Drew University

College of Liberal Arts

INVESTIGATING THE ROLE OF GRANULOCYTE COLONY STIMULATING FACTOR AS A REGULATOR OF THE STRIATAL PROTEOME

A Thesis in Neuroscience

By

Tanner J. Euston

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Abstract

Pathologic use of illicit drugs represents a major public health concern and creates significant economic and social costs. Addiction to cocaine and other psychostimulants remains a major cause of this morbidity. The pathophysiological mechanisms that lead to persistent and dysregulated drug use remain incompletely understood, and there are currently no FDA-approved pharmacotherapies for treatment of psychostimulant use disorders. There is growing evidence that dysregulation of the immune system plays a role in the pathophysiology of multiple psychiatric disorders including major depressive disorder and schizophrenia. While cocaine is known to have immunomodulatory effects, the link between these immune interactions and pathological use behaviors has only recently been investigated. We recently identified granulocyte-colony stimulating factor (G-CSF) as a cytokine that is increased in serum and brain by chronic cocaine. Peripheral administration of G-CSF enhances cocaine place preference and cocaine intake in a self-administration model, and also facilitates cocaine-induced neuronal activation in the nucleus accumbens and prefrontal cortex. While G-CSF has clear effects on synaptic and behavioral plasticity, the molecular mechanisms underlying these effects remains unclear. To interrogate changes induced by G-CSF in the setting of active cocaine treatment a 2 x 2 design was utilized with animals injected with Saline or Cocaine (7.5mg/kg) and PBS or G-CSF once daily for seven days. This was followed by discovery proteomics analysis of the nucleus accumbens (NAc) using data-independent acquisition mass spectrometry analysis. As expected, there were many proteins that were significantly altered by one week of cocaine treatment. However, treatment with G-CSF alone resulted in regulation of

an even larger number of proteins, and co-treatment with the two resulted in the largest number of significantly regulated proteins – suggesting a significant interaction between the two. Gene ontology analysis of samples from G-CSF + Cocaine treated animals showed that there was a strong upregulation of proteins associated with the synaptic compartment, with a number of both pre and postsynaptic proteins demonstrating significant alterations. Confirmatory Western blot analysis demonstrate similar increases in levels of PSD-95 and GluR1 by G-CSF + cocaine in a separate cohort of animals. These findings complement our previous work and suggest that in the presence of G-CSF the ability of cocaine to cause synaptic remodeling is enhanced.

1. Introduction

1.1 Epidemiology of Substance Use Disorders

Drug addiction is a chronic neurological disorder with high rates of relapse. Drug abuse disorders, like any other clinically operationalized disorder, can be diagnosed through standards presented in the Diagnostic and Statistical Manual of Mental Disorders (DSM-V) (American Psychiatric Association, 2013) and the International Statistical Classification of Diseases and Related Health Problems (ICD-10) (adopted 2015). The previous edition of the DSM (DSM-IV) split diagnoses for drug abuse disorders into two separate categories: substance dependence and substance abuse (American Psychiatric Association, 1994). A diagnosis of substance dependence required a minimum of three of the following criteria over a 12-month period: tolerance; withdrawal; impaired control (taking the drug over a longer period of time than intended); unsuccessful quit attempts; excessive time spent obtaining, using, and recovering

from the drug; neglect of important social, occupational, or recreational activities; and continued use despite recurrent or persistent physical and/or psychological problems. The diagnosis of substance abuse omitted those who met criteria for substance dependence, and required one of the following four criteria to be met over a 12-month period: failure to fulfill major role obligations at work, school, or home, hazardous use (driving or operating machinery under the influence, legal problems such as arrests for disorderly conduct related to substance use, and continued use despite social problems. In the DSM-V, these two categories were combined into a single diagnosis called "substance use disorder," while omitting legal problems as a criterion and adding drug craving to the diagnostic checklist (for a total of 11 criteria) (American Psychiatric Association, 2013). These disorders are not the same as occasional drug use, as they entail unbalanced seeking of drugs, altered motivational states, and chronic health problems secondary to the drug abuse. In general, substance use disorders are classified clinically by three levels of severity: mild (2-3 DSM-5 criteria), moderate (4-5 criteria) and severe (6+ criteria). Also, the etiology of drug addiction usually develops from occasional use, to recreational use, to chronic use and then finally addiction. In a 2016 survey by Grant et al. (2016), the 12-month and lifetime incidence of drug abuse disorders in the United States alone was 3.9% and 9.9%, respectively. Not only was there a high prevalence of clinically defined drug abuse disorders in the United States, a follow-up survey determined that of those who responded to the original survey, a large majority had comorbid diagnoses of major depressive disorder, bipolar disorder, post-traumatic stress disorder, antisocial personality disorder, or borderline personality disorder. Drug addiction also confers a higher risk for infectious diseases such as HIV and hepatitis.

One barrier to assessing the national prevalence of substance use disorders, as defined by the DSM-5, is the lack of information surveyed about adolescents. The large bulk of national statistics on substance use disorders comes from the National Epidemiologic Survey on Alcohol and Related Conditions-III (NESARC-III) (Grant et al., 2016). The NESARC-III only surveys the US population who are ages 18+. Information about drug abuse statistics for ages 12-18 are available through the annual National Survey on Drug Use and Health (NSDUH) (Center for Behavioral Health, 2014). However, this survey uses DSM-IV criteria for assessment, which may causes some discrepancies in comparative analyses between adolescents and adults. The NSDUH data combines both substance abuse and substance dependence, which can be diagnosed independently from each other in a clinical setting through DSM-IV criteria. Therefore, comparing adolescent drug abuse statistics from the NSDUH to adult usage statistics from NESARC-III using modern DSM-5 criteria could be incongruous. Before strategizing a plan to stop substance use disorders, researchers must understand the developmental stages of involvement in drug abuse and doing so would benefit greatly from expanding the standards of the population surveyed to include adolescents aged 12-17.

Addiction carries with it a large burden for the people whom it directly affects, their families, their friends, and society at large. Recently, the national yearly economic cost of drug abuse has ballooned to $500 billion in medical, counseling, humanitarian, and correctional costs (Volkow et al. 2016). People who suffer from drug addiction are highly likely to see impairments in normal life functioning, such as maintaining both romantic and non-romantic relationships, keeping a steady job, and practicing smart financial decision-making. All of these addiction-associated issues are further compounded by increasing acceptance of recreational drug use

and legalization, leading to higher rates of poverty, homelessness, and incarceration in the United States. Ultimately, drug abuse disorders are not just a public health issue, but also a political issue as many of the problems caused by addiction challenge policy makers who are trying to determine the best course of action to ameliorate the societal effects. Previous policy decisions and public health initiatives intended to stall the increasing costs of addiction have been ineffective, as drug use rates across races, genders, and ages have remained relatively constant since the 1990s regardless of policy and public health changes. One starting point to solving the countless comorbid health and societal problems associated with drug abuse disorders is to find a drug therapy that effectively cures the disorder.

1.2 Differences Between Psychostimulant and Opioid Abuse Disorders

1.2.1) Reward Circuitry and Drug Addiction

In 1950, the World Health Organization recognized the growing epidemic of drug abuse across the world, and attempted to define the condition to provide a framework for studying the disorder. They stated that drug addiction is characterized by psychological dependence, independent of the class of drug. From a neuropsychological perspective, the disorder also presented with an increased tolerance to the drug and difficulties in maintaining abstinence from the drug. This makes sense, as “abuse” suggests increased drug intake and “addiction” suggests an inability to remain abstinent from taking the drug. In 1953, electrical stimulation studies by James Olds and Peter Milner in primates and rodents showed that electrical stimulation of the mesocorticolimbic system (Fig. 1) may be the mechanism through which reward learning occurs. Thomas et al. (2001) demonstrated that drugs of abuse cause addictive

phenotypes through changes in neurons in the mesocorticolimbic pathway that release dopamine, which is the substrate of reward learning in drug abuse disorders. It is very important to note the similarities in drug abuse disorders regardless of drug class, as the unified view of addiction as a psychological disorder states that all addiction disorders are classified by incentive sensitization (salience towards drug-seeking) (Robinson & Berridge, 2008), aberrant learning (increased reward memory toward substances of abuse) (Torregrossa et al., 2011), and hedonic allostasis (negative reinforcement of drug taking as avoidance of unpleasant withdrawal symptoms) (Koob, 2008). The development of these diagnostic symptoms mirrors the biological disease model of addiction (Volkow et al., 2016), which has three stages: binge and intoxication, withdrawal and negative effect, and preoccupation and anticipation. In the binge and intoxication stage, drug use engages dopamine reward circuitry leading to addictive behaviors through a feeling of pleasure (Kelley, 2004), which causes incentive sensitization. In the withdrawal and negative effect stage, primary rewarding stimuli (such as food, water, and sex) elicit lower reward compared to taking drugs, which causes aberrant learning of drug use. Lastly, preoccupation and anticipation is caused by anhedonia during withdrawal from drug use, which increases drug seeking and use in order to decrease unpleasant feelings. When drug seeking is driven by prevention of unpleasant feelings instead of by seeking pleasurable feelings of drug use, hedonic allostasis is achieved.

