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DREADD Targeting Projections from the Nucleus Accumbens to the Ventral Pallidum with
Nicotine Self-Administration

A thesis
presented to
the faculty of the Department of Biological Sciences
East Tennessee State University

In partial fulfillment
of the requirements for the degree
Master of Science in Biology with a concentration in Biomedical Sciences

by
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December 2018

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Keywords: nucleus accumbens, ventral pallidum, mesolimbic pathway, motivation, GABA

ABSTRACT

DREADD Targeting Projections from the Nucleus Accumbens to the Ventral Pallidum with

Nicotine Self-Administration

by

Amanda L. Smith

Projections from the Nucleus Accumbens (NAc) to the Ventral Pallidum (VP) play a critical role in motivation and reward. Rewards and reward-associated cues are thought to alter this pathway by suppressing GABA release to the VP, however, the role of the NAc to VP pathway has never been investigated with regard to nicotine self-administration. We hypothesized that increasing GABAergic signaling from the NAc to the VP would decrease the reinforcing effects of nicotine. To increase GABA release, CRE dependent DREADD was expressed in the NAc of male rats. Administration of low dose clozapine activated the DREADD receptor and showed a reduction in responding for nicotine suggesting that activation of the NAc to VP pathway reduced reinforcement. However, a nonspecific effect was observed leading to future investigation of locomotor activity. Immunohistochemistry and microdialysis were used to confirm DREADD expression in the NAc and increased extracellular GABA in the VP.

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TABLE OF CONTENTS

	Page
ABSTRACT	2
ACKNOWLEDGEMENTS.....	3
LIST OF FIGURES	7
Chapter	
1. LITERATURE REVIEW	8
Project Overview	8
Animal Model	9
Nicotine.....	9
Reward Center	10
Indirect and Direct Pathway.....	11
Innervation of Indirect Pathway onto VP	12
Neuronal Manipulation	12
2. DREADD Targeting Projections from the Nucleus Accumbens to the Ventral Pallidum with Nicotine Self-Administration	15
Introduction	15
Hypotheses	17
Materials and Methods	18
Subjects	18
Apparatus	18
Conditioning Chambers	18
Drugs and Solutions.....	19
Intraperitoneal Solution	19
Intravenous Solution.....	19

Surgery	19
Surgery 1: Intravenous Catheter and Retro AAV Placement	19
Intravenous Catheter	19
Retro AAV	20
Surgery 2: AAV DREADD and Control Vector Placement	20
DREADD or Control	20
Procedures	21
Experiment	21
Training	21
Intravenous Nicotine or Saline Self-Administration	21
Microdialysis	21
Experiment 2	21
Microdialysis Surgery	21
Microdialysis Collection Assembly	22
GABA Data Collection	22
Microdialysis Solution	23
Artificial Cerebrospinal Fluid	23
GABA Mobile Phase	23
GABA Standard	23
Tissue Collection and Storage	24
Experiment 1	24
Perfusion	24
Tissue Fixation	24
Experiment 2	24
Histology	25
Tissue Slicing	25
Tissue Staining	25

Fluorescence Imaging	26
Statistical Analysis.....	26
Experiment 1: Nicotine Self-Administration	26
Experiment 2: Microdialysis.....	26
Results	27
Experiment 1.....	27
Acquisition.....	27
Treatment	28
Immunohistochemistry	29
Experiment 2.....	31
Discussion.....	32
Nicotine Self-Administration.....	33
Future Directions	36
Conclusion	36
REFERENCES	38
VITA	41

LIST OF FIGURES

Figure	Page
1. Active Lever Presses for Acquisition	27
2A. Active Lever Presses with Treatment	28
2B. Inactive Lever Presses with Treatment	29
3A. DREADD Localization	30
3B. DREADD Expression	30
4. Amount of GABA (%Baseline)	31

CHAPTER 1

LITERATURE REVIEW

Project Overview

Substance dependence is widely considered to be a ‘brain disease’ – or, more specifically a disorder of maladaptive neuroplasticity (Nestler 2014). External stimuli, cognition, behavior, and emotion can remap the brain to alter its responses to future circumstances (Fuchs and Flügge 2014). Drugs of abuse can directly alter neuroplasticity in circuits that mediate motivation and approach behaviors, distorting the reward and reinforcement derived from the addictive drug and its associated stimuli (Lüscher and Malenka 2011). These synaptic changes in brain circuits can lead to craving, unsuccessful quit attempts, and taking the drug in increasing amounts. Dependence (used interchangeably with ‘addiction’) is traditionally defined as ‘loss of control’ over drug taking - most addicts will continue to use a substance despite persistent problems related to taking the drug (American Psychiatric Association 2014). A common example is a chronic smoker continuing to smoke tobacco cigarettes after the diagnoses of a chronic respiratory disease, like emphysema. Even with negative consequences, motivation to seek and consume the drug still persists. Drugs of abuse are portrayed to ‘hijack’ the brains reward system promoting changes that lead to impair judgement, memory, and decision making (Pfaus and Blaustein 2015; National Institute of Drug Abuse 2018). A large amount of funding and research been invested into understanding why humans continue to desire a drug. Clinical trials use imaging, like fMRI or functional magnetic resonance imaging, to show what areas of the brain are activated during cravings, reward, or withdrawal. However, it is not plausible to use a human model when converging the neurobiology behind the behavior. The best alternative option is using an animal model which allows for more diverse methods when investigating dependence.

Animal Model

Animal models of substance dependence have provided more perspective about the complex neurobiology of this disorder. Conditioned place preference, intracranial self-stimulation, and drug self-administration are a few of the developed models that have allowed researchers to observe behavior and further understand the function of reward and motivation in non-human animals (Feltenstein and See 2008). Rodents Rats have the ability to perform multifaceted cognitive tasks which allow for highly translational data and invasive manipulations (Iannaccone and Jacob 2009). Not only has rodent behavioral testing shown relevance to clinical studies but has also provided validity of the similarities in neurochemical and neuroanatomy involved in drug-associated behaviors between rodents and humans. Rodents will not only voluntarily self-administer the same abusive drugs but will do so in comparable ways including intravenous and orally self-administration (Spanagel 2017). The animal model is an excellent way to further understand the changes in the reward pathway associated to drug use specifically the more common consumed drug, nicotine.

Nicotine

Nicotine is a readily available psychostimulant found in tobacco cigarettes, smokeless tobacco, and vaping products. In the United States, smoking tobacco cigarettes is one of the leading causes of smoking-related premature deaths including cancer, cardiovascular disease, and pulmonary diseases. An estimated 45 million Americans smoke with only 3% successfully quitting (Benowitz 2010). Health risks of smoking or the use of smokeless tobacco are well known and yet many still persist. Evidence has shown that nicotine alters the brains natural reward system leading to dependence (Tomkins and Sellers 2001). The reward system is referred to as the mesolimbic dopaminergic system and is a common target among all drugs of abuse

(Lüscher and Malenka 2011). Nicotine controls the mesolimbic system by binding to $\alpha 4\beta 2$ nicotinic acetylcholine receptors expressed on dopaminergic neurons increasing pleasure and mood (Benowitz 2010). The psychostimulant effects of nicotine may not be comparable to cocaine or other drugs of abuse. It does, however, activate the same dopaminergic pathways making nicotine a reliable drug when investigating dependence.

