esigner *R*eceptor *E*xclusively *A*ctivated by *D*esigner *D*rug) are expressed in a cell-type-specific fashion to remotely control cellular signaling (Armbruster *et al*, 2007).

In prior work, the ability of the rM3Ds DREADD to remotely control Gs signaling in an inducible and cell-type specific fashion was demonstrated by transgenic expression of rM3Ds in pancreatic β-cells (Guettier *et al*, 2009). Here, I validate the rM3Ds in a neuronal context *in vivo* by creating a bacterial artificial chromosome (BAC) transgenic mouse line carrying the Gs-DREADD (rM3Ds) downstream of the adora2A (adenosine A2A receptor) promoter. Adora2A-rM3Ds transgenic mice afford a unique pharmacosynthetic means to specifically modulate $G\alpha_{s/olf}$ signaling *in vivo* in a spatially and temporally-controlled fashion using the pharmacologically inert, designer drug clozapine N-oxide (CNO; see (Armbruster *et al*,

2007)). Herein, I use this novel transgenic line to validate the rM3Ds by measuring the biochemical, electrophysiological, and behavioral consequences of CNO administration to adora2A-rM3Ds transgenic mice.

2.3. METHODS

Plasmids: The plasmid map of the p-rM3Ds-IRESmCherry construct is detailed in Figure 2.

Drugs: Clozapine N-oxide (CNO) was obtained from the NIH as part of a Rapid Access to Investigative Drug Program funded by the National Institute of Neurological Disorders and Stroke (NINDS). D-amphetamine (AMPH) and isoproterenol were purchased from Sigma (St. Louis, MO). For experiments in mice, CNO was first dissolved in DMSO then brought to final concentration with 0.9% saline and a final concentration of DMSO of 0.5%. Amphetamine was dissolved directly into 0.9% saline. For all experiments, the appropriate (e.g., 0.9% saline for amphetamine experiments and 0.5% DMSO in saline for CNO experiments) vehicle controls were utilized. Unless otherwise noted, the dose of CNO was 1.0 mg/kg and the dose of amphetamine was 2.0 mg/kg. Drugs were injected intraperitoneally (i.p.) at a volume 100 ul /10 g body weight. For *in vitro* studies, drugs were dissolved in DMSO at 10 mM as stocks and then diluted into sample buffer.

In vitro studies - cAMP accumulation in neurons: The IRES sequence was cloned from the pIRES-neo vector (Clontech, Palo Alto, CA) into an mCherry vector (Shu *et al*, 2006) using the Xi-clone High-Speed Cloning Kit (Gene Therapy Systems, Inc., San Diego, CA) to generate pIRES-mCherry by Ying Pei. The coding region for rM3Ds was subsequently subcloned by Ying Pei into pIRES-mCherry upstream of the IRES sequence to generate p-rM3Ds-IRESmCherry.

Lentiviral studies were done as previously described with modification (Abbas *et al*, 2009; Alexander *et al*, 2009) by Ying Pei. To generate a lentiviral construct, the coding region for rM3Ds-IRESmCherry was subcloned into the lentiviral expression vector FUGW (Lois *et al*, 2002), a gift from Dr. Guoping Feng (Duke University). Fugene6 (Roche Applied Science, Indianapolis, IN) was used to co-transfect seven 150 cm^2 dishes of HEK293T cells with the FUGW plasmid and two viral packaging constructs (Δ8.9 HIV-1 and VSVG) in a ratio of 3.3:2.5:1. Lentivirus-containing media was collected 48 hours post-transfection. Virus was concentrated by centrifugation and Amicon ultra-15 centrifugal filter devices (Millipore, St. Louis, MO), aliquoted, and frozen at -80°C until use. Rat cortical neurons were infected with FUGW-rM3Ds-IRES-mCherry as previously described (Alexander *et al*, 2009). Two days following infection, cells were exposed to increasing concentrations of CNO, and cAMP accumulation was quantified using the Catchpoint assay per manufacturer's instructions (Molecular Devices).

Assessment of cAMP production in HEK293T cells: Agonist-induced cAMP production was measured in living cells as described previously (Abbas *et al*, 2009; Kimple *et al*, 2009) by Vincent Setola. HEK293T cells were maintained in DMEM with L-glutamine, 1 g/l glucose, 10% fetal bovine serum (all from Cellgro) (C/H medium). The day before transfection, the cells were seeded in 10-cm dishes (Greiner) in C/H medium (4 million cells/plate). The next day, cells were transfected (using the calcium phosphate method) with 2 μg of the pGloSensor-22F cAMP biosensor (Promega) and various amounts of expression vectors for the G_α subunits and the hM3/turkey beta1AR chimer DREADD (rM3Ds) at the indicated ratios. Empty pcDNA3.1(+) was used as an inert vector so that each plate was transfected with similar amounts of DNA (12 μg). The next day, the cells were harvested

with dilute trypsin, resuspended in 1X HBSS (with calcium and magnesium) (Invitrogen) supplemented with 20 mM HEPES, pH 7.4 (drug buffer), counted, and diluted to 15,000 cells/20 microliters. The cell suspension was added to white 384-well plates (Greiner) (20 microliters/well). After a 1-2 hr incubation, the cells were challenged with 10 microliters/well of 3X working dilutions of CNO (for concentration-dependent activation of rM3Ds) or isoproterenol (for concentration-dependent activation of endogenously expressed beta2AR). The 3X working dilutions were prepared in drug buffer containing 6% (i.e., 3X) GloSensor reagent (Promega). Ten minutes after agonist challenge, the luminescence was counted (1 s/well) on a TriLux (Perkin Elmer) microbeta/luminescence plate reader. For each transfection condition (rM3Ds +/- G_{α}), the luminescence per well was expressed as a function of the log [agonist], and the data were fit using a three-parameter logistic equation as described previously (Alexander *et al*, 2009). Best-fit pEC50 and Emax values +/- SE were compared across transfection conditions and between agonists.

Animal Subjects: Behavioral, biochemical, and electrophysiology experiments were performed at the University of North Carolina and Duke University in accordance with the National Institutes of Health's guidelines for the care and use of animals and with approved animal protocols from the Institutional Animal Care and Use Committees of the aforementioned institutions.

Generation of adora2A-rM3Ds mice: Transgenic mice were created by the Duke Neurotransgenic Core (Durham, NC) using standard techniques previously described (Gong *et al*, 2003). The rM3Ds-IRES-mCherry construct was recombineered into the adenosine2A bacterial artificial chromosome (BAC; GENSAT1-BX868, BAC address: RP24-238K3) downstream of the endogenous ATG codon, and this adora2A-rM3Ds-IRES-mCherry BAC

was then injected into the pronucleus of B6SJLF1/J mouse oocytes. Genotyping was performed by PCR of genomic DNA extracted from tail clips using the following primers for mCherry: FW 5′-GTGAGCAAGGGCGAGGAGG-3′ REV: 5′-GTCGGCGGGGTGCTTCAC-3′ using the following cycle: Initial denaturation: 94 – 4 minutes, followed by 30 cycles of 94°C 30s / 65°C 30s / 72°C 30s, followed by 72°C 4 minute final extension. PCR products were analyzed using gel electrophoresis (1%, Aqua Por LE, National Diagnostic, Atlanta, GA), and rM3Ds positive mouse samples present a clear band at 200 bp (Figure 2d). From this screen, 9 genotype-positive mice were found. These mice were bred to wild-type (C57BL/6J) mice, and their offspring were screened for mCherry expression via immunohistochemistry for mCherry following the methods detailed below. Three mCherry-positive founder lines were identified and named AD6, AD8, and AD10. The AD6 line was used in the present study. Following initial screening, AD6 mice were bred onto the C57BL/6J background. The breeding strategy had consistent pairing with an AD6 mouse always paired with a C57BL/6J mouse; thus, all mice used in these studies were hemizygous for the adora2A-rM3Ds gene. The AD6 line of mice is referred to as adora2A-rM3Ds mice in this manuscript. Mice used for behavioral studies were bred in large cohorts using a harem-breeding strategy, and littermate pairings between conditions were used. All behavioral studies were performed on mice of the F3 generation or later. The amphetamine behavioral sensitization studies and behavior core screen were performed on the F3 generation. Electrophysiology studies were performed on the F6 generation or later.

Other lines of mice used: Drd1a-EGFP (000297-MU) and Drd2-EGFP (000230-UNC) reporter mice were obtained from the Mutant Mouse Regional Resource Centers and crossed with adora2A-rM3Ds mice for quantification of expression of rM3Ds in D1 and D2-

expressing neurons. C57BL/6J mice were obtained from Jackson laboratories (Bar Harbor, ME).

Immunohistochemistry and image analysis: Mice were anaesthetized with tribromoethanol (Avertin) and then transcardially perfused with 20 ml PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, 2 mM KH2PO4, pH 7.5) followed by 40 ml 4% paraformaldehyde (PFA) in PBS. Brains were removed and placed in 4% PFA overnight at 4°C gentle rocking. The following day, brains were placed in 30% sucrose PBS solution and continued to rock at 4°C. On day 3, when brains had sunk to the bottom of the tube, the brains were frozen on dry ice. Sections were obtained using a cryostat at 30 μm. Slices were processed either thaw-mounted to the slides or in a free-floating fashion. Samples were initially incubated in 0.5% TritonX-100 in PBS for 30 minutes RT, followed by a 30 minute incubation in blocking buffer (3% BSA 0.5% TritonX-100 in PBS) at RT. Samples were then incubated with primary antibodies overnight at 4°C in blocking buffer. The following day, samples were washed 4 X 10 with PBS 0.5% TritonX-100, followed by 1 hour RT incubation with secondary fluorescent-conjugated antibodies in blocking buffer. Primary antibodies used: anti-RFP, ab65856, 1:1000, AbCam, Cambridge, MA; Anti-GFP, A11122, 1:1000, Invitrogen, Carlsbad, CA. Secondary antibodies used: goat anti-rabbit AlexaFluor-488 and goat anti-mouse AlexaFluor-594 antisera (1:250, Invitrogen, Carlsbad, CA). Hoechst stain was added to secondary incubation at 1:2000 for some experiments. Fluorescent images were collected on a Nikon 80i Research Upright Microscope (Nikon, Tokyo, Japan) equipped with Surveyor Software with TurboScan (Objective Imaging, Kansasville, WI). Tiled images were collected with a Qimaging Retiga-EXi camera (Qimaging, Surrey, BC, Canada). For colocalization studies, images of coronal slices were analyzed using ImageJ software (NIH).

A region of interest in the body of the striatum was selected for N=3 mice, and the number of EGFP positive and mCherry positive cell bodies was quantified.

DARPP-32 study: Mice were injected i.p. with CNO (5.0 mg/kg), cocaine (20.0 mg/kg) or vehicle and then sacrificed 15 min later by cervical dislocation. The ventral striatum was isolated using a rapid head-freeze dissection technique as described previously (Beaulieu *et al*, 2004). Frozen tissue samples were probe sonicated in 95°C 1% SDS buffer containing 1X Halt phosphatase inhibitor (Halt, Pierce, 87786) and 1X protease inhibitor (Roche Diagnostics, Complete, no. 11697498001). Protein concentration of sample was determined using the BCA method (Pierce). Samples were boiled in Laemmli buffer, and 25 ug of protein were loaded into 10% SDS-PAGE gels, transferred onto nitrocellulose membranes, and incubated with antibodies to pT34 DARPP-32 (Phosphosolutions, Aurora, CO, p1025-34, 1:300, TBST / 3% BSA), total DARPP-32 (BD Transduction, San Diego, CA, 611520, 1:1500, TBST / 5% BSA), pERK1/2 (Cell Signaling, Danvers, MA, 9101, 1:500, TBST / 5% milk), total ERK1/2 (9107, 1:500, TBST / 5% milk), pAKT308 (2965, 1:100, TBST / 5% BSA), or total AKT (2920, 1:1500, TBST / 5% BSA). Blots were imaged on the LI-COR Odyssey instrument (LI-COR Biosciences, Lincoln, NE). The phospho-specific probe band intensity was measured using NIH ImageJ software and was normalized to total probe band intensity. The fold stimulation was determined by normalizing these values to the average of the vehicle treatment group.

Behavioral Phenotype of adora2A-rM3Ds mice: Twelve littermate pairs (7 male pairs, 5 female pairs) of adora2A-rM3Ds transgenic (rM3Ds) mice and wild-type (WT) mice underwent a broad survey of behavioral testing. Mice were approximately three months old when testing began. Procedures were conducted by an experimenter blind to mouse

genotype. Data were analyzed using one-way or repeated measures Analysis of Variance (ANOVA) to determine effects of genotype. Fisher's protected least-significant difference (PLSD) tests were used for comparing group means only when a significant F value was determined in the overall ANOVA. Within-genotype comparisons were conducted to determine side preference in the social approach test, and quadrant preference in the water maze. For all comparisons, significance was set at $p < 0.05$.

Testing Regimen: Mice were tested in the following procedures, with at least one or two days between each assay: elevated plus maze test for anxiety-like behavior, neurobehavioral screen, activity in an open field, accelerating rotarod (2 tests, 48 hours apart), social approach test, acoustic startle test, buried food test for olfactory ability, visual cue test in the Morris water maze, hidden platform test for spatial learning, reversal learning in the Morris water maze, hotplate test for thermal sensitivity.

Elevated plus-maze test: Mice were given one 5-min trial on a metal plus-maze, which had two closed arms, with walls 20 cm in height, and two open arms. The maze was elevated 50 cm from the floor, and the arms were 30 cm long. Animals were placed on the center section (8 cm x 8 cm), and allowed to freely explore the maze. Arm entries were defined as all four paws entering an arm. Entries and time in each arm were recorded during the trial by a human observer via computer coding. Percent open arm time was calculated as 100 x (time spent on the open arms/time in the open arms + time in the closed arms). Percent open arm entries was calculated using the same formula.

Neurobehavioral screen for reflex, sensory, and motor impairment: The neurobehavioral screen consisted of several measures to assay overall appearance and behavior of the mice. Measures included general observations on coat condition, body

posture, and normality of gait. Normal reflexive reactions to a gentle touch from a cotton-tipped swab to the whiskers on each side of the face, and the approach of the swab to the eyes, were assessed. Each subject was placed in a small, empty plastic cage, and ability to remain upright when the cage was moved from side-to-side or up-and-down was noted. Locomotor coordination was assayed by allowing the mouse to walk across an elevated dowel (wrapped in nylon rope to facilitate grasping) and to climb down a similar pole. Each subject was also placed on a wire grid and allowed to hang for one minute. Reaction to 20 seconds of tail-suspension was recorded.

Buried food test for olfactory function: Several days before the olfactory test, an unfamiliar food (Froot Loops, Kellogg Co., Battle Creek, MI) was placed overnight in the home cages of the subject mice. Observations of consumption were taken to ensure that the novel food was palatable to the mice. Sixteen to twenty hours before the test, all food was removed from the home cage. On the day of the test, each mouse was placed in a large, clean tub cage (46 cm L x 23.5 cm W x 20 cm H), containing paper chip bedding (3 cm deep), and allowed to explore for five minutes. The animal was removed from the cage, and one Froot Loop was buried in the cage bedding. The animal was then returned to the cage and given fifteen minutes to locate the buried food. Measures were taken of latency to find the food reward and whether it was consumed.

