1. BACKGROUND AND SIGNIFICANCE

1.1 Background:

The use of drugs to achieve an altered mental state has existed for millions of years [10]. Although humans have used substances for such an extended period of time, science still lacks a complete understanding of the mechanisms behind drug abuse. The field of study associated with drug addiction has primarily focused on dopaminergic and serotonergic pathways in the brain, which are proved to be highly linked with reinforcement and reward systems [10, 11]. These studies have focused on finding specific genes of interest involved in the pathways targeted by drugs, but most gene sets produced by them have been broad, generalized gene sets. There has been little exploration into the theory that genes exist in discrete subsets highly correlated with abuse of an individual substance, rather than being part of a more generalized addiction gene structure [3]. To address this**, the broad goal of our project is to determine if there exist sets of highly co-expressed genes that are localized to certain brain structures and specific to particular drugs of abuse.** We will do this by investigating co-expression profiles of substance use disorder (SUD) related genes and their location of expression in the brain.

Because of the highly variable phenotypic expression of genotypes, along with the fact that one's chance of addiction is also influenced by environmental interactions on gene expression (epigenetics) and heritability, there are too many variables to compile a list of genes guaranteed to increase susceptibility to addiction. However, by using gene sets from various studies attained through GeneWeaver and Brainarchitecutre [26, 27], two cross species gene set integration platforms, we are able to generate more targeted gene sets. We compiled a set of general addiction genes (n=418) and subsets of more specialized genes linked with the pathways targeted by specific drugs of abuse, such as methamphetamines, opiates and cannabinoids. These gene sets (ranging from n=22 to n=3) are genes upregulated during use of the drug or are receptors and transports along the targeted pathway. These sets will be compared and analyzed with the data collected and curated by the Allen Brain Institute, which will allow us to delve deeply into the genetic variations implicated in increased susceptibility to addiction.

The reason we will compare our data sets to data within the Allen Brain Institute is due to the fact that the atlas allows us to study gene expression over a standardized three-dimensional brain. This brain was produced by compiling data from thousands of in situ hybridization experiments into one, averaged, mouse brain. The brain was then spliced into voxels, further categorized into non-overlapping regions and subregions with various levels of granularity [1]. This format gives us the ability to compare expression of addiction related genes with gene-expression throughout the entirety of the brain. Furthermore, we can see what regions (or subregions) show high co-expression with our substance-specific gene sets. This, in conjuncture with the variable annotations schemes enables for very fine granularity for defining physical regions within the brain.

1.2 Significance

In 1992, the estimated total economic cost of drug abuse in the United States by the National Institute on Drug Abuse and the National Institute on Alcohol Abuse and Alcoholism was $97.7 billion [10]. More recently, the annual spending by the US government against the proliferation of drugs hovers around $51 billion [12]. This estimate, however, doesn't include crime costs, direct healthcare costs, and loss of worker productivity, which add up to over $600 billion each year [13]. Together, **current drug addiction and substance abuse spending in the United States is over $650 billion every year, which is an increase of 665% from 1992**.

Substance abuse is not only fiscally costly, but takes a huge toll on the abuser's health. Addiction can increase susceptibility to cardiovascular disease, stroke, cancer, mental disorders, and a variety of other illnesses [14]. Addictions are particularly harmful due to the fact that they can negatively impact not only the user, but those around them as well. The physical and mental health of individuals coupled with the high economic toll of substance abuse is driving research on addiction. By ascertaining whether or not addiction is particular to each independent drug of abuse we will be able to shift our current approaches to treatment of addiction. If drug abuse is related to general addiction genes and reward mechanisms, we can broaden drugs to target all pathways evenly. If addiction is specific to the drug of abuse, we will be able to tailor treatments to specific pathways, contributing to more effective and efficient recoveries. In this manner, we can limit the economic and social losses caused by frequent drug use, and re-assimilate people suffering from addiction back into the community and the work force.