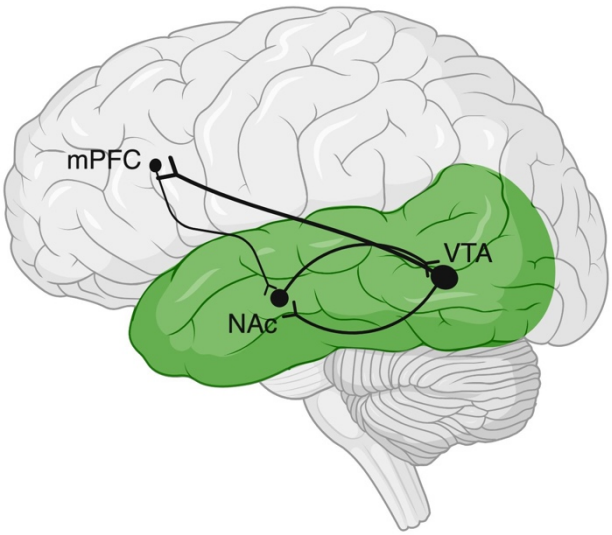


Figure 1. Diagram of the mesocorticolimbic pathway. Targets in green region are subcortical. VTA – ventral tegmental area; NAc – nucleus accumbens; mPFC – medial prefrontal cortex

1.2.2) Cellular and Molecular Mechanisms of Action

When trying to approach treatment options and further research into therapeutic targets for drug addiction, the neurological and psychological differences between psychostimulant abuse disorder and opioid abuse disorder are often overlooked. The most controversial disparity between addictions in these two classes of drugs was in mid 1980s, when rodent studies showed that lesions to the mesocorticolimbic pathway or blockade of dopamine receptors decreases the reward for psychostimulants, but not opioids (Badiani et al., 2011). This challenged the unified view of addiction that was widely accepted. At this point in time, it was clear that clinically diagnosing patients with drug abuse disorders could benefit from utilizing a unified theory of addiction. However, drug abuse presents clinically in a very similar manner, regardless of drug class. It is clear that a new cellular and molecular model of addiction would better explain the pharmacodynamic differences in functioning between the classes of drugs.

Chronic use of psychostimulants and opioids induce conflicting changes in neuroplasticity across key regions in the mesocorticolimbic pathway. The dominating neurobiological framework for the development of addictive behaviors in the 1990s was that drug abuse and relapse were behaviors reinforced by intracellular and synaptic changes to the dopaminergic neurons in the mesocorticolimbic pathway, as well as the glutamatergic targets that they project to (Badiani et al., 2011). Scientists believed that these changes were independent from drug class. Psychostimulants and opioids do cause some similar changes in intracellular signaling, and these changes induce long-term potentiation (LTP) in glutamatergic synapses in the VTA from the NAc and short-term depression of dopamine levels in the NAc following withdrawal (2011). Neurobiologically, however, drug-induced synaptic plasticity in other key regions of the mesocorticolimbic pathway differs between the two classes of drugs. Withdrawal from repeated cocaine intake increased LTP of glutamatergic projections from the NAc to the medial prefrontal cortex (mPFC), while withdrawal from opioids like heroin and morphine did not induce LTP in those neurons (2011). Repeated injections of cocaine lead to increases in spine density and dendritic branching of mPFC and NAc neurons, which is exactly the opposite of what is observed in repeated morphine injection (Russo et al, 2010). Physiologically, changes in spine density and branching are correlated with functional synaptic plasticity leading to LTP or LTD (long-term depression), and these changes in neuronal morphology have been shown to be experience-dependent (Tada & Sheng, 2006). This means that the classes of drugs may produce similar addictive phenotypes, but the experience of taking those drugs produces opposing cellular changes at the synapse.

(A) (B)

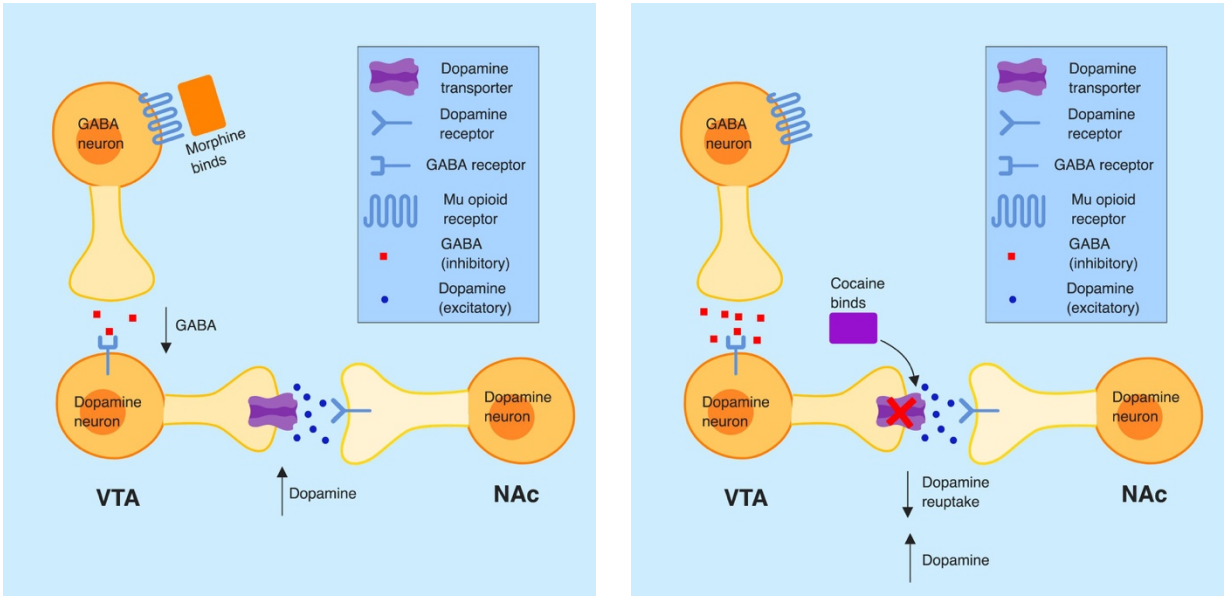


Figure 2. Molecular mechanisms of action of morphine and cocaine. (A) Morphine binds to opioid receptors on GABAergic interneurons in the VTA. This decreases GABA release, which disinhibits dopaminergic VTA neurons and increases dopamine release into the NAc. (B) Cocaine binds to presynaptic dopamine transporters on dopaminergic neurons in the VTA, leading to increased release of dopamine into the NAc. Adapted from Badiani et al. 2011.

There are major differences between psychostimulants and opioids in terms of molecular mechanisms of addiction. While both classes of drugs increase the release of dopamine into the nucleus accumbens (NAc) in the mesocorticolimbic pathway, they do so through different mechanisms (Fig 2). Presynaptic dopamine reuptake in the VTA acts as a feedback system to decrease neuronal firing and dopamine release following a rewarding stimulus. Psychostimulants, such as cocaine and amphetamine, block the reuptake of dopamine in the ventral tegmental area (VTA), causing continued release of dopamine into the NAc (Kleven & Koek, 1998). Conversely, opioids bind to receptors on GABAergic neurons that project to the VTA, causing decreased inhibitory input to VTA dopaminergic neurons and subsequent

increased dopamine release to NAc neurons. Therefore, when searching for pharmacotherapeutic targets, these classes of drugs must be approached differently.