Hotplate test for thermal sensitivity: Individual mice were placed in a tall plastic cylinder located on a hotplate, with a surface heated to 55^{o}C (IITC Life Science, Inc., Woodland Hills, CA). Reactions to the heated surface, including hindpaw lick, vocalization, or jumping, led to immediate removal from the hotplate. Measures were taken of latency to respond. The maximum test length was 30 sec, to avoid any type of paw damage.

Activity in an open field: Exploratory activity in a novel environment was assessed by a one-hour trial in an open field chamber (40 cm x 40 cm x 30 cm) crossed by a grid of photobeams (VersaMax system, AccuScan Instruments). Counts were taken of the number of photobeams broken during the trial in five-minute intervals, with separate measures for ambulation (total distance traveled), fine movements (repeated breaking of the same set of photobeams), and rearing movements. Time spent in the center region of the activity chamber was used as a measure of anxiety-like behavior in a novel environment.

Rotarod: Subjects were tested for motor coordination and learning on an accelerating rotarod (Ugo Basile, Stoelting Co., Wood Dale, IL). For the first test session, animals were given three trials, with 45 seconds between each trial. Two additional trials were given 48 hours later. Rpm (revolutions per minute) was set at an initial value of 3, with a progressive increase to a maximum of 30 rpm across five minutes (the maximum trial length). Measures were taken for latency to fall from the top of the rotating barrel.

Sociability: The three-chamber social approach test was designed to assess whether mice will approach or avoid an unfamiliar stranger mouse. Each session consisted of two ten-minute phases: a habituation period and a test for sociability. For the sociability assay, mice were given a choice between being in the proximity of an unfamiliar conspecific (stranger 1), versus being alone.

The social testing apparatus was a rectangular, three-chambered box fabricated from clear polycarbonate. Dividing walls had doorways allowing access into each chamber. Photocells were embedded in each doorway to allow automatic quantification of entries and duration in each side of the social test box. The chambers of the apparatus were cleaned between each trial.

The test mouse was first placed in the middle chamber and allowed to explore for ten minutes, with the doorways into the two side chambers open. After the habituation period, the test mouse was enclosed in the center compartment of the social test box, and an unfamiliar C57BL/6J male (stranger 1) was placed in one of the side chambers. The stranger mouse was enclosed in a small wire cage, which allowed nose contact between the bars, but prevented fighting. An identical empty wire cage was placed in the opposite side of the chamber. Following placement of the stranger and the empty wire cage, the doors were re-opened, and the subject was allowed to explore the entire social test box for a ten-minute session. Measures were taken of the amount of time spent in each chamber and the number of entries into each chamber by the automated testing system.

Acoustic startle: The acoustic startle test can be used to assess auditory function and sensorimotor gating. The test is based on the measurement of the reflexive whole-body flinch, or startle response, that follows exposure to a sudden noise. Assessments can be made of startle magnitude and of prepulse inhibition, which occurs when a weak prestimulus leads to a reduced startle in response to a subsequent louder noise. For this study, animals were tested with a San Diego Instruments SR-Lab system. Briefly, mice were placed in a small Plexiglas cylinder within a larger, sound-attenuating chamber. The cylinder was seated upon a piezoelectric transducer, which allowed vibrations to be quantified and displayed on a computer. The chamber included a house light, fan, and a loudspeaker for the acoustic stimuli. Background sound levels (70 dB) and calibration of the acoustic stimuli were confirmed with a digital sound level meter (San Diego Instruments).

Each session consisted of 42 trials that began with a five-minute habituation period. There were 7 different types of trials: the no-stimulus trials, trials with the acoustic startle

stimulus (40 msec; 120 dB) alone, and trials in which a prepulse stimulus (20 msec; either 74, 78, 82, 86, or 90 dB) occurred 100 ms before the onset of the startle stimulus. Measures were taken of the startle amplitude for each trial across a 65-msec sampling window, and an overall analysis was performed for each subject's data for levels of prepulse inhibition at each prepulse sound level (calculated as 100 - [(response amplitude for prepulse stimulus and startle stimulus together / response amplitude for startle stimulus alone) x 100].

Morris water maze - Visible platform test: The Morris water maze task was used to assess spatial learning in the mice. The water maze consisted of a large circular pool (diameter = 122 cm) partially filled with water (45 cm deep, 24-26^{o} C), located in a room with numerous visual cues. Mice were first tested using a visible platform. In this case, each animal was given four trials on one day to swim to an escape platform cued by a patterned cylinder extending above the surface of the water. For each trial, the mouse was placed in the pool at one of four possible locations (randomly ordered), and then given 60 seconds to find the visible platform. If the mouse found the platform, the trial ended, and the animal was allowed to remain 10 seconds on the platform before the next trial began. If the platform was not found, the mouse was placed on the platform for 10 seconds, and then given the next trial. Measures were taken of latency to find the platform, swimming distance, and swimming speed, via an automated tracking system (Noldus Ethovision).

Acquisition in the hidden platform test: In the week following the visual cue task, mice were tested for their ability to find a submerged, hidden escape platform (diameter = 12 cm). As in the procedure for visual cue learning, each animal was given four trials per day, with one minute per trial, to swim to the hidden platform. Criterion for learning was an average latency of 15 seconds or less to locate the platform on one day. Mice were tested until

criterion was reached, with a maximum of nine days of testing. When criterion was reached, mice were given a one-minute probe trial in the pool with the platform removed. In this case, selective target search was evaluated by measuring percent time spent in each quadrant, and the number of crossings for the target location where the platform had previously been located versus corresponding locations in each quadrant of the pool.

Reversal learning: Following the acquisition phase, mice were tested for reversal learning, using the same procedure as described above. In this phase, the hidden platform was located in a different quadrant in the pool, diagonal to its previous location. On the eighth day of testing, the platform was removed from the pool, and the group was given a probe trial to evaluate reversal learning.

Locomotor behavior studies: Locomotor activity was assessed in photocell-based activity chambers under standardized environmental conditions using an AccuScan activity monitor (AccuScan Instruments, Columbus, OH) with a 41 cm x 41 cm x 30 cm Plexiglas chamber and a beam spacing of 1.52 cm as described (Abbas *et al*, 2009). Horizontal activity was measured as the total distance covered in centimeters as the total of all vectored X-Y coordinate changes and recorded in 5 minute bins.

Spontaneous Locomotor Activity: Effect of CNO on spontaneous locomotion was measured during the dark phase of the light cycle to monitor behavior during a period of relatively high basal activity. Mice were placed in dark locomotor activity boxes at 8 pm. At 9 pm, mice were injected with a dose of CNO and returned to the locomotor chamber for two hours. *Novelty-Induced Locomotor Activity:* A separate cohort of adora2A-rM3Ds mice and wild-type mice were injected with CNO (1.0 mg/kg) or vehicle or CGS 21680 (0.1, 0.5

mg/kg) and placed in activity boxes 20 min. later. Locomotor activity was recorded for 1 hour.

Amphetamine sensitization study

Development Phase: Mice were placed in locomotor activity boxes for 1 hour to acclimate. Mice were then injected (i.p.) with drug(s) and/or vehicle and then returned to the activity chamber for 2 hours. This was repeated once daily for 5 days. Drug doses were 2.0 mg/kg amphetamine and 1.0 mg/kg CNO. The following conditions were tested in separate cohorts: Cohort 1 (all adora2A-rM3Ds mice): amphetamine + CNO vs. amphetamine + vehicle; Cohort 2 (all wild-type mice): amphetamine + CNO vs. amphetamine + vehicle. *Incubation phase:* On days 6 – 14, mice were left in their home cages with no drug treatment. *Expression phase:* On day 15, mice were placed in locomotor chambers. One hour later, mice were injected with amphetamine (2.0 mg/kg) and returned to the activity chamber for 2 hours. Locomotor activity chambers were located in a room separate from the mouse colony. All behavioral sensitization sessions were conducted from 1 pm to 5 pm. *Data Analysis:* For each individual mouse, total distance travelled during the hour post injection was summed. Day 1 distance of a cohort was averaged, and this value was used to calculate each mouse's percentage of Day 1 distance travelled. Data are presented as the average of these percentages. Significance was determined using a Student's *t* -test on Day 15 data between the two conditions for each cohort.

*Electrophysiology***:** P23 mice were subject to the amphetamine behavioral sensitization procedure as detailed above. On day 15, mice were sacrificed (without drug challenge) and tissue preparation and recording were performed as previously described (Thomas *et al*, 2001). Sagittal striatal slices (240 um in thickness) were cut containing the nucleus

accumbens shell. After at least one hour recovery, slices were transferred to a recording chamber where they were continuously perfused with oxygenated artificial cerebrospinal fluid containing (in mM) 124 NaCl, 2.5 KCl, 1.2 NaH2PO4, 1 MgCl2, 2.0 CaCl2, 26 NaHCO3, and 10 glucose. Whole-cell recordings were made from rM3Ds-containing cells (identified by their mCherry fluorescent signals) at room temperature (24-25° C) in the presence of 50 uM picrotoxin and 1 uM glycine. Recording pipette resistances were 2.5–3.5 MΩ with internal solution containing (in mM) 103 cesium gluconate, 2.8 NaCl, 5 TEA-Cl, 20 HEPES, 0.2 EGTA, 5 lidocaine N-ethyl chloride, 4 Mg-ATP, 0.3 Na-GTP, 10 Na-phosphocreatine and pH 7.2–7.3. Experiments were discarded if series resistance (typically 15-20 MΩ) changed by more than 20%. Signals were low-pass filtered at 2 kHz and sampled at 10–20 kHz with an Axopatch 200B amplifier and a Digidata 1440A (Axon Instruments) for subsequent off-line analysis. To evoke excitatory postsynaptic currents (EPSCs), tungsten bipolar electrodes were placed at the prelimbic cortex-NAc border to stimulate afferents preferentially from prelimbic cortex. Stimuli with 150 μs duration were delivered at 0.05 Hz. AMPAR/NMDAR ratio is the ratio of the peak of the EPSC at -70 mV to the magnitude of the EPSC at +40 mV at 60 ms following stimulation.

2.4. RESULTS

2.4.1. Generation and characterization of adora2A-rM3Ds mice

The rM3Ds is an engineered and evolved muscarinic receptor which as originally described: (1) selectively couples to $G\alpha_s$-type G-proteins (2) is activated by the inert designer drug CNO and (3) is insensitive to the native ligand acetylcholine (Guettier *et al*, 2009). To determine whether the rM3Ds can activate canonical $G\alpha_s$-type signaling in a neuronal environment, cAMP accumulation in response to increasing concentration of CNO was

measured in cultured neurons infected with a lentivirus expressing rM3Ds by Ying Pei (Figure 2b). CNO did not cause cAMP accumulation in uninfected, wild-type neurons as measured by Xi-Ping Huang (Figure 2e,f). Because striatal neurons express $G\alpha_{olf}$, a $G\alpha_s$-like G-protein enriched in striatum (Corvol *et al*, 2001; Drinnan *et al*, 1991; Zhuang *et al*, 2000), HEK-T cells were transfected with a 1:0, 1:1, and 1:3 ratio of rM3Ds to $G\alpha_{olf}$ and their cAMP accumulation in response to CNO measured by Vincent Setola. In Figure 2c, it can be seen that rM3Ds induces cAMP accumulation through the endogenous $G\alpha_s$ present in HEK-T cells in the 1:0 condition, and this accumulation is increased when $G\alpha_{olf}$ is co-transfected at ratios of 1:1 and 1:3. These experiments verify the functionality of rM3Ds in neurons and demonstrate that it can couples to both $G\alpha_s$ and $G\alpha_{olf}$.

To create striatopallidal-targeted rM3Ds transgenic mice, the adora2A BAC (GENSAT1-BX868) - a gene preferentially expressed in striatopallidal MSNs (Brown *et al*, 2008; Chen *et al*, 2001) - was used to create a transgene carrying the rM3Ds construct. The adora2A BAC was recombineered to include an rM3Ds – IRES – mCherry construct (Figure 2a) downstream of the adora2A start site, and mouse oocyte pronuclei were injected with the recombineered and purified BAC to create mice expressing rM3Ds under control of the adora2A BAC by Bernd Gloss at the Duke University neurotransgenic core. Three founder lines had detectable and essentially identical patterns of mCherry fluorescence, and the line denoted "AD6" was used for subsequent studies, referred to as "adora2A-rM3Ds mice".

Immunofluorescence microscopy revealed an expression pattern limited to the dorsal and ventral striata and consistent with striatopallidal projection (Figure 3a and Figure 4). Cell-type specificity was subsequently determined by crossing the adora2A-rM3Ds mice with the GENSAT Drd1a-EGFP and Drd2-EGFP reporter mice. Drd2-EGFP / adora2A-

rM3Ds double transgenic mice displayed 82.15% (± 5.14, n=695 D2 cells in 3 mice) colocalization between adora2A-rM3Ds-mCherry cells and Drd2-EGFP cells (Figure 3b), while Drd1a-EGFP / adora2A-rM3Ds double transgenic mice show a 2.51% (±1.262, n=671 D1 cells in 3 mice) colocalization between adora2A-rM3Ds-mCherry cells and Drd1a-EGFP cells (Figure 3c). Images for the above studies were obtained by Noah Sciaky. As expected, there was no colocalization between parvalbumin- containing interneurons and mCherry (Figure 3d). These data demonstrate that the adora2A-rM3Ds mice express rM3Ds selectively in striatopallidal D2-dopamine receptor-expressing MSNs.

A key signaling protein in MSNs is DARPP-32 (dopamine- and cyclic AMP-regulated neuronal phosphoprotein of molecular weight 32 kD) (Svenningsson *et al*, 2004). DARPP-32 is directly phosphorylated at threonine 34 by protein kinase A (PKA), the canonical downstream effector of the $G\alpha_{s/olf}$ signaling cascade. Additionally, DARPP-32 phosphorylation at threonine 75 is modulated by multiple extracellular signals and Thr75 modulation can inhibit PKA. To determine whether the rM3Ds activates canonical $G\alpha_{s/olf}$ signaling pathways in MSNs, Tanya Daigle of Marc Caron's laboratory tested whether CNO caused phosphorylation of DARPP-32 at threonine 34 and threonine 75. Mice were treated with CNO (5.0 mg/kg) or vehicle and striatal tissue was analyzed for pT34 DARPP-32 levels by Western blot analysis. It was found that CNO increased pT34 DARPP-32 levels in adora2A-rM3Ds mice, but not wild-type mice (Figure 5a), and no effect on pT75 DARPP-32 levels was observed (Figure 5b). Additionally, CNO decreased pErk1/2 levels in adora2A-rM3Ds mice, but not wild-type mice and that CNO did not cause a change in pAKT308 levels (Figure 6). To determine whether the rM3Ds was interfering with canonical $G\alpha_s$-type signaling in these neurons, the biochemical response to cocaine administration in adora2A-

rM3Ds and wild-type mice was measured. No difference in cocaine-induced DARPP-32 Thr34 levels between wild-type and adora2A-rM3Ds mice (Figure 7) was observed. These findings demonstrate that rM3Ds activates canonical $G\alpha_s$-type signaling pathways *in vivo* and indicate that the endogenous signaling mechanisms are not disturbed.