Reward Center

Perhaps the most commonly studied target of substance dependence is the mesocorticolimbic dopamine system, which mediates incentive motivation and approach behaviors evoked by natural rewards (e.g., sex, food, and social interactions; Feltenstein and See 2008). The mesocorticolimbic system is constructed from two overlapping circuitries called the mesolimbic and mesocortical dopaminergic systems that stem from dopaminergic neurons within the ventral tegmentum area (VTA) in the midbrain which sends projections to various forebrain structures (Chinta and Andersen 2005). The mesolimbic system is comprised of reciprocal projections from the VTA to the nucleus accumbens (NAc), amygdala, hippocampus, olfactory tubercle. While projections from the VTA to the prefrontal, cingulate and perirhinal cortices comprise the mesocortical system (Chinta and Andersen 2005), the dopaminergic projection from the VTA to the NAc is commonly referred to as the ‘final common pathway’ of dependence because inhibition of dopamine (DA) release or blockade of DA receptors in the NAc reduces or abolishes the reinforcing effects of drugs, drug-associated cues, and relapse-like behavior evoked by drug-associated cues (Feltenstein and See 2008). Increased DA in the NAc has been implicated in associative learning, incentive salience, reward prediction, and memory (Kelley and Berridge 2002). The NAc is also a point of convergence in the mesolimbic pathway; in addition to the dopaminergic projections from the VTA, the NAc receives glutamatergic

afferents from the prefrontal cortex (PFC) and hippocampus, which presumably also alter neuroplasticity in response to drugs of abuse (Lüscher and Malenka 2011).

Indirect and Direct Pathways

The NAc is located ventrally in the corpus striatum of the basal ganglia and is comprised of two parts: the core and shell (Dafney and Rosenfeld 2017). While DA is one of the main neurotransmitters released in the NAc, glutamate and GABA are also thought to help regulate goal-directed behaviors through integration of the limbic system (Dafney and Rosenfeld 2017). The neuronal structure of the ventral striatum is predominantly medium spiny neurons (MSNs) that synthesize the inhibitory neurotransmitter, GABA (Perreault et al. 2011). MSNs are subcategorized by their expression of dopamine D1 and D2-like G protein-coupled receptors and corresponding involvement in the indirect and direct pathways, although some overlapping exists (Lobo and Nestler 2011). These two distinct pathways have been identified and used in understanding integration of reward and drug-seeking behaviors.

The striatopallidal “indirect” pathway consists of MSNs that predominantly express dopamine D2-like receptors and project from the striatum to the external globus pallidus (GP) (Perreault et al. 2011). The second striatonigral “direct” pathway is known for dopamine D1-like receptor expression and projections to substantia nigra reticulata and internal segment of the GP (Perreault et al. 2011). The direct and indirect pathways are thought to combine to control thalamic output – with the indirect pathway serving as a ‘brake’ on the direct pathway (Perreault et al. 2011). DA is believed to have opposing effects on dopamine D1 and D2-like receptors within these two pathways (Gallo et al. 2018). Recent evidence has suggested that dopamine D1-like receptors are low affinity (e.g., activated primarily by large increases in DA release) and activated in response to delivery of rewards and anticipation of rewards. In contrast, DA D2-like

receptors are low affinity (activated by small increases in DA tone) and appear to be associated with drug seeking mechanisms (Lobo and Nestler 2011). While addiction research of the NAc has allowed for insight into the mechanism of drug-associated reward and reinforcement, its efferents to thalamocortical circuits and their contributions to regulation of reward and approach behavior have been under-studied in the substance dependence literature (Smith et al. 2009).

Innervation of Indirect Pathway onto the VP

The ventral pallidum (VP) receives the primary GABA-ergic efferent from the NAc MSNs in the indirect pathway (Gallo et al. 2018). The VP was once considered part of the GP, but has more recently been identified as its own brain region (Smith et al. 2009). GABAergic neurons are the primary neuronal component of the VP and are associated with inputs of GABA and non-GABA transmission from the striatum (Root et al. 2015). Because of the location and reciprocating projections from various brain regions, it is hypothesized that the VP plays a crucial role in motor output for limbic motivation signals (Smith et al. 2009). The MSNs of the NAc project primarily to the VP which allows for incorporation of the direct and indirect pathways through D1 and D2-like receptor activity (Creed et al. 2016). A focus has been placed on the innervation of the MSNs expressing D2-like receptors and the VP. Activation of these D2-like receptors are thought to reduce inhibitory transmission to the VP enhancing the motivation or willingness to work for a rewarding external stimuli (Gallo et al. 2018). Modifications to the indirect pathway could theoretically alter the incentive motivation for the reward. These types of manipulations can be accomplished using a chemogenetic technique called DREADD.

Neuronal Manipulation

Chemogenetics is becoming a more valued method when attempting to understand neuronal signal transduction. It can manipulate cells via alterations to proteins, G protein-

coupled receptors, or ligand-gated ion channels (Roth 2016). Designer Receptors Exclusively Activated by Designer Drugs, or DREADD, is a form of a chemogenetic technique gaining acclamation because of its ability to distort neuronal transmission using a mutated G protein-coupled receptor (GPCR) that only responds to a synthetically designed ligand. The design for the DREADD receptor evolved from the human seven-transmembrane domain muscarinic receptor. The developed receptor was mutated to specifically respond to clozapine-n-oxide (CNO) and metabolites but not to the endogenous ligand, acetylcholine (Zhu and Roth 2014). Recent evidence has shown that CNO metabolizes back into clozapine showing conflicting results of both CNO and clozapine activating DREADD receptors (Gomez et al. 2017) (Zhu and Roth 2014). Three DREADD signaling pathways have been produced and behave in different ways: hM3Dq induces neuronal transmission, hM4Di inhibits neuronal transmission, and rM3Ds which modulates neuronal activity (Zhu and Roth 2014). DREADD expression has shown to be high in injected brain regions because of the cell-type promoter associated with the DREADD genome and is tagged with a fluorescent protein. This also means that expression is not specific (Roth 2016). To increase specificity, a Cre-recombinase dependent DREADD was produced.

Cre-recombinase is an enzyme that recognizes specific regions within the DREADD genome. Expression of Cre-recombinase can be implicated through animal genetic modification and breeding or through viral administration discussed in detail below. DREADD expression became more selective with the production of a modified double-floxed open reading frame in an antisense configuration (Smith et al. 2016). Located on both sides of the reading frame are LoxP sites which are recognized by Cre-recombinase. Cre-recombinase cuts and flips the inverted gene transforming the reading frame into a sense configuration and allowing for DREADD expression

(Smith et al. 2016). This method increases selective targeting by restricting DREADD expression in neurons that do not already express Cre-recombinase.

As previously mentioned the genes responsible for Cre-recombinase and DREADD expression can be administered through viral applications. The common virus used is the Adeno Associated-Virus (AAV). AAV is not autonomously replicating unless the assistance from a helper virus occurs. While a virus may seem dangerous, to administer to animals or humans, researchers have constructed different AAVs to have little or no immune response (Mingozzi and High 2013). AAVs can infect cells through normal endocytosis or retrograde activity making them perfect for neuronal targeting. The Roth lab has combined the AAV and DREADD allowing for a molecular manipulation of neuronal transmission (Roth 2016).

