Real-time detection of dopamine kinetics in opioid-dependent rats following treatment with intranasal GDNF DNA nanoparticles

A thesis submitted by

Urvi K Vani

in partial fulfillment of the requirements for the degree of

Master of Science

in

Pharmacology and Drug Development

Tufts University

Graduate School of Biomedical Sciences

May 2021

Advisor: Emmanuel N. Pothos, PhD

Co-Advisor and Reader: Amy Yee, PhD

Abstract

Opioid Use Disorder (OUD) refers to the physical and psychological dependence on opioids despite several deleterious and life-threatening consequences. Opioid medications can be legal or illicit but have the ability to be highly addictive. Legal opioids include prescription drugs like oxycodone to manage chronic pain while illegal opioids include heroin. The consumption rate of opioids has skyrocketed in recent years which has caused a global public health crisis now defined as the opioid epidemic. Timely treatment and management of this condition are of top-most priority. In the past decade, numerous studies have been carried out to find a suitable treatment option for OUD. The present work investigates a potential therapeutic role for Glial-Derived Neurotrophic Factor (GDNF). It is known to play a role in the survival and protection of adult dopaminergic neurons. It can be abundantly found in the peripheral and central nervous system (CNS). There is some prior evidence that GDNF has the ability to prevent the biochemical and behavioral responses caused by drugs of misuse. The present work combines the use of DNA nanotechnology and GDNF as a means for the treatment of OUD. Our hypothesis centers around pGDNF amplifying and stabilizing dopamine release when the animal undergoes craving for or withdrawal from oxycodone. In our experimental design, four groups of Sprague Dawley rats were compared. The first set of rats underwent treatment with self-administration of oxycodone and intranasal treatment with GDNF nanoparticles (pGDNF), group 2 had access to self-administered oxycodone and were treated with intranasal vehicle, the third group was only self-administering oxycodone, and the last group was saline-yoked, where the rats received a saline injection when their oxycodone partner pressed the lever for oxycodone. Identification of changes in dopamine kinetics in real-time in the dorsal striatum and nucleus accumbens region (the drug reward centers) of the rat brain was carried out using brain slice electrophysiology. According to the preliminary results from this thesis, oxycodone increased electrically evoked dopamine release in both sites,

while abstinence of oxycodone decreased it. An increase in statistical power and more follow-up studies would be required to understand the role of pGDNF in the treatment of opioid addiction.

Acknowledgments

I would like to express my thanks and gratitude to all the people who helped me write this thesis.

Firstly, I would like to thank my mentors, Dr. Emmanuel Pothos, Dr. Eric Paulson, and Dr. Amy Yee, for their patience and guidance during these past few months. I am appreciative of all that they taught me. From training me in specific laboratory techniques to improving my understanding of academic writing, I am deeply grateful. Secondly, I would like to extend my thanks to Dr. Daniel Volchok, who helped me edit and format this thesis.

Lastly, I would like to thank my family, especially my mom, dad and brother, and friends for their loving words of encouragement throughout these two years. It would not have been possible without them. I am also grateful to my classmates, specifically, Helia Mojahedyazdi, for being with me every step of the way. I would like to thank my lab mates, Qingchen Zhang and Iziegbe Omorodion, for making my lab experience worthwhile.

Thank you.

Table of Contents

Title Page i

Abstract ii

Acknowledgments iv

Table of Contents v

List of Tables vii

List of Figures viii

List of Abbreviations ix

Chapter 1: Introduction 1
- 1.1 What is Drug Addiction? 1
- 1.2 How does reward transition into addiction? 1
- 1.3 Opioid Use Disorder 2
- 1.4 Addiction mechanism for other illicit substances 3
  - 1.4.1 Alcohol 3
  - 1.4.2 Cannabis 3
  - 1.4.3 Cocaine 4
  - 1.4.4 Amphetamines 4
  - 1.4.5 Nicotine 5
  - 1.4.6 Hallucinogens 5
- 1.5 DNA Nanotechnology and its advantages 5
- 1.6 Blood Brain Barrier and the Intranasal Drug Delivery System 6
- 1.7 What is GDNF? 7
- 1.8 Role of GDNF in Addictive Disorders and Parkinson's disease 8

Chapter 2: Methods and Materials 10
- 2.1 Experimental Animals 10
- 2.2 Brain Slice Preparation 11
  - 2.2.1 Brain Slicing 11
- 2.3 Carbon Fiber Electrodes 12
- 2.4 Slice Electrophysiology 13
- 2.5 Statistical Analysis for Brain Slice Electrophysiology 13
- 2.6 Rat issue storage to isolate RNA for RT-PCR 13
- 2.7 RT-PCR 13
- 2.8 Gel Electrophoresis 16
- 2.9 Tyrosine Hydroxylase (TH) Immunohistochemistry (IHC) Technique 16

Chapter 3: Results 18
- 3.1 Preliminary studies on central DA Neurotransmission 18

3.2 GDNF enhances DA release in cultured midbrain dopaminergic neurons 18
3.3 Carbon Fiber Amperometry 19
3.3.1 Effect on Amplitude, T ½, Area Under the Curve, Molecules Released, and Width 19
3.3.1.1 Amplitude Analysis for Dorsal Striatum 19
3.3.1.2 Area Under the Curve 20
3.3.1.3 T ½ 21
3.3.1.4 DA Molecules released per event 22
3.3.1.5 Width 22
3.3.2 Amperometric Event analysis for NAc 23

Chapter 4: Discussion 25

Chapter 5: Bibliography 27

List of Tables

Table 2.1: Treatment groups. 10

Table 2.2: Primers used for RT-PCR to measure mRNA for regulators of DA exocytosis. 16

List of Figures

Figure 2.1: Carbon Fiber Amperometry. 14
Figure 2.2: Amperometric Traces. 15
Figure 3.1: Opioid withdrawal leads to reduction in extracellular DA levels. 19
Figure 3.2: Amperometric release of DA molecules from cultured midbrain DA neurons are enhanced by GDNF treatment (90 microgram). 20
Figure 3.3: Amplitude of DA release in dorsal striatum. 21
Figure 3.4: Area Under the Curve in dorsal striatum. 21
Figure 3.5: T ½ for DA release in Dorsal Striatum. 22
Figure 3.6: DA molecules released per event in Dorsal Striatum. 23
Figure 3.7: Width of the DA release in Dorsal Striatum. 23
Figure 3.8: Amperometric events in Nucleus Accumbens. 24

List of Abbreviations

aCSF: Artificial Cerebrospinal Fluid
AUC: Area Under the Curve
ATP: Adenosine Triphosphate
BBB: Blood Brain Barrier
CaCl2: Calcium Chloride
cAMP: Cyclic Adenosine Monophosphate
CDC: Centers for Disease Control and Prevention
cDNA: Complementary DNA
CNS: Central Nervous System
CSTC: Cortico-Striatal Thalamocortical Pathway
DA: Dopamine
DAB: Diaminobenzidine
DNA: Deoxyribonucleic Acid
ERK: Extracellular Signal Regulated Kinase
GABA: Gamma Aminobutyric Acid
GC-AT: Guanine-Cytosine and Adenine-Thymine Base Pair
GDNF: Glial-Cell Derived Neurotrophic Factor
GFR: GDNF Family Receptor
HHS: Health and Human Services
IACUC: Institutional Animal Care and Use Committee
IHC: Immunohistochemistry
KCl: Potassium Chloride
KH2PO4: Potassium Dihydrogen Phosphate
LSD: Lysergic Acid Diethylamide
MAPK: Mitogen-Activated Protein Kinase
MAT: Medical Assisted Treatment
MDMA: Methylenedioxymethamphetamine
MgSO4: Magnesium Sulphate
MOR: Mu-opioid Receptor
NA: Noradrenaline
NAc: Nucleus Accumbens
NaHPO4: Monosodium Phosphate
NAS: Neonatal Abstinence Syndrome
NCAM: Neural-Cell Adhesion Protein
NGS: Normal Goat Serum
NIDA: National Institute of Drug Abuse
NIH: National Institute of Health
NMDA: N-methyl-D-aspartate
ORN: Olfactory Receptor Neuron
OUD: Opioid Use Disorder
PBS: Phosphate Buffered Saline
PD: Parkinson’s Disease

PFA: Paraformaldehyde
PI3K: Phosphatidylinositol 3 Kinase
PLC-gamma: Phospholipase C Gamma
PR: Progressive Ratio
RET: Rearranged during Transfection
RNA: Ribonucleic Acid
RT - PCR: Reverse Transcriptase Polymerase Chain Reaction
SA Oxycodone: Self-Administered Oxycodone
SLC6a3: Soluble Carrier Family 6, Member 3
SUD: Substance Abuse Disorder
TAE: Tris Acetate - EDTA
THC: Tetrahydrocannabinol
TH: Tyrosine Hydroxylase
VMAT2: Vesicular Monoamine Transporter 2
VTA: Ventral Tegmental Area

Chapter 1: Introduction

1.1 What is Drug Addiction?

Drug addiction, clinically defined as Substance Use Disorder (SUD), is a chronic brain disorder characterized by uncontrollable drug craving and seeking despite its deleterious consequences [41]. Addiction can be to various illicit drugs, namely, opioids, cocaine, hallucinogens (phencyclidine, LSD), amphetamines, marijuana, and alcohol. Addiction to drugs obtained from the opium poppy seed plant is known as opioid addiction. Heroin, morphine, fentanyl, methadone, codeine, oxycodone, and hydrocodone are some drugs that are highly addictive in nature, all of which, belong to the class of opioids. While some opioids are legal, oxycodone, hydrocodone, and methadone to name a few, they can still lead to drug addiction with continued use. Anxiety, depression, anhedonia, and irregular sleep patterns are some of the symptoms of opioid addiction. According to National Institute on Drug Abuse (NIDA), NIH, about 21%-29% of patients misuse the prescribed opioids, 8%-12% develop an addiction, 4%-6% of patients who misuse prescribed opioids start taking heroin, and about 80% of the people who use heroin start by misusing prescription opioids [2, 24]. The misuse and overuse of opioids have led to a global crisis called the opioid epidemic. In 2017, the U.S Department of Health and Human Services (HHS) declared opioid addiction a public health emergency. About 128 people die from opioid overuse every day. About one baby is born every 15 minutes in the United States that suffers from neonatal abstinence syndrome (NAS). Thus, the management and timely treatment of this disorder are of pertinence [24].