To determine whether the adora2A-rM3Ds transgene had any CNO-independent activity that significantly altered behavior, transgenic mice and litter-mate controls were tested in a battery of behavioral tests in their naïve state without CNO treatment by Randal Nonneman of Sheryl Moy's laboratory. In comparison to wild type litter-mates, adora2A-rM3Ds mice did not have significant differences in weight, locomotion, rotarod performance, prepulse inhibition, Morris water maze performance, sociability, elevated plus maze performance, olfaction or thermal sensation (Figure 8 tables 2,3). To determine whether the presence of the striatopallidal rM3Ds influenced endogenous $G\alpha_s$-type signaling, adora2A-rM3Ds and wild-type mice were injected with vehicle, 0.1, or 0.5 mg/kg CGS21680, a selective agonist for the adenosine A2A receptor, and placed in a locomotor chamber 20 minutes later to record novelty-induced locomotor activity (Figure 9). A two-way ANOVA indicated a significant effect of dose ($p < 0.05$) but not genotype or interaction ($p=0.66$ and $p=0.51$, respectively). These data indicate that in the absence of designer drug activation (CNO), the presence of the adora2A-rM3Ds transgene does not significantly alter mouse behavior. Combined, these control studies establish the suitability of adora2A-rM3Ds mice to activate $G\alpha_s$-type signaling in striatopallidal neurons.

2.4.2. CNO-induced modulation of locomotion in adora2A-rM3Ds mice

Striatopallidal medium spiny neurons of the indirect pathway are thought to exert an inhibitory effect on locomotor behavior when activated (Albin *et al*, 1989; Alexander and Crutcher, 1990; DeLong, 1990; Kravitz *et al*, 2010). To determine whether rM3Ds activation in striatopallidal MSNs inhibits locomotion, adora2A-rM3Ds mice were injected with CNO (1.0 mg/kg) and placed into a novel open field testing chamber 20 minutes later. CNO treatment of transgenic, but not wildtype, mice robustly decreased locomotion (Figure 10a). Similar effects and a dose dependency were demonstrated by testing spontaneous locomotion during the active period (dark phase) of the diurnal cycle (Figure 10b,c). These two data sets indicate that $G\alpha_{s/olf}$-activation in striatopallidal neurons of adora2A-rM3Ds mice is sufficient to inhibit locomotion.

2.4.3. CNO-induced modulation of amphetamine sensitization

In rodent models, repeated exposure to psychostimulants produces an enhanced behavioral responsiveness, known as behavioral sensitization (Steketee and Kalivas, 2011). Amphetamine causes an increase in synaptic dopamine release, thereby inducing excessive stimulation of the D1- and D2-dopamine receptors (McKenzie and Szerb, 1968; Sulzer, 2011). The increased locomotor effects produced in behavioral sensitization may thus arise as a result of $G\alpha_{s/olf}$ signaling in D1R-containing striatonigral MSNs, $G\alpha_i$ signaling in D2R-containing striatopallidal MSNs, or a combination of these two effects. A potential role for $G\alpha_s$-type signaling in striatopallidal neurons in amphetamine-induced behavioral sensitization is suggested by pharmacological studies in rodents in which an A2AR agonist during the development phase inhibits sensitization (Shimazoe *et al*, 2000). Given these observations, I tested whether rM3Ds activation during development of amphetamine

sensitization significantly altered behavioral sensitization. Mice were administered amphetamine (2.0 mg/kg) with or without CNO (1.0 mg/kg) for five days and their locomotor activity recorded. Mice were then given a 10 day "break" period and the expression of sensitization was tested on day 15 by the administration of amphetamine (2.0 mg/kg) and vehicle. When CNO was co-administered with amphetamine in adora2A-rM3Ds mice during development, behavioral sensitization was inhibited (Figure 11a, red line and box). In contrast, wild-type mice treated with amphetamine, wild-type mice treated with amphetamine and CNO, and adora2A-rM3Ds mice treated with amphetamine alone all had normal sensitization (Figure 11a). These data indicate that (1) rM3Ds activation blocks development of amphetamine sensitization, (2) in the absence of CNO adora2A-rM3Ds mice sensitize normally to amphetamine, and (3) that CNO does not have off-target effects. Lastly, these findings were replicated in an independent founder line of adora2A-rM3Ds mice (Figure 12).

In the nucleus accumbens, behavioral sensitization to psychostimulants can be accompanied by long-lasting changes in fast glutamatergic synaptic transmission (Bowers *et al*, 2010; Thomas *et al*, 2001; Wolf and Ferrario, 2010). Thus, we investigated whether repeated rM3Ds activation during the amphetamine sensitization protocol induced long lasting changes in glutamatergic synaptic transmission in adora2A MSNs of adora2A-rM3Ds mice. Three week old mice (to facilitate whole cell recordings) were treated with amphetamine daily for 5 days along with CNO (1mg/kg) or vehicle as done in the behavioral sensitization paradigm described above. On day 15, mice were sacrificed (without amphetamine challenge) and excitatory postsynaptic currents (EPSCs) of mCherry-expressing (transgene marker) MSNs in the nucleus accumbens shell of acute brain slices

were measured by Yehong Wan of Nicole Calakos's laboratory. Under recording conditions in which responses of AMPA- (2-amino-3-(5-methyl-3-oxo-1,2- oxazol-4-yl) propanoic acid) and NMDA- (N-Methyl-D-aspartic acid) type glutamate receptors (AMPAR and NMDAR, respectively) could be compared, it was found that CNO treatment during induction of behavioral sensitization caused a significant and long-lasting increase in the AMPAR/NMDAR ratio compared to amphetamine treatment alone ($p=0.006$, t-test; $p=0.016$, rank test) (Figure 11b). Of note, relative to non-sensitized (saline) controls, the effects of amphetamine on the AMPAR/NMDAR ratio trended in opposite directions depending on the presence or absence of CNO. Treatment with amphetamine alone trended to decrease the AMPAR/NMDAR ratio compared to saline ($p = 0.108$, two-tailed t-test; $p=0.100$, rank test), whereas treatment with amphetamine and rM3Ds activation by CNO trended to increase the ratio compared to saline ($p=0.108$, two-tailed t-test; $p=0.184$, rank test). These data demonstrate that concurrent activation of rM3Ds during amphetamine sensitization is accompanied by long lasting changes in synaptic strength of adora2A MSNs that oppose the effects of amphetamine alone.

CHAPTER 3. GENERAL DISCUSSION, IMPLICATIONS, AND FUTURE DIRECTIONS

3.1. DISCUSSION

Although striatal GPCR signaling has been studied for decades, the role of whole-striatum, cell type-specific, GPCR-mediated signaling on behavior remains unknown and has been heretofore unknowable. Using a newly developed mouse model in which I selectively and stably expressed a Gs-DREADD (rM3Ds) in striatopallidal neurons, I have validated the use of the DREADD technology to non-invasively control G_s-type signaling in a neuronal context *in vivo*. Because of the non-invasive spatio-temporal control afforded, DREADD technology has far-reaching applicability to test the role of GPCR activity in a broad array of neuronal and non-neuronal contexts. In this report, I further demonstrate that Gs-DREADD is well-tolerated when expressed long-term in a transgenic context and this construct can be used in future studies to reveal new insights on the cellular and behavioral significance of $G\alpha_s$ signaling in defined cellular populations.

Since the introduction of the DREADD technology, several independent groups have reported success activating and silencing neurons with hM3Dq and hM4Di respectively (Alexander *et al*, 2009; Ferguson *et al*, 2011; Garner *et al*, 2012; Krashes *et al*, 2011; Ray *et al*, 2011; Sasaki *et al*, 2011), but have not explored the slow neurotransmission-type $G\alpha_{s/olf}$-mediated signaling. In this report, I demonstrate the suitability of DREADD technology to manipulate the $G\alpha_{s/olf}$-mediated signaling pathway in neurons and thereby modulate

behavior. My evidence for this is as follows: adora2A-M3Ds mice, when administered CNO, (1) show increased DARPP-32 phosphorylation indicative of MSN $G\alpha_{s/olf}$ activity; (2) show a dose-dependent decrease in locomotor activity; and (3) show a blunted behavioral sensitization response to amphetamine indicative of long-term $G\alpha_s$-induced modulatory effects in striatopallidal MSNs. Additionally, these findings complement recent evidence that selective perturbation of D1 MSN activity influences behavioral sensitization (Pascoli *et al*, 2012). In this dissertation, I found that manipulations specifically targeting A2AR MSNs are also sufficient. Moreover, the findings of the two studies are parsimonious with the idea of opposing effects of D1R and A2AR MSNs on motor activity. D1 MSN synaptic weakening inhibited expression of behavioral sensitization (Pascoli *et al*, 2012), and we find that A2AR MSN synaptic strengthening is sufficient to block behavioral sensitization.

It is important to note the differences between the pharmacosynthetics and the optogenetic approaches to remotely control neuronal activity. Differences in the technical / methodological considerations have been previously discussed, as have differences in the type of signaling afforded by these technologies (Alexander *et al*, 2009; Rogan and Roth, 2011). A key difference not fully appreciated is the nature of the neuronal modulation provided by these two technologies. Pharmacosynthetics provides a system that more closely resembles the hormonal signaling mechanisms found in the brain, whereas optogenetics more closely resembles electrochemical signaling phenomena. These differences should fundamentally affect the type of experimentation performed with the respective technologies. To date, the implementation of pharmacosynthetics as "synthetic pharmacology" has yet to occur, in that the modulation afforded by peripheral CNO administration – bathing the brain with modulator – and the corresponding widespread DREADD expression afforded by

genomic transgene expression closely resembles pharmacotherapeutic drug action. Potential applications of this aspect can be further appreciated by the fact that DREADDs are engineered GPCRs, and GPCRs are the target for more than 50% of currently prescribed psychiatric therapeutics (Roth *et al*, 2004). In this regard, the differences between the two technologies can be observed as differences in scientific objectives: pharmacosynthetics is best suited for the study of how drugs modulate the function of the brain (neuropharmacology), whereas optogenetics is best suited for the study of brain function itself (neurophysiology).

In conclusion, I here provide the first evidence that the Gs-DREADD technology can afford modulation of *in vivo* neuronal populations in a reproducible and non-invasive manner, and thereby providing the neuroscience community with a new tool to selectively and non-invasively modulate $G\alpha_s$ signaling in a neuronal cell type -specific manner.

3.2. IMPLICATIONS

3.2.1. Regarding Technological Validation

As mentioned before, the nature of modulation afforded via pharmacosynthetics is more nuanced than merely turning "on" or "off" a neuron. This is especially true regarding Gs type G protein signaling, which cannot claim the relatively straightforward biochemical signaling pathway towards neuronal excitation or inhibition of the other DREADDs (hM3Dq ► Gq ► PLC ► IP3 ► Ca2+ ► neuronal firing; hM4Di ► Gβ/γ ► GIRK ► K+ ► neuronal silencing). The findings of my research prove that the rM3Ds is an effective means to control neuronal Gs-type signaling, and this validation will enable further research into Gs-type modulation of neurons.

3.2.2. Regarding Neurobiological Findings

The observation that selective striatopallidal Gs signaling can inhibit the development of behavioral sensitization caused by repeated amphetamine administration suggests that coactivation of striatopallidal Gs signaling may be an effective means to prevent the development of drug addiction. Indeed, adenosine A2A receptors have been investigated as targets for drug addiction pharmacotherapeutics (Filip *et al*, 2012). This previous research, in addition to the findings of my dissertation research, led me to test the ability of striatopallidal rM3Ds signaling to block another behavioral model of drug addiction, conditioned place preference (CPP). In CPP, the pairing of a rewarding drug with a unique environment causes an animal to seek that particular environment in a drug free state, suggesting that the drug has produced a pleasurable effect in the animal. Compared to behavioral sensitization, CPP is thought to have greater construct validity as it recapitulates the rewarding properties of drug administration (Bardo and Bevins, 2000). Preliminary results suggest that same concurrent striatopallidal Gs signaling utilized in my behavioral sensitization experiments does not block the development of an amphetamine conditioned place preference. The correspondence between behavioral sensitization and conditioned place preference has been a cornerstone of addiction research for decades as a key behavioral manifestation of the "Incentive-Sensitization Theory" of drug addiction (Robinson and Berridge, 1993). These preliminary results suggest that selective activation of striatopallidal MSN $G\alpha_s$ signaling differentially affects these two behavioral paradigms for drug addiction. These findings suggest that enhanced striatopallidal $G\alpha_s$-signaling can separate the behavioral-sensitization aspects from the rewarding aspects of amphetamine, a finding that could have implications for drug addiction. For instance, if similar findings are observed when studying opiate-induced

phenomena, these findings could lead to the development of analgesic adjuvants that prevent the development of prescription opiate addiction.

In the literature, the leading hypothesis of adenosine A2A receptor modulation on addiction-type behaviors is centered on a proposed dopamine D2 receptor / adenosine A2A receptor heterodimer (Ferre *et al*, 2008). The findings of the amphetamine sensitization studies suggest that striatopallidal Gs-type signaling is sufficient to block amphetamine behavioral sensitization independent of a heterodimer. Although I did not directly test this hypothesis, for example, by determining whether the rM3Ds forms heterodimers with dopamine D2 receptors, the nature of the DREADD manipulation implies that the effects on amphetamine sensitization were due to downstream biochemical signaling events as opposed to steric receptor influences. This avenue of inquiry could be a fruitful direction of future research DREADDs in general, and the adora2A-rM3Ds mice in particular.

3.3. FUTURE DIRECTIONS

3.3.1. Future technological development

My dissertation research was focused on the validation of a new technology. Therefore, I believe that one of the most relevant future directions is that of further development of the pharmacosynthetic approach. These future developments will permit this technology to have broader and greater impacts in the field of neuropharmacology.

3.3.1.1. A non CNO-based DREADD

First and foremost, the development of a second, non CNO-based DREADD would be the most advantageous development to further our neuropharmacological understanding of the brain. The availability of an additional DREADD could permit the mapping of functional

neuronal circuits, for example, by placing an excitatory non CNO-based DREADD upstream of a nuclei modulated by the hM4Di. In this fashion, one could determine the functional involvement of a series of nuclei posited to be integral for a given neuronal circuit. Indeed, the Roth laboratory has recently created a new Gi-biased DREADD to this end (Vardy et al, *in preparation*).