Dopamine-mediated changes in reward feelings occur for both natural rewards and drugs of abuse. For natural rewards like food and water, repeated exposure to these stimuli will eventually reduce the release of dopamine in the mesocorticolimbic system. Drugs of abuse, however, increase dopamine signaling with each independent exposure (Koob & Vokow, 2010). This difference is important to note because it explains why drug-addicted individuals attribute higher salience to drugs than natural rewards which are necessary for life. The first stage of substance use disorders (binge and intoxication) is caused by increased salience of drug taking caused by elicited feelings of pleasure from dopamine release. In the second stage of addiction (withdrawal and negative effect), occasional use turns into regulated relapse characterized by the disruption of glutamate homeostasis (Kalivas & O'Brien, 2008). Decreased extracellular glutamate in the NAc is caused by chronic self-administration of cocaine and has been shown to disrupt synaptic strength (Kalivas, 2009). In contrast, chronic administration of opioids does not elicit this response. Lower levels of glutamate in the extracellular space in the NAc means that non-synaptic glial uptake of glutamate and subsequent inhibition of glutamatergic release decreases. The end target of this homeostatic disruption is more excitation of NAc neurons following chronic cocaine use. The mPFC plays a protective role by initially preventing this change in glutamate homeostasis. By the last stage of addiction (preoccupation and anticipation), dysregulation and synaptic damage to mPFC neurons disrupts the protective mechanism maintaining glutamate homeostasis, and the addicted individual loses the ability to use executive control to refrain from drug seeking. While the cellular

mechanisms underlying this loss of executive control are still unclear, addicted individuals have shown lower baseline levels of metabolic activity in the mPFC (Volkow et al., 2007).

1.2.3) Genetic Basis of Substance Use Disorders

Substance use disorders develop, like most other psychiatric conditions, from the complex interplay of genetics and environment. The inheritance of these disorders from parent to child ranges from moderate (40%) to high (70%) (Ducci & Goldman, 2012). Genetic vulnerability to substance use disorders can either be drug-specific (i.e. genetic variants that mediate drug metabolism) or non-specific (i.e. variants that control stress resilience). Clinically measuring environmental risk factors for addiction is manageable. Understanding the influence of genetics in addiction is more challenging. The predictive value of any single allele or variant

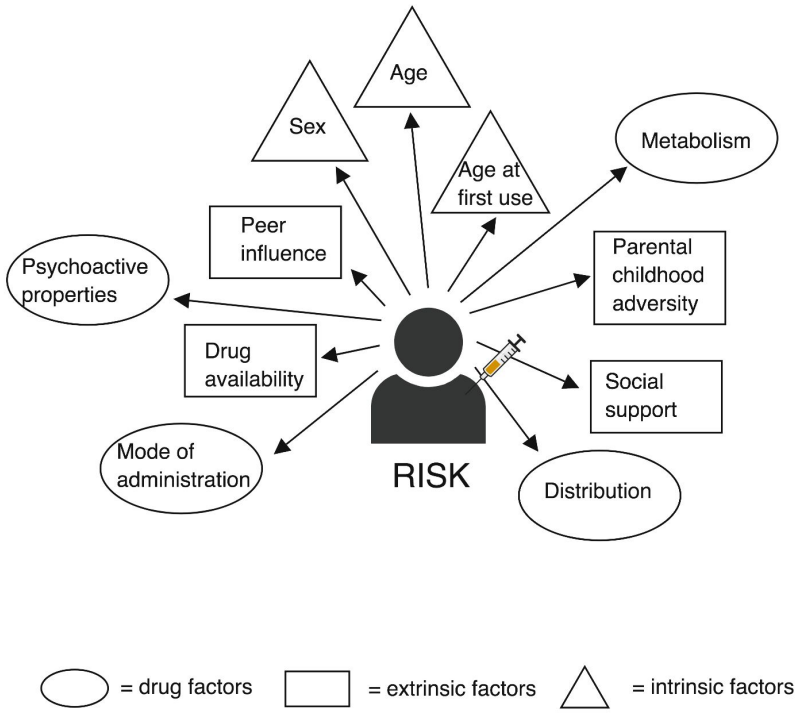


Figure 3: Risk factors for the development of substance use disorders.

in determining a diagnosis of substance use disorders is very low. On top of that, about 95% of the genetic variance observed between patients is unexplained. The "missing heritability" is explained by high interpersonal variance in physiological processes like drug metabolism, sensation, reward learning, and cognitive control. All of these processes are polygenetic, and the interaction of these factors exponentially complicates the understanding of genetics in determining diagnostic outcome. Secondly, substance use disorders are known as "end-stage" diagnoses, meaning that the role of genetics in conferring risk for these disorders is diluted or lost in the sea of other predictive phenotypes (Fig. 3).

Researchers often utilize genome-wide association studies (GWAS) to study the genetic variants that can influence the onset of certain phenotypes and diseases. These studies query large databases of genetic information from many subjects through hypothesis-free analysis to probe for associations between phenotypes and recurrent genetic markers. In the case of addiction, this methodology has not yielded many loci of interest except for a variant believed to be involved in vulnerability to nicotine addiction (Ducci & Goldman, 2012). The prominent approach to finding genes of interest in addiction is through functional pathway analysis. This approach entails a comparison of gene expression to databases of known phenotypic presentation caused by specific genes. Studies that query pathway and protein-protein interaction databases help identify the collective effects on phenotype expression based on differential expression of genes. Previously, this method of pathway analysis has led to the discovery of metabolic pathways involved in alcohol dependence (Han et al., 2013).

Epigenetic mechanisms also play a role in the onset of substance use disorders. Epigenetics involves the non-genetic modification of chromatin at histones and other proteins

that control DNA transcription. In the case of addiction, chronic exposure to cocaine causes global increases in acetylation of histones H3 and H4 in the NAc (Nestler, 2014). This increase in acetylation initially causes an increase in reward learning toward cocaine, but if the acetylation is continued then this reward learning decreases. Research suggests that this decrease in reward learning after prolonged acetylation of histones in the NAc could be due to methylation of the same histones. Since acetylation usually "opens" chromatin to make it more available for recruitment of transcription factors, and methylation "closes" chromatin, these behavioral effects make sense (Nestler, 2014). Acetylation of histones allows for higher levels of protein expression and therefore more changes due to cocaine administration. Methylation of histones lowers the level of protein expression, which can act as a protective mechanism against neuronal changes caused by cocaine exposure. Ultimately, genetic and epigenetic factors play a large role in the inheritance of substance use disorders, but more research is required to characterize and find the "missing heritability" of genetics in the etiology of these disorders

1.3 Novel Approaches to Substance Abuse Disorder Treatment

Psychostimulants and opioids act on similar brain regions and produce similar behaviors, but the area of treatment is where these two classes of drugs differ the most. Opioid craving is a symptom that has no ideal pharmacological treatment. The only available drugs marketed for opioids are intended to treat and reverse overdoses (naloxone) or curb craving by replacing the drug dependence by administering other addictive agents (methadone and buprenorphine). The issue with this is that after reversing an overdose, opioid abuse disorders can only be treated presently by giving drugs that are themselves addictive. While methadone and

buprenorphine use is definitely preferable to opioid abuse, the ultimate goal of treating substance use disorders is to fully remove dependence from the drug of abuse without prescribing medications that require chronic use to work optimally. Psychostimulants such as cocaine have no marketed drugs intended to curb craving and abuse. This poses a problem as substance use disorders are biological and should be treated as illnesses, but doctors are lacking medications to prescribe to cocaine addicts.

The field of drug discovery has shown little interest in developing novel drugs to treat psychostimulant abuse disorders. The reasons for this are numerous. Firstly, new drug development takes 15 years from start to finish on average and costs at minimum 1.5 billion dollars. The market of substance use disorder treatment is not very sizable, since only about 20% of people suffering from these disorders actually seek treatment. Secondly, if a new drug were to be developed, a 2011 SAMSHA survey estimated that a treatment cost of ~$700 a month per person would only reap an annual revenue of $4.2 billion. While this seems like a lot of money, and it certainly outweighs the initial investment of drug discovery, current treatment options like methadone and buprenorphine are more expensive and bring in more revenue without requiring a new time and money investment. Therefore, the largest area of research in drug development for substance use disorders is in repurposing approved drugs. Recent research, summarized by Hofford (2018), indicates that substance abuse disorders cause distinct changes in neuroimmune responses. Modulation of these neuroimmune pathways shows promise in treating substance abuse disorders, since they approach treatment without targeting neurotransmitter systems. Drugs that have been tested to target dopaminergic and glutamatergic systems have not been approved due to undesirable side effects, difficult routes

of delivery, and even potential for abuse. Many drugs that target immune cells and responses have already been approved by the FDA to treat various disorders and assist in recovery from certain surgical procedures without the slew of negative side effects or abuse potential. Our lab recently identified one cytokine, granulocyte colony stimulating factor (G-CSF) as a potent regulator of the behavioral and neurological response to cocaine abuse. G-CSF is a 25 kD glycoprotein and is very important in immune signaling in response to infection (Roberts, 2005). In the healthy brain, G-CSF and its corresponding receptor (G-CSFR) are robustly expressed in microglia (Diederich et al., 2009). During inflammatory responses, G-CSF signaling causes an increase in neutrophil production through stem cell proliferation and maturation (Roberts, 2005). G-CSF has been previously shown to be neuroprotective in the event of a stroke, delay neurodegenerative disease progression, and even play a key role in learning and memory processes (Calipari et al., 2018). Recombinant G-CSF drugs are commonly used following chemotherapy and post-surgery for bone marrow transplants in order to stimulate production of immune cells (Schmitz et al., 1996). Repurposing of drugs and antibodies that block G-CSF function have eliminated cocaine craving in mice without affecting general motivation and locomotion (Calipari et. al, 2018).