1.2 How does reward transition into addiction?

Mesolimbic pathway is associated with the dopaminergic cell bodies in the ventral tegmental area of the midbrain. It extends to parts of the brain like ventral striatum, nu-

cleus accumbens, amygdala, bed nucleus of stria terminalis, lateral septal area and the lateral hypothalamus [1, 11]. The mesolimbic system is responsible for the mediation of the reward system. Drugs of misuse, food and sex increase extracellular levels of dopamine (DA) in the mesolimbic system. The need to elicit high DA concentrations in the brain is drives a person to get into a cycle of drug-seeking, drug-taking, drug-craving and relapse to drug-seeking behavior [17, 11, 1].

1.3 Opioid Use Disorder

Opioid drugs interact with the endogenous opioid system (usually with polypeptides like proopiomelanocortin, proenkephalin, and prodynorphin) by acting as mu-opioid receptor agonists (MOR). This receptor is responsible for the increased functioning of the reward system [1, 32]. These receptors are found in areas associated with pain, reward, and emotions. Long-term use of opioids can, hence, affect the mood and lead to depression and anxiety. In opioid addiction, there is an increase in the dopaminergic neurons in the Ventral Tegmental Area (VTA) and the nucleus accumbens (NAc). Repeated use of opioids can lead to changes in the mRNA expression of prodynorphin and the kappa receptor genes. Out of the three receptors, mu and delta are responsible for producing positive reinforcements when they interact with their respective agonists. Kappa is responsible for producing negative reinforcements like hallucinations and malaise.
It has been concluded that the nucleus accumbens, a region in the basal forebrain below the striatum, plays a very important role in drug addiction [9, 1]. It is known as the pleasure center since it has a role in the reward circuit of the brain. In drug misuse, there is a sudden increase of dopamine influx in the nucleus accumbens. Because of this event, the reward system and motivation processing in the brain is disturbed [22].
In normal circumstances, a chemical entity attaches itself to the mu receptor on the neuron which leads to the activation of an enzyme that helps in the conversion of ATP to cAMP which further leads to the release of monoamine neurotransmitters. When

heroin or any other opioid is taken, it attaches to the mu receptor and inhibits the conversion of ATP to cAMP. Due to that, less monoamine neurotransmitters are released in the brain. With continuous use, the neurons compensate by producing more ATP which can then be used to make cAMP and ultimately resulting in a normal levels of monoamine neurotransmission. At the same time, there is an increase in DA release. With repeated use, less and less DA is released, and the OUD patient or animal model takes more opioids to compensate for the reduced DA [22, 36, 25].

1.4 Addiction mechanism for other illicit substances

Apart from opioids, the substances that are commonly misused in the United States and share cellular and molecular substrates in their mechanism of action with opioids include alcohol, marijuana, cocaine, amphetamines, nicotine, and hallucinogens.

1.4.1 Alcohol

Alcohol reduces the activity of the NMDA receptor that belongs to the Glutamate (an excitatory neurotransmitter) and it is also known to increase the activity of the GABA-A receptor that belongs to GABA (an inhibitory neurotransmitter). There is a surge of dopamine in the nucleus accumbens when alcohol is taken. Apart from nucleus accumbens, endogenous opioid peptide neurotransmitters also play a role in alcohol addiction [10, 3].

1.4.2 Cannabis

Delta9-tetrahydrocannabinol (THC) may be responsible for the addictive nature of cannabis. THC is known to have effects on the dopaminergic neurons in the brain. Repeated use of cannabis can lead to less and less DA response to the cannabinoid receptor agonists. Continuous use can also affect the endocannabinoid system and reward system in the brain. The effects of chronic use also downregulate the cannabi-

noid receptors. Apart from the blunted response and release of DA in the brain, there is also a reduction in the glucose metabolism in the brain which correlates with craving. Cannabis withdrawal can often lead to anxiety, insomnia, tremors, fever, chills, depression, anorexia, aggression, and restlessness [40].

1.4.3 Cocaine

DA reuptake can be blocked by cocaine administration. When DA reuptake is blocked, synaptic DA levels increase. Metabolism and excretion of DA take place in the presence of cocaine before it can be stored and recycled for the subsequent release. This deficit forces the brain to produce more dopamine in order to maintain its normal physiological levels. After chronic cocaine use, DA synthesis in the brain cannot compensate and DA levels run low. This leads to symptoms like irritation, depression, and paranoia [7].

1.4.4 Amphetamines

Amphetamine represents a class of psychostimulant drugs that acts on the central nervous system by redistributing catecholamines from synaptic vesicles to the cytosolic and extracellular space. Amphetamine blocks the transport of neurotransmitters through plasmalemmal transporters. It also disrupts the activity of monoamine vesicular transporters as a transporter substrate competitive to endogenous ligands; inhibits monoamine oxidase which leads to an increase in the expression of monoamines; and increases the level of tyrosine hydroxylase (TH). The above effects induce an increase in libido, euphoria and anorexia. Withdrawal from amphetamines can lead to insomnia, aggression, paranoia, and hallucinogenic episodes [4].

1.4.5 Nicotine

Nicotine is the addictive component of tobacco. Cholinergic receptors in the brain release neurotransmitters upon interaction with nicotine. It is responsible for the release of DA in the midbrain and the frontal cortex terminals. The DA neurons in the midbrain are responsible for the reward induced by the drug. Nicotine also plays a role in the release of GABA which is responsible for the inhibition of dopamine. With chronic use, cholinergic receptors become desensitized. The activity of GABA subsides over time, but the activity of glutamate induced by nicotine is longer which leads to excitation of DA neurons. During withdrawal, nicotine misusers experience stress and anxiety which causes them to start smoking again [18].

1.4.6 Hallucinogens

A most common hallucinogen and stimulant is MDMA (methylenedioxymethamphetamine) which is also known as ecstasy. Other hallucinogens include lysergic acid diethylamide, phencyclidine, mescaline, and psilocybin. Chronic use of these neurotoxins causes a depletion in serotonin which can lead to depression, anxiety and memory loss [38].

As all aforementioned misusable substances share a common mechanism in central DA systems, the treatment investigated here for OUD may apply to several more addictive disorders.

1.5 DNA Nanotechnology and its advantages

Viral vectors have been used extensively in the field of medicine for therapeutic purposes. While widely used, they present certain potential concerns, including safety issues, inflammation, and possible adverse effects. Detailed studies have been done on nonviral vectors for the treatment of novel diseases since they have a better safety

profile and a low production and manufacturing cost. Nonviral vectors can be plasmid DNA, lipoplexes, or polyplexes. Nanoparticles have been also used for gene delivery since they have a large nucleic acid loading capacity, and they prevent risks caused by adenoviral transfection [39].

Nanotechnology is an emerging field that produces particles that can increase drug targeting and reduce drug toxicity. Nanoparticles tailored in different ways can appease different drug properties and their interactions on a cellular level. In recent years, DNA nanoparticles have emerged as a budding treatment option for various diseases. They are permeable, biocompatible, and nonimmunogenic. They can protect DNA molecules from degradation and since they are small in size (range from 30 mm to 500 mm), they can travel through membranes and reach the site of the target efficiently [16]. Many studies have been done to elucidate the role of DNA nanoparticles in the treatment of neurodegenerative diseases. A group of our collaborators at Copernicus Therapeutics designed a plasmid with GDNF DNA encoded in it. This is used to overexpress GDNF in the rat brain. The presence of plasmid GDNF nanoparticles has been noted even after three weeks post intranasal treatment. It was confirmed that it is nonimmunogenic and a continuous delivery option for a long duration of desired effects. Since GDNF is a neurotrophic factor that helps in the protection and survival of DA neurons, it serves as a potentially effective treatment option for disease states that involve central dopaminergic deficits like addictive and neurodegenerative disorders [39, 29].