3.3.1.2. Enhanced genetic expression strategies

The cell-type specificity afforded by the pharmacosynthetic approach is dependent upon the available genetic targeting approaches. As mentioned above, virally mediated gene transfer provides for the most effective targeting of small nuclei, whereas genomic modification approaches are the most non-invasive means to obtain cell-type specific DREADD expression, though the expression patterns obtained can be off-target (due to the nature of the gene promoter used). Furthermore, existing technologies have been designed to be versatile as opposed to specific, creating scenarios in which multiple transgenes must be present in a mouse to confer DREADD expression or generations of germline recombination must be undertaken to obtain a useable mouse. Ideally, a DREADD could be expressed in the intended neuronal population using as few transgenes as possible. This both simplifies mouse breeding requirements and would "future proof" a given mouse line. E.g., if a single transgene mouse was created that expressed a DREADD in a specific population, a second transgenic mouse carrying a non CNO-based DREADD could be crossed with this mouse. Thus, one could have two different DREADDs expressed using only two transgenes.

3.3.1.3. Complete experimental control of signal transduction

The supposition that signal transduction can be separated from the physiological response, in addition to the implications of functional selectivity, necessitate a further

rightward shift in our control and understanding of the pharmacological equation. Whereas the pharmacosynthetic state of the art currently provides for control of the ligand-receptor pair, the physiological response is still dependent on the effectors present in a given cell type. In the future, it may be possible to control the ligand-receptor-effector triplet (or the ligand-receptor-effector-effector quartet, etc), providing unprecedented depth of pharmacological manipulation as was recently described by Yagi et al, (2011). For instance, one can imagine creating polycistronic transgenes that contain custom effectors designed to solely interact with designer receptors (Figure 13c). In this manner, one could truly synthesize signaling states in specific cell populations to create an end-goal for pharmacotherapeutic development. These custom signaling cascades will provide an unprecedented level of signaling control and definitively determine the type of signaling required for a particular physiological response.

3.3.2. Future Applications of Pharmacosynthetic Technology

Pharmacosynthetics has untapped potential. The utilities not yet applied are inherent to the nature of GPCR signaling in general and that of the pharmacosynthetic approach itself. With the advent of more specific cell-type expression and measurement systems, DREADD technology can be utilized to probe the mechanisms of pharmacotherapeutic efficacy and the nature of GPCR-induced neuronal modulation. Here I will discuss currently underutilized aspects of pharmacosynthetics.

3.3.2.1. Non-interfering modulation

An overlooked aspect of the pharmacosynthetic approach is the lack of interference with endogenous signaling. Whereas the noninvasive aspect relates to the physiologically

benign approaches of DREADD expression (transgenic mice), this lack of interference relates to the nature of the experimental manipulation.

It has been posited elsewhere that the ultimate function of the brain arises from the collection, transmission, and integration of information (deCharms and Zador, 2000; Rolls and Treves, 2011). The brain encodes this information in the biochemical and electrochemical phenomena of neurons, with the transmission and integration occurring through the function of action potentials, neurotransmitters, and receptors. Various nuclei in the brain have been implicated in the etiology of disease and the mechanism of action of therapeutics. Due to the limitations of conventional approaches, it is difficult to separate the role of a nuclei's transmission, integration, or generation of information in the etiology of the associated diseases. For example, in a standard pharmacological approach, a small molecule ligand would be used to modulate a particular receptor. In addition to inherent off-target confounds of this approach, receptor theory posits that any small molecule will compete with the endogenous ligand for that receptor, ultimately functioning as an antagonist of the endogenous tone. This confound applies to allosteric modulation as well. Whereas measurements from such a study would implicate the role of receptor-mediated changes in the postsynaptic neuron, the phenomena observed may be due, in part, to interference with the endogenous tone of ligand-receptor signaling. Thus, the interpretation of such a study would not be able to resolve whether the experimental manipulation modulated the integrator and transmitter of information (the post-synaptic cell receiving input) or the information itself (the endogenous tone).

Pharmacosynthetics circumvents this confound by utilizing an exogenous receptor, leaving the endogenous tone intact. This aspect of the DREADDs has been studied in vitro,

where the hM3Dq (with or without CNO) had minimal or no effect (dependent on measurement system used) on the quaternary organization of wild-type and DREADD variants (hM3 and hM3Dq) of the human muscarinic receptor (Alvarez-Curto *et al*, 2010). Furthermore, the inert clozapine *N*-oxide does not interfere with endogenous receptor signaling. In this manner, the experimenter can specifically modulate the neuronal nuclei in question, independent of the information transfer. This non-interference of experimental manipulation has yet to be explicitly utilized or considered in experimental design using the DREADDs.

3.3.2.2. Cell-type specific GPCR signaling vs. "activation" and "silencing"

A majority of the studies to date have been designed and the data interpreted in the context of DREADD-induced activation or silencing of neuronal activity. Whereas a result of $G\alpha_q$-coupled GPCR activation is depolarization and a result of $G\alpha_i$-coupled GPCR activation is hyperpolarization, these electrophysiological endpoints are only one result of GPCR signaling pathways (Allen and Roth, 2011; Beaulieu *et al*, 2011). In the pharmacosynthetic field, other physiological endpoints have heretofore been, for the most part, overlooked. G-protein pathways are involved in a myriad of neuronal functions, including gene regulation (West *et al*, 2002). Indeed, whereas the straightforward interpretation and design of $G\alpha_q$-mediated depolarization and $G\alpha_i$-mediated hyperpolarization is pragmatic for studies to date, the inherently metabotropic nature of GPCR signaling needs to both be utilized and taken into account when considering pharmacosynthetics for experimental manipulation.

An application of pharmacosynthetics that would utilize this facet of DREADDs is determining the particular G-protein signaling necessary for therapeutic efficacy. The advent of functional selectivity has caused a sea change in our understanding of GPCR function, in

that the simple distinction of ligands as agonists, antagonists, and inverse agonists no longer exists (Allen *et al*, 2011; Urban *et al*, 2007). Instead, it is now appreciated that a particular small molecule can impart intracellular signaling entirely dependent on the signaling machinery present in a given cell type. Thus, a given "agonist" to a receptor in cell population *A* can induce receptor-mediated signaling, whereas the same "agonist" at the same receptor in cell population *B* can have no effect or a different effect entirely. Furthermore, the observed phenomena to date suggest that the signaling induced upon ligand binding is dependent on the small molecule - a particular "agonist" X to a receptor can induce a particular GPCR-mediated signaling phenomenon (such as cAMP accumulation), whereas another "agonist" Y at the same receptor can cause entirely different GPCR-mediated signaling phenomenon (such as beta-arrestin signaling). This "functional selectivity" of small molecule ligands opens a new chapter in small molecule drug discovery for G-protein coupled receptors, an already well-validated drug targeted.

This new effort is hindered by an unfortunate fusion of the phenomena to be exploited and the nature of modern drug discovery efforts. To date, a majority of drug discovery efforts use cultured cell populations as a model system to study GPCR signal transduction. In what is termed "reverse pharmacology", a receptor is isolated from the organism and its signal transduction properties are studied in the cultured cell populations (Figure 14, top). Due to the implications of functional selectivity, these cultured cell populations, in combination with the type of GPCR being studied and the chemical space of the drug, synergize to produce the signaling phenomena observed. Thus, the observed phenomena are dependent upon the model cell system used. Any subsequent translation of a drug's function to the whole organism is due to a fortuitous similarity of cellular phenotype between the model system

and the whole organism. While reverse pharmacology has created a wealth of information regarding the relationships between the structure of a small molecule ligand and the response of the receptor, the translation of these findings to the whole organism, in terms of therapeutic efficacy, has been less fruitful. A well-known contributor to inverse pharmacology's lack of success is that these chemicals can have off-target effects when reintroduced to the whole organism. While this confound is measurable and perhaps rectified with further compound development, a second unmeasurable confound is the differential receptor function in the native cellular environment compared to the cultured cell. Whereas a chemical may induce a unique signaling state when it is bound to a receptor in the model cell culture system, the native neuronal environment of the receptor may not have cellular factors capable of recognizing the signal being transduced by the chemical-receptor complex (for review see Allen and Roth 2011).

Pharmacosynthesis creates a means to overcome this confound. The opposite approach to reverse pharmacology can be termed "directed" pharmacology, in which the signaling of a particular receptor is designed and characterized in the cultured cell system and then introduced into the whole organism (Figure 14, bottom). An aspect of the DREADDs not fully appreciated is the fact that mutations used to engender CNO modulation and rectify endogenous neurotransmitter modulation are functionally benign. That is, the receptor behaves for all intents and purposes identical to the wild-type receptor (Alvarez-Curto *et al*, 2011). This facet of DREADDs permits the manipulation of intracellular receptor components that can affect the coupling of the receptor to downstream effectors. Guettier et. al. (2009) recognized this and swapped the intracellular loops of the hM3Dq with the loops of the turkey β1-adrenergic receptor to confer Gs-coupling of the DREADD. Although the

exact mechanism of ligand induced GPCR activation is unknown, it has been hypothesized to be a shared mechanism across GPCRs. For instance, many groups have reported creating chimeras of the transmembrane and extracellular portions of GPCR type A with the intracellular portions of GPCR type B to confer type B receptor signaling following application of ligands for receptor type A (Kim *et al*, 2005; Marion *et al*, 2006; Oh *et al*, 2010). If this holds true across receptor types, one can hypothesize that the intracellular components of a DREADD can be replaced with those of a different receptor to confer CNO modulation of that receptor's signaling. DREADDs could be designed to modulate unique and specific signal transduction pathways in cell culture systems, and then these DREADD variants could be inserted *in vivo* to determine whether the designed functional selectivity translates into usefully different neuronal signaling and function (Figure 14E-G). Because the specificity of CNO for the DREADD is pharmacologically unprecedented, this approach would allow us to introduce designer signaling into specific cell types and study the subsequent physiological response to this signaling. Indeed, recent efforts have culminated in the creation of optically activated β-arrestin functionally-selective DREADDs (Lee and Roth, *in preparation*; Nakajima and Wess (2012)) which allow for the precise spatio-temporal control of arrestin signaling.

In one sense, this potential application can invert our standard means of molecular pharmacology. Instead of isolating the signal transduction device (the GPCR) and studying its effects on non-native signal transduction cascades (Figure 14A,B), we can now study the ligand-receptor complex as a single entity. The results of these studies will be correlative information between the signaling transduction induced by CNO/DREADDs in model cell systems and the physiological response of the CNO/DREADDs in the whole organism

(Figure 14E,F). These data could then be used to design small-molecule ligands (or collections of small molecule ligands) that mimic the CNO/DREADD signaling phenomena observed in the cultured cell system (Figure 14I). This approach would essentially invert the process of drug discovery: instead of designing a chemical to elicit an efficacious physiological response, we could directly design an efficacious signaling state and then create a chemical (or chemicals) that can recapitulate the designed state. To our knowledge, only the DREADD technology provides this utility, capitalizing on the drug-like modulation of selective but dispersed neuronal populations.

3.3.3. Future Neurobiological Directions

The findings related to drug addiction warrant further investigation utilizing DREADDs to determine the role of cell type specific G protein signaling in addiction etiology. The preliminary conditioned place preference findings should be followed, and the effects of striatopallidal Gs signaling on drug self-administration should be studied to further clarify the effects of this signaling on drug addiction phenomena. By studying the effects of striatopallidal Gs signaling in this suite of addiction paradigms using the adora2A-rM3Ds mice, we can advance our understanding of addiction etiology.

3.4. FINAL WORDS:

Pharmacosynthetic technology provides the means to advance our understanding of drug pharmacology and the underlying physiology of pharmacologically tractable systems. This dissertation provides neuronal validation of the rM3Ds, a Designer Receptor Exclusively Activated by Designer Drug (DREADD) that specifically modulates G_s-type G-alpha proteins. The further development of pharmacosynthetic tools will enable more precise

manipulation of biochemical signaling phenomena, allowing neuropharmacologist to advance towards connecting the signal to the physiological response. Ultimately, these advances may lead to the development of better and more efficacious therapeutics.

APPENDIX A. TABLES

Table 1: Summary of neuronal DREADD applications to date.

This table provides a quick reference for all neuroscience research performed to date that utilize DREADD pharmacosynthetic technology.

Publication	DREADD used	Expression System	Vector Tissue Targeting Mechanism	Expression Localization	Dose used (mg/kg)	Behavior effect at highest dose	Physiological effect reported at highest dose (in vivo)	Longest *Measured* Duration of Activity
Alexander 2009	hM3Dq	genomic transgene	CamKII-alpha promoter	Cerebral neurons	0.03 - 5.0	Hyperlocomotor activity (0.3)	Status epilepticus (>0.5)	9 hours
Ferguson 2010	hM4Di	Viral	Enkephalin promoter in virus	Striatopallidal MSNs	1.0	Enhanced amphetamine sensitization development and expression	Decreased number of evoked action potentials	2 hours
			Dynorphin promoter in virus	Striatonigral MSNs	1.0	No effect on development, but mice did not express sensitization on challenge day		
			Localized injection	VTA	3.0		Dopamine transmission inhibition	
Geuttier 2009	hM3Dq	Genomic transgene	650 bp fragment of Rat insulin	pancreatic Beta cells	0.0001 - 10.0	None reported	Decreased blood glucose, increased plasma insulin levels	8 hours
	rM3Ds			pancreatic Beta cells				
Krashes 2011	hM3Dq	Viral transgene hybrid	Cre-dependent virus in Agrp-ires-Cre transgenic mice	Agouti-related peptide (AGRP) expressing neurons in arcuate nucleus (ARC)	0.3	Increased feeding, increased breakpoint for food reward, increased food-seeking behavior	Increased c-fos	4 hours
	hM4Di					Decreased feeding		4 hours
Sasaki 2011	hM3Dq	Viral transgene hybrid	Cre-dependent virus in orexin-cre transgenic mice	Orexin expressing neurons of lateral hypothalamis area	5.0	Increased wakefulness	Increased c-fos, decreased NREM and REM sleep time.	1-2 hours
	hM4Di				5.0	Decreased wakefulness	Decreased c-fos, increased NREM sleep time	1-2 hours
Ferguson 2012	hM4Di	Viral	Enkephalin promoter on virus	Striatopallidal MSNs	1.0	Impaired action-outcome learning	None reported	1 hour
Garner 2012	hM3Dq	Genomic transgene	c-fos driver	Diffuse c-fos expressing neurons	5.0	Memory interference	Increased spiking activity	1 hour
Farrell 2012	rM3Ds	Genomic transgene	adora2A-Bac	Striatopallidal MSNs	0.01 - 5.0	Decreased locomotor activity, inhibited amphetamine sensitization development	Increased DARPP-32 phosphorylation	2 hours
Kozorovitskiy 2012	hM4d	Viral transgene hybrid	D1-Cre, D2-Cre, adora2A-Cre mice, Rbp4-cre	Striatonigral, striatopallidal, corticostriatal neurons	1.0	None reported	Decreased synaptogenesis	
Ray 2011	hM4Di	Genomic transgene	Slc6a4-Cre	Serotonergic neurons	10.0	None reported	Reduced ventilation response, hypothermia	10 hours
Kong 2012	hM3Dq	Viral transgene hybrid	Rip-Cre	Non-POMC, non-AgRP neurons of ARC	0.3	No effect on food intake	Increased oxygen consumption, brown adipose tissue consumption	9 hours
Atasoy 2012	hM4Di	Viral transgene hybrid	POMC-Cre	POMC neurons of ARC	3 * 5.0 / 24 hr	Increase in food intake over 24 hours	None reported	2 hours
			Sim1-Cre	Paraventricular hypothalamic nucleus	0.3, 5.0	Increase in food intake, increased break point for food reinforcement		
	hM3Dq		AGRP-Cre	AGRP in ARC	0.3	Increase in food intake		

Table 2: Baseline Behavior Activity

adora2A-rM3Ds transgenic mice and wild-type mice show similar behavioral activity in the elevated plus maze, olfactory test, and hotplate test. Data shown are means ± SEM, n=12 for each genotype.