In this study, Cre-dependent DREADD was used to manipulate and further understand the motivational pathway of the brain with nicotine. Currently addiction treatment consists of medication that mimics abusive drugs, behavioral counseling, treating withdrawal, and long-term follow up appointments (NIDA 2018). The brain is complex and is not fully understood. With that being said, this research combined with the studies previously mentioned could provide a more clear understanding about neuronal pathways associated with motivation and habitual drug use. Therefore providing new therapeutic opportunities and further insight into substance abuse disorder.

CHAPTER 2

DREADD Targeting Projections from the Nucleus Accumbens to the Ventral Pallidum with Nicotine Self-Administration

Introduction

The nucleus accumbens (NAc) has been a major target in understanding the intricacy of drug dependence. Various research has shown the effects of commonly abused drugs on dopamine levels and receptors expressed on neurons within the NAc. With an increasing amount of data implicating the importance of the NAc to the reward of a drug, a new interest in incentive motivation has emerged. This has shifted the focus to the ventral pallidum (VP) and its role in drug incentive motor integration.

The NAc primarily consists of GABA transmitting medium spiny neurons (MSN) expressing D1-like and D2-like dopamine receptors. Cells that express D1-like receptors are part of the direct pathway – the receptors have lower affinity for dopamine (e.g., require more dopamine release before they are activated), therefore, stimulation of these receptors increases reward-based motivation and approach to incentive stimuli (Day and Carelli 2007). In contrast, D2-like receptors are high affinity (e.g., require less dopamine release to be activated) and are part of the ‘indirect pathway’ which regulates reward-based motivation and reduces approach to incentive stimuli (Hamada et al. 2004). Unlike D1 receptors which activate the direct pathway, D2-like receptors inhibit the indirect pathway. Inhibition would decrease the incentive motivational inhibitor and may have different effects on behavior. This would inhibit the indirect pathway and reduce inhibition on incentive behaviors, causing an increase in unfocused search behaviors. The indirect pathway includes projections from the NAc to the Ventral Pallidum (VP). Stimulation of NAc D2-like receptors decrease GABA release in the VP, which leads to a

‘release from inhibition’ on incentive motivation (Smith et al. 2009). All drugs of abuse activate the incentive motivational systems of the brain and either increase DA release in the NAc or mimic the effects of increased DA release in the NAc (Lüscher and Malenka 2011).

The effects of nicotine on the direct and indirect pathway are currently unclear. For example, naïve rats exposed to nicotine injections showed approximately 100% increase in extracellular DA in the NAc (Rada et al. 2001), but this increase pales in comparison to other drugs of abuse such as cocaine, which can cause over 500% increases in extracellular DA (Andrews and Lucki 2001). Theoretically, nicotine induced DA release activates the indirect pathway implying that after brief activation by nicotine, a tobacco user’s incentive system might be released from inhibition – making them more sensitive to non-drug rewards because the indirect pathway has essentially been tuned-down. Such effects could make the act of smoking, the smoking-associated stimuli (flavors, smells, actions associated with smoking) and social/contextual cues (friends, alcohol drinking, bars, parties, etc.) more salient incentives and more likely to increase behavior in the future. Therefore, if the indirect pathway could be activated, a reduction in nicotine self-administration should be observed.

To alter the indirect pathway, Designer Receptors Exclusively Activated by Designer Drugs, or DREADDs, was used to selectively target neurons projecting from the NAc to the VP pathways and control cellular function. DREADD is a single DNA strand containing the makeup for a designer G-coupled muscarinic receptor that, in this study, is dependent on Cre-Recombinase (CRE) for expression (Kwon and Schaffer 2008). CRE, like DREADD, is not endogenous to neurons and must be implanted using an adeno-associated virus (AAV) that induces retrograde expression of CRE into the VP. All neurons with projections to the VP should be targeted and express CRE. The synapsin 1 promoter ensured that selectivity occurred of

targeted neurons and not glial cells (Madisen et al. 2015). After 2-3 weeks, CRE-dependent DREADD was injected into the NAc. By doing so, only neurons that project from the NAc to the VP should express the DREADD. Once the DREADD is expressed it can be activated by a low dose of clozapine, which was too low to bind to dopamine receptors (Gomez et al. 2017). The DREADD used changed the neuronal function by causing increased transmission. In this study, the DREADD was predicted to increase GABA release from the MSNs in the NAc to the VP, with the goal of increasing activity in the indirect pathway and reducing nicotine self-administration.

Hypotheses

1. Activation of DREADD in the NAc will cause an increased GABA concentration in the VP.
2. The administration of clozapine will not affect nicotine self-administration of control animals.
3. Increased GABA release to the VP via activation of the indirect pathway will cause a reduction in nicotine self-administration.

Materials and Methods

Subjects

Adult male Sprague Dawley rats (Charles River) were used for the experiment. Rats were individually housed and on a 12:12 hour light and dark cycle. All experiments took place during the dark cycle. Upon arrival, each rat had unrestricted access to food and water until weight exceeded 200 g. Food was then restricted to 15-20 g daily with unrestricted water for the remainder of the study.

Apparatus

Conditioning Chambers

Self-administration occurred in ten Med-Associates (Georgia, VT) operant chambers (ENV-008CT) measuring $30.5 \times 25.4 \times 30.5$ (w \times l \times h). Each chamber individually housed a rat in a ventilated, sound and light attenuated enclosure. A receptacle containing a liquid dipper was located on the right side of the chamber. The dipper had a 0.1 ml cup attached to a motorized arm that delivered a sucrose reinforcer. The sucrose reinforcer was only used during training. Two retractable levers (ENV-112CM) were located on each side of the receptacle, 12 cm above the grid flooring. Pressing the active lever, left lever, earned a reinforcer. While pressing the inactive lever, right lever, resulted in no reinforcer earned. All lever presses were recorded. A stimulus light was located above each lever that would turn on when lever was pressed and retracted. During conditioning a house light located at the top of both sides of the chamber, approximately 28.5 cm above the floor, remained on until reinforcer was earned. House light would then turn off for a 30 second time out. Each operant conditioning chamber was equipped with a drug delivery system with a syringe pump (Med-Associates, model PHM100 – 10rpm)

that would deliver intravenous drug throughout all experiments. Recorded data collection from the operant chambers took place on a computer using MED-PC V software programming.

Drugs and Solutions

Intraperitoneal Solution

Clozapine (Cayman Chemical) was dissolved into 1 ml of 1M HCl. NaOH was used to pH the solution to 7.0 (± 2). The clozapine concentrations were tested between 0.03 - 0.75 mg/kg to find the most affective dose. Clozapine was administered into the peritoneal cavity (ip) 30 minutes prior to the animal being placed into the operant chamber.

Intravenous Solution

Nicotine hydrogen tartrate salt (Stigma, St. Louis, MO) was diluted with 0.9% sterile saline to form a 30 $\mu\text{g}/\text{kg}$ concentration. NaOH was used to pH solution to 7.0 (± 2). Nicotine was self-administered through the animal's intrajugular catheter. The volume self-administered by the rat was weight dependent. For a control solution, 0.9% sterile saline was used.