1.6 Blood Brain Barrier and the Intranasal Drug Delivery System

The blood-brain barrier is the biggest limiting factors for the delivery of drugs in the CNS. The blood-brain barrier is a highly selective semipermeable border made from endothelial cells surrounding the central nervous system. It prevents foreign particles and even biological therapeutics from entering the brain. Large systemic doses are required for the drugs to cross the BBB, leading to adverse effects in the body. Intracere-

broventricular and intraparenchymal injections are some ways of introducing drugs in the CNS but they are often regarded as risky and invasive. Intranasal drug delivery systems can cross the blood-brain barrier and help in the treatment of many neurological and psychiatric diseases and disorders as a better alternative compared to other invasive drug delivery systems [8, 37].

Drugs can enter the CNS through the olfactory nerve pathways from the nasal cavity. Olfactory receptor neurons (ORNs) play an important role in the mediation of the sense of smell by transferring information from the surrounding environment. ORNs are dispersed in and around the supporting cells, microvillar cells, and basal cells. One end of the ORNs, the dendritic end, extends into the mucosal layer of the olfactory epithelial layer, and the other end, the axonal end, extends through the lamina propria and the cribriform plate of the ethmoid bone. These neurons project into areas like the amygdala, hypothalamus, piriform cortex, and olfactory tract. Intranasal GDNF nanoparticles can move past the BBB and induce their effects in the brain through the aforementioned process [8, 13, 31].

1.7 What is GDNF?

GDNF is the neurotrophic factor responsible for promoting the survival of dopaminergic neurons and the nervous system's growth and development. It is encoded by the GDNF gene. GDNF is a small, secreted protein first found in the B49 glial cell line. GDNF belongs to the GDNF family ligands (GFLs), including bio-molecules like persephin, neurturin, and artemin. During developmental stages, GDNF can be found abundantly throughout the CNS, especially in the striatum, cortex, thalamus, hippocampus, and astrocytes [12, 5]. The adult brain, more specifically the midbrain, where most of the dopaminergic cell bodies reside, shows the presence of GDNF. Apart from survival, growth, and development, GDNF also plays a role in preventing apoptosis, and promoting kidney development, hair follicle growth development, and cutaneous wound heal-

ing. GDNF is also crucial in the release of neurotransmitters, therefore suggesting its important role in CNS plasticity. RET activation is the first step of the major pathway involved in the activity of GDNF. GDNF attaches to the GFR-alpha-1 receptor (a subtype of GFR-alpha receptor, mainly a glycosylphosphatidylinositol linked membrane-associated protein). There is ligation of the GDNF-GFR-alpha-1 complex with RET, which leads to the autophosphorylation of RET, rendering its activation. Activation mitogen-activated protein kinase (MAPK) pathway, extracellular signal-regulated kinase ½ (ERK ½) pathway, phosphatidylinositol 3 kinase (PI3K) pathway, and phospholipase C-gamma (PLC-gamma) pathway is seen. One of the other pathways indicates an absence of RET and the action of GDNF in conjunction with a neural cell adhesion protein, NCAM. GFR-alpha and NCAM bind to each other and activate the Src family kinase Fyn, associated with neuronal development and T-cell signaling. GDNF-NCAM complex holds importance in maintaining the nervous system, plasticity, forming memories, and learning. [5]

1.8 Role of GDNF in Addictive Disorders and Parkinson's disease

Recent studies and experiments suggest that GDNF plays a significant role in the treatment of SUDs. GDNF regulates the action of drugs of misuse by reducing their effects. The mechanism of action of GDNF associated with SUDs is not known entirely. The possible mechanisms of action are the MAPK effect of GDNF on the DA neurons in the brain and GDNF's ability to alter TH levels. These changes mediated by GDNF help counter the effects of drugs of misuse [21]. Experiments and research on GDNF also suggest its potential in the treatment of Parkinson's disease. GDNF protects the dopamine neurons in the midbrain against cell death caused by toxins. It stimulates the axonal regeneration in the nigrostriatal DA neurons as well as aids in the recovery of function by increasing the DA release, content, and turnover. It also increases the amount of TH [30, 15].

The present work primarily focuses on identifying changes in the neurotransmitter DA kinetics in real-time in the rat brain after intranasal treatment with GDNF DNA nanoparticles and in a rat model of oxycodone self-administration.

Statement: All of the work included in this chapter was carried out by me in collaboration with other members (Helia Mojahedyazdi) of the Pothos and Yee laboratories unless otherwise stated.

Chapter 2: Methods and Materials

2.1 Experimental Animals

2–3-month-old male Sprague Dawley rats were used. The animals were housed in a 14:10 light cycle and were given a 22% fat chow diet with water. All animal studies were approved by IACUCs at Northeastern University, Tufts University Grafton, and Tufts University Boston campuses and were carried out adhering to NIH guidelines. Each of the animals processed belonged to the experimental group referred to Table 2.1.

Subject	Treatment group
1	Saline Yoked
2	Oxycodone (IV Self Administration – SA)
3	Saline Yoked
4	Oxycodone SA
5	Oxycodone SA
6	Oxycodone SA
7	Saline Yoked
8	Saline Yoked
9	Oxycodone SA + GDNF
10	Oxycodone SA + GDNF
11	Oxycodone SA + Vehicle
12	Oxycodone SA + Vehicle

Table 2.1: Treatment groups.

All oxycodone self-administration work was done in the laboratory of Dr. Elizabeth Byrnes at Tufts Cummings School of Veterinary Medicine in Grafton, MA. All treatments with pGDNF were done through the methods employed by the Waszczak laboratory at Northeastern University. In subjects 2, 4, 5 and 6, SA oxycodone was given in 6-hour sessions for 12 days in the dose of 90 microgram followed by a progressive run session which was proceeded by an extinction period starting on day 14. In subjects, 1,3, 7 and 8, saline injection was received by a rat when its partner pressed the lever for oxycodone. In subjects 9 and 10, the rats were given GDNF (90 microgram) on day

14 after a 6-hour session for 12 days of SA oxycodone and a PR session on day 13. In subjects 11 and 12, SA oxycodone was given in 6-hour session for 12 days followed by a PR session proceeded by an intranasal vehicle delivery on day 14.

2.2 Brain Slice Preparation

The rats were sacrificed using ketamine: xylazine (1:1 ratio) cocktail. The brain was dissected from the rat and placed in ice-cold sucrose bicarbonate solution (components: 210nM sucrose, 10mM glucose, 3.5mM KCl, 1mM Cacl2, 4mM MgSO4, 1.25 mM NaH2PO4) that was continuously oxygenated. The cerebellum was discarded and then the brain was divided into two hemispheres. The two halves supported by sturdy agar gels (8-gram agar dissolved in 100 ml nanopore water) were then glued to a metallic base of Leica VT1000S Vibratome (Leica Microsystems) to cut them into 300 micrometer slices (using stainless steel blades: Campden Instruments Limited) of 300 micrometers. The slices were promptly transferred to a petri dish containing cold artificial cerebrospinal fluid (components: 124mM NaCl, 2mM KCl, 1.25 mM KH2PO4, 2mM MgSO4, 25 mM NaHCO3, 2 mM CaCl2, and 11 mM glucose) with pH ranging from 7.3-7.4. Slices were allowed to acclimate for an hour in the oxygenated aCSF solution. Slices containing the dorsal striatum and nucleus accumbens were chosen to carry out slice electrophysiology.

2.2.1 Brain Slicing

The brain was kept ventral side up (brain upside down) on the brain blocker in ice for about a minute. A new blade (Campden Instruments Limited) was taken to cut the brain right on the Sulcus pontocruralis (the fissure in front of the pons) [27]. The cut part was cerebellum and was stored in RNA-free tube at -80 °C. A cut was made 2 mm anterior to the sulcus pontocruralis. This tissue was the midbrain. The sample from this cut was stored for IHC and RT-PCR. Cut further with blade anterior to the optic chi-

asm. The tissue between this cut and the previous cut should be flat on both sides and it should be immediately processed on the vibratome for brain slicing. The remaining tissue anterior to the previous and last cut can be used for prefrontal cortex slices for further analysis.

2.3 Carbon Fiber Electrodes

To measure DA release, we employed carbon fiber amperometric microelectrodes. The insertion of single carbon fiber into the capillary tubes of 1.0 mm x 0.75 mm 4" (A.M Systems, Inc.) was first carried out using a vacuuming system. These capillary tubes were then pulled using the Flaming/Brown Micropipette Puller (Model 97 by Sutter Instrument Co.) The halved capillary tubes were glued using 301 1LB Part A: 301 1LB Part B in 3:1 ratio (Epoxy Technology, Epo-tek) at the tip. They were then baked overnight in an oven at 90°C. To remove the excess glue, the glued and baked tips of the electrodes were dipped in a hot acetone solution for approximately 30 seconds. Next, the electrodes were retrieved from the oven and were beveled at the tip using K.T Brown Type Micropipette Beveler (Model BV-10 by Sutter Instrument Co.) at 45 degrees angle. 3M KCl (22.368 grams of oven-dried KCl was weighed and dissolved in 100 ml of nanopure water) was used to back-fill the electrodes (using containing MicroFil Nonmetallic syringe needle by World Precision Instruments) with carbon fiber. The electrodes were tested to measure the favorable electrical noise between 0.8 and 1.2 using Axopatch 200B Integrating Patch Clamp. 1mg/ml dopamine solution (1 mg of dopamine dissolved in a mixture of perchloric acid and nanopore water) was used to check the validity of the electrodes. The validity of the electrode is confirmed when it shows the right artifact as seen in Figure 2.2B.