2. SPECIFIC AIMS:

The current genetic model of addiction is still developing, however it is being developed from a set perspective – that addiction is a uniform concept independent of the abused substance. This view implies that addiction is composed of a set of genes that increase susceptibility to substance abuse, regardless of the substance. This is demonstrated in part a) of the schematic to the right. Our proposed hypothesis, that there **exists sets of highly co-expressed genes that are localized to certain brain structures and specific to particular drugs of abuse,** is demonstrated in part b) of the schematic – subsets of addiction genes that are independent of general addiction.

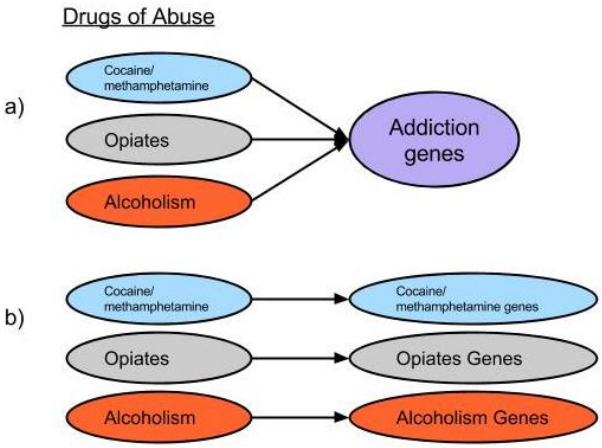


Figure 1. Schematic of the hypothesis being tested. See text.

2.1 Specific Aim 1:

To determine whether there exists specific subsets of genetic modifications that correspond to different types of addiction, different drugs of abuse, or a combination of both. The **first step** to achieving this aim is to compile a list of genes associated with drug addiction as well as subsets of genes associated with addiction to individual drugs. We will use these delineations as a first approximation of potential clique formations. **Second**, we will use the previously developed MatLab toolbox, Brain Gene Expression Analysis (BGEA), to carry out gene co-expression analysis. This will include Monte Carlo simulations and calculating cumulative distribution functions of co-expression coefficients (as outlined in detail by Grange et al [4, 6]) to determine if our addiction genes hold a significant level of co-expression that is greater than expected by chance.

2.2 Specific Aim 2:

To determine whether or not there are discrete regions of the brain that have unique gene expression profiles highly co-expressed with mechanisms specific to one drug of abuse. To do this, we will sort our genes into small groups that we call cliques, based on fitting scores drawn from graph theory, calculated using the BGEA toolbox [4,6]. These scores tell us to what extent a

specific clique of genes aligns with regional expression in particular areas of the brain [4]. The **main task** is to use these, in conjunction with the results from the analyses used above in our first specific aim, to determine if the regions defined by our gene clusters overlap with traditional neuroanatomy and current understanding of where addiction physically resides in the brain. This will allow us to confirm known regions of the brain implicated in drug addiction, such as the striatum [2], and second, to encounter new regions that could influence susceptibility to drug abuse that have not yet been uncovered.

2.3 Health relevance:

To the immediate user, recreational drugs have been shown to be highly detrimental to health, and the deficiencies they cause have been well documented and studied. These health complications depend on the drug of abuse, but can range from slight attention deficits to lung cancer, heart problems, seizures, and death. Tobacco use killed around 100 million people during the 1900's and is projected to kill around 1 billion in this coming century if the current, abundant usage continues [14]. Alcohol damages parts of the cerebral cortex and hippocampus which are vital in decision-making and the consolidation of memory, respectively [14, 17, 18]. Methamphetamines are highly addictive, causing users to binge use the drug for extended periods of time, generally on the order of several days [14], which not only decreases their health, but also their productivity as a worker in society. It is known that cocaine use during pregnancy impairs uterine growth of the fetus and causes deficits in the cognitive and information processing performance of the offspring [14]. All of these are just a few of the examples of the ways in which disorders cost our society; monetarily through hospital and rehab facility admissions, economically through loss of productivity, and societally by lessening future generations' mental and cognitive preparedness.