Behavioral Measure	Wild-type	Adora2A-rM3Ds
Elevated plus maze		
Percent time in the open arms	19.1% ± 3.8	20.7% ± 3.5
Percent entries into the open arms	24.5% ± 3.7	27.3% ± 3.1
Total number of entries	19.9 ± 1.7	21.3 ± 2.0
Olfactory test		
Latency to find the buried food (sec)	24.8 ± 5.3	20.2 ± 4.6[a]
Percent of group finding the food	100%	92%
Hotplate test		
Latency to respond (sec)	15.3 ± 1.7	14.6 ± 1.2

[a]The data from one adora2A-rM3Ds mouse with an extreme score (900 sec) was removed from the analysis.

Table 3: Baseline Morris Water Maze:

adora2A-rM3Ds transgenic mice and wild-type mice show similar behavioral performance in the Morris water maze. Data shown are mean of four trials ± SEM, n=12 for each genotype.

Measure	Wild-type	adora2A-rM3Ds
Latency to escape (sec)	12.0 ± 1.2	15.4 ± 1.4
Swimming distance (cm)	202.7 ± 18.2	245.7 ± 26.8
Speed (cm/sec)	18.5 ± 0.6	17.2 ± 0.8

APPENDIX B. FIGURES

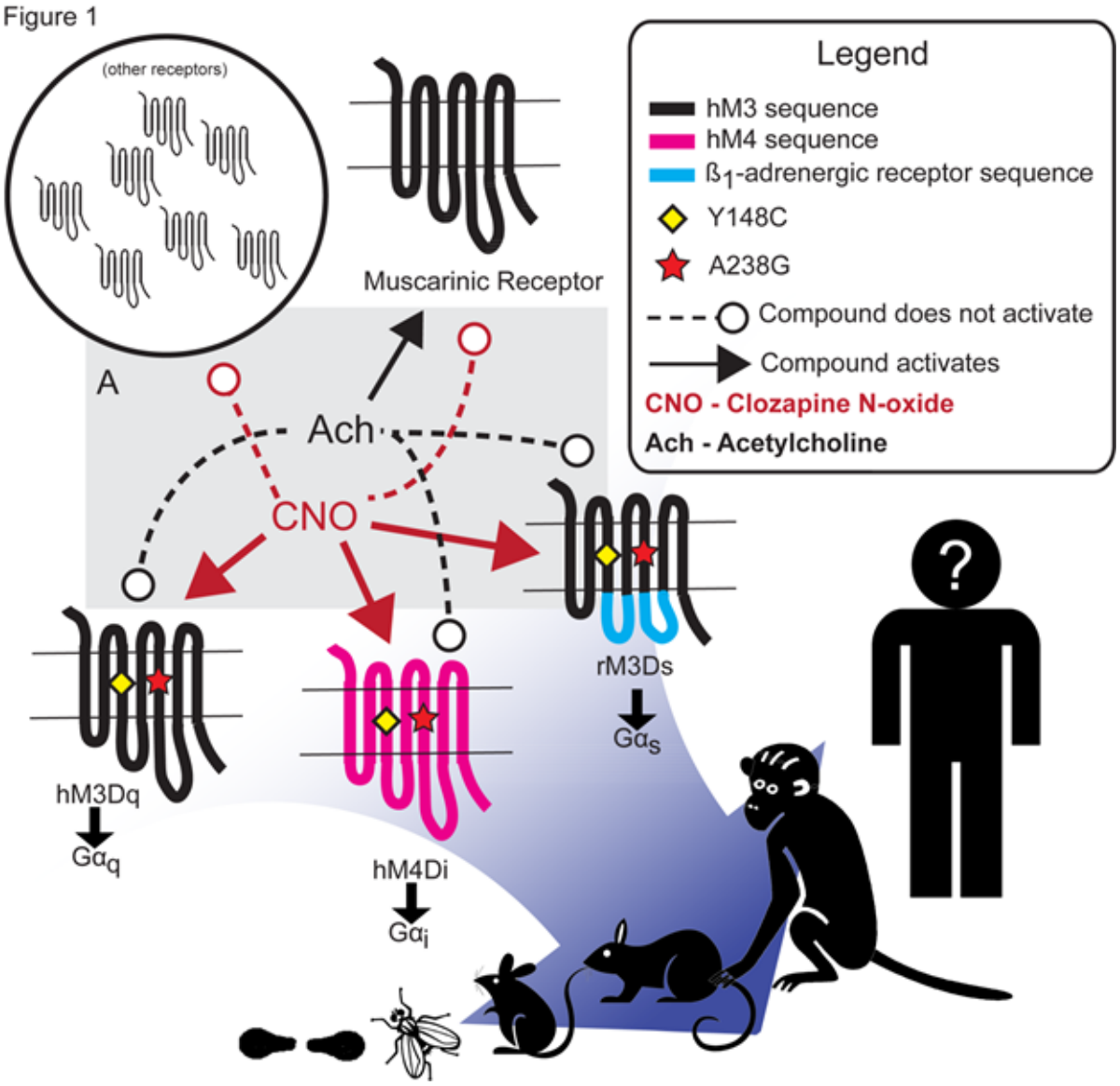


Figure 1: Properties and Composition of Currently Utilized DREADDs.

(A) Gray box indicates two-way selectivity component of DREADDs. Solid lines with arrows indicate that the source compound activates the receptor at the arrow's target. Dashed lines with circles indicate that the source compound does not activate the targeted receptor.

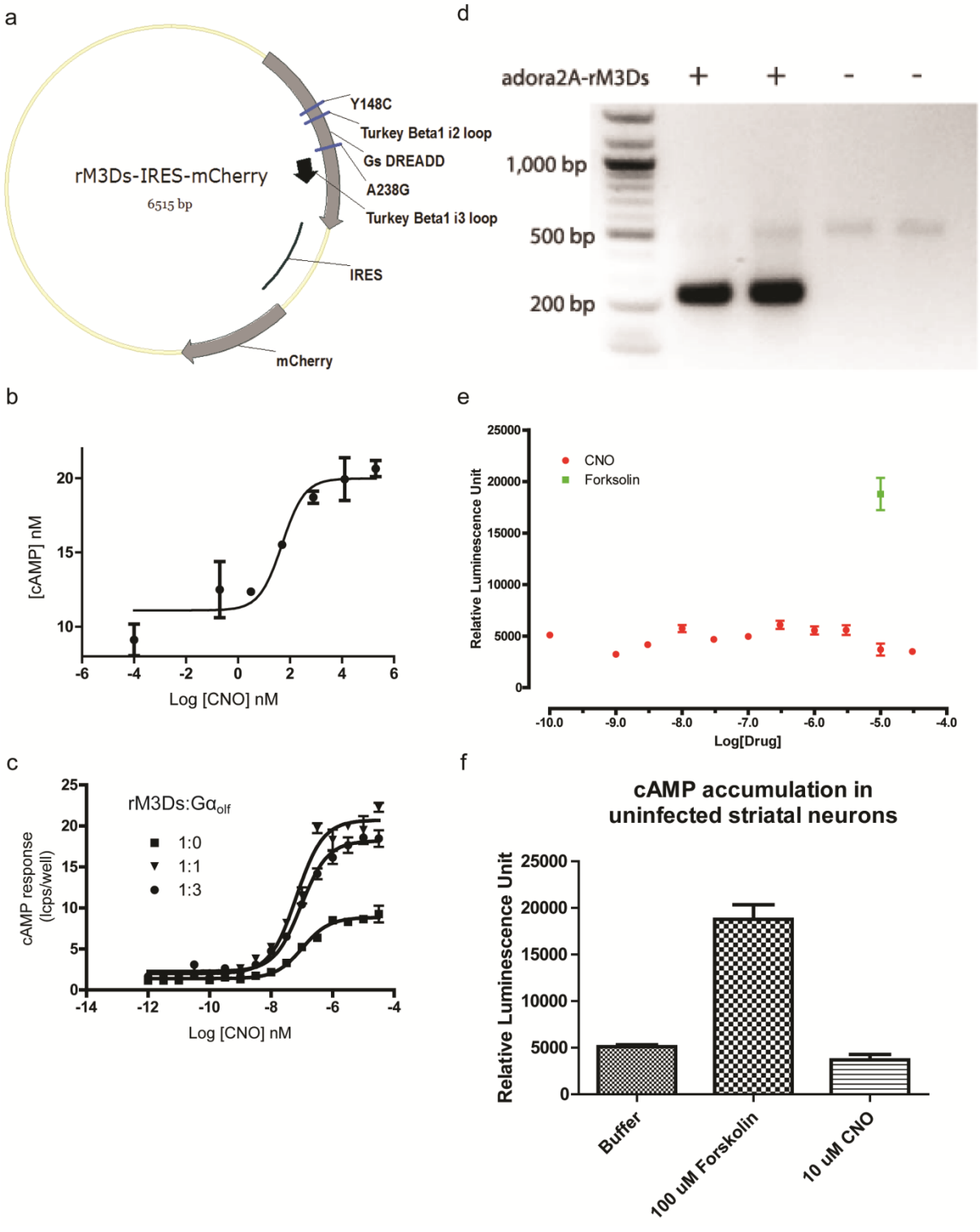

a
rM3Ds-IRES-mCherry
6515 bp
Y148C
Turkey Beta1 i2 loop
Gs DREADD
A238G
Turkey Beta1 i3 loop
IRES
mCherry
d
adora2A-rM3Ds + + - -
1,000 bp
500 bp
200 bp
b
[cAMP] nM
Log [CNO] nM
e
Relative Luminescence Unit
CNO
Forksolin
Log[Drug]
c
rM3Ds:Gα olf
1:0
1:1
1:3
cAMP response (lcps/well)
Log [CNO] nM
f
cAMP accumulation in uninfected striatal neurons
Relative Luminescence Unit
Buffer
100 uM Forskolin
10 uM CNO

Figure 2: Creation and validation of adora2A-rM3Ds mice.

(a) Schematic of rM3Ds-IRES-mCherry construct that was recombineered into the adora2A BAC. (b) Activation of rM3Ds by CNO in rat cortical neurons infected with FUGW-rM3Ds-IRES-mCherry causes a concentration-dependent increase in cAMP accumulation (n=3). (c) Increasing concentrations of $G\alpha_{olf}$ plasmid relative to rM3Ds-IRES-mCherry plasmid increase cAMP accumulation in response to CNO application (n=3). (d) Genotyping band for adora2A-rM3Ds mice. Lanes 1 & 2 are of adora2A-rM3Ds mice. Lanes 3 & 4 are of wild-type mice. A band in adora2A-rM3Ds mice is seen at approximately 250 bp. (e&f) cAMP accumulation in uninfected striatal neurons.

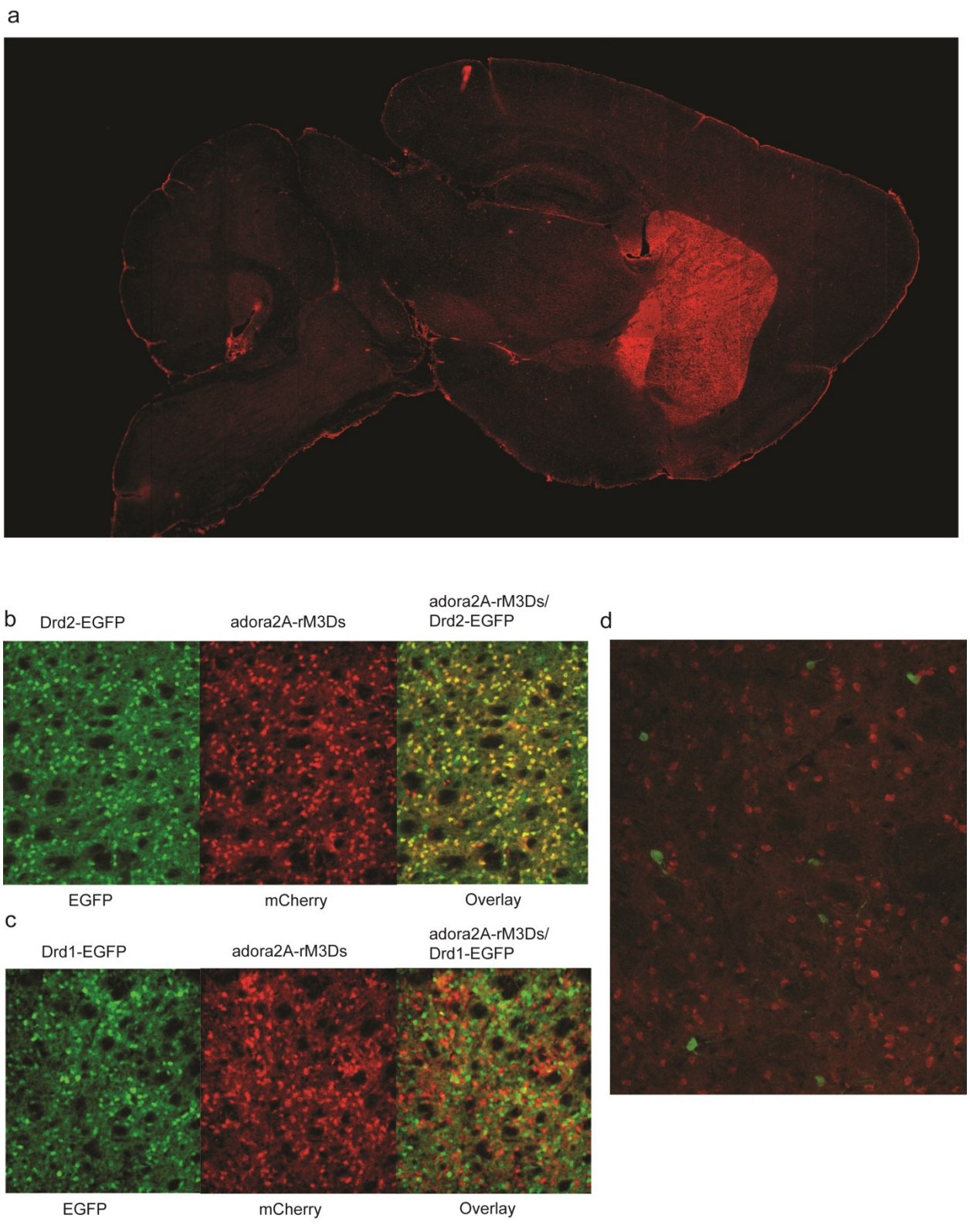

a
b
Drd2-EGFP
adora2A-rM3Ds
adora2A-rM3Ds/
Drd2-EGFP
EGFP
mCherry
Overlay
c
Drd1-EGFP
adora2A-rM3Ds
adora2A-rM3Ds/
Drd1-EGFP
EGFP
mCherry
Overlay
d

Figure 3: rM3Ds is expressed in striatopallidal neurons in adora2A-rM3Ds mice.