2.4 Slice Electrophysiology

For recording, brain slices were bathed in a recording/perfusion chamber (Model: RC-26GLP by Warner Instruments). This chamber maintains a temperature of 37°C at all times using a heater controller system (Model CC-28 by Warner Instruments). The chamber is also supplied with flowing oxygenated aCSF. The recording electrode is attached to a CV 203BU headstage by Axon Instruments) and a bipolar stimulating electrode (Plastics One) is delivering electrical stimulatons using a pulse stimulator (Master 8 by A.M.P.I.). This is complemented by a stimulus isolator that can be used to get results with a negative phase as well as a positive phase (Iso-Flex by A.M.P.I.). A current of 5 milliamperes is applied every five minutes for five times on DA-releasing sites. The output is acquired and subsequently analyzed using Axograph [19].

2.5 Statistical Analysis for Brain Slice Electrophysiology

Statistical analysis was done using Graph-pad Prism software (version 9). Amplitude, peak, half-life, area, and DA molecules were obtained for each DA signal through this analysis. One-way ANOVA was done. 5 stimulations per site were done to get a more statistically accurate result.

2.6 Rat issue storage to isolate RNA for RT-PCR

The rat tissue was flash-frozen in liquid nitrogen, labelled, and then stored at -80°C until the day of the assay. It was retrieved later to isolate RNA.

2.7 RT-PCR

For the RT-PCR, the isolated RNA was used to make a cDNA library. This was done using the Bio-Rad cDNA synthesis Kit (Mixture of 4 microliters of iScript Reaction mixture, 1 microliter of iScript Reverse Transcriptase, 2 microliters of isolated

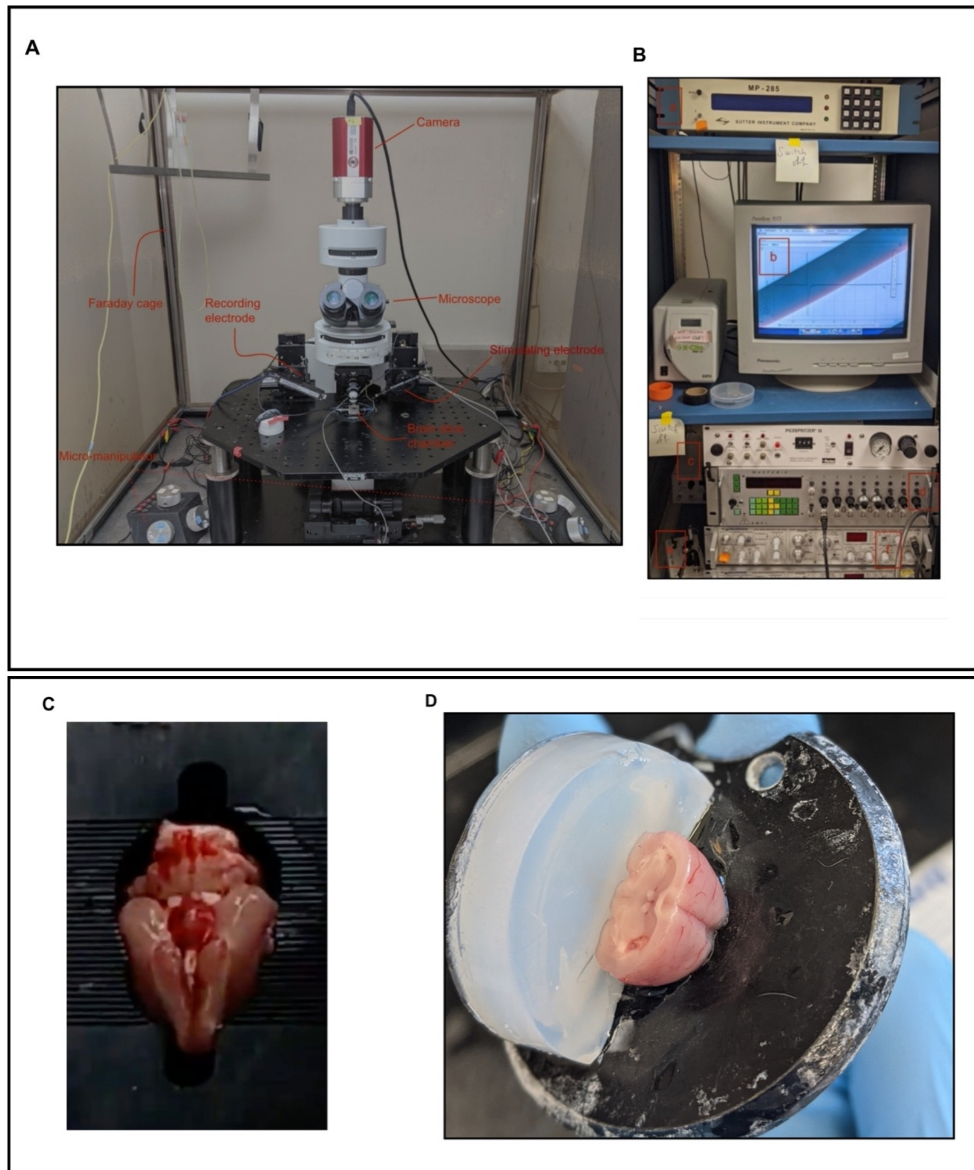

Figure 2.1: Carbon Fiber Amperometry: A) the electrophysiology set-up including an upright BX-40 Olympus microscope, electrode holders and micromanipulators on a Gibraltar stage. B) a – MP-285 micromanipulator instruments to precisely move the stimulating and recording electrodes, b – Computer running the Axograph application, c – Heater: to keep the temperature 37C at all times to mimic physiological conditions d – Master 8: Pulse stimulator, e – Isoflex: stimulus isolator, f – Axopatch 200B amplifier C) Rat brain (ventral view) D) Assembly of the brain section against agar gel on the stage of Leica Vibratome for brain slicing.

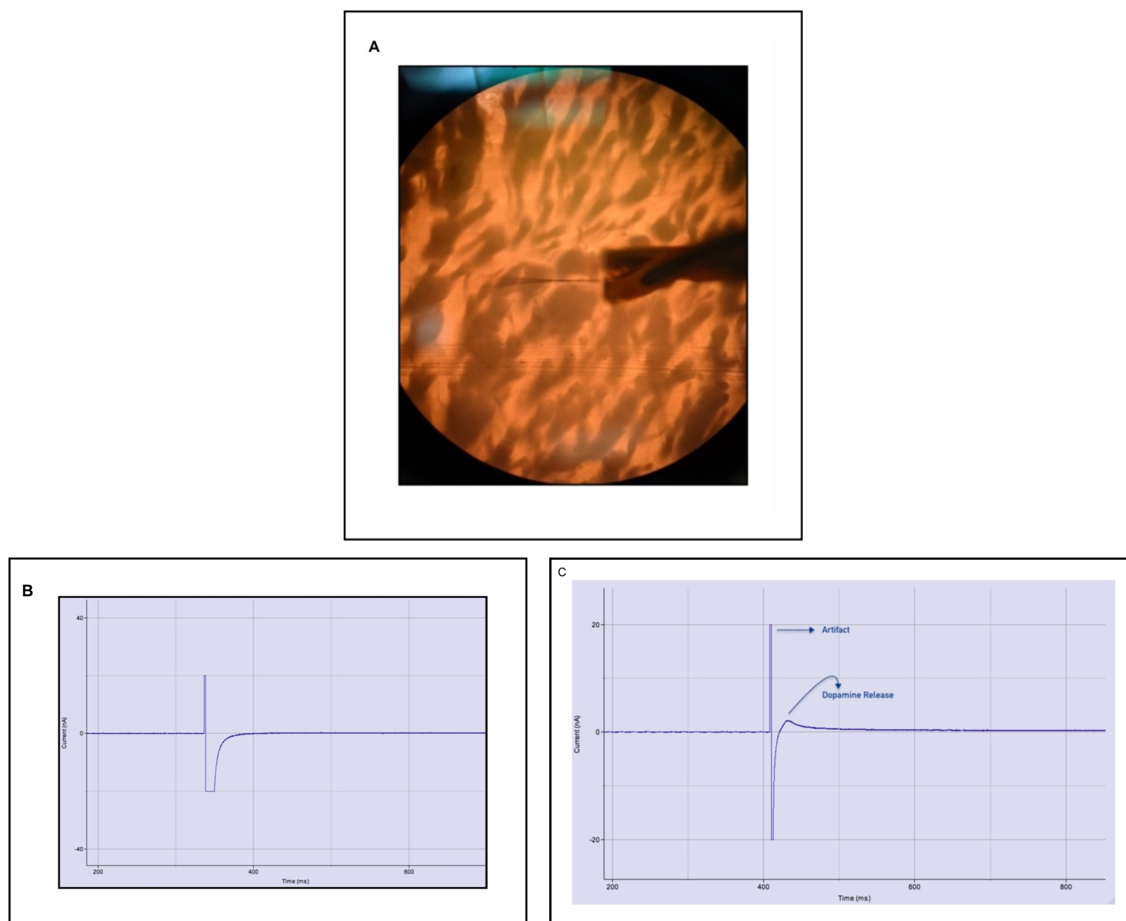

Figure 2.2: Amperometric Traces: A) Assembly of recording (left) and bipolar stimulating (right) electrode seen at 20X magnification and recording DA release from a coronal dorsal striatum slice. B) The artifact obtained when the electrode is valid and can record DA release. C) The right artifact followed by DA release.