Surgery

Surgery 1: Intravenous Catheter and Retro AAV Placement

Intravenous Catheter. Each catheter was constructed using a blunt 22 gauge needle bent to form a 90° angle that was attached to a silastic tube (Dow Corning, Michigan) (0.51 ID x 0.94 mm OD) 175 mm in length. To secure the tubing to the needle, a nylon washer (.09 x .25 x .062) (Fastenal, Minnesota) positioned on both sides of a round patch of polyester fabric (37.5 mm diameter) was placed at the base of the needle and fastened by medical device epoxy adhesive (LOCTITE EA M-21 HP, Henkel, North Carolina). Lastly, a silicone ball (3 mm diameter) was attached 40 mm from end of tubing using 100% RTV silicone (DAP, Maryland). All surgical equipment and catheters were sterilized by an autoclave treatment prior to surgery. Each rat was

placed under an inhaled anesthetic, Isoflurane USP (Henry Schein) and equipped with a chronic jugular vein catheter. The catheter exited between the two scapulae while the tubing was placed under the right front extremity. The tubing was then positioned into the right jugular (IV) and secured using two sutures. All incisions were closed using tissue staples. Ketoprofen (0.3mg/kg) was administered subcutaneously on day 0 and followed two days after surgery. Timentin (Ticarcillin and Clavulanate) diluted in heparinized saline was administered IV daily to prevent infection and platelet clot formation. The animals were allowed one week for recovery before nicotine self-administration began. Patency was checked consistently by injecting 0.2 mL PropoFlo (200mg/20mL) IV.

Retro AAV. After catheter placement, each animal was placed in a stereotaxic apparatus and remained under Isoflurane. An estimated one inch incision was made exposing the rat's skull. Bregma was measured and noted. The first bilateral intracranial injections targeted the rostro medial VP (1.6 AP, \pm 1.8 ML, -7.9 DV) at a 10 degree angle. The second bilateral intracranial injection targeted the caudal lateral VP (-0.5 AP, \pm 2.6 ML, -8.2 DV). The viral infusion rate was set to 0.1 μ L/min with a total infusion of 0.5 μ L for each injection. Incision was closed using tissue staples. Two weeks were given to allow for retrograde activity of the AAV and viral genomic expression of CRE-Recombinase.

Surgery 2: AAV DREADD and Control Vector Placement

DREADD or Control. Each animal was anesthetized using Isoflurane and placed in a stereotaxic apparatus. Incision and closing were the same as previously demonstrated. The animals were divided into three groups: DREADD Nicotine, DREADD Saline, Control Nicotine. Both DREADD Nicotine and DREADD Saline groups were injected with AAV expressing CRE dependent DREADD into the NAc through bilateral intracranial injections (1.6 AP, \pm 1.3 ML, -

7.3 DV). The Control Nicotine group received the control AAV into the NAc with the same coordinates stated above. For all intracranial injections into the NAc, the viral infusion rate was set to 0.1 μ L/min with a total infusion of 1.0 μ L for each injection.

Procedures

Experiment 1

Training. Prior to any surgery, rats were trained to lever press for a reinforcer of sucrose access through the receptacle. To begin, the left lever was inserted into the operant chamber for 15 s followed by activation of the liquid dipper allowing 0.1 ml sucrose access for 0.5 s. If the lever was pressed during presentation, responses were recorded and the animal was given two sucrose reinforcers separated by 5 s. The animal needed to earn 60 reinforcers within a 1-h session to be considered successfully trained.

Intravenous Nicotine or Saline Self-Administration. After IV catheter implantation and viral expression, rats were individually placed into the operant chambers and connected to the syringe pump containing nicotine 30 μ g/kg or 0.9% sterile saline. Once the session began, both retractable levers presented and operated on a fixed ratio (FR) for 1 h. Self-administration began on a FR2 and increased to ten over a period of sessions. An increase in the FR was dependent on data consistency. Once animals reached a FR10, Clozapine was administered chronically until data collection was complete.

Microdialysis

Experiment 2

Microdialysis Surgery. Male Sprague Dawley rats (5n) that were placed under Isoflurane and stereotaxically implanted ipsilaterally with the Retro AAV containing CRE-Recombinase into the rostro medial VP (1.6 AP, \pm 1.8 ML, -7.9 DV) at a 10 degree angle. Retro AAV was

allowed two weeks for expression. Rats were then ipsilaterally implanted with the AAV containing DREADD into the NAc (1.6 AP, \pm 1.3 ML, -7.3 DV). Both viral infusions were 0.1 μ L/min with a total infusion of 1.0 μ L for each injection. An 18 gauge, cut 11 mm below pedestal cannula (PlasticsOne, Roanoke, VA.) was placed, on the same side as previous injections, into rostro medial VP (1.6 AP, \pm 1.8 ML, -6.7 DV) at a 10 degree angle. Dummy stylets were placed into the cannula following surgery and removed until the microdialysis collection session. Animals were allowed one week to recover. During recovery rats were placed into microdialysis chambers (Dr. Gerald Deehan PhD., East Tennessee State University) for a three day habituation. A loop style microdialysis probe was inserted 2 mm below cannula into the VP one day prior to microdialysis data collection.

Microdialysis Collection Assembly

Gamma-Aminobutyric Acid, GABA, dialysate was collected by connecting the microdialysis probe directly to a microdialysis collection assembly. The assembly was constructed with two pieces of tubing. One side connected to a swivel that perfused aCSF towards the probe in the rat's brain. This side was constructed 29 cm of PE20 tubing. The other side collected aCSF coming from the microdialysis probe and was constructed of 29 cm of PE10 tubing with a ball 3 cm from the end. The plastic ball secured an amber vial and cap to the line.

GABA Data Collection

One day following probe placement, the rats were placed into microdialysis chambers and connected to the collection assemblies. For the first 90 minutes one washout sample was collected. This helped eliminate any potential contaminants. After the first 90 minute sample, each sample was collected at 20 minute intervals. Baseline activity of GABA was collected over a period of 100 minutes. Immediately after the fifth sample was collected, 0.75 mg/kg Clozapine

was administered through intraperitoneal (IP) injections to each animal. Following the injection, five more samples were collected. Clozapine was injected IP at 1.50 mg/kg succeeding collecting ten more samples. This resulted in 400 minutes of total data collection. All samples were stored in a -80°. GABA dialysate was analyzed through electrochemical detection via graphite electrode using a EiCOM HTEC-510 HPLC machine (San Diego, CA). EiCOM AS-700 autosampler (San Diego, CA) controlled by the Envision software detected and quantified GABA dialysate samples using a one-sample t test.

Microdialysis Solution

Artificial Cerebrospinal Fluid. Artificial cerebrospinal fluid (aCSF) microdialysis perfusion medium was composed of 145.0 NaCl, 2.7 KCl, 1.0 MgCl₂, 2.5 CaCl₂, and 2.0 Na₂HPO₄. aCSF was filtered through a sterile filter (pore size, 0.2 µM), and prepared the day of microdialysis sessions. When necessary, the pH was adjusted to 7.4 with 0.1 N acetic acid. aCSF was perfused through 2 mm loop-style probes at a rate of 1.0 µl/min.

GABA Mobile Phase. GABA mobile phase used for GABA dialysate analysis via HPLC was composed of first dissolving 10.76 g NaH₂PO₄(2H₂O), 3.95 g Na₂HPO₄(12H₂O) in 800 mL of HPLC grade water with an end pH of 6.0. Next 50 mL of methanol was added with 130 mL acetonitrile, 5 mg EDTA(2Na). Solution was mixed until all particulate matter was dissolved.