While G-CSF has been shown to affect behavioral and biological response to cocaine administration, there is still very little understood about its mechanisms of action in treating cocaine addiction. In this study, we utilize an unbiased proteomics analysis to characterize the changes in protein expression in the NAc caused by G-CSF administration both alone and together with cocaine administration. We then query pathway analysis and protein-protein interaction databases to predict the regulation of signaling cascades that may modulate the

differential protein expression observed through mass spectrometry analysis. Lastly, we quantify Western blot analysis of predicted targets of interest to confirm differential expression based on G-CSF and/or cocaine treatment. We demonstrate that G-CSF likely modulates signaling cascades controlling synaptic morphology and plasticity as well as RNA binding and function, and we suggest a biased, two-fold nuclear transcriptomic and synaptosome analysis to more clearly and definitively characterize the mechanisms of action of G-CSF in regulating behavioral and neuronal responses to cocaine abuse.

2. Materials and Methods

2.1 Animals and Drug Treatments

Male C57BL/6J (8 weeks old, ~20-25 g; Jackson Laboratories, Bar Harbor, ME, USA) were housed in the animal facilities at the Icahn School of Medicine at Mount Sinai. The mice were maintained on a 12 hour light/dark cycle and were given food ad libitum throughout the experiment. Drug treatments were administered based on a 2 x 2 experimental design. The first group (n = 5) received injections of PBS followed by saline, the second group (n = 5) received 50 µg/kg G-CSF (obtained from GenScript Biotech, Piscataway NJ) followed by saline, the third group (n = 6) received 7.5 mg/kg injections of cocaine hydrochloride (obtained from NIDA) with a PBS vehicle, and the fourth group (n = 8) received both G-CSF and cocaine hydrochloride (premixed 50 µg/kg and 7.5 mg/kg, respectively) (Fig. 4). The injections were performed once daily in a novel environment for 7 days. Mice were decapitated 24 hours following the final injection, and NAc accumbens tissue was harvested using a reference brain atlas and kept frozen until tissue digestion.

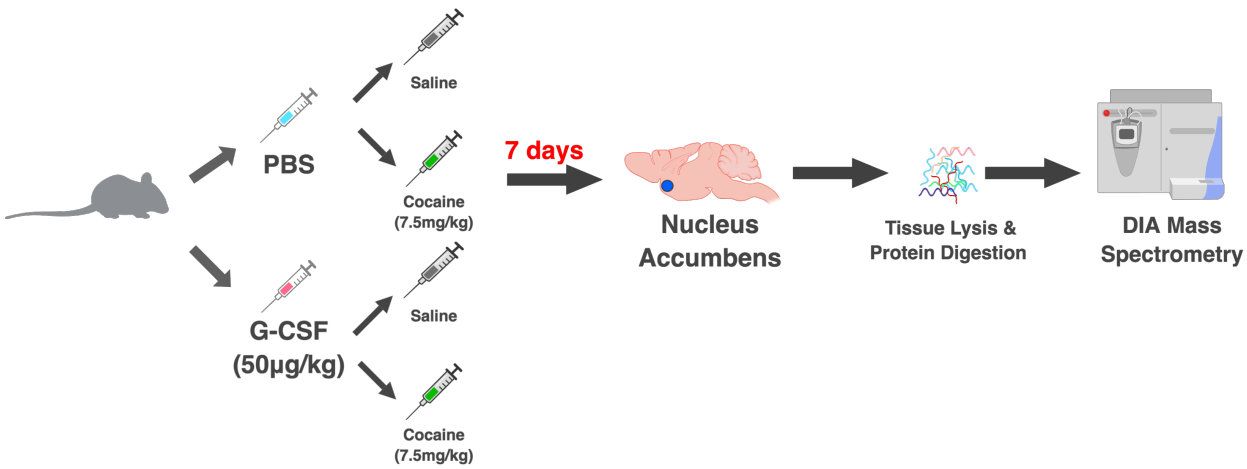


Figure 4: Experimental design and implementation. Mice received injections of either PBS or 50 µg/kg G-CSF followed by injections of either saline or 7.5 mg/kg cocaine. Following 7 days of treatment, NAc tissue was harvested and lysed before protein digestion and DIA mass spectrometry.

2.2 Data Independent Acquisition (DIA) LC-MS/MS

For each mouse, the NAc tissue was sonicated in 50 mL of RIPA buffer (50 mM Tris [pH 8.0], 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 110 mM NaCl & Halt Protease and Phosphatase Inhibitor Cocktails [Fisher]). These tissue samples were then sent to the Yale/NIDA Neuroproteomics Center (New Haven, CT) for protein digestion. The purpose of this analysis is to quantify protein expression of any detectable protein in the NAc.

At Yale: Data-independent acquisition (DIA) LC-MS/MS was performed using a nanoACQUITY UPLC system (Waters Corporation, Milford, MA, USA) connected to an Orbitrap Fusion Tribrid (ThermoFisher Scientific, San Jose, CA, USA) mass spectrometer. After injection, samples were loaded into a trapping column (nanoACQUITY UPLC Symmetry C18 Trap column, 180 µm × 20 mm) at a flow rate of 5 µL/min and separated with a C18 column (nanoACQUITY

column Peptide BEH C18, 75 µm × 250 mm). The compositions of mobile phases A and B were 0.1% formic acid in water and 0.1% formic acid in ACN, respectively. Peptides were eluted with a gradient extending from 6% to 35% mobile phase B in 90 min and then to 85% mobile phase B in another 15 min at a flow rate of 300 nL/min and a column temperature of 37◦C. The data were acquired with the mass spectrometer operating in a data-independent mode with an isolation window width of 25 m/z. The full scan was performed in the range of 400–1,000 m/z with "Use Quadrupole Isolation" enabled at an Orbitrap resolution of 120,000 at 200 m/z and automatic gain control (AGC) target value of 4 × 10 5 . Fragment ions from each peptide MS2 were generated in the C-trap with higher-energy collision dissociation (HCD) at a collision energy of 28% and detected in the Orbitrap at a resolution of 60,000.

2.3 DIA Data Analysis

DIA spectra were searched against a Mus musculus proteome database exported from Uniprot using Scaffold DIA software v. 1.1.1 (Proteome Software, Portland, OR, USA). Within Scaffold DIA, raw files were first converted to mzML format using ProteoWizard v. 3.0.11748. The samples were then aligned by retention time and individually searched against the proteome database with a peptide mass tolerance of 10 ppm and a fragment mass tolerance of 10 ppm. The data acquisition type was set to "Non-Overlapping DIA", and the maximum missed cleavages was set to 1. Fixed modifications included carbamidomethylation of cysteine residues (+57.02). Peptides with charge states between 2-3 and 6-30 amino acids in length were considered for quantitation, and the resulting peptides were filtered by Percolator v. 3.01 at a threshold false discovery rate (FDR) of 0.01. Peptide quantification was performed by

Encylopedia v. 0.6.12, and 6 of the highest quality fragment ions were selected for quantitation. Proteins containing redundant peptides were grouped to satisfy the principles of parsimony, and proteins were filtered at a threshold of 2 peptides per protein and an FDR of 1% compared to PBS/saline controls.

At Mount Sinai: Raw data (including t-tests for each identified protein) was entered into a Microsoft Excel spreadsheet. Protein expression levels obtained from proteomics analysis were averaged among all subjects in each experimental group to obtain a group mean. Group-specific fold change (FC) over saline was calculated by comparing the mean saline protein expression levels to the mean protein expression of each experimental group. Log_2 and log_{10} FC values were calculated to clarify directionality and relative magnitude, respectively, of FC values over saline. Identified proteins with a $p < 0.05$ were considered significantly regulated.