RNA sample, Nuclease free water to make up the volume to 20 microliter - about 13 microlite). The RT-PCR machine (iQ5 multicolor Real-Time PCR Detection System) was set to i) 5 min at 25C, ii) 30 min at 42C, iii) 5 min at 85C, iv) Hold at 4C to be carried out in a sequence. The cDNA library was labeled and stored in -70C. On the next day, the cDNA library was amplified using reagents in the following amounts: 1 microliter of forward-primer, 10 microliters of 2x Taq, 1 ul of cDNA, and nuclease-free water to make up the volume up to 20 microliter - about 8 microliters for one reaction. The amplification mixture was made for nine reactions. It was then transferred to RNA-free pipes in a way that was bubble-free. After this, the samples were then run through

Name	Forward primer	Reverse primer
D1 Receptor	GCATGGCTTGGATTGCTACG	CCAGTTGCTGCCTGGACTAA
D2 Receptor	TGAAGACACCACTCAAGGGC	TGGGAGGGATGGGGCTATAC
D3 Receptor	CTACGTTCCCTTCGGGGTGA	CCAGTTGCTGCCTGGACTAA
TH	CTACTGTCCGCCCGTGATTT	GGTCAGCCAACATGGGTACA
SLC6a3	TCTGGAGGTTTCCCTACCTGT	ACAGTGAAGCCCACACCTTTC

Table 2.2: Primers used for RT-PCR to measure mRNA for regulators of DA exocytosis.

a gel [28].

2.8 Gel Electrophoresis

For making a gel, agarose (Denville Scientific Inc.) and TAE buffer (Boston Bio-Products) were used. (1.5% agarose in 150ml of TAE buffer). Ethidium Bromide is a red fluorescent dye that intercalates with the GC-AT base pairs when it undergoes fluorescence, about 15 microliters of this dye is used to add into the gel for the detection of proteins/DNA. This gel is added to a tray and then a comb is inserted to create wells. It is left to set for 20-30 mins. Once the gel becomes opaque, samples are loaded into each of these wells. A molecular weight marker is also loaded into the well for reference. The gel can run to ¾ of its length. The gel is then seen through a fluorescent light to detect the expression of proteins/DNA [28].

2.9 Tyrosine Hydroxylase (TH) Immunohistochemistry (IHC) Technique

The midbrain section (containing DA cell bodies) is placed in 4% methanol free paraformaldehyde for approximately 72 hours. It is then transferred to a scintillation vial containing 30% sucrose solution and can stay in it until the brain tissue sinks at the bottom. The brain sections are then washed with PBS for three times (10 minutes each). After this, the sections are pretreated with 0.3% hydrogen peroxide in mesh wells for 30 minutes for two times. This process is followed by other three consecutive washes with PBS of 5 minutes each. 5% NGS in PBS is used to block the sections

to reduce non-specific binding for 60 minutes at room temperature. The sections are then placed in scintillation vials containing 1.6 ml of anti-TH antibody (primary antibody) which are then incubated overnight at 4 °C on a rotating wheel. On the next day, the incubated sections are washed with PBS three times, each wash lasting for 10 minutes. After the last wash, the midbrain sections are again transferred to scintillation vials containing 1.6 ml of secondary antibody (biotinylated goat anti-rabbit IgG, Vector BA-1000) of a dilution of 1:200 in PBS. Incubation period for secondary antibody is an hour at room temperature. Later, the sections are washed with PBS three times for 10 minutes. After this, the sections are placed in vials containing the ABC solution on a shaker for 1 hour. Washing of sections is done yet again with PBS for three times.

Incubation of the brain sections is done in 1.6 ml of 3,3' diaminobenzadine DAB solution in scintillation vials until they turn brown. The sections are transferred to mesh wells containing PBS after discarding the DAB solution. The sections are washed with PBS three times, every 5 minutes. Lastly, the sections are mounted on a droplet of water. To flatten the sections, they can air dry overnight before placing a coverslip on them [14].

Statement: All the work in this chapter is being done collaboratively with members of the Pothos (Helia Mojahedyazdi), Yee and Waszczak (Northeastern) laboratories. RT-PCR (will be done by me) and IHC (will be done by Helia Mojadyazdi) results beyond initial tissue processing are pending due to restrictions and delays imposed by the COVID-19 pandemic.

Chapter 3: Results

3.1 Preliminary studies on central DA Neurotransmission

Drugs of misuse have a direct effect on DA release kinetics, quantal size (number of DA molecules released by a single vesicle during exocytosis) and synaptic plasticity in terminal areas like the nucleus accumbens, prefrontal cortex and the dorsal striatum. The DA deficiency induced by chronic drug use and repeated stimulation of DA release leads to a reward deficit which leads to addictive behavior [34]. In the Pothos Lab, this hypothesis was put to test by treating rats with morphine, an opioid drug, which lead to an increase in DA release in the nucleus accumbal area (Figure 3.1A). Opioid withdrawal in morphine dependent rats decreased extracellular levels of DA in the same area (Figure 3.1B). To treat the withdrawal symptoms, rats were given clonidine, which reduced the decrease in extracellular levels of DA (Figure 3.1C) [35].

3.2 GDNF enhances DA release in cultured midbrain dopaminergic neurons

Similar findings have also been observed in Parkinson's and dietary obesity animal models. Food seeking behavior and drug seeking behavior may be a means of overcoming the DA deficit in the brain. In attempts of studying mechanisms of neuroprotection for DA neurons in the brain, the Pothos Lab concluded that a single dose of GDNF (90 microgram) increases the survival rate of these cells in culture. GDNF also augments DA sequestration in synaptic vesicles and quantal DA release in real time. Therefore, GDNF is restoring DA release (Figure 3.2B) in comparison to control neurons (Figure 3.2A) and as measured by carbon fiber amperometry. The quantal size of the release of DA is increasing significantly in GDNF treated neuronal cells (Figure 3.2C). After carrying out TH immunohistochemistry (IHC), it was found that GDNF has a neuroprotective effect on DA neurons (Figure 3.2D).

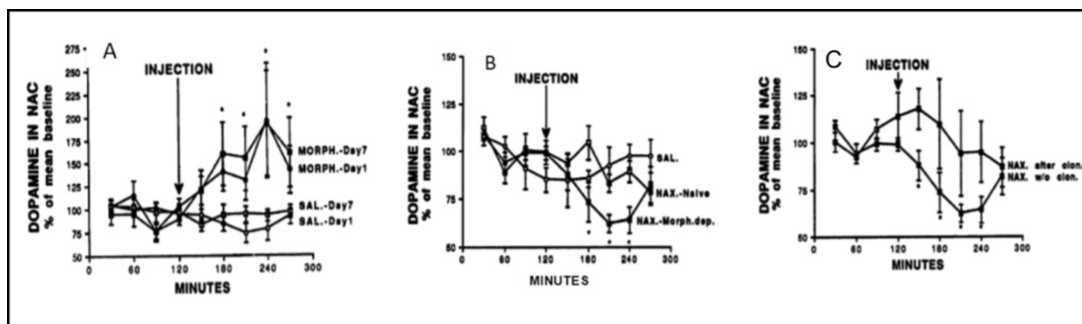

Figure 3.1: Opioid withdrawal leads to reduction in extracellular DA levels. A) Opioid drug, morphine, increases DA levels in the nucleus accumbens region. B) Opioid withdrawal induced by naloxone which leads to a reduction in extracellular DA levels in NAc. C) Pre-treatment of withdrawal with help of clonidine reduces the decrease in extracellular DA in NAc of opioid dependent rats following naloxone.

3.3 Carbon Fiber Amperometry

The effect of intranasal pGDNF nanoparticles on central DA release in real time was measured with carbon fiber amperometry on acute coronal brain slices containing mesolimbic and nigrostriatal pathway DA terminals. Four treatment groups as mentioned in Table 2.1 were employed to measure the effect of GDNF on oxycodone dependent rats.