Obtaining a full understanding of SUD is vital because it is prolific in our society. According to the Substance Abuse and Mental Health Services Administration (SAMHSA), 24.6 million persons over the age of 12 were currently using illicit drugs. Furthermore, 1.4 percent of adolescents were diagnosed with join substance use disorder and major depressive episodes [19]. This indicates that addiction and substance abuse are likely linked strongly with mental illness. This hypothesis is also supported by the fact that "3.2 percent of adults had co-occurring AMI [any mental illness] and SUD [substance use disorder]." Thus, in addition to the costs of direct substance abuse and addiction described in the preceding paragraph, the effects of these disorders are much farther reaching. They influence mental health and stability as well, generating more losses to both the individual and the society in which they reside.

3. PRELIMINARY DATA

3.1 Construction of addiction-related genes datasets

To begin our study of drug-specific, addiction related genes, we needed to identify and compile a list of genes associated with drug addiction, with emphasis on genes implicated in particular substance use disorders. To accomplish this task, we took advantage of a searchable, web-based gene database generated as part of the brainarchitecture project initiated by the Cold Spring Harbor Laboratory [27]. Although we were able to identify over 800 addiction related genes, we restricted our research to a subset of the Allen Genes Expression Atlas (AGEA) to exclude genes with low transcription levels and retain genes that meet certain statistical criteria. Thus, only 418 mouse genes were available for study in the subset of AGEA, which we demarcated as our comprehensive addiction-related genes.

In our preliminary investigation, we wanted to extract meaningful substructure from this dataset pertaining to drug-specific addictions through two converging lines of analysis: (1) studying co-expression patterns within this broad addiction gene dataset to identify gene cliques and (2) comparing these gene cliques to select subsets of genes identified by the literature as drug-specific.

We employed GeneWeaver, another web-based gene set database and integration platform to form the drug-specific subsets [26]. The advantage to using GeneWeaver is that it allowed us to combine cross-species data and evaluate the intersection of a collection of gene sets produced by numerous experiments. After collecting these gene subsets and isolating the genes available to study in AGEA, we had genes sets specific to marijuana, methamphetamines, morphine and cocaine (Table 1).

Table 1. Drug Specific gene sets generated by Gene Weaver, a web-based, cross-species gene set database and integration platform.

GS185035		GS122980		GS33944		GS87486	
Marijuana		Methamphetamines		Morphine		Cocaine	
Name	Entrez ID	Name	Entrez ID	Name	Entrez ID	Name	Entrez ID
Cnr1	197	Prdx5	1333	Calm1	115	Ctnnb1	130
Mgll	1147	Dlst	2000	Dlk1	267	Neurod1	677
Abhd6	1506	Maoa	623	Pcnt	738	Ascl1	625
		Gad2	374	Papola	783	Mxi1	663
		Got2	425	Ltbp3	612	Og9x	721
		Ctnnb1	130	Synj2	1017	Pbx3	737
		Tk2	1416	Rgs6	1251	Chgb	171
		Aldoc	33	Syncrip	1369	Dpysl3	1097
		Per2	761	Csrp2bp	2535	Fdft1	332
		Gls	409	Pogk	1789	Pole4	1575
		Clic4	1231	Senp2	1935	Camk1g	2402
		Bdnf	89	Pnpo	2132	Adrbk2	2839
		Ptgs2	830	Ebf4	2533	Esrra	1167
		Arc	54	Ogfod1	2796	Ramp3	1346
		Th	1046	Rims3	2703	Asb13	2291
		Rgs4	882			Serpini1	990
		Cs	228			Amot	1214
		Acsl5	2958				
		Mif	643				
		Mrpl12	1355				
		Cnr1	197				

3.2.a. Cumulative distribution functions (CDFs) of co-expression coefficients of addiction related genes