(a) Sagittal whole-brain expression pattern of adora2A-rM3Ds mice. (b) Representative immunohistochemistry for mCherry (red) and EGFP (green) in adora2A-rM3Ds / Drd2-EGFP double transgenic mice. (c) Representative immunohistochemistry for mCherry (red) and EGFP (green) in adora2A-rM3Ds / Drd1a-EGFP double transgenic mice. (d) Immunohistochemistry for mCherry (red) and parvalbumin interneurons (green).

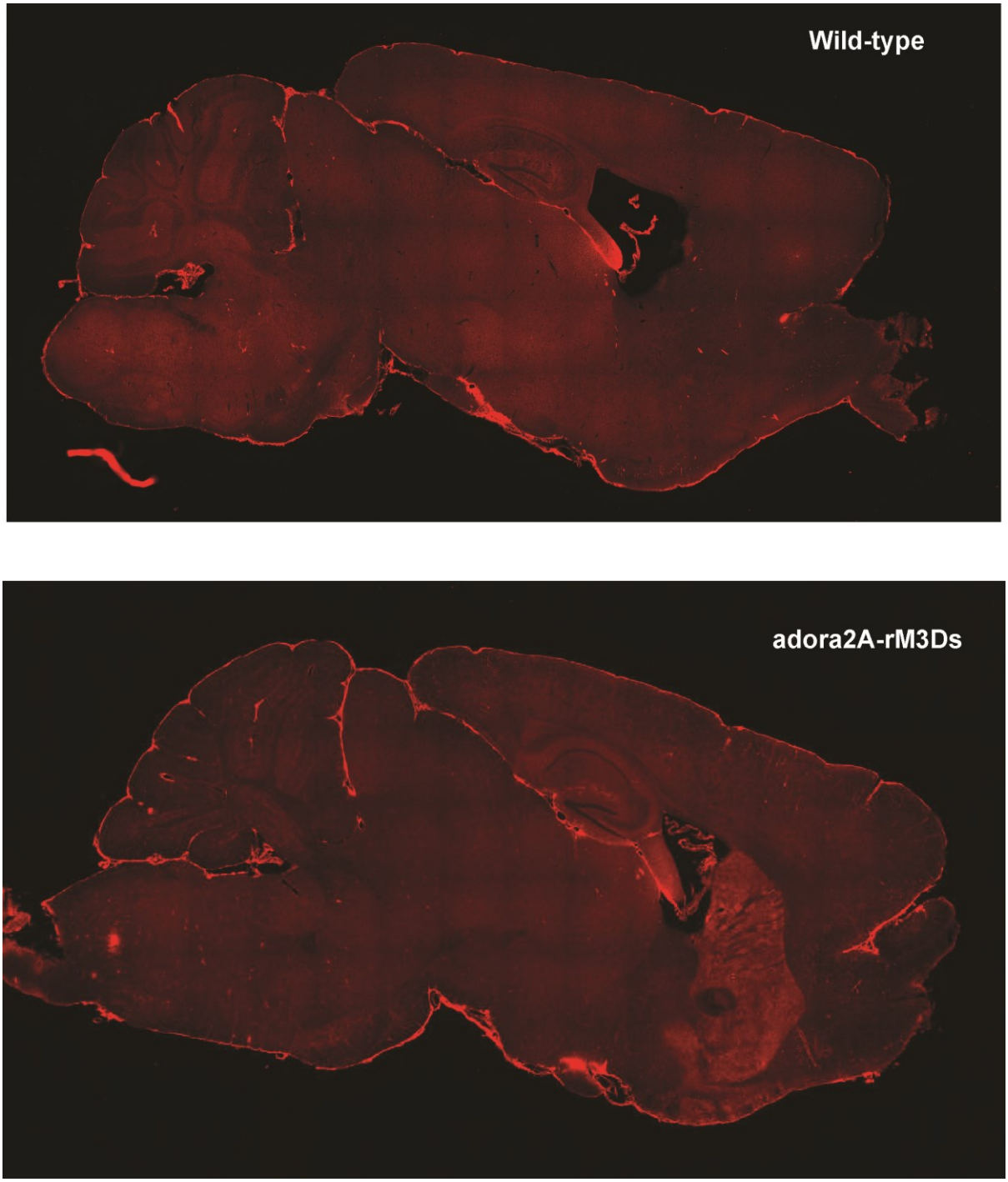


Figure 4: Additional immunohistochemistry images comparing a wild-type mouse with an adora2A-rM3Ds transgenic mouse.

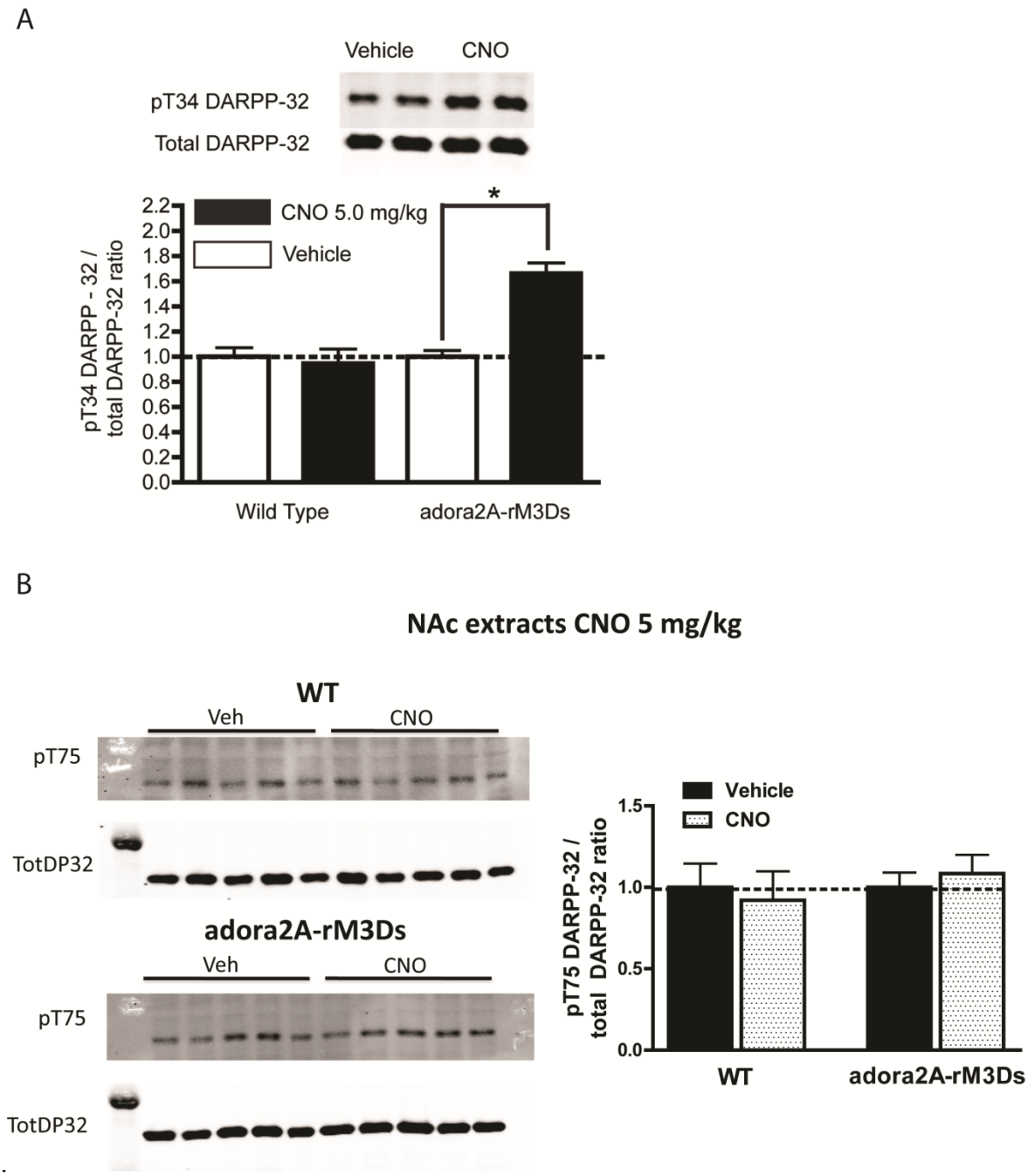


Figure 5: CNO activates canonical Gα_s-type signaling in adora2A-rM3Ds mice.

CNO (5.0 mg/kg) increases pT34 DARPP-32 levels in the ventral striatum of adora2A-rM3Ds mice but not WT mice. Representative Western blots of pT34 DARPP-32 levels in adora2A-rM3Ds transgenic mice administered CNO (5.0 mg/kg) or vehicle are shown. *p<0.01, two-tailed t-test, n=4-5.

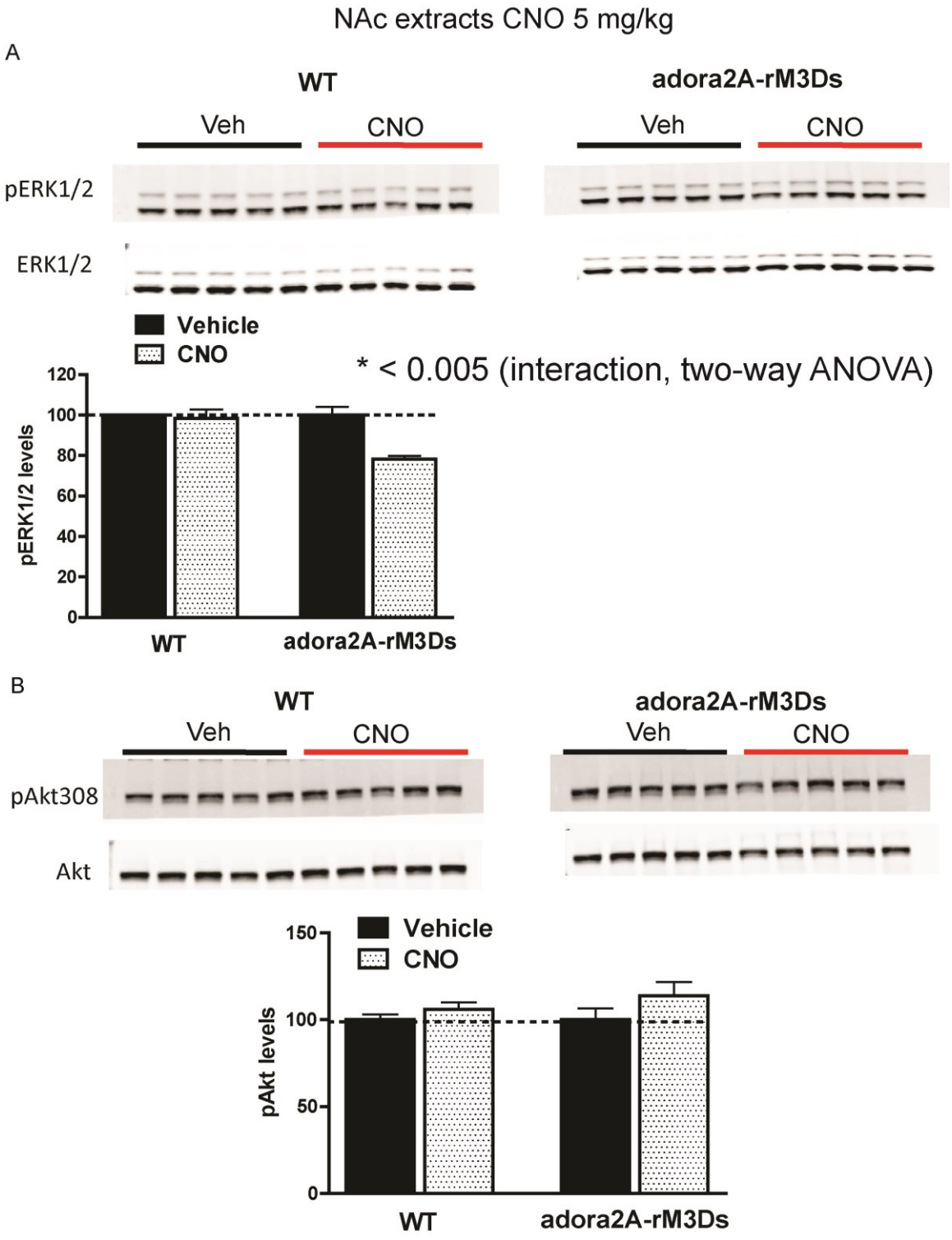

NAc extracts CNO 5 mg/kg
A
WT
adora2A-rM3Ds
Veh
CNO
Veh
CNO
pERK1/2
ERK1/2
Vehicle
CNO
* < 0.005 (interaction, two-way ANOVA)
pERK1/2 levels
120
100
80
60
40
20
0
WT
adora2A-rM3Ds
B
WT
adora2A-rM3Ds
Veh
CNO
Veh
CNO
pAkt308
Akt
Vehicle
CNO
pAkt levels
150
100
50
0
WT
adora2A-rM3Ds

Figure 6: pErk1/2 and pAkt308 signaling in adora2A-rM3Ds mice.

Western blot analysis of (A) pErk1/2 and (B) pAkt308 levels from adora2A-rM3Ds (GsD) and wild-type (WT) mice administered CNO 5.0 mg/kg or vehicle. Data are presented as % total ERK and AKT, respectively.