3.3.1 Effect on Amplitude, T ½, Area Under the Curve, Molecules Released, and Width

3.3.1.1 Amplitude Analysis for Dorsal Striatum

The highest efflux of DA release is measured from the baseline to represent the amplitude of the peak. For oxycodone + vehicle, the mean amplitude for the baseline peak was 233.9 ± 37.54 pA (± SEM), the mean amplitude for SA Oxycodone group was 12.74 ± 2.909 pA, for Saline Yoked group, it was 22.93 ± 4.600 pA, and, lastly, for the oxycodone + GDNF group, the mean amplitude for the baseline peak was 23.72 ± 0.6299 pA. Each point represents the average of 1-5 stimulations on the same site with an interstimulus interval of 5 min to ensure recovery of DA in terminals (n=2 per each group).

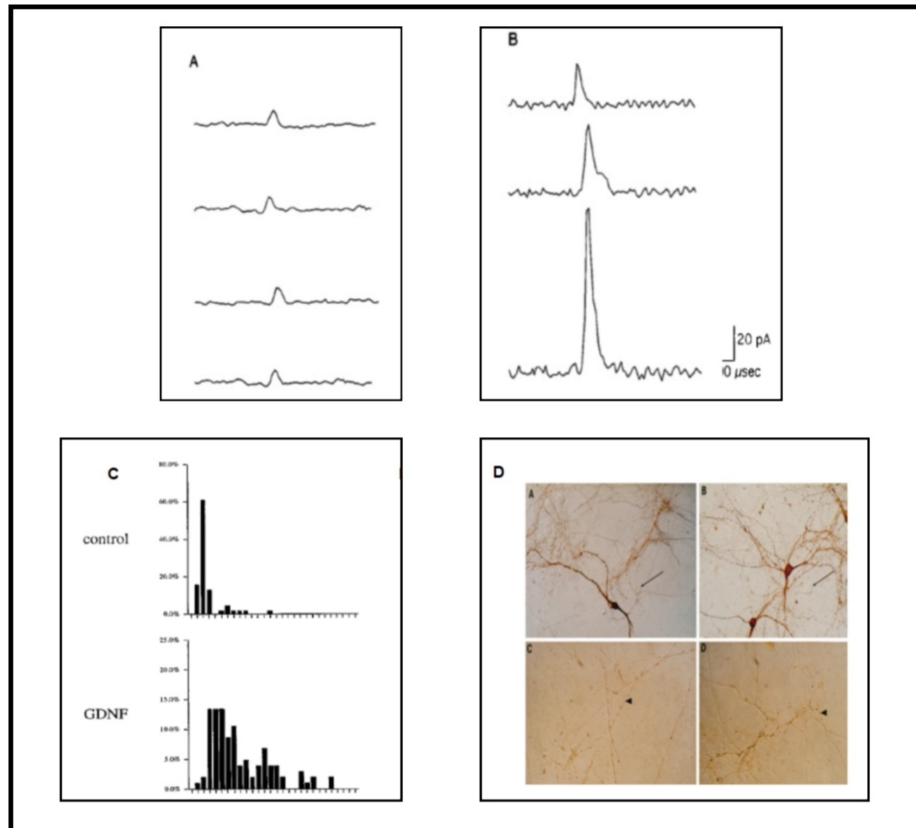

Figure 3.2: Amperometric release of DA molecules from cultured midbrain DA neurons are enhanced by GDNF treatment (90 microgram). A) Amperometric measurement of the events from control neurons, B) Amperometric measurement of the events from GDNF treated neurons. C) Quantal sizes of DA; GDNF treated group presented higher values than the control group. D) Morphological effects of GDNF, a) Arrow shows the presence of a dense plexus of neurite outgrowth, b) GDNF treated neurons show two-fold more stained cell bodies along with a dense neurite plexus, c) the arrow indicates the presence of neuronal vesicular monoamine transporter VMAT2 in the control cells, d) increased number of VMAT2 varicosities in GDNF treated cells.

3.3.1.2 Area Under the Curve

The area under curve here is an index of DA release as a function of amplitude and time. For oxycodone + Vehicle, the ± SEM was 6.140 ± 0.3489. The mean area under curve for SA Oxycodone group was 25.86 ± 8.187, it was 48.47 ± 10.03 for the saline yoked group and it was 102.2 ± 4.744 for the oxycodone + GDNF treatment group.

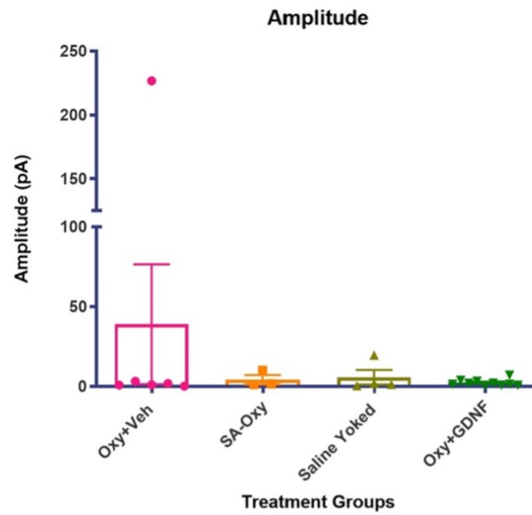

Figure 3.3: Amplitude of DA release in dorsal striatum.

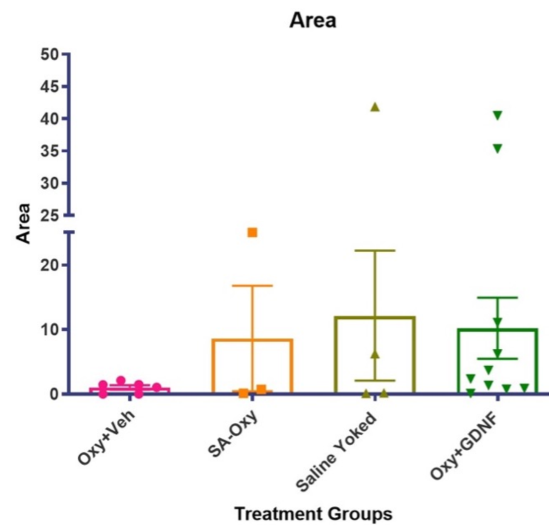

Figure 3.4: Area Under the Curve in dorsal striatum.

3.3.1.3 T ½

The mean± SEM for oxycodone + vehicle, SA oxycodone, saline yoked, and oxycodone + GDNF groups was calculated to be 7.690 ± 0.7254, 1.119 ± 0.1571, 23.70 ± 3.920, and 40.04 ± 1.624 msecs respectively.

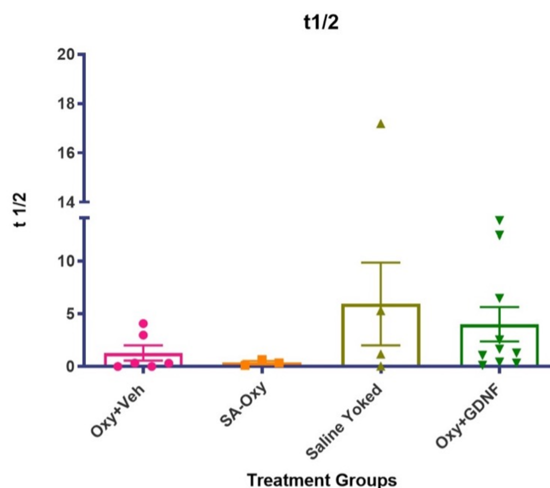

Figure 3.5: T ½ for DA release in Dorsal Striatum.

3.3.1.4 DA Molecules released per event

The average number of DA molecules released per amperometric event was measured converting AUD to DA molecules through the Faraday equation, i.e N = Q / nF (Q= charge of the spike, N= the number of moles, n= number of electrons transferred, and F= Faraday's constant) [19]. Oxycodone + Vehicle, SA Oxycodone, Saline Yoked and Oxycodone + GDNF had $1.916\times10^7 \pm 1.089\times10^6$, $8.072\times10^7 \pm 2.555\times10^7$, $1.513\times10^8 \pm 3.129\times10^7$, and $3.191\times10^8 \pm 1.481\times10^7$ DA molecules respectively.

3.3.1.5 Width

The mean width of each detected DA signal was also calculated for all four groups. For oxycodone + Vehicle, it was 44.83 ± 3.303 msec. For SA Oxycodone, the mean width was 16.36 ± 4.652 msec. For Saline Yoked group, the average dopamine width was calculated to be 151.6 ± 13.81 msec, and for oxycodone + GDNF treatment group, the average width was 248.3 ± 9.506 msec.

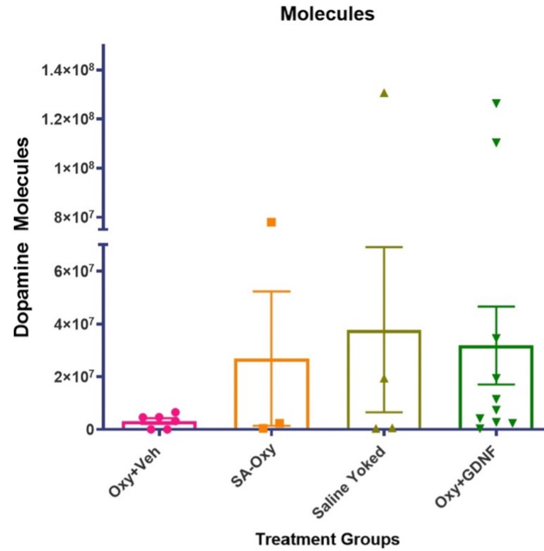

Figure 3.6: DA molecules released per event in Dorsal Striatum.