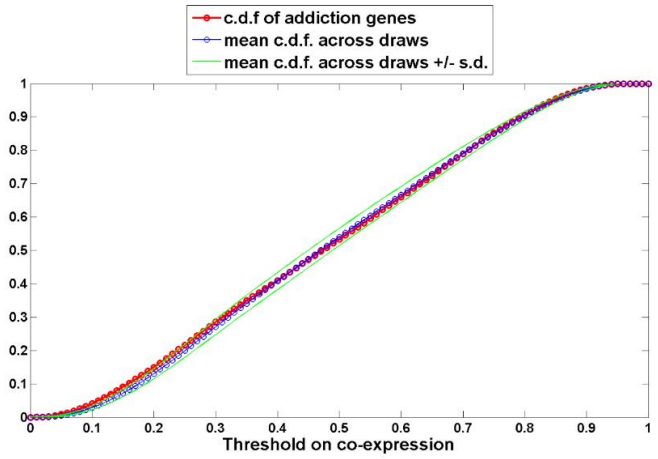


Figure 2. Cumulative distribution functions for 1000 random draws from AGEA and our addiction gene subset. The CDF is defined as the fraction of co-expression coefficients smaller than a given number between 0 and 1. A two sample Kolmogorov-Smirnov test revealed that both CDFs were drawn from the same probability distribution

We began our investigation of co-expression profiles by examining the cumulative distribution function of our comprehensive set of addiction related genes. First, it was necessary that we compute a gene-by-gene, atlas-wide co-expression matrix, which for any two given genes is defined as the cosine similarity between the gene-expression profiles with values ranging from zero to one by construction [4]. This matrix is symmetric and ones populate the diagonal. Based on the indices of our addiction related genes in AGEA, we extracted a special co-expression submatrix of our addiction related genes and investigated its properties.

We now asked whether our addiction genes were more co-expressed than other

genes expected by chance. Here we employed Monte Carlo simulations, randomly drawing sets of genes of the same size as our addiction set from the atlas-wide co-expression matrix to generate a mean CDF. With this mean CDF, we made qualitative and quantitative comparisons to the addiction genes CDF. We found that the addiction genes CDF did not deviate significantly from the mean CDF. We speculated that this result could potentially stem from two reasons: (1) the fact that addiction is mostly involved in diffuse, generalized systems of the brain such as the dopaminergic system, or (2) that our addiction gene data set, because it contains approximately one sixth of genes in the AGEA, overshadowed underlying significant co-expression. To explore this second theory and to encounter any significant deviations, we needed more specific gene subsets that are comprised of very highly co-expressed genes that meet a high threshold requirement. To achieve this, we changed our approach and decided to run CDFs for our substance specific gene sets.

3.2.b. CDF coefficients of subsets of specific drug related addiction genes reveal interesting co-expression properties.

Through the same processes outlined in 3.2.a above, we again calculated the CDF coefficients for addiction related genes; however, we restricted our genes of interest to a subset of our addiction related genes specifically associated with methamphetamine addiction. For highly co-expressed genes, we would expect growth of CDF to aggregate at higher values of the argument. Indeed, we began to observe this behavior with methamphetamine genes. Comparing the distribution of co-expression coefficients of methamphetamine genes with the other genes of the AGEA revealed that the largest deviation between these distributions was 26.7 % at a threshold of co-expression of 0.64 (Fig. 3). This deviation indicates that, with refinement of the gene set, our methamphetamine genes could be pulled from a different distribution than our general addiction gene set, hinting toward a possibility for specialization of addiction genes to a particular substance of abuse.