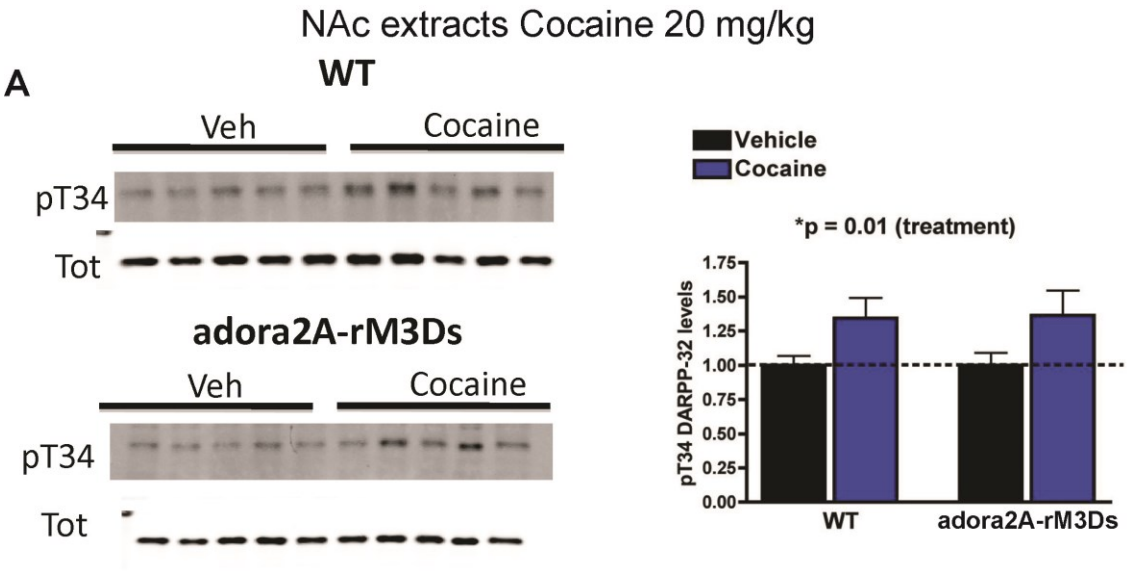


Figure 7: Cocaine-induced signaling in wild-type and adora2A-rM3Ds mice.

Western blot analysis of (A) pT34 DARPP-32 levels from adora2A-rM3Ds (GsD) and wild-type (WT) mice administered cocaine 20.0 mg/kg or vehicle. Data are presented as % total DARPP-32.

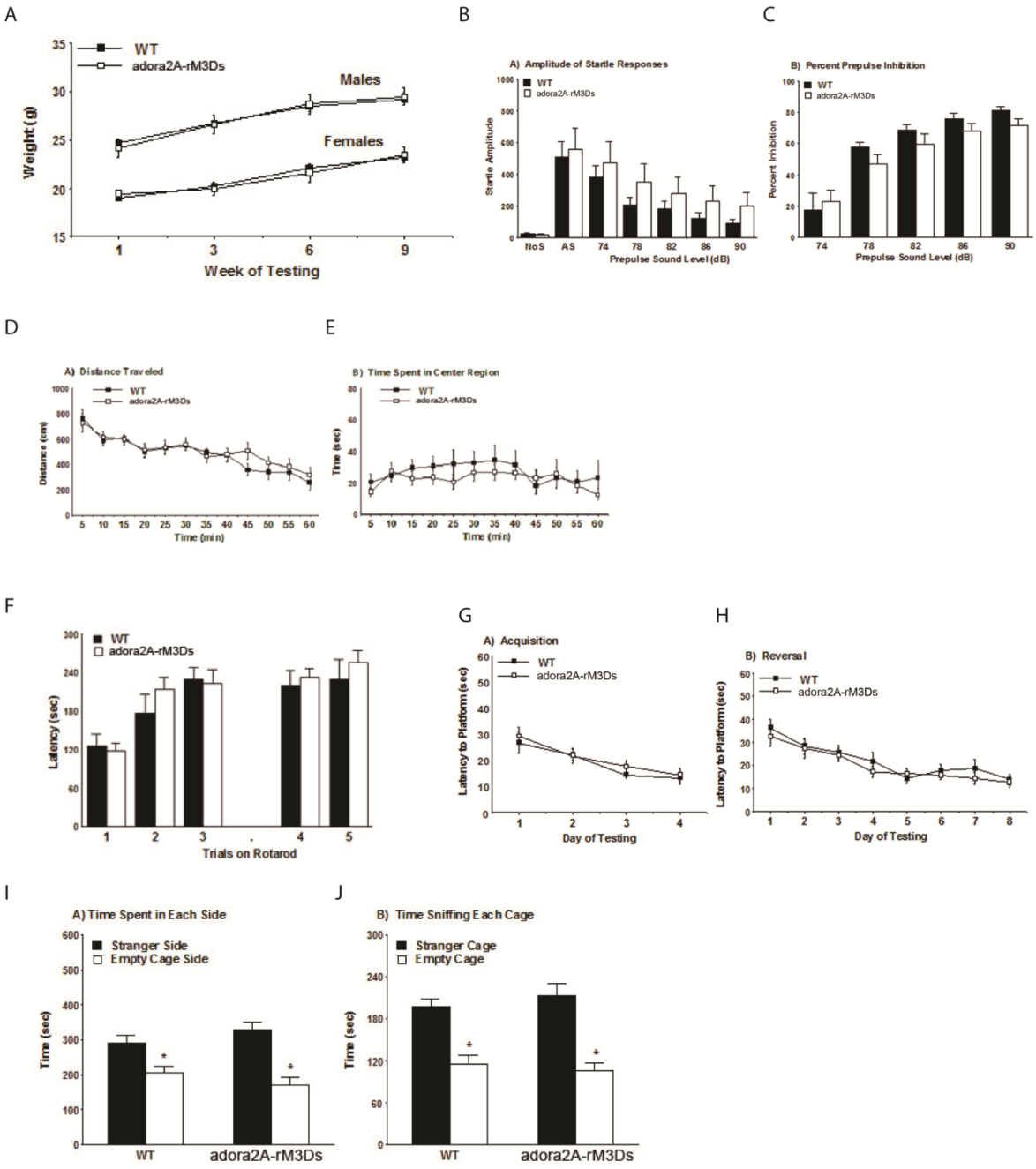
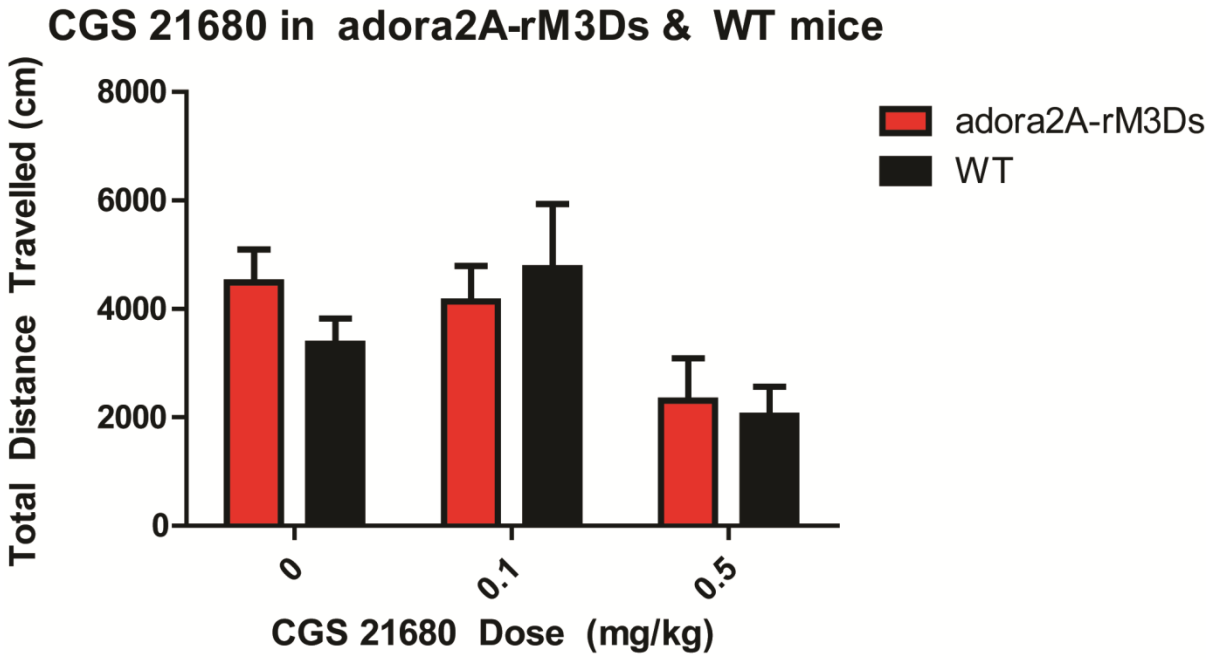


Figure 8: Results of baseline behavior screen.

(A) Weights of mice in grams during behavioral testing. Data shown are means (± SEM) for each group. (B) Amplitude of the startle response and (C) prepulse inhibition following presentation of acoustic stimuli. Data shown are means (+ SEM) for each group. Trials included no stimulus (No S) trials and acoustic startle stimulus (AS) alone trials. (D)

adora2A-rM3Ds (Ds TG) mice have normal locomotion and (E) anxiety-like behavior in a novel environment. Data shown are means (± SEM) for a one-hour test session. (F) Latency to fall from an accelerating rotarod. Data shown are means (+ SEM) for each group. Trials 4 and 5 were given 48 hours after the first three trials. (G) Acquisition and (H) reversal learning in the Morris water maze. Data shown are mean (± SEM) of four trials per day. (I) Time spent in each of the side chambers and (J) sniffing the two cages during the test for sociability. Data shown are mean + SEM for each group for a 10-min test. * $p < 0.05$, within-group comparison between the stranger cage and the empty cage.



Two-way ANOVA		
Source of Variation	% of total variation	P value
Interaction	2.54	0.5100
Genotype	0.36	0.6639
CGS 21680 Dose	19.01	0.0103
Source of Variation	P value summary	Significant?
Interaction	ns	No
Genotype	ns	No
CGS 21680 Dose	*	Yes

Figure 9: Endogenous Gs signaling is intact in adora2A-rM3Ds mice.

Wild-type and Adora2A-rM3Ds mice show no difference in novelty-induced locomotor activity suppression caused by the adenosine A2A agonist CGS 21680. Data are presented as total distance travelled over 40 minutes. $p < 0.05$, dose effect, n=8.

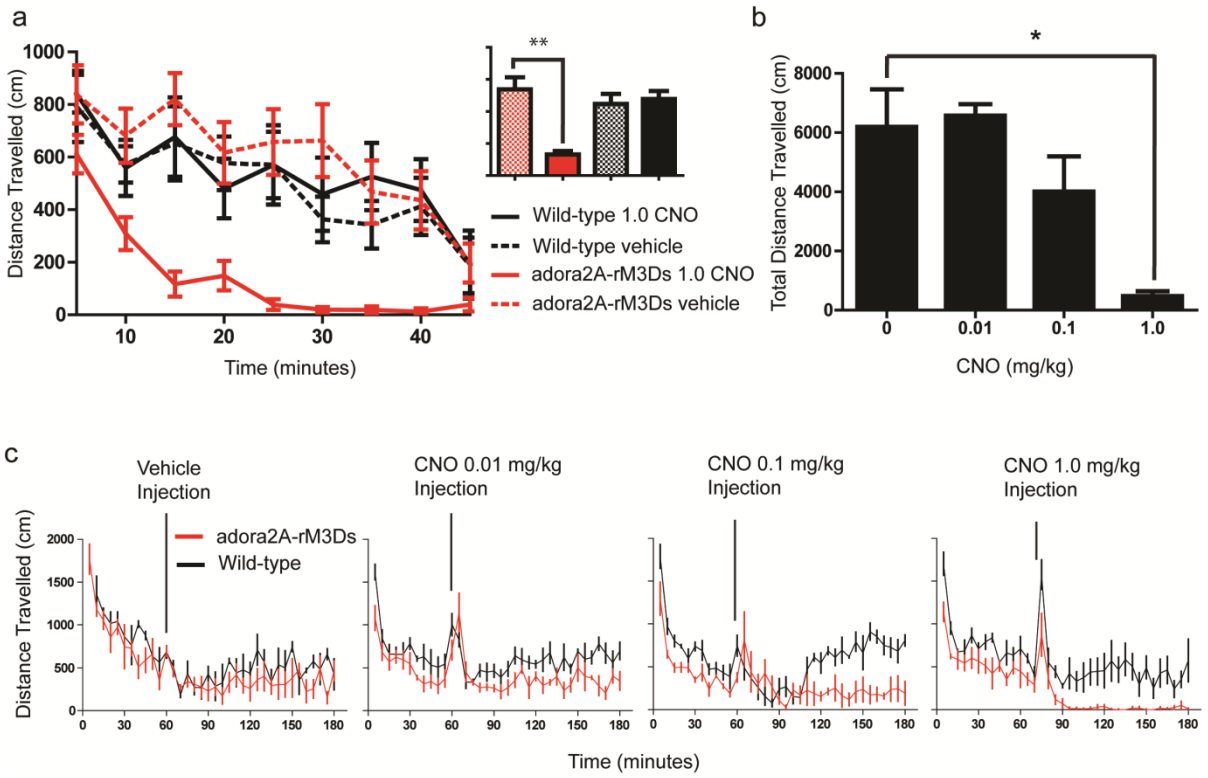


Figure 10: CNO administration inhibits locomotor activity in adora2A-rM3Ds mice.

(a) CNO blocks novelty-induced locomotor activity. Inset – bar graph representation of data summed over time. Data are presented as distance travelled (cm) in 5 minute bins ± s.e.m. starting 5 minutes after placement into chamber. Histogram is the total distance travelled as summed from minute 5 to minute 45 ± s.e.m. (b) Bar graph representation of dark-phase locomotor activity summed for 100 minutes post-injection. Data are presented as total distance travelled as summed for 100 minutes starting 10 minutes after injection ± s.e.m. (c) CNO inhibits dark-phase spontaneous locomotor activity, time course. Data are presented as distance travelled (cm) in 5 minute bins ± s.e.m. *$p<0.05$; two-tailed t test, n=4. **$p<0.005$; two-tailed t test, n=8

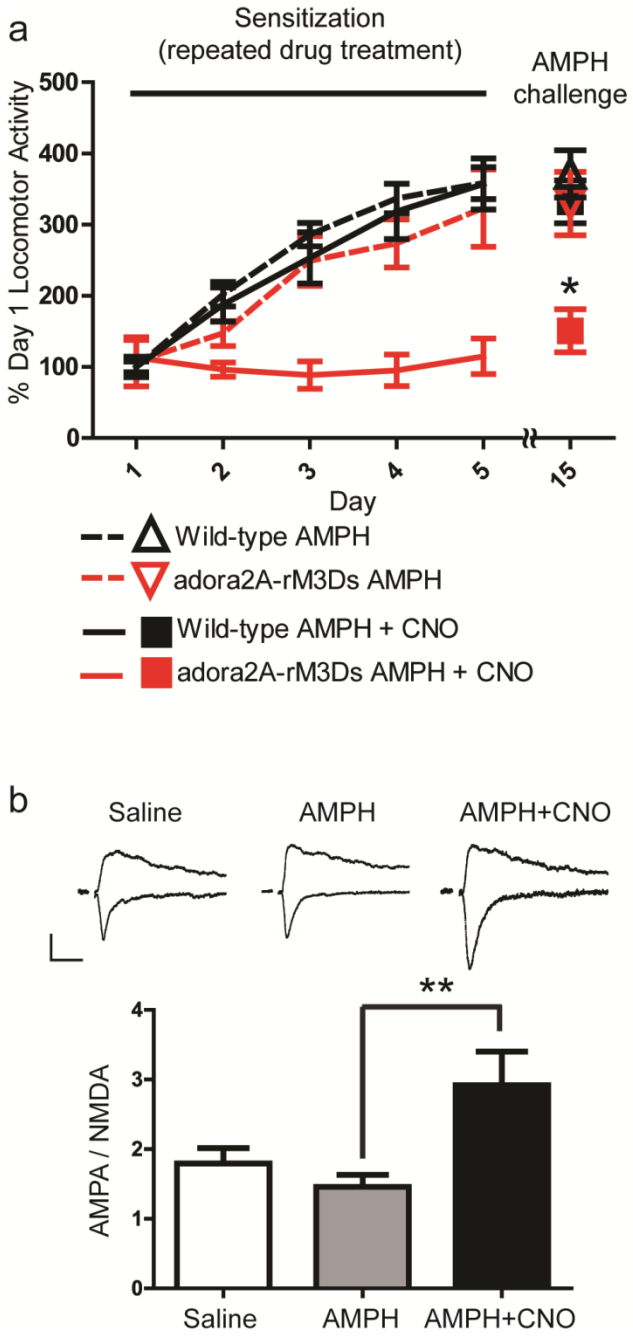


Figure 11: CNO administration modulates amphetamine-induced physiological changes.