GABA Standard. GABA/Glutamate standards were constructed for HPLC calibration and controlled GABA and Glutamate detection. Standards were composed of γ -Aminobutyric acid and L-Glutamic acid (Sigma) and diluted to a concentration of 100 nM GABA and 10 µM Glutamate using aCSF.

Tissue Collection and Storage

Experiment 1

Perfusion. Concluding behavioral testing, animals were sacrificed by an overdose of Isoflurane. During anesthesia, the animal was placed on its back and the thoracic cavity was opened to fully expose the heart. A 30 gauge needle with tubing connected to a pump was inserted into the left ventricle. A small laceration to the right atrium was placed to allow for blood to flow out of the circulatory system. The descending abdominal aorta is clamped to increase preservation of superior tissues. Once the needle had been placed, phosphate buffered saline (PBS) 1x was perfused throughout the tissue for 20 min followed by 4% paraformaldehyde. Once the blood had cleared from exposed tissues, the brain was then extracted.

Tissue Fixation. The brains were placed in 4% paraformaldehyde in 4° for 24 h. The tissue was then transferred into PBS 1x and stored in 4° for a minimum of 24 h. To cryoprotect the tissue, brains were put in 30% sucrose and stored in 4° until tissue descended fully into solution. Next, each brain was rinsed in O.T.C compound (Fisherbrand) and placed in a new O.T.C for freezing. Brains were stored in a -80°.

Experiment 2

Directly after microdialysis, animals were sacrificed and tissue was preserved using the same perfusion procedure and tissue fixation as defined above. However prior to tissue extraction, Bromophenol blue (1%; 0.5µl) was perfused through the microdialysis probe to stain the VP to confirm correct probe placement. The tissue then underwent the same histology as experiment 1 described below.

Histology

Tissue Slicing

The fixed brain tissue was cryopreserved and coronally sliced at 20 μm . The slices were immediately placed on colorfrost plus microscope slides (Fisherbrand) and stored at -20° .

Tissue Staining

Slides were kept in microscope slide box to reduce light exposure and allowed 30 min to dry at room temperature prior to staining. Tissue borders were defined using a hydrophobic pen and allowed to dry. The tissue was covered with a blocking solution containing PBS 1x, 2% bovine serum albumin, and 0.3% Triton x100 was added to the tissue for 1 hr at room temperature to increase tissue permeability. The blocking solution was then removed. Next the primary antibody, rabbit anti-mCherry antibody from Abcam, was diluted to 1:800 with the blocking solution defined above and added to fully cover sliced tissue. The primary antibody was incubated overnight at 4° Celsius.

The following day, slides were washed (3 x 5 min) with a solution that contained PBS 1x with 3% Triton x100 (PBS-T). The secondary antibody, Alex 594 donkey anti-rabbit IgG from Jackson ImmunoResearch, diluted to 1:1000 in PBS-T was added to the tissue for a 2 h incubation at room temperature. Slides were then washed (2 x 15 min) with PBS-T. A third wash of PBS 1x for 15 min completed the staining process. The PBS 1x was removed and a fluorescence mounting medium, Fluro-Gel (Electron Microscopy Sciences) was added followed by a coverslip. After slides had dried overnight, a clear fingernail polish was applied to the corners to secure the coverslips. Slides were stored at 4° Celsius in the dark until imaging.

Fluorescence Imaging

Tissue was viewed using an Olympus BX41 microscope (Olympus America, Inc.) at a wavelength of 594 nm (Dr. Donald Hoover, East Tennessee State University Biomedical Sciences). Imaging was used to confirm bilateral DREADD expression in the NAc.

Statistical Analysis

Experiment 1: Nicotine Self-Administration

To investigate the effect of Virus (Cre vs. DREADD) and Drug (nicotine vs. saline) on operant responding we conducted a 4-way mixed factors ANOVA with Virus and Drug as between groups factors and Lever (Active vs. Inactive) as within subjects factor. For acquisition Schedule (FR1, FR2, FR5, FR10) was a repeated measure. For clozapine challenge and saline recovery tests, Test (day of treatment) was the repeated measure. Because not all levels of Virus and Drug were represented in our design (e.g., no Cre-Saline group), some of the interactions (any including Drug and Virus) were not calculated. Drug and Virus conditions included were DREADD Nicotine (30 $\mu\text{g}/\text{kg}$), Cre Nicotine, and DREADD Saline (0.9% sterile saline). Alpha criterion was set at $p \leq 0.05$, significant interactions were investigated with second-orders contrasts using Bonferroni's correction for alpha inflation.

Experiment 2: Microdialysis

The % baseline was calculated for each sample and compared to 100% using a one-sample t-test. The analysis was able to detect the difference in % baseline extracellular GABA concentration after the first clozapine (0.75 mg/kg) and second clozapine (1.25 mg/kg) IP injections.

Results

Experiment 1

Acquisition. Responding significantly increased during acquisition, but only on the active lever and only for rats that were self-administering nicotine. This was confirmed by a significant Drug x Lever x Schedule interaction, $F(3,18)= 7.893, p= 0.001$. Second-order contrasts confirmed that all groups showed greater active lever responding, relative to the inactive lever ($p \leq 0.012$) on the FR1 schedule as seen in Figure 1. However, during the FR2, FR5, and FR10 tests, this difference was observed in rats self-administering nicotine ($p < 0.001$) but not rats self-administering saline ($p \geq 0.26$). The difference between active and inactive levers in the Saline group under the FR1 schedule was most likely driven by prior food shaping (e.g., only the active lever was shaped). All animals were food shaped on an FR1 to promote lever pressing.

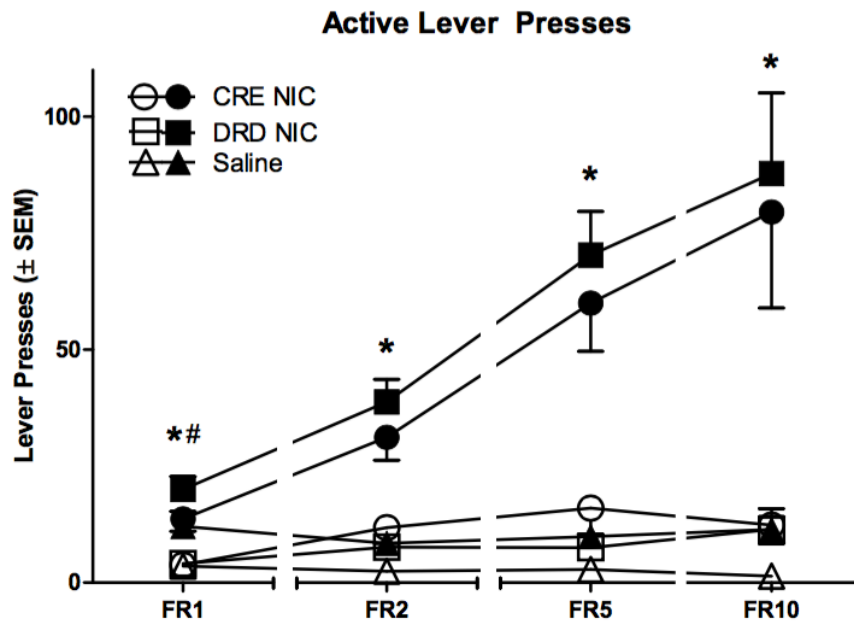


Figure 1: Active Lever Presses for Acquisition. Nicotine enhanced acquisition of the active lever as FR increased. The DREADD Saline group decreased in active lever presses between FR1 and FR2 represented by the pound sign (#). Asterisks (*) represent the significant increase in active lever presses for nicotine as the FR increases.