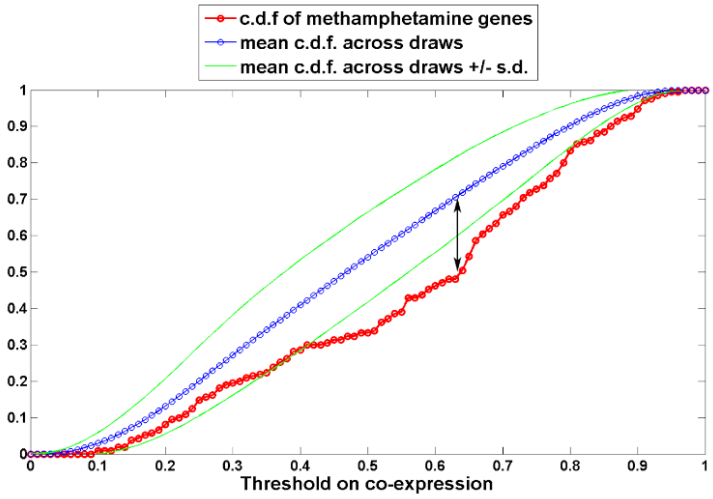
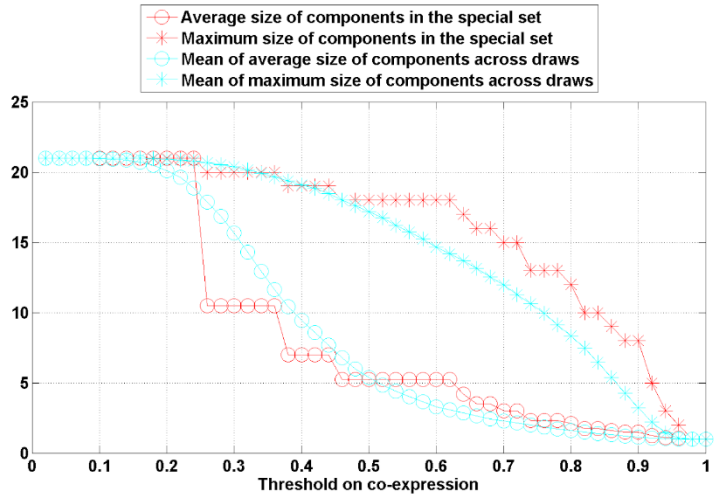


Figure 3. Cumulative distribution functions for 1000 random draws from ABA gene set and our specific methamphetamine gene subset. The black arrow indicates the maximal difference (26.7%) between the two CDFs.



3.3 Connected components/gene clique statistics

Next, we took a systematic approach in identifying interesting statistical properties of our addiction genes dataset by studying the weighted gene network graph underlying its co-expression matrix. In this

Figure 4. Average and maximum sizes of connected components as measured at different co-expression thresholds are shown for the addiction gene network (Red) and 1000 randomly generated gene networks containing 418 genes (Blue).

graph theory formalism, each node represents an addiction gene and the length of an edge connecting any two genes signifies their co-expression. We then applied a thresholding procedure to isolate connected components, which are subsets of genes whose pairwise co-expression exceeded a given criteria [5, 6]. By incrementally increasing our co-expression threshold and computing the sizes of the gene cliques, we studied certain statistics of our addiction gene dataset, such as average and maximum size of the connected components. Again, for comparison, we relied on Monte Carlo simulations to generate random co-expression gene networks of the same size.

Evaluation of addiction gene connectivity across a range of co-expression thresholds revealed that the maximum size of connected components was consistently larger than the mean maximum size of components across 100 draws (Fig. 4). This would suggest that there are subsets of highly co-expressed genes (> 0.5) in our addiction gene set that contain more genes than expected by chance, indicating the possibility for specification of gene cliques amongst the larger, addictions gene set. These specifications could either be pathway specific or drug specific, and will require further biological and computational analysis to discriminate between the two.

3.4 Neuroanatomical properties of gene cliques

Lastly, as part of our preliminary investigation, we aimed to isolate specific genes cliques exhibiting the highest degree of co-expression and study their locations within the brain. Using a procedure similar to the one described above, we isolated and examined gene cliques characterized by addiction-related genes connected with co-expression values above 0.5. We identified a total of 23 overlapping gene cliques containing, on average, 199.52 genes.