Volcano plots were created using GraphPad Prism version 8. Pathway analysis was determined using Ingenuity Pathway Analysis (IPA) by Qiagen. Analysis was sorted to show the top 5 predicted upregulated and downregulated mechanisms. Significantly regulated proteins were also queried against the STRING protein-protein interaction (PPI) database through Cytoscape to create network diagrams. PPI network diagrams

2.4 Western Blot Confirmation

For Western blot analysis animals were treated identically to those above, and NAc tissue was dissected and frozen on ice until further processing. Samples were sonicated in SDS lysis buffer (1% SDS, 50 mM Tris [pH 8.0], 130 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM PMSF, protease and phosphatase inhibitor cocktails from ThermoFisher). Sample concentrations were

determined using a Bradford colorimetric assay (ThermoFisher) according to manufacturer protocols, and 10μg of protein from each animal in every group was run on a 4–12% gradient gel. Proteins were transferred to PVDF membranes. Membranes were blocked using LiCor blocking buffer with a TBS base mixed 1:1 with standard TBS for one hour at room temperature. Primary antibodies were incubated with mixing at 4°C overnight with constant agitation. Primary antibodies used were PSD95 (Cell Signaling #36233S, 1:1000, mouse), GluA1 (Cell Signaling #13185S, 1:1000, rabbit), mTOR (Cell Signaling #2983, 1:1000, rabbit) & actin (Cell Signaling #3700, 1:10,000, mouse). Membranes were washed with TBS + Tween-20 before incubation with secondary antibodies (anti-rabbit and anti-mouse green and red fluorophores) raised against the appropriate species (LiCor, 1:10,000) for one hour at room temperature. Membranes were then washed with TBS + Tween-20, rinsed with TBS without Tween, and imaged using a LiCor Fluorescent imager. Image quantification was performed using freely available ImageJ software (NIH). Western blot data from the 24-hour protocol subjects were compared with NAc tissue samples from mice that followed the same treatment protocol, but were dissected 1 hour after their last injection. Changes in expression level over between 1-hour and 24-hour mice were graphed in GraphPad Prism version 8.

3. Results

3.1 Experimental Design

Our lab previously determined that peripheral injections of G-CSF lead to changes in gene expression in the NAc (Calipari et al., 2018), changes in dopamine release into the VTA (Kutlu et al., 2018), and changes in the proteomic landscape of the VTA (Mervosh et al., 2018).

Therefore, we hypothesized that peripheral injections of G-CSF would cause changes in the proteomic landscape of the NAc. In order to assess the function of G-CSF (both alone and in conjunction with cocaine) in the NAc, we designed a 2 x 2 study in which animals were being treated with cocaine (7.5 mg/kg), G-CSF (50 μg/kg), or both, with the proper PBS/saline vehicle controls. We chose the cocaine and G-CSF dosages based on previous research that showed optimal behavioral interaction between the two substances at those doses (Calipari et. al., 2018) The mice received injections in a novel cage once daily for 7 days before dissection (Fig. 4). The effects of cocaine administration, including psychomotor sensitization, have been shown to be greater outside of the home cage (Mattson et. al, 2007) and in a novel environment (Li et al., 2004). Therefore, we decided to inject the mice in a novel environment to best model the symptoms of human cocaine abuse.

To obtain higher statistical power and detect smaller changes in protein expression, we utilized a DIA mass spectrometry analysis. DIA mass spectrometry has benefits over DDA (data-dependent acquisition) mass spectrometry, including the ability to detect protein expression at a smaller level (Doerr, 2014). DDA mass spectrometry identifies proteins by taking a sample of digested protein, pushing it through a mass spectrometer, and sorting the resulting peptide fragments by mass/charge (m/z) ratio. DIA mass spectrometry, instead, sorts the digested proteins by m/z ratio before analysis in the mass spectrometer, and analyzes each “bin” of resulting peptide fragments individually in order to detect proteins expressed at low levels.

3.2 Proteomic Effects of G-CSF and Cocaine in the NAc

For our proteomic analyses, we compared the changes in protein expression between each comparison group. DIA mass spectrometry identified 1,477 proteins in the NAc tissue. Based on fold change over saline, the G-CSF treated mice expressed 222 proteins at a significance of $p < 0.05$. The cocaine-treated mice showed differential expression of 57 proteins at $p < 0.05$, and the combinatory G-CSF w/ cocaine-treated mice (GC) showed differential expression of 227 proteins at $p < 0.05$ (Fig. 5). We also looked at differential protein expression of the GC group compared to the cocaine group, since this may reveal molecular mechanisms of G-CSF administration as a modulator of the biological effects of cocaine administration. When

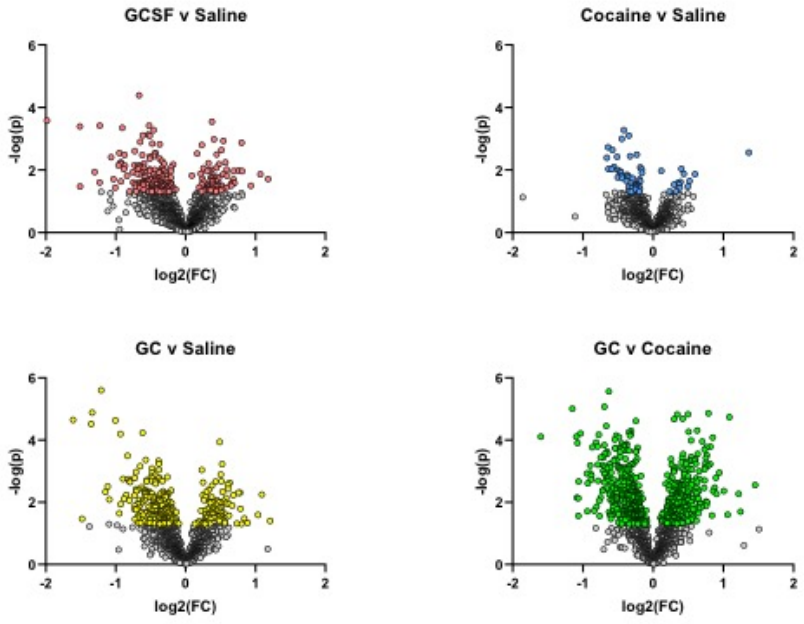


Figure 5: Volcano plots of differential protein expression. Dots represent proteins detected to be differentially expressed over saline. Colored dots are significantly regulated if -log(p) > 1.30 ($p < 0.05$). Proteins with a $\log_2$ fold change (log2(FC)) < 0 are down-regulated, and those > 0 are upregulated.

comparing the GC group to the cocaine group, we identified 597 differentially expressed proteins ($p < 0.05$).

The primary purpose of these experiments was for protein discovery, so it is important to note that we used uncorrected p-values throughout this study. While this may produce slightly biased results through higher levels of false discovery, we felt that it was necessary to include all proteins detected with an uncorrected p-value < 0.05. However, we believe this approach allows for broader analysis in this study which could lead to more strict, mechanistic future studies. All subsequent analyses incorporated all proteins that were significantly regulated regardless of the magnitude of fold change. Again, we believe this is an appropriate

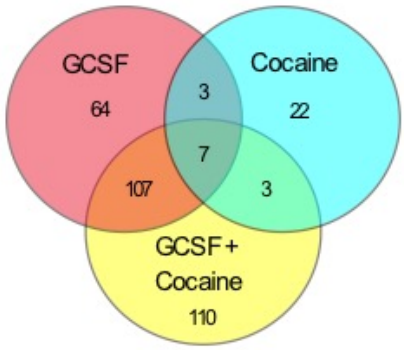
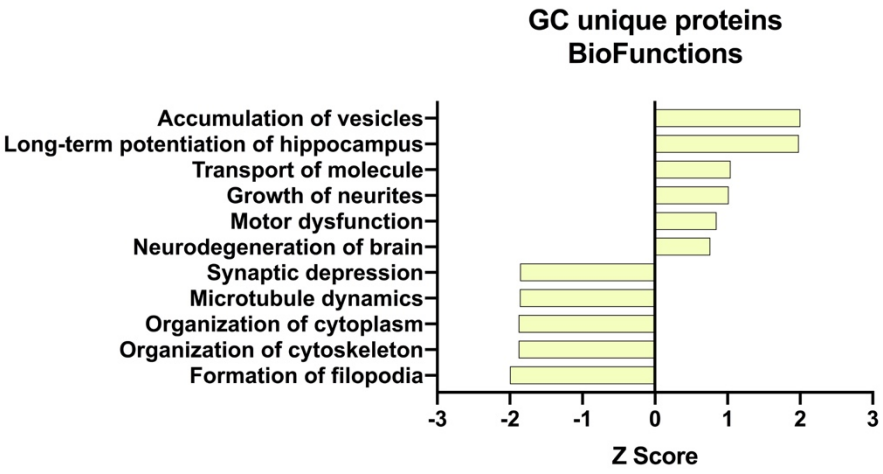


Figure 5: Venn diagram of significantly regulated proteins. Overlapping sections indicate identical protein expression between comparison groups. G-CSF, Cocaine, and G-CSF w/ Cocaine protein expression numbers are compared against saline controls.

approach since some proteins (i.e. membrane proteins) are more resistant to large-scale changes at this 24-hour timeframe. By including all significantly regulated proteins regardless of fold change, further pathway analyses should predict more changes in molecular and cellular mechanisms that otherwise would not be predicted to change following treatment.