(a) Co-administration of CNO (1.0 mg/kg) blocks the development of behavioral sensitization caused by amphetamine (2.0 mg/kg) in adora2A-rM3Ds mice. Data shown are mean percentage of day 1 total horizontal distance traveled over 60 min (+/- SEM). (b) CNO

+ AMPH increases the AMPAR/NMDAR receptor ratio in adora2A MSNs of the NAc shell relative to AMPH alone treated mice on day 15. Stimulus intensity was set to obtain an evoked AMPAR EPSC of about 70 pA at -70 mV for all three conditions. Scale bar: 25 pA and 20 ms. Sample traces shown on top. All data are presented as means ± s.e.m. *$p<0.05$; two-tailed t test, n=7. **$p<0.01$; two-tailed t test, CNO/AMPH n=12, AMPH n=14, Saline n=12.

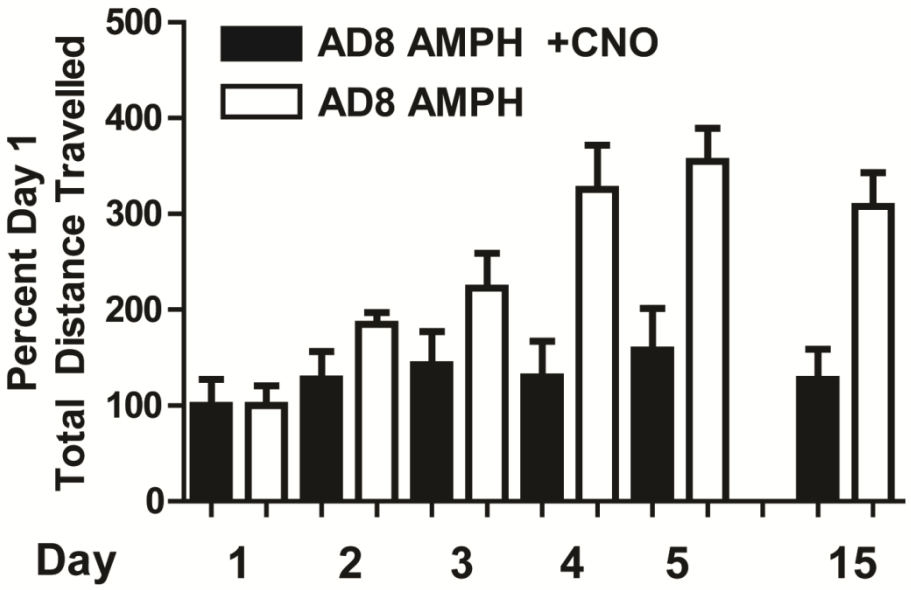


Figure 12: Replication in second founder line.

A second founder line (AD8) of the adora2A-rM3Ds transgenic mouse line exhibits a similar phenotype in the amphetamine (2.0 mg/kg) behavioral sensitization paradigm in response to CNO (1.0 mg/kg) administration. Data are presented as percent day 1 average total distance travelled from t60 – t120.

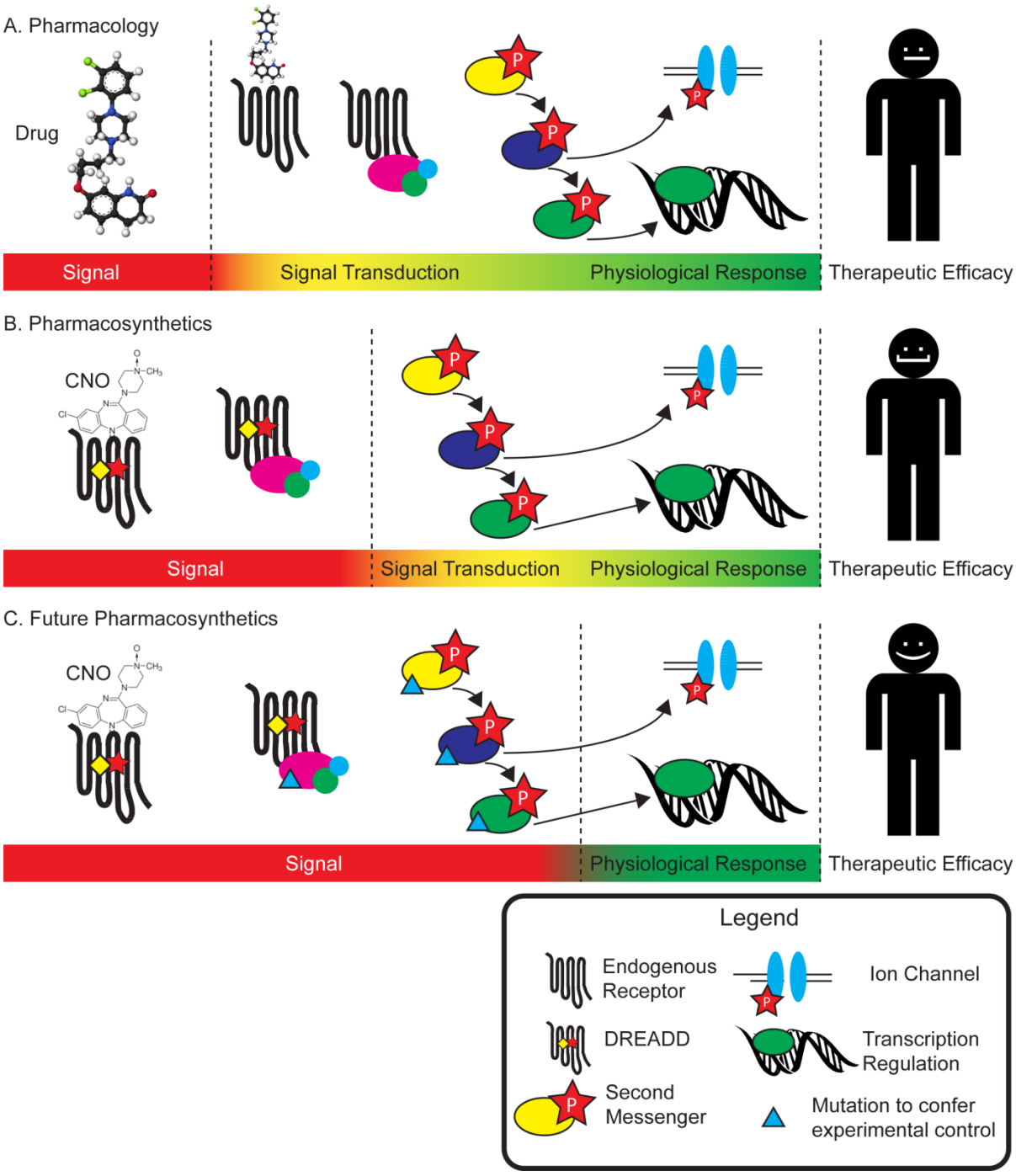

A. Pharmacology
Drug
Signal
Signal Transduction
Physiological Response
Therapeutic Efficacy
B. Pharmacosynthetics
CNO
Signal
Signal Transduction
Physiological Response
Therapeutic Efficacy
C. Future Pharmacosynthetics
CNO
Signal
Physiological Response
Therapeutic Efficacy
Legend
Endogenous Receptor
Ion Channel
DREADD
Transcription Regulation
Second Messenger
Mutation to confer experimental control

Figure 13: Shifting the pharmacological equation.

(A) The conventional understanding of pharmacology posits that drugs interact with physiological systems to produce a physiological response. This interaction takes place through the transduction of a signal (either an endogenous chemical or an exogenous drug) via signal transduction mechanisms. The current depth of pharmacology defines the signal as the ligand itself, freeing the signal transducer and the signal transduction cascade to blend with the physiological response. **(B)** Pharmacosynthetic depth of pharmacology. Using pharmacosynthetics, both the ligand and the signal transducer (receptor) shift to the left side of the equation, creating a more defined signal with less signal transduction noise. **(C)** Future Pharmacosynthetics. With further development, a deeper level of pharmacosynthetic manipulation can be obtained. With the entirety of the signal transduction cascade under experimental control, we could potentially understand the type of signal needed to evoke a particular physiological response.

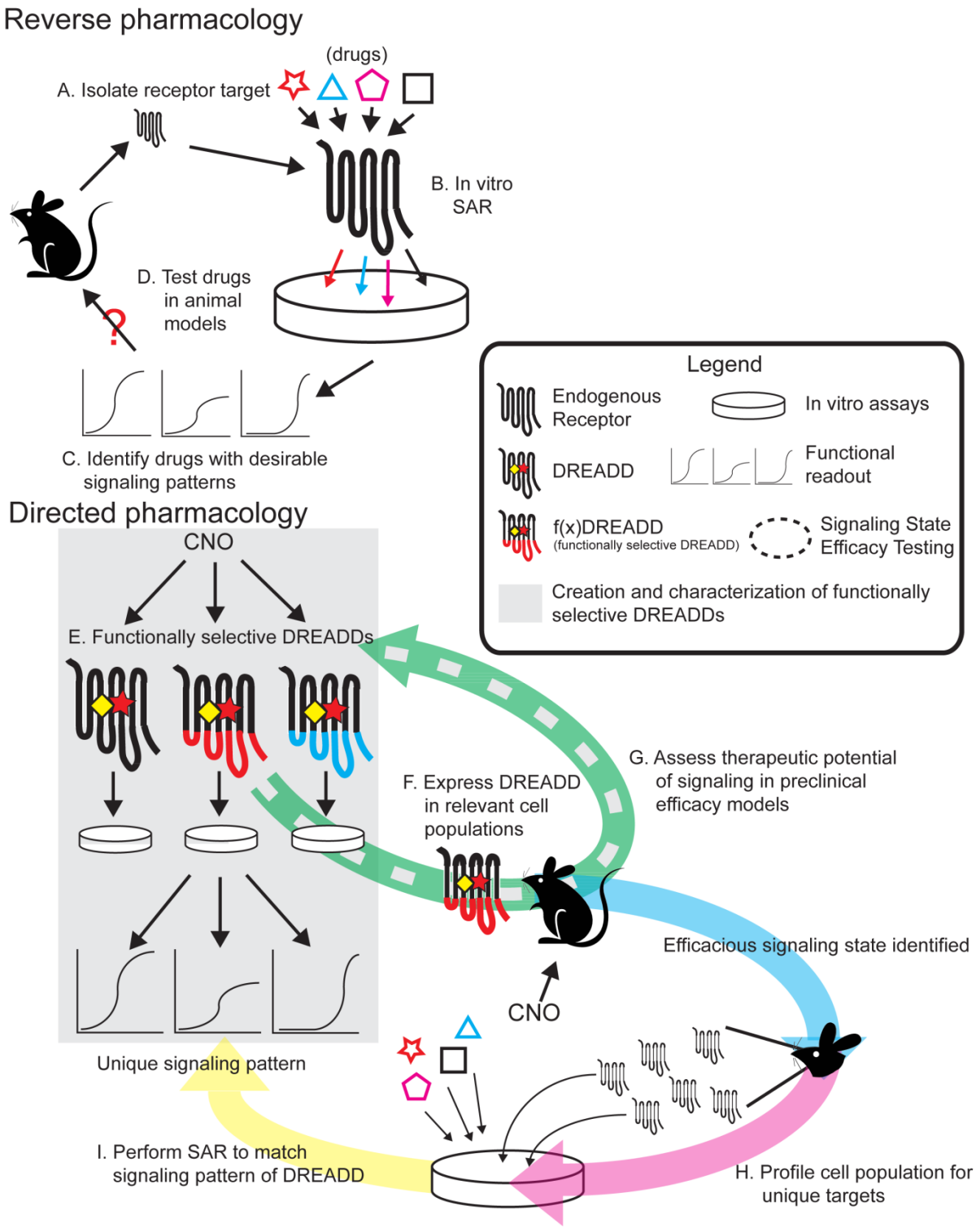

Reverse pharmacology
(drugs)
A. Isolate receptor target
B. In vitro SAR
D. Test drugs in animal models
C. Identify drugs with desirable signaling patterns
Legend
Endogenous Receptor
In vitro assays
DREADD
Functional readout
f(x)DREADD
(functionally selective DREADD)
Signaling State Efficacy Testing
Creation and characterization of functionally selective DREADDs
Directed pharmacology
CNO
E. Functionally selective DREADDs
F. Express DREADD in relevant cell populations
G. Assess therapeutic potential of signaling in preclinical efficacy models
Efficacious signaling state identified
CNO
Unique signaling pattern
I. Perform SAR to match signaling pattern of DREADD
H. Profile cell population for unique targets

Figure 14: An application of pharmacosynthetics in drug discovery.

(A-D) Conventional drug discovery efforts – reverse pharmacology. In this approach, a drug target is selected (usually based on previous success at that particular target) and isolated from the organism. A particular type of signaling is hypothesized to be efficacious, and chemicals are created that cause the receptor to produce that signaling state in cultured cells. The drug is then reintroduced to the model organism and tested for therapeutic efficacy. **(E-I)** A theoretical workflow of drug discovery efforts using pharmacosynthetics, here termed direct pharmacology. In this approach, functionally selective DREADDs (f(x)DREADD, where x=signaling type) are created by modifying DREADDs to modulate distinct signaling phenomena in cultured cells while maintaining their DREADD properties. These f(x)DREADDs are then expressed in therapeutically relevant neuronal populations. These mice are then tested in animal models of therapeutic efficacy. If a particular f(x)DREADD in a particular neuronal population is efficacious, then that cell population is profiled to find druggable targets. These druggable targets are then isolated (expressed in cultured cells) and chemicals are created that modulate these targets to reproduce the signaling state created by the f(x)DREADD. Chemicals that successfully recapitulate the f(x)DREADD-induced signaling are then tested in animal models of efficacy.

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