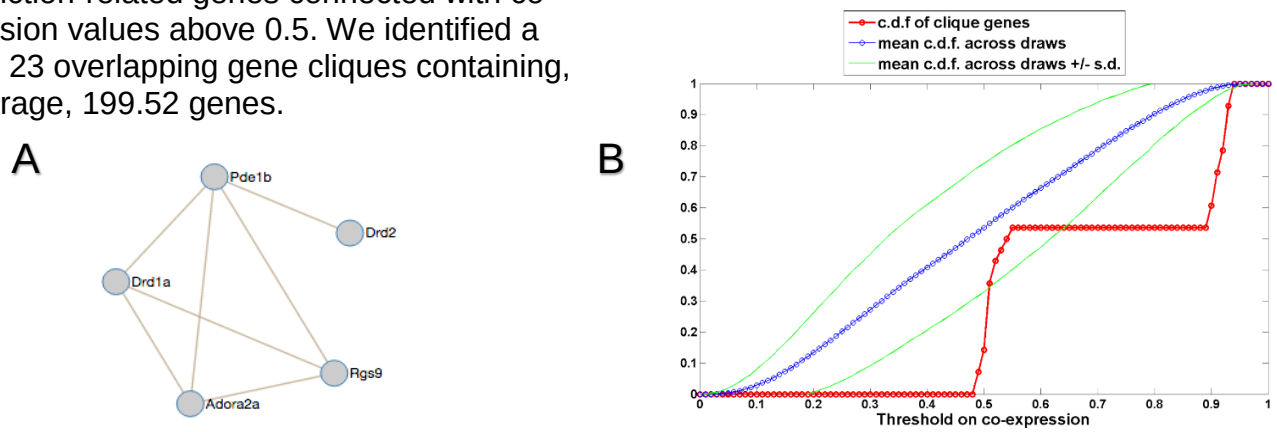
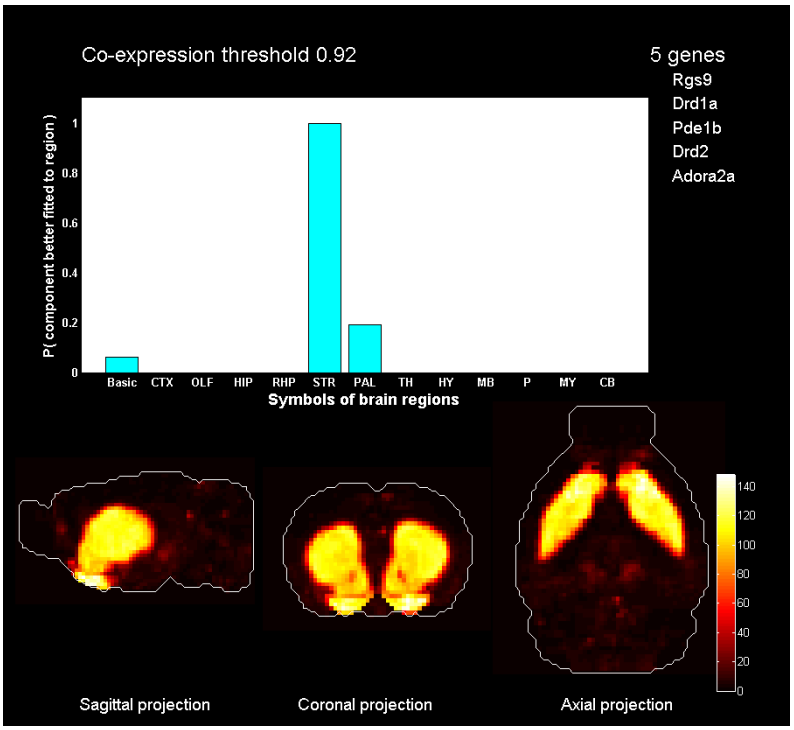


Figure 5. Identity and properties of top ranked gene clique. (A) Graph of co-expression connectivity. (B) Cumulative distribution functions for 1000 random draws and our gene clique. A two sample Kolmogorov-Smirnov test revealed that the CDFs were drawn from different probability distributions (p = 1.0295x10^{-10}).

Our analysis revealed that the top ranked gene clique maintained a co-expression threshold of 0.92 and contained the addiction related genes: *Rgs9, Drd1a, Pde1b, Drd2* and *Adora2a*, whose functions are described in table 2. Neuroanatomical investigation of these genes across 12 brain areas delineated by the Allen Reference Atlas' 'big 12' annotation scheme indicated significant overexpression of this