3.3 Pathway Analysis of Differentially Regulated Proteins

In order to provide context for the large-scale changes in protein expression in the NAc, we ran the significantly regulated proteins for each comparison group through Qiagen's Ingenuity Pathway Analysis (IPA) software. This software queries databases of predicted and experimentally-determined mechanisms of intracellular signaling and compares them to the proteins involved in each signaling pathway. The G-CSF w/ cocaine group differentially



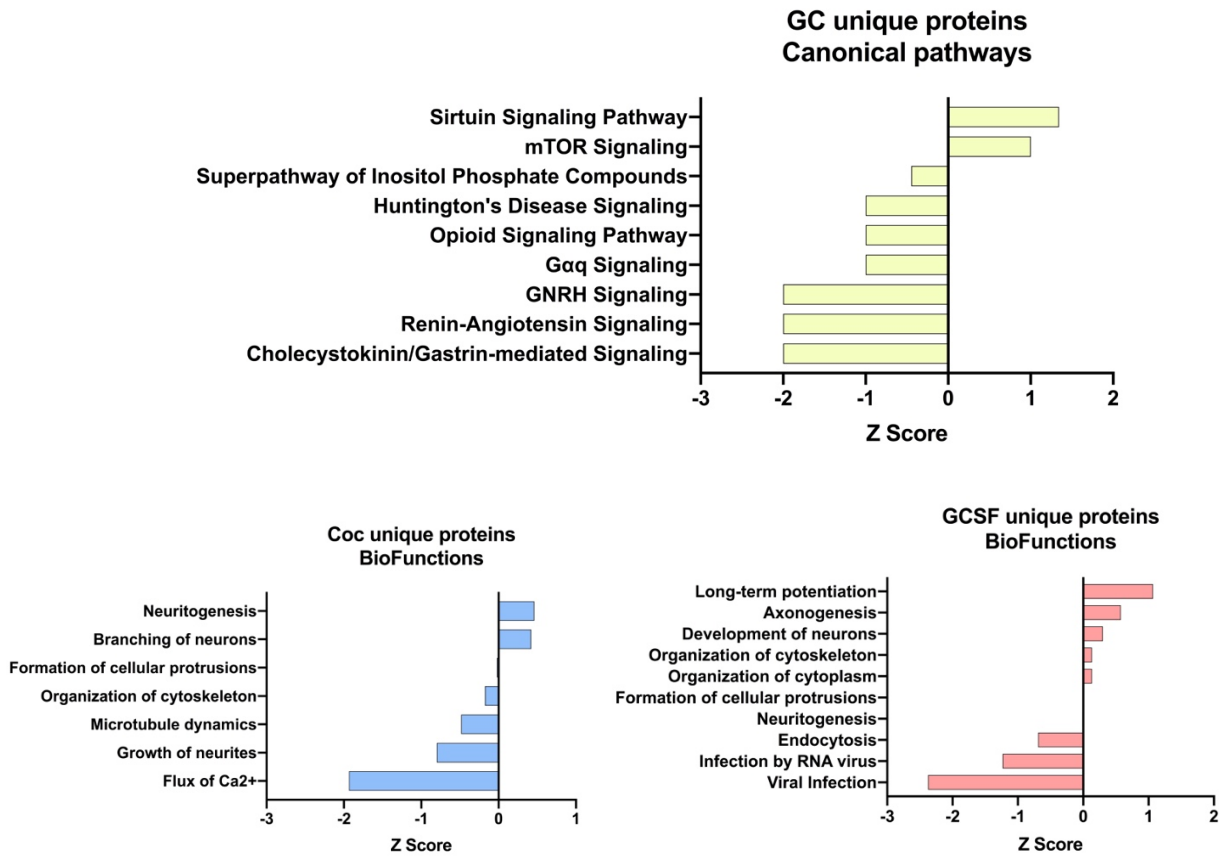


Figure 6: IPA predicted pathway changes from uniquely expressed proteins in the G-CSF, cocaine and GC treatment groups. A positive Z score indicates a predicted upregulation of canonical pathways or biological functions. A negative Z score indicates a predicted downregulation.

expressed proteins that are predicted to be involved in increased vesicle accumulation, intracellular molecule transport, and neurite growth. (Fig. 6). This group also showed a downregulation of synaptic depression. The changes in biological functioning closely resemble those predicted in the cocaine group and the G-CSF group canonical pathways involved in the G-CSF w/ cocaine group also show a predicted increase in mTOR signaling.

Figure 7: PPI network diagram for all proteins significantly regulated between GC and cocaine-treated mice. Each node represents a protein. Node size represents p-value from DIA mass spectrometry analysis. Node color represents FC over cocaine-treated mice. Darker hues of red indicate higher predicted upregulation of proteins, and darker hues of blue indicate higher predicted downregulation. Edges connecting nodes indicate protein-protein interactions, based on STRING databases for Mus musculus.

To further investigate these predictions, we queried the proteins that were differentially regulated in our GC vs cocaine comparison group against the STRING database of protein-protein interactions (PPI) to characterize the interplay of these proteins in causing the predicted pathway analyses. STRING PPI results were visualized using the Cytoscape software to create a network diagram (Fig 7). Enrichment data, including PANTHER gene ontologies and KEGG pathways were loaded into Cytoscape from the STRING database for comparison to IPA pathway analyses. The tightness of node clusters indicates the level of interaction. Enrichment

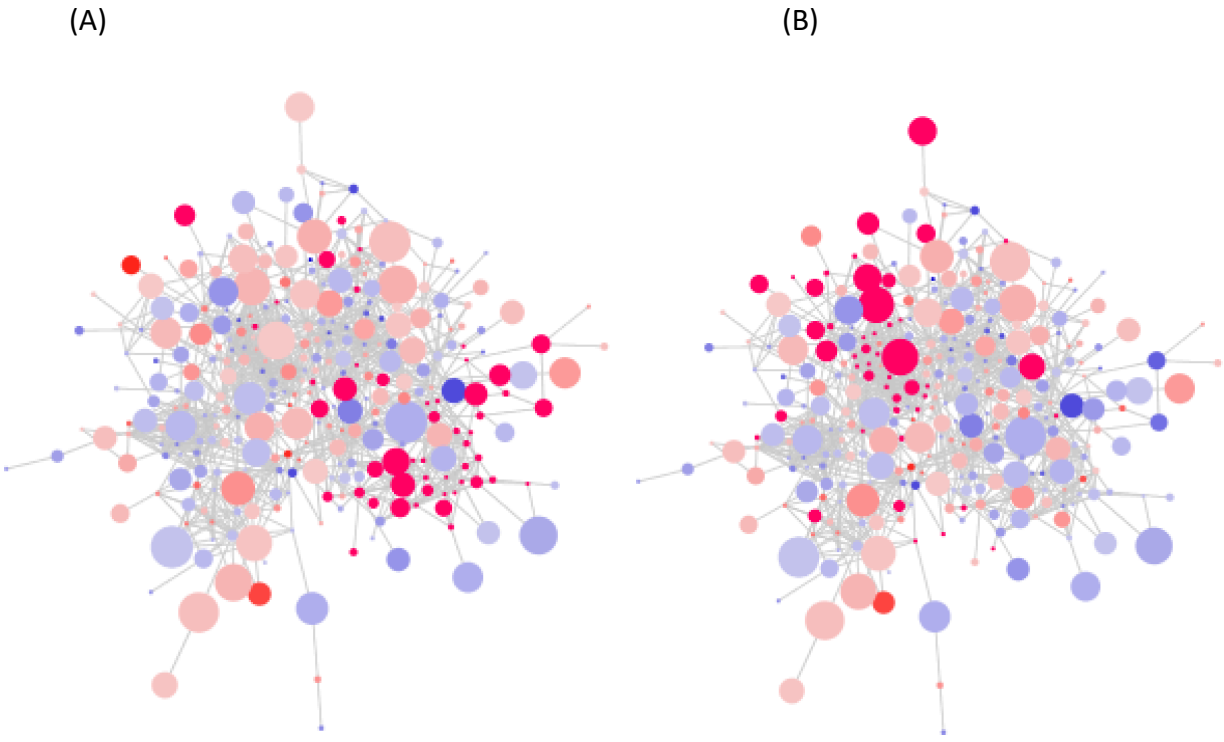


Figure 8: Enrichment data overlays for GC vs cocaine network diagrams. Node size and color represent the same information as in Figure 7. **(A)** The cluster of dark red nodes are proteins predicted by PANTHER databases to be involved in vesicle-mediated transport. **(B)** This cluster of dark red nodes is predicted by PANTHER databases to be involved in poly(A) RNA binding.

data overlays for poly(A) RNA binding and vesicle-mediated transport indicate clusters of tightly connected proteins with a high level of predicted and experimentally-determined interaction (Fig. 8).