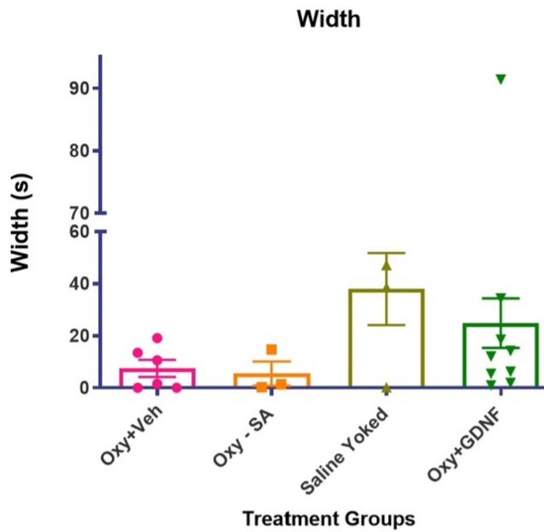

Figure 3.7: Width of the DA release in Dorsal Striatum.

3.3.2 Amperometric Event analysis for NAc

The effect of oxycodone and the GDNF treatment was also tested in the nucleus accumbens. Drugs of misuse mainly target and affect the NAc. NAc extends its dopaminergic projections into the ventral tegmental area and the substantia nigra. Due to COVID-19, the results below only indicate results for amplitude, area under the curve, T ½ and

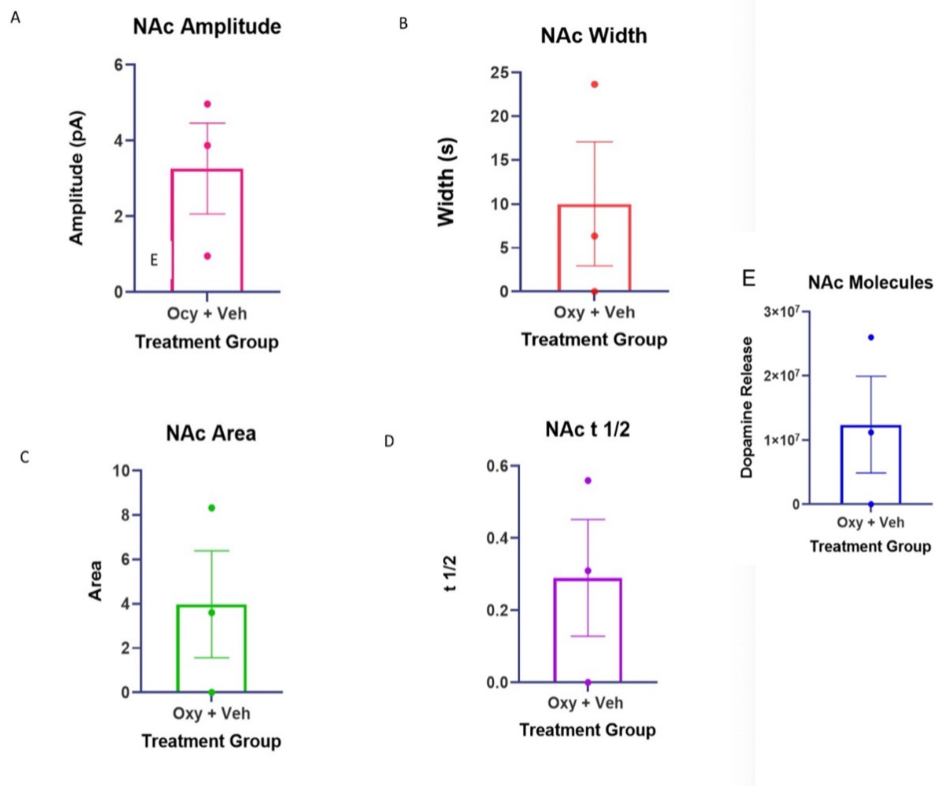

Figure 3.8: Amperometric events in Nucleus Accumbens: A) Amplitude of DA release, B) Width of dopamine release (seconds), C) Average area under the curve of the DA release, D) Average T ½ of the peak, E) Average number of DA molecules released per event calculated according to the Faraday Equation – All data represented in the figure are shown as mean ±SEM.

dopamine molecules for n=1 (group with oxycodone + Vehicle). More studies will be further carried to gain a more statistically accurate insight.

Statement: All of the work in this chapter was done collaboratively. Figures 3.1 and 3.2 were taken from previous work done in the Pothos Lab. Figures 3.3, 3.4, 3.5, 3.6, 3.7 and 3.8 were collaboratively done by me and Helia Mojahedyazdi.

Chapter 4: Discussion

According to the Centers for Disease Control and Prevention (CDC), about 450,000 people have died from opioid overdose in the last decade. Approximately 41 people die as a result of prescription opioid misuse. The most commonly misused opioids include oxycodone (Oxycontin, Percocet), hydrocodone (Vicodin), oxymorphone (Opana), morphine (Avinza), codeine, and fentanyl. While treatments like naloxone (Narcan) are available for the treatment of overdose, not many medications exist to treat OUD [20, 26].

Medications used in the treatment of OUD mainly focus on preventing relapse and managing withdrawal symptoms. Some of the medications include buprenorphine (Suboxone, Subutex), naltrexone (Vivitrol) and methadone. According to WHO, buprenorphine and methadone are efficient in controlling opioid use disorders. These two drugs are also given in a combination. These drugs along with behavioral counseling are known as Medication Assisted Treatment (MAT). While MAT is effective in decreasing opioid use and opioid related overdoses, it comes with its own set of drawbacks [23, 33]. Methadone and buprenorphine, themselves, can be misused. Newer and improved medications like Sublocade, a buprenorphine injection, have also been approved by the U.S. FDA for treating moderate to severe opioid addiction. Implantable formulation of buprenorphine, Probuphine, was also approved by the U.S. FDA. NIH HEAL (Helping to End Addiction Long-term Initiative), is an initiative taken on by NIH to test, among other treatments, whether vaccines can target opioids and prevent them from entering the brain. Apart from medications and vaccines, transcranial stimulation devices like NSS-2 Bridge are also being used to treat OUD. NSS-2 Bridge sends electric pulses to specific brain nerve cells to reduce withdrawal symptoms caused by opioids [23, 6].

In the present work, we carried out preliminary studies to evaluate intranasal pGDNF nanoparticles as a treatment option for OUD. Four groups of rats self-administering

oxycodone through iv catheters and receiving the pGDNF treatment or vehicle were subsequently processed with brain slice carbon fiber amperometry to assess the effects of pGDNF treatment on central electrically evoked DA release in real time in the dorsal striatum and nucleus accumbens, the main terminal regions for coding drug reward in the brain. It should be emphasized that all results are preliminary with low statistical power and currently not appropriate for parametric statistical analysis. The following descriptions and statements refer to trends of data only and are highly speculative. For amplitude of each DA event, the oxycodone + vehicle-treated group showed a higher efflux of dopamine in comparison with the other three groups, consistent with higher DA release associated with the use of oxycodone. After measuring the area under the curve of the dopamine release, the three groups other than the oxycodone + vehicle-treated showed a larger area, pointing to the possibility that treatment with pGDNF stabilizes DA release. T ½ was longer for the pGDNF-treated group which could further corroborate the stabilizing effect of the treatment along with the overall higher number of DA molecules released per event in the same group.

OUD is thought to induce DA deficiency in the brain and pGDNF should be able to upregulate DA release to counteract one of the most impacting adverse effects of OUD. It should also prevent premature cell death of dopaminergic neurons due to drug toxicity. pGDNF should also suppress drug-taking, drug-seeking, relapse, and withdrawal symptoms.

Amplifying statistical power in ongoing experiments and completion of the work on RT-PCR and IHC assays will help us reach more definite conclusions on the efficacy and safety of intranasal pGDNF in the treatment of OUD.

Statement: This chapter includes work done by me in collaboration with members (Helia Mojahedyazdi) of the Pothos laboratory. The third paragraph of this chapter was written collaboratively with Helia Mojahedyazdi while the rest was written by me.

Chapter 5: Bibliography

[1] Bryon Adinoff. Neurobiologic processes in drug reward and addiction. Harvard review of psychiatry, 12(6):305–320, 2004.

[2] AAo Anesthesiologists. Opioid abuse: Statistics, signs & symptoms - when seconds count. Available from: https://www.asahq.org/whensecondscount/pain-management/opioid-treatment/opioid-abuse/.

[3] Niladri Banerjee. Neurotransmitters in alcoholism: A review of neurobiological and genetic studies. Indian journal of human genetics, 20(1):20, 2014.

[4] Alasdair M Barr, William J Panenka, G William MacEwan, Allen E Thornton, Donna J Lang, William G Honer, and Tania Lecomte. The need for speed: an update on methamphetamine addiction. Journal of psychiatry & neuroscience, 2006.