Treatment. An analysis of Virus x Levers x Tests between nicotine receiving groups revealed a decrease in DREADD Nicotine lever presses with administration of clozapine (0.75mg/kg) $F(2,28) = 7.670, p = 0.002$. The significance can be observed in Figure 2A on Test3 of clozapine between Cre and DREADD Nicotine $F(1,14) = 8.195, p = 0.013$. However, further analysis of inactive lever presses also showed a significant decrease in DREADD Nicotine ($p \leq 0.027$) represented in Figure 2B. Overall, clozapine decreased active and inactive lever responding in the DREADD Nicotine group. Lastly in reference to Figure 2A and 2B, clozapine injections were replaced with 0.9% sterile saline. While the DREADD Nicotine group appeared to gradually increase active lever responding, saline1 and saline2 showed a significant difference in both active $F(1,20) = 8.718, p = 0.008$; $F(1,20) = 4.481, p = 0.047$, and inactive levers ($p < 0.001$) when compared to Cre Nicotine.

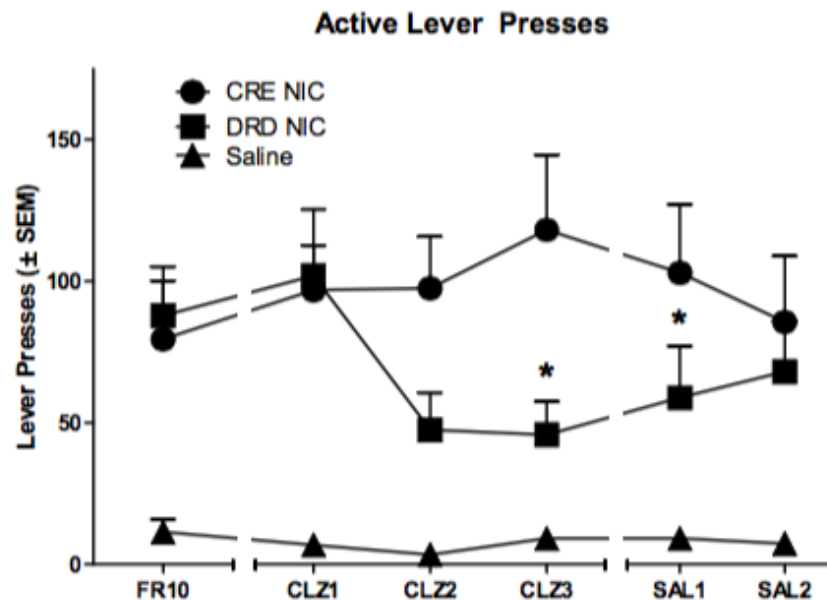


Figure 2A: Active Lever Presses with Treatment. DREADD Nicotine decreased active lever responding for nicotine in comparison to Cre Nicotine. Asterisks (*) represent the significant difference between both groups. Active lever presses are also represented when clozapine was replaced by saline.

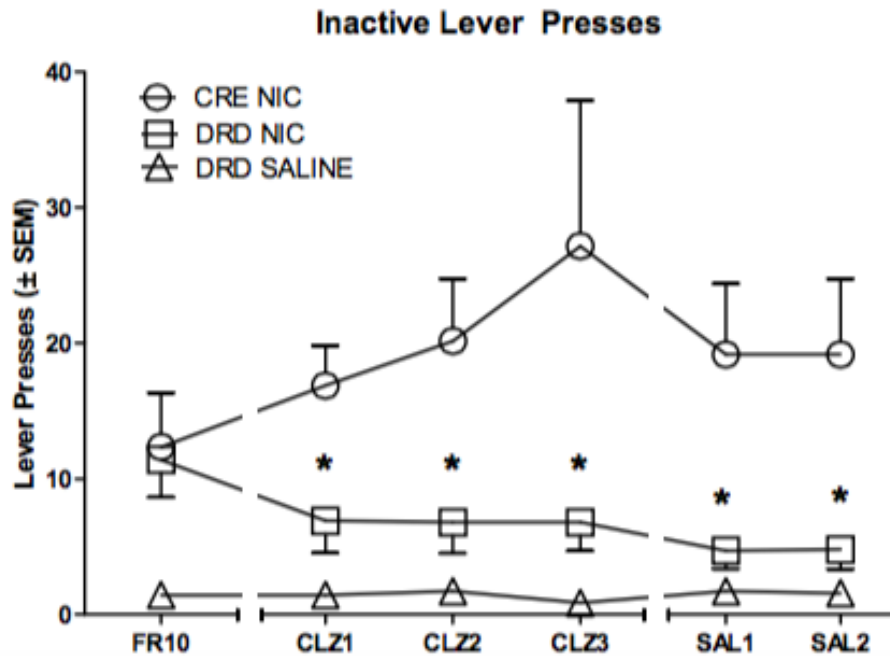


Figure 2B: Inactive Lever Presses with Treatment. A significance, denoted by Asterisks (*), can be observed in inactive lever presses between Cre and DREADD Nicotine. Differences between inactive responding can also be seen in regards to saline replacement.

Immunohistochemistry. The technique of immunohistochemistry (IHC) was performed to ensure expression of the DREADD receptor and expression in correct anatomical location. Perfused brain tissue was sliced at 20 μ m and stained using a primary and secondary antibody. The secondary antibody fluoresced the marker mCherry that was seen using a Olympus BX41 fluorescent microscope at the of wavelength 594 nm. The fluorescent mCherry indicated DREADD expression in the NAc shown in Figure 3A. Expression was also present throughout the entire neuron including cell body and projections represented in Figure 3B.