Table 2. Gene functions of top-ranked gene clique from the addiction gene

Gene	Function
Rgs9	Regulates dopamine and opioid signalling in the basal ganglia
Drd1a	Encodes the D1 subtype of the dopamine receptor which stimulates adenylyl cyclase and activates cyclic AMP-dependent protein kinases
Drd2	Encodes the D2 subtype of the dopamine receptor which inhibits adenylyl cyclase activity
Pde1b	Protein encoded by this gene belongs to the cyclic nucleotide phosphodiesterase
Adora2a	G protein-coupled adenosine receptor (A2A subtype), which uses adenosine as preferred endogenous agonist to increase intracellular cAMP levels



gene clique in the striatum, and to a lesser extent the pallidum (Fig. 6). These initial findings are consistent with previous research, which has implicated these genes in key components of the reward-signaling pathways [10, 11]. This indicates that all addiction genes and pathways do in fact converge at the striatum and pallidum. This calls for further research to uncover if there are discrete sub regions in these brain structures that house unique expression profiles highly correlated to the mechanisms of individual drugs of abuse. We will use PCA and clustering analyses based on the co-expression profiles of our subsets of addiction specific gets to determine these regions.

Figure 6. Expression and neuroanatomical properties of top ranked gene clique (co-expression threshold of 0.92). (A) Fitting score of gene clique is highest in striatal and pallidal regions. (B) Maximal-intensity projections of gene cliques

4. FUTURE DIRECTIONS

Addiction has been rooted in human societies for millennia, ranging from use of mild drugs to intensely mind-altering, hallucinogenic substances. The current foundations of addiction-based research are focused on profuse pathways in the brain such as the dopaminergic and serotonergic systems. Although this may explain segments of general addiction, it amalgamates addiction to each drug despite the fact that they affect the brain in fundamentally different ways. Methamphetamine and cocaine work by similar mechanisms; binding to dopamine transporters, blocking reuptake channels, and limiting degradation or monoamines, which all directly lead to increased dopamine levels [20]. Cannabis, however, does not target the dopaminergic system at all, but rather the specific cannabinoid receptors [21]. Similar to cannabis, opium has not been shown to target or influence the dopaminergic pathway in any way, but has its own, unique neurological and synaptic effects [22, 23]. These dissimilar physiological interactions indicate that general addiction cannot be explained solely by researching dopamine pathways and reward systems, because it can be grounded in a wide array of differing receptors and signaling projections - addiction lies in more complicated pathways than general reinforcement and reward. Once we reach the conclusion of our specific Aims (described above in section 2), we will be able to gain a more comprehensive insight into pathways of addiction and their genomic and neuroanatomical characteristics and assign segments of them to individual substances of abuse. This will allow for a revolutionary change of perspective on how drugs of abuse interact within our brain, and help future researchers tailor studies to determine ways to **mitigate addiction**, **aid recovery** using gene therapy, and develop more **effective rehabilitation programs**.

After achieving our specific aims through computational analysis, further research will be focused on confirming the conclusions we obtained in animal models. One way in which we plan to do this is using DNA microarray analysis for both control and addicted mouse phenotypes. Within the addicted mouse phenotype, there will be subgroups addicted to only one particular drug of abuse, and an extra, general addiction control group as well, addicted to multiple drugs of abuse. This will

allow us to determine whether or not the differing gene expression we predict to see in specific regions in mice addicted to different drugs of abuse is in fact expressed in vivo. If we find alternate gene expressions or new candidate genes, we will use this data to tailor our gene sets for re-analysis.

Substance use disorder affects millions of individuals and their families stemming from all walks of life and is profoundly damaging to all layers of society. Our research is vital to fining a more efficient and effective way to help these individuals recover and reintegrate into society. Gaining the ability to understand the general model behind addiction will give us the basis needed to help those struggling with SUD. Furthermore, once the mechanisms behind addiction are established, novel research can begin on new gene therapies, more specific diagnoses, and rehabilitation programs that target root causes rather than symptoms. Moreover, current determinations of susceptibility are loosely based on heritability and familial medical history. Knowing the specific genotypes that increase susceptibility to addiction (or even susceptibility to specific drugs) will give us the ability to more concretely ascertain personal risk. In these ways, our research will lead to savings of hundreds of billions of dollars yearly in the form of decreased medical costs, increased worker productivity, increased happiness, and increased health of the general population.

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