[5] Sebastien Carnicella and Dorit Ron. GDNF — a potential target to treat addiction. Pharmacology & therapeutics, 122(1):9–18, 2009.

[6] Redonna K Chandler, Jennifer Villani, Thomas Clarke, Elinore F McCance-Katz, and Nora D Volkow. Addressing opioid overdose deaths: The vision for the HEALing communities study. Drug and Alcohol Dependence, 217:108329, 2020.

[7] Charles A Dackis and Mark S Gold. New concepts in cocaine addiction: the dopamine depletion hypothesis. Neuroscience & Biobehavioral Reviews, 9(3):469–477, 1985.

[8] Shyeilla V Dhuria, Leah R Hanson, and William H Frey II. Intranasal delivery to the central nervous system: mechanisms and experimental considerations. Journal of pharmaceutical sciences, 99(4):1654–1673, 2010.

[9] Marco Diana. The dopamine hypothesis of drug addiction and its potential therapeutic value. Frontiers in psychiatry, 2:64, 2011.

[10] Center for Substance Abuse Treatment. Substance abuse treatment for persons with co-occurring disorders. Rockville (MD): Substance Abuse and Mental Health Services Administration (US); 2005. (Treatment Improvement Protocol (TIP) Series, (42.), 2005. 9 Substance-Induced Disorders. Available from: https://www.ncbi.nlm.nih.gov/books/NBK64178/.

[11] E.L. Gardner. Addiction and brain reward and antireward pathways. Adv Psychosom Med, 30:22–60, 2011.

[12] Udi E. Ghitza, Haifeng Zhai, Ping Wu, Mikko Airavaara, Yavin Shaham, and Lin Lu. Role of BDNF and GDNF in drug reward and relapse: A review. Neuroscience & Biobehavioral Reviews, 35(2):157–171, 2010. Novel Perspectives on Drug Addiction and Reward.

[13] Leah R Hanson and William H Frey. Intranasal delivery bypasses the blood-brain barrier to target therapeutic agents to the central nervous system and treat neurodegenerative disease. BMC neuroscience, 9(3):1–4, 2008.

[14] BT Harmon, AE Aly, L Padegimas, O Sesenoglu-Laird, MJ Cooper, and BL Waszczak. Intranasal administration of plasmid DNA nanoparticles yields successful transfection and expression of a reporter protein in rat brain. Gene therapy, 21(5):514–521, 2014.

[15] Murray Hong, Karim Mukhida, and Ivar Mendez. GDNF therapy for parkinson's disease. Expert review of neurotherapeutics, 8(7):1125–1139, 2008.
[16] Qinqin Hu, Hua Li, Lihua Wang, Hongzhou Gu, and Chunhai Fan. DNA nanotechnology-enabled drug delivery systems. Chemical reviews, 119(10):6459–6506, 2018.
[17] Satoshi Ikemoto. Brain reward circuitry beyond the mesolimbic dopamine system: A neurobiological theory. Neuroscience & Biobehavioral Reviews, 35(2):129–150, 2010. Novel Perspectives on Drug Addiction and Reward.
[18] Saba Kassim, Sharif Islam, and Ray E Croucher. Correlates of nicotine dependence in uk resident yemeni khat chewers: a cross-sectional study. Nicotine & Tobacco Research, 13(12):1240–1249, 2011.
[19] Andre Kleinridders, Weikang Cai, Laura Cappellucci, Armen Ghazarian, William R Collins, Sara G Vienberg, Emmanuel N Pothos, and C Ronald Kahn. Insulin resistance in brain alters dopamine turnover and causes behavioral disorders. Proceedings of the National Academy of Sciences, 112(11):3463–3468, 2015.
[20] Christine L Mattson, Lauren J Tanz, Kelly Quinn, Mbabazi Kariisa, Priyam Patel, and Nicole L Davis. Trends and geographic patterns in drug and synthetic opioid overdose deaths—united states, 2013–2019. Morbidity and Mortality Weekly Report, 70(6):202, 2021.
[21] Chad J Messer, Amelia J Eisch, William A Carlezon Jr, Kim Whisler, Liya Shen, Daniel H Wolf, Heiner Westphal, Frank Collins, David S Russell, and Eric J Nestler. Role for GDNF in biochemical and behavioral adaptations to drugs of abuse. Neuron, 26(1):247–257, 2000.
[22] B.S. Muntean, M.T. Dao, and K.A. Martemyanov. Allostatic changes in the cAMP system drive opioid-induced adaptation in striatal dopamine signaling. Cell Rep, 960(e2), 2019-10. PMID: 31644915; PMCID: PMC6871051.
[23] N.I.D.A. Effective treatments for opioid addiction, 2016. Retrieved from https://www.drugabuse.gov/publications/effective-treatments-opioid-addiction on 2021, March 24.
[24] N.I.D.A. The science of drug use and addiction: The basics. National Institute on Drug Abuse, 2020. Accessed 28 Jul. 2020.
[25] Janne Nielsen, Kamil Gotfryd, Shizhong Li, Nikolaj Kulahin, Vladislav Soroka, Kim K Rasmussen, Elisabeth Bock, and Vladimir Berezin. Role of glial cell line-derived neurotrophic factor (GDNF)–neural cell adhesion molecule (NCAM) interactions in induction of neurite outgrowth and identification of a binding site for NCAM in the heel region of GDNF. Journal of Neuroscience, 29(36):11360–11376, 2009.
[26] Eric M Ossiander. Using textual cause-of-death data to study drug poisoning deaths. American journal of epidemiology, 179(7):884–894, 2014.
[27] Çağdaş Oto and R Merih Hazıroğlu. Macro-anatomical investigation of encephalon in donkey. 2009.
[28] Kewu Pan, Yifan Chen, Mendel Roth, Weibin Wang, Shuya Wang, Amy S Yee, and Xiaowei Zhang. HBP1-mediated transcriptional regulation of DNA methyltransferase 1 and its impact on cell senescence. Molecular and cellular biology, 33(5):887–903, 2013.

[29] Inmaculada Posadas, Silvia Monteagudo, and Valentín Ceña. Nanoparticles for brain-specific drug and genetic material delivery, imaging and diagnosis. Nanomedicine, 11(7):833–849, 2016.

[30] Suresh Babu Rangasamy, Katherine Soderstrom, Roy AE Bakay, and Jeffrey H Kordower. Neurotrophic factor therapy for parkinson's disease. Progress in brain research, 184:237–264, 2010.

[31] James E Schwob. Neural regeneration and the peripheral olfactory system. The Anatomical Record: An Official Publication of the American Association of Anatomists, 269(1):33–49, 2002.

[32] J. Staszkiewicz, M.T. Skowronski, T. Kaminski, G. Siawrys, B.E. Krazinski, M. Kusmider, J. Przala, and S. Okrasa. Expression of proopiomelanocortin, proenkephalin and prodynorphin genes in porcine theca and granulosa cells. Anim Reprod Sci, Sep;101(1-2):97-112. PMID: 17023126.

[33] Angela L Stotts, Carrie L Dodrill, and Thomas R Kosten. Opioid dependence treatment: options in pharmacotherapy. Expert opinion on pharmacotherapy, 10(11):1727–1740, 2009.

[34] David Sulzer. How addictive drugs disrupt presynaptic dopamine neurotransmission. Neuron, 69(4):628–649, 2011.

[35] David Sulzer and Emmanuel N Pothos. Regulation of quantal size by presynaptic mechanisms. Reviews in the neurosciences, 11(2/3):159–212, 2000.

[36] Nora D Volkow, Emily B Jones, Emily B Einstein, and Eric M Wargo. Prevention and treatment of opioid misuse and addiction: a review. JAMA psychiatry, 76(2):208–216, 2019.

[37] Zian Wang, Guojun Xiong, Wai Chun Tsang, Andreas G Schätzlein, and Ijeoma F Uchegbu. Nose-to-brain delivery. Journal of Pharmacology and Experimental Therapeutics, 370(3):593–601, 2019.

[38] Li-Tzy Wu, Christopher L Ringwalt, Paolo Mannelli, and Ashwin A Patkar. Hallucinogen use disorders among adult users of MDMA and other hallucinogens. American Journal on Addictions, 17(5):354–363, 2008.

[39] David M Yurek, Anita M Fletcher, George M Smith, Kim B Seroogy, Assem G Ziady, Joseph Molter, Tomasz H Kowalczyk, Linas Padegimas, and Mark J Cooper. Long-term transgene expression in the central nervous system using DNA nanoparticles. Molecular Therapy, 17(4):641–650, 2009.

[40] Amna Zehra, Jamie Burns, Christopher Kure Liu, Peter Manza, Corinde E Wiers, Nora D Volkow, and Gene-Jack Wang. Cannabis addiction and the brain: a review. Journal of Neuroimmune Pharmacology, 13(4):438–452, 2018.

[41] Zhiling Zou, Huijun Wang, Federico d'Oleire Uquillas, Xiaomei Wang, Jianrui Ding, and Hong Chen. Definition of Substance and Non-substance Addiction, pages 21–41. Springer Singapore, Singapore, 2017.