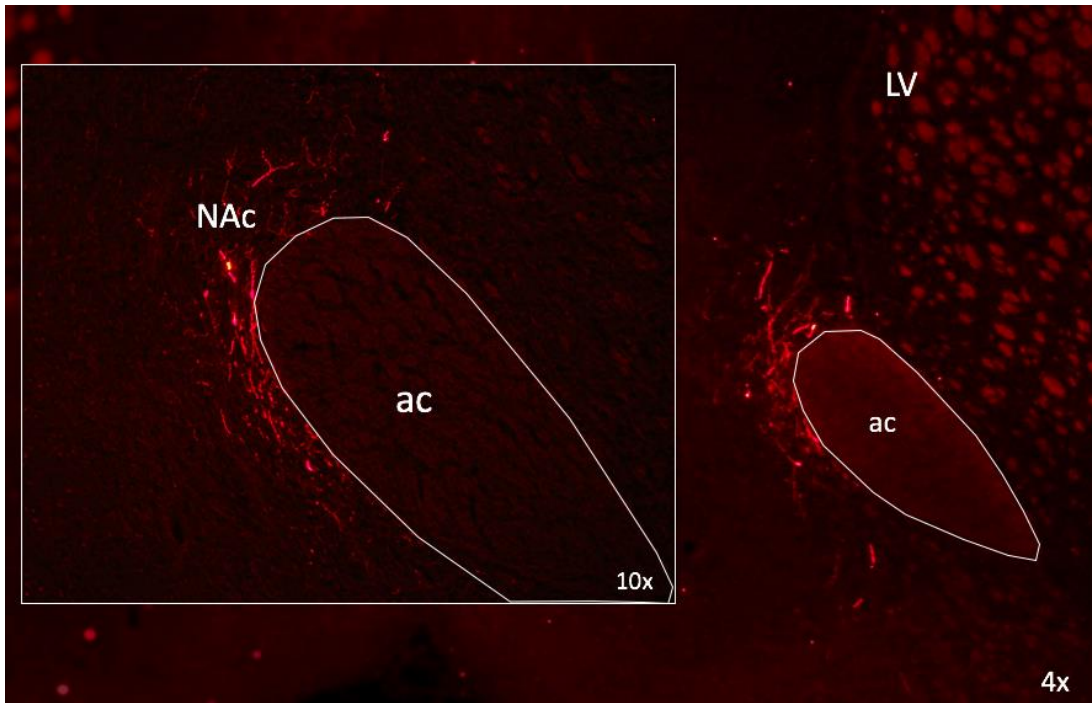


Figure 3A: DREADD Localization. The fluorescent mCherry marker for DREADD expression can be observed in the nucleus accumbens denoted NAc. Other anatomical landmarks are present – anterior commissure (ac) and the lateral ventricle (LV) seen on 4x magnification.

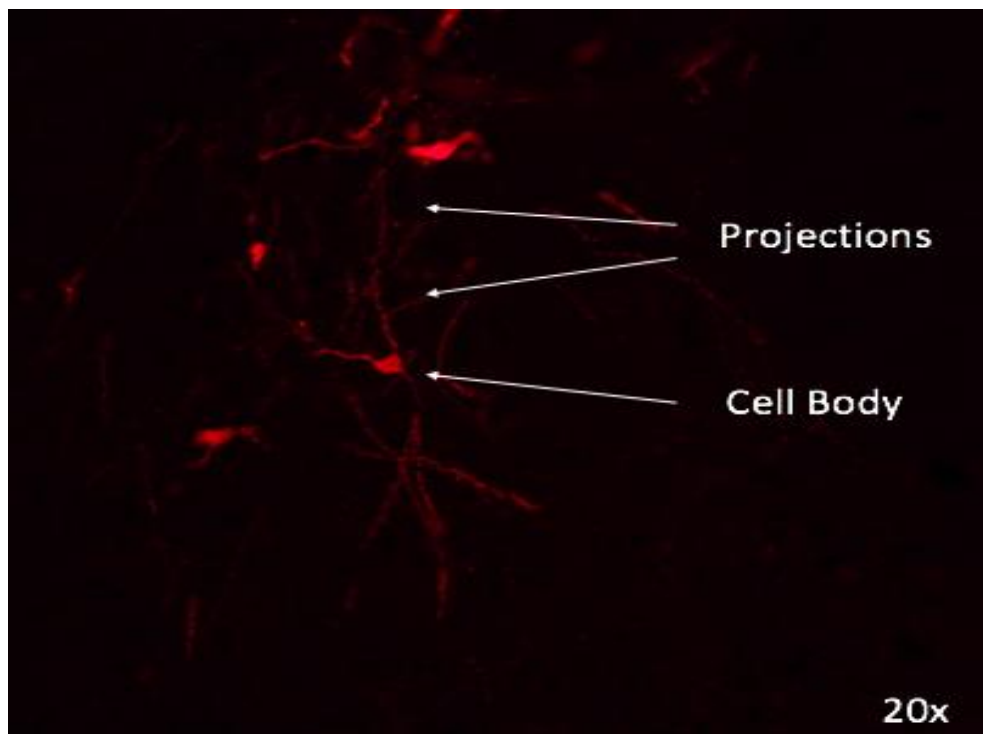


Figure 3B: DREADD Expression. Expression throughout the neuron indicated by the mCherry marker.

Experiment 2

Microdialysis was performed to confirm that the administration of clozapine activated the DREADD receptor and caused an influx of GABA into the VP. Figure 4 shows that the first injection of clozapine (0.75 mg/kg) (M=143.948, SD=29.08422) significantly increased the concentration of GABA in the VP; $t(4) = 3.379$, $p = 0.028$. Lastly, no significance was found with the second injection of clozapine (1.25 mg/kg), ($p = 0.538$).

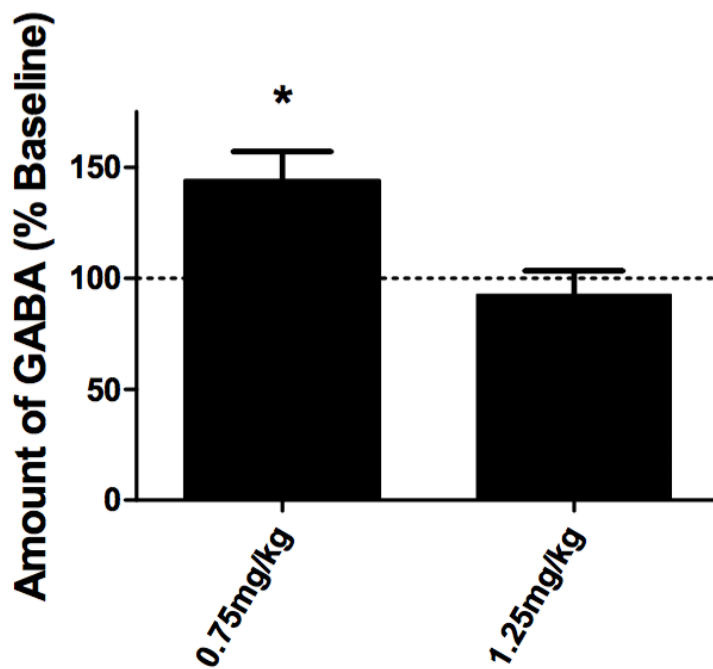


Figure 4: Amount of GABA (%Baseline). The effects of the two different injections of clozapine on % Baseline of GABA are shown. Asterisk (*) represent significance.

Discussion

These experiments investigated the role of ventral striato-pallidal projections in nicotine self-administration in rats using a chemogenetic strategy to increase GABA inhibition in the indirect pathway of cortico-striato-thalamic circuits. To do so, a retrograde Cre-recombinase virus was injected into the ventral pallidum (VP), followed several weeks later by injection of a Cre-dependent DREADD receptor (hM3-DIO, cite) in the ventral striatum (nucleus accumbens, or NAc). This strategy should allow injections of the designer drug (clozapine) to cause burst firing of neurons projecting from the NAc to the VP, increasing GABA release, and activating the indirect pathway. After we established reliable intravenous nicotine self-administration, we found that clozapine administration increased GABA into the VP and caused a reduction in active lever presses for nicotine. However, we also observed a decrease in inactive lever responses, suggesting that the reduction of responding could have been a non-specific motor deficit. During a recovery phase (saline injections substituted for clozapine), the DREADD-Nicotine group took two sessions for nicotine self-administration to return to control levels. Most importantly, we found that the effects of clozapine were not based on off-target effects (e.g., dopamine D2 receptors) as a control group that did not receive DREADD receptors maintained high rates of responding for nicotine. We also confirmed that the DREADD receptor was expressed where we anticipated (NAc projection neurons) using IHC and that injection of clozapine increased extracellular GABA in the VP using HPLC. Several of the findings deserve further consideration, including the pattern of reduced nicotine self-administration and recovery after clozapine injections, the putative non-specific motor effects of clozapine injection, and the biochemical change in GABA observed in the microdialysis control experiment.