3.4 Protein Validation

Due to the relatively small sample sizes of our treatment groups (n = 5-8 mice per group), as well as the large number of identified proteins, we performed Western blot confirmations for three protein targets of interest predicted by IPA to be : mTOR, PSD-95, and GluA1. mTOR, the mammalian target or rapamycin, has been predicted in previous proteomic

analyses of G-CSF function in the striatum to be differentially regulated by G-CSF administration (Mervosh et al., 2018). mTOR is also involved in producing locomotor sensitization in response to cocaine administration through activation by D1 receptors (Sutton & Caron, 2015). PSD-95 (post-synaptic density protein 95) is an anchoring protein mostly expressed at the post-synaptic terminal and is responsible for anchoring glutamate receptors (both AMPA and NMDA receptors) as well as their associated ion channels and messenger molecules to the cell membrane. GluA1 is one isoform of the AMPA receptor, and GluA1 receptors in the NAc are responsible for receiving glutamatergic input from the mPFC and are crucial for regulating glutamatergic homeostasis (Quintero, 2013). After running Western blots for these targets, we quantified the protein concentrations by measuring fluorescence of bands using ImageJ software.

Analysis of total mTOR, PSD-95, and GluA1 levels did not show any significant changes by treatment group. Two-way ANOVA showed no significant effect of G-CSF, cocaine, or a G-CSF x cocaine interaction (Fig 9). Since mTOR is involved in neuronal growth and plasticity (Jaworski & Sheng, 2006) and PSD-95 and GluA1 are membrane-associated proteins, regulation of expression of these proteins may be a slow process. Therefore, we compared the GC and cocaine mice that were euthanized 24 hours after their last injection to mice who underwent the same treatment protocols but were euthanized 1 hour after their last injection in order to analyze the potential effect of time on protein expression levels. Mixed-effects analysis showed no significant effect of time for either the GC or cocaine groups on total mTOR levels. There was a main effect of time on GluA1 ($p = 0.0176$) and PSD-95 ($p = 0.0048$) for our GC group (Fig. 10). There was no significant effect of treatment or treatment x time on these protein levels. This

shows that levels of PSD-95 and GluA1 are increased significantly in mice that were administered G-CSF w/ cocaine between the 1 hour and 24 hour time mark.

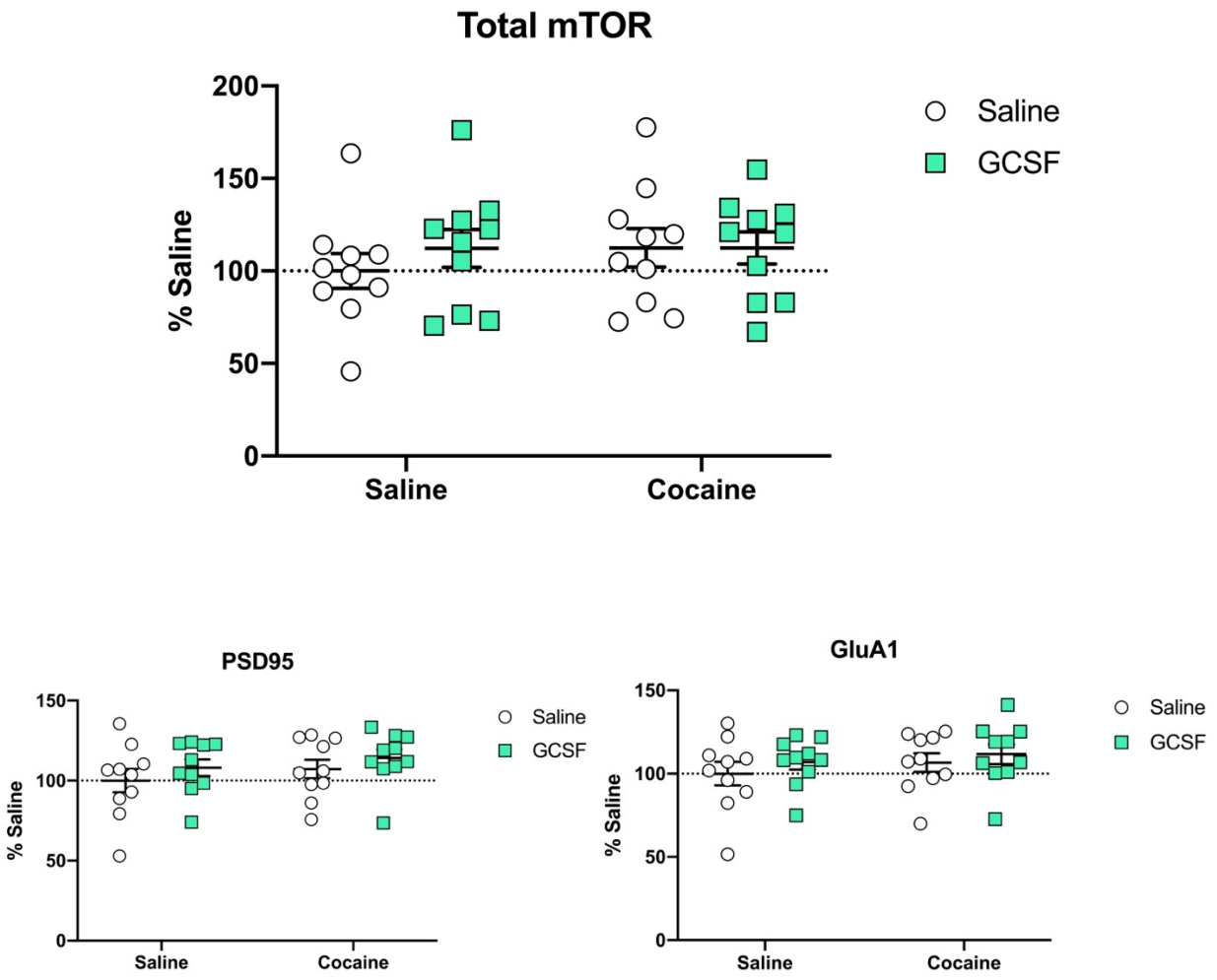


Figure 9: Western blot quantification of protein targets of interest. Each point represents the mice in each treatment group. Protein levels were determined through analysis of fluorescence and compared as a percentage to the mean protein expression of saline controls. Mean expression for saline controls is indicated by the dashed line. There is no main effect of cocaine, G-CSF, or an interaction between the two treatments on protein expression levels.