Nicotine Self-Administration

We hypothesized that by increasing GABA into the VP, the reinforcing effects of nicotine would decrease. Behavior consistent with the hypothesis was observed in active lever presses related to clozapine administration. Surprisingly, responding declined gradually suggesting that the activation of the DREADD receptor (e.g. increased GABA into the VP) decreased the reinforcing effects of nicotine through extinction. ‘Extinction’ refers to a pattern of operant responding that occurs when a reinforcer is removed – behavior gradually declines as the subject learns a new contingency (that the response no longer leads to a reinforcer). Similar experiments have increased GABA activity in the VP and revealed comparable results. Muscimol, a GABA_A agonist, was injected directly into the VP resulting in a decrease in cocaine self-administration (Root et al. 2015). Also microinjections of neurotensin, a neuropeptide thought to increase GABA, into the VP correspondingly decrease cocaine self-administration (Torregrossa and Kalivas 2008). With the combined evidence of the current and previous studies, an increase in GABA into the VP decreased non-specific drug self-administration. However, further analysis of the current experiment depicted non-specific decrease in responding, as inactive lever responses also decreased during testing.

The VP is a critical component of the indirect pathway of cortico-striato-thalamic circuits. While any manipulation of striatal pathways could impact motor activity, the consequences of increasing GABA in the VP during nicotine self-administration on motor function has not been investigated. Our manipulation of increasing GABA by DREADD activation could have induced mild locomotor deficits as evidenced by decreased inactive lever responses. Inhibition of GABA receptors in the VP increases the initiation of activity in an open-field test (Mogenson & Nielsen 1983). In addition, GABA injections in the VP reduce

hyperactivity caused by dopamine injections into the NAc (Mogenson & Nielsen 1983). Further experiments investigating locomotor activity and GABA concentration within the VP will be necessary to determine if the activation of the DREADD receptor decreased nicotine reinforcement or caused inhibition of motor activity. However, another study used neurotensin, a neuropeptide thought to increase GABA transmission by colocalizing with GABA release. Neurotensin was placed in the NAc and caused an increase in GABA release in the VP, extracellular GABA was measured with microdialysis. They found that the increase in GABA did not have any effect on locomotor activity (Torregrossa and Kalivas 2008). The GABA concentrations observed in our experiment were much lower when compared to the GABA influx caused by neurotensin. Yet, we found a decrease in inactive lever presses. This could be a result of the DREADD not being as specific as originally thought. The activation of D1 and D2-like receptors may have had opposing effects.

Another interesting finding was how the active and inactive levers differed in reduced responding with the administration of clozapine. Two days of clozapine injections were needed to see an observable reduction in active lever presses (for nicotine infusions) of the DREADD Nicotine group. Not until the third day was the group significantly different from Cre Nicotine in active lever presses. Additionally, two days of saline were necessary for the DREADD Nicotine to return to baseline nicotine responding. However, an immediate reduction in inactive lever presses was observed in response to the first day of clozapine injections of both DREADD Nicotine and Saline groups. Interestingly, the activity of inactive lever presses did not recover for either group during the two day saline recovery. Interpretation of inactive lever behavior can become complicated when considering that they may not be motivationally 'neutral'. The inactive lever could obtain low motivational value because of its association with the active

lever. To further elaborate, with the DREADD Nicotine group, the inactive lever is physically comparable to the active lever that resulted in nicotine delivery. Similarly, the active lever for the DREADD Saline group historically resulted in sucrose delivery and is physically similar to the inactive lever. Because the possible connection between the two levers, inactive lever presses may not be an accurate measurement of spontaneous behavior when investigating motivation for a reward. Therefore, it is possible that increasing GABA in the VP also reduces motivation for a stimulus of low incentive value (inactive lever). Since this hypothesis is less parsimonious than simply effecting motor activity, it should be tested in an open-field locomotor task as well.

Expression of the DREADD was confirmed at the anatomical location of interest (NAc) with immunohistochemistry. Expression was observed in the both the core and shell of the NAc. This pattern of expression is consistent with anatomical tracing data showing that the NAc core and shell both send efferents to the VP. These subregions of the NAc both integrate in the direct and indirect pathways. MSNs expressing DA D1-like receptors are thought to be localized within the NAc core, while the MSNs expressing D2-like receptors are located in the shell (Root et al. 2015). DA has opposing effects on D1 and D2-like receptors and has been studied extensively during drug self-administration. Other experiments have found that increasing D1 activity (exciting the direct pathway) or antagonizing D2-like receptors (inhibiting the indirect pathway) increases drug-seeking behaviors (Gallo et al. 2018). Nicotine is thought to alter the activity of both resulting in habitual use (Benowitz 2010). No research has shown the effects of increasing GABA using DREADD on D1 and D2-like receptor activity during nicotine self-administration. Indeed, one reason for the slow-recruiting effect of clozapine in DREADD Nicotine rats may be a lack of anatomical specificity. The DREADDs could have activated both direct and indirect pathway projections from the accumbens. According to Kupchik and Kalivas (2016),

differentiating the indirect and direct pathways based off D1 and D2-like receptors has been over simplified and needs further investigation into more distinguishing factors. The gradual decrease in nicotine self-administration observed in the presented data could have been a direct result of DREADD activating both pathways. With that being said, we would need to negate the possible counteractivity of nicotine and DREADD on the indirect and direct pathway.

Future Directions

To confirm DREADD expression and microdialysis probe placement, the tissue from Experiment 2 will be sliced and stained. The implanted probe was injected with methylene blue prior to tissue collection. The tissue will be sliced and the NAc will be stained for DREADD expression while the methylene blue should be visible in the VP confirming correct probe placement.

Locomotor testing will be completed to investigate the activity of DREADD induced GABA release on the VP. The open field testing has been a reliable measure of locomotor activity (Metz et al. 2000) and will be applied to measure the locomotor activity of rats implanted with DREADD. Methods for viral implantation will be an exact replication of the current study. Further investigation of increasing GABA into the VP on motor activity will disclose if the observed decrease in inactive lever presses was overall decrease in motivation or induced locomotor deficit.

Conclusion

The mesolimbic dopaminergic pathway is essential in substance abuse research. More and more evidence has revealed the importance of certain nuclei, within the mesolimbic pathway, in drug-associated behaviors. Investigating these projections has shown that the motivational role of the NAc to VP pathway may be an important regulatory afferent from the

mesolimbic system as part of the 'indirect' pathway. More recently this pathway has been targeted by various manipulations resulting in the further understanding of its effects on drug self-administration. This is the first time the NAc to VP pathway has been investigated for its role in the reinforcing effects of nicotine with chemogenetic techniques. In conclusion, future data combined with current will provide a clearer representation of the effects GABA in the VP during drug-seeking behaviors.

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