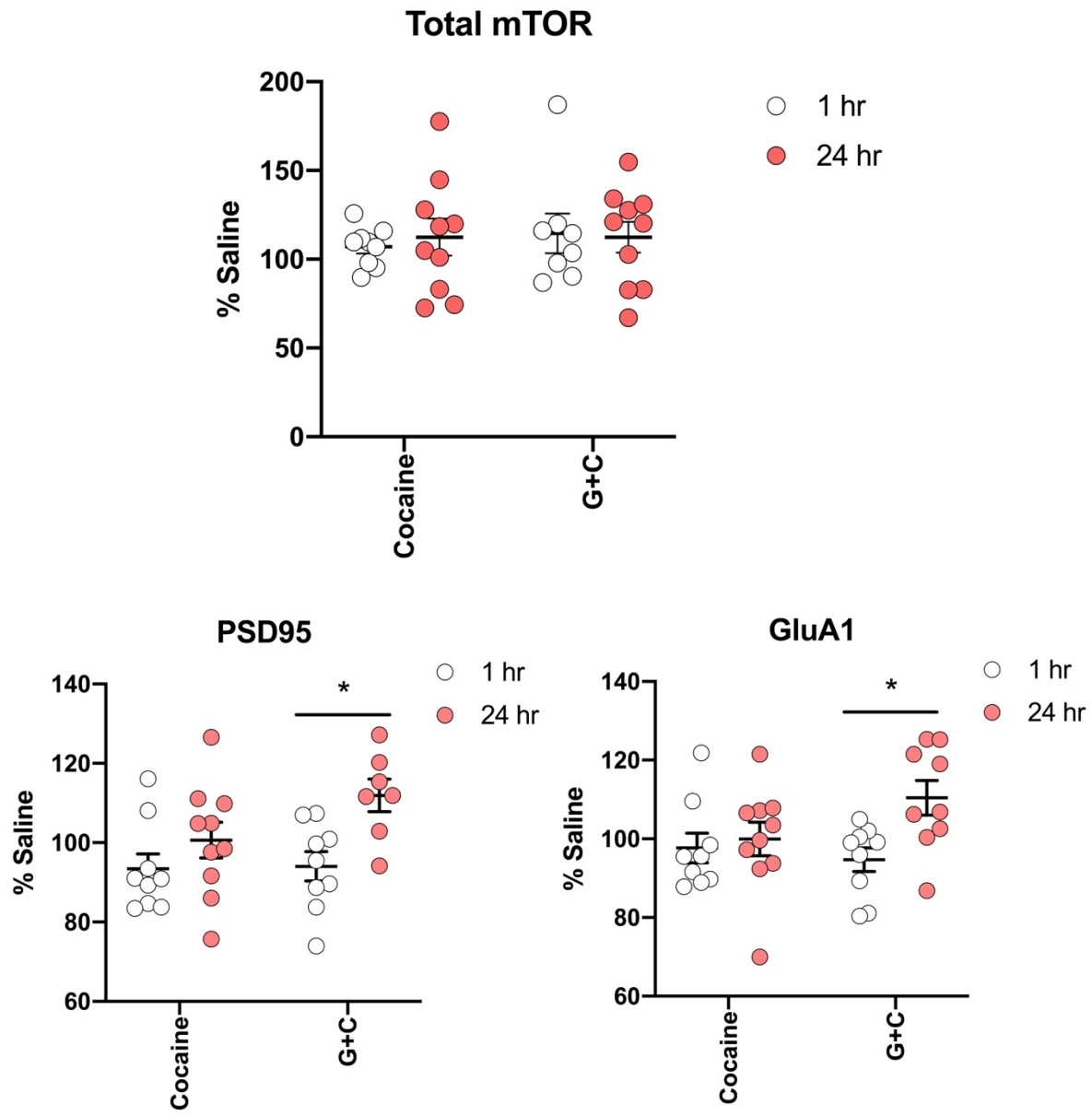


Figure 10: Western blot quantification comparisons of time effects on protein expression levels. White dots represent mice from the 1 hour post-treatment euthanasia group. Red dots indicate mice from the 24 hour post-treatment euthanasia group. mTOR levels are not significantly affected by time, treatment, or a time x treatment interaction. There is a main effect of time on PSD-95 ($p = 0.0048$) and GluA1 ($p = 0.0176$) expression levels.

4. Discussion

Our lab recently identified G-CSF as a regulator or behavioral and neurological response to cocaine administration (Calipari et al., 2018). This study utilized an unbiased proteomics

analysis with the purpose of identifying differentially expressed proteins in the NAc in response to G-CSF treatment, cocaine treatment, or a combination of the both. G-CSF has been shown to cause changes in protein expression in the VTA (Mervosh et al., 2018), while also potentiating the release of dopamine from the VTA to the NAc during cocaine administration (Kutlu et al. 2018). Therefore, in order to understand the behavioral and neuroplastic changes that occur during G-CSF administration, both alone and in conjunction with cocaine administration, it is important to characterize the proteomic landscape of the NAc. Analysis of the proteomic changes in the NAc can elucidate further, more mechanistic studies into specific regulators of protein expression.

This study highlighted proteins and intracellular signaling cascades involving changes in neuronal morphology, post-synaptic formation and mRNA functioning may be modulated by G-CSF w/ cocaine administration. This is important, since numerous previous studies have demonstrated that changes in synaptic density and neuronal morphology are induced by cocaine and are important in the formation of substance use disorders (Hofford, 2018). This suggests that G-CSF may cause changes in synaptic plasticity that primes animals for changes in reward learning and memory for cocaine. Since recent research from Calipari et al. (2018) and Mervosh et al. (2018) characterized the molecular and proteomic changes caused by G-CSF and cocaine independently, this study focused on the interaction between G-CSF and cocaine treatment. Initial proteomic analysis of DIA mass spectrometry data showed the largest number of differentially expressed proteins in our combinatory GC treatment group. Therefore, we hypothesized that since G-CSF potentiates the behavioral and neuronal response to cocaine, the interaction of the two molecules may cause characteristically different changes in

proteomic expression compared to G-CSF administration by itself. We decided, based on this hypothesis, to focus most heavily on the effects of the combination of those two treatments together.

This study is a great starting place for more mechanistic analyses of G-CSF action in potentiating the behavioral and molecular changes causes by cocaine administration. Even though self-administration paradigms are used more often when modelling the behavioral phenotypes of substance use disorders, we used an experimenter-administered paradigm for this study. We believe this is the best method for analyzing the cellular and molecular changes observed in our treatment groups, since behavior was not a focus. However, repeating this study using a conditioned place preference self-administration design may show different changes in proteomic expression in the NAc that could be useful if interpreted in coordination with the findings of this study and previous studies.

There are also a few potential caveats to the results of these experiments. We utilized a 2 x 2 experimental design for the purpose of discovery of protein regulation in the NAc. While the results provide a good starting place for future studies in characterizing the effects of G-CSF w/ cocaine treatment, we still utilized uncorrected p-values from the DIA mass spectrometry analysis. This leads to a higher likelihood that some of our results are indeed false positives and some of the transient changes in protein expression may not be indicative of the mechanisms of action of G-CSF and cocaine. Additionally, while IPA and STRING analyses provide a good general idea of what pathways and upstream regulators may be involved in the changes in observed proteomic expression, these databases are not based entirely on experimentally-determined molecular interactions. Instead, to provide a broader approach for discovery, these

databases are built on large cross-sections of experimentally-defined interactions as well as predicted interactions from previous research. Additionally, these databases often pool data from multiple tissues in the brain and body of mice, which means results of IPA and STRING queries may not entirely be applicable to the NAc since different tissues and brain regions express different levels of different proteins. The data derived from these queries may be useful as a general prediction of mechanistic action, but regulation of intracellular pathways from other tissues than the NAc may lead to premature conclusions on the mechanisms of action.

The results from our Western blot quantifications do not indicate that some targets of interest such as mTOR, PSD-95 and GluA1 are actually differentially regulated in response to G-CSF or cocaine administration. There are a few reasons why this may be so. Firstly, as previously mentioned, pathway analyses are useful but do not conclusively implicate regulators of protein interaction due to their high level of false discovery rates and cross-sectional approach that utilizes data from multiple brain tissues. So even though IPA and STRING predicted that these proteins may be involve in the changes in intracellular signaling due to our treatments, this may not be true. Secondly, the targets of interest that we explored are all involved in changes to mRNA function and synaptic changes, which may have slower mechanisms of action that take place over a longer period of time than the 24 hours following the last treatment. The results of our Western blot quantification analysis of time suggest that this may in fact be why we do not see significant changes in these proteins. Longer periods of abstinence from cocaine administration have been previously shown to incubate cocaine craving and cause longer-lasting changes to neuronal signaling and plasticity in the striatum (Hofford, 2018). The

mechanisms of action of G-CSF, both alone and in combination with cocaine administration, may have similar long-term effects that are not observable at 24 hours post-treatment. We believe that more studies of this kind that focus on longer periods of abstinence may show increased regulation of our proteins of interest.

The best next step to more accurately characterize potential changes in synaptic and mRNA function would be to employ a targeted, biased approach that analyzes only the nucleus and synapse. This is achievable by lysing and sonicating NAc neurons to separate the synapse and nucleus from the rest of the cellular components and analyzing those individually. This would allow for low-noise analysis of nuclear transcriptomics and synaptic morphology following G-CSF w/ cocaine administration. Further research in this manner would provide a more accurate, full-picture analysis of the mechanisms of action of G-CSF w/ cocaine in the striatum and NAc in particular.

In summary, our lab has identified G-CSF as a neuroimmune regulator of behavioral and neuronal response to cocaine administration. The original Calipari et al. (2018) study showed that G-CSF is a promising therapeutic target for manipulation in order to treat cocaine craving and dependence. However, much remains to discover about the molecular mechanisms of action of G-CSF in regulating the response to cocaine administration. This study, along with the Mervosh et al. (2018) study, help to characterize the changes in proteomic expression in the VTA and NAc following G-CSF and/or cocaine administration. Here, we employ an unbiased proteomic analysis of the NAc of mice treated with either or both of these molecules. We identified potential key regulators of molecular action of G-CSF w/ cocaine in the striatum that lays the groundwork for further, more mechanistic studies in this field.

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