APPLYING A CANDIDATE GENE APPROACH TO INVESTIGATE THE MOLECULAR
CHANGES THAT ACCOMPANY THE DEVELOPMENT OF ADDICTION-LIKE
BEHAVIORS TO OPIOIDS IN RATS

A Dissertation in
Pharmacology
by
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ABSTRACT

Opioid addiction is a devastating chronic disorder characterized by compulsive drug seeking and a loss of control over drug use. Activation of the mu-opioid receptor (MOR) is critical for mediating the rewarding and addictive properties of opioids. Recent research suggests that MOR signaling is modulated by proteins that interact with MOR to form multiprotein signalplexes. The goal of my thesis research is to understand how MOR interacting proteins contribute to the molecular adaptations induced by opioid exposure and addiction. To achieve this goal, I developed a rodent model for "addiction-like" behaviors for heroin that serves as a platform for investigating potential underlying neural substrates involved in the development of opioid addiction. My central hypothesis is that the transition from opioid use to opioid addiction is accompanied by differential regulation of MOR interacting proteins.

Criteria for "addiction-like" behaviors in rats have been established for cocaine (Deroche-Gamonet et. al., 2004), and I evaluated whether similar behaviors can be observed in rats following the intake of an opioid drug. I used a rat model of heroin self-administration that included key features of human addiction. Thus, responding for heroin was examined in 43 male Sprague-Dawley rats given the opportunity to intravenously self-administer heroin for 27 sessions. Criteria for addiction-like behaviors included greater drug seeking during periods of signaled non-availability, an increased willingness to work for drug when tested with a progressive ratio schedule of reinforcement, and greater lack of satisfaction and increased drug-seeking during timeout periods. My study revealed that although all rats self-administered approximately the same amount of heroin, only 9.3% of rats met all criteria for "addiction-like" behaviors. Additionally, behavior during the initial timeout and signaled non-availability periods
(onset activity) was found to be an early predictor of the severity of "addiction-like" behavior that a rat would develop by the end of the study.

To gain an understanding of the molecular changes that accompany the development of behavioral criteria for "addiction-like" behavior, I analyzed the protein expression of known MOR interacting proteins and the D_{2} dopamine receptor. Western blotting was used to assess protein expression in the prefrontal cortex, hippocampus, and nucleus accumbens from 14 rats from the heroin self-administration study. I found a differential expression of the D_{2} dopamine receptor, spinophilin, and wntless that correlated with behavioral evidence of "addiction-like behaviors." These changes in protein expression may reflect molecular correlates of the development of opioid addiction.

Using a modified membrane yeast two-hybrid screen, our laboratory recently identified VAMP-associated protein A (VAPA) as a candidate MOR interacting protein. VAPA is highly expressed in the endoplasmic reticulum and Golgi apparatus and functions in vesicular docking and exocytosis. In this dissertation, I confirmed that VAPA is a bona fide MOR interacting protein in vivo using co-immunoprecipitation and immunoelectron microscopy. To determine if VAPA plays a role in the transition to opioid dependence, I examined VAPA expression in morphine-treated mice harboring a common genetic variant of MOR (A112G allele). In both the cerebral cortex and hippocampus of mice expressing the variant allele, an increase in VAPA protein expression was found. These results suggest that changes in VAPA expression may potentially contribute to the development of opioid addiction.

The research in this thesis is important because it uses a novel model of "addiction-like" behavior in rats self-administering heroin to analyze the expression of MOR interacting proteins in key brain regions that mediate reward and addiction. My data suggest that spinophilin and wntless are potential molecular substrates that accompany the transition from opioid exposure to opioid addiction. Future research is needed to demonstrate a cause and effect relationship
between the expression of MOR interacting proteins and "addiction-like" behaviors. To test
whether wntless or spinophilin are important neural substrates for opioid addiction, experiments
can be designed to determine if the development of "addiction-like" behaviors can be prevented
by blocking the interaction of the MOR with wntless or spinophilin. Further studies are also
required to test my working hypothesis that VAPA is involved in the intracellular trafficking of
MOR along the vesicular pathway and that this process is altered in the presence of a chronic
opioid agonist. Understanding the role of VAPA in MOR translocation to the cell surface will
provide insight into additional mechanisms of receptor regulation.
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<th>Full Form</th>
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<tbody>
<tr>
<td>A</td>
<td>adenine</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>BCA</td>
<td>bicinchoninic acid</td>
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<tr>
<td>BLAST</td>
<td>basic local alignment search tool</td>
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<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CAPS</td>
<td>calcium-dependent activator protein for secretion</td>
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<tr>
<td>CI</td>
<td>confidence interval</td>
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<tr>
<td>co-IP</td>
<td>co-immunoprecipitation</td>
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<tr>
<td>CPP</td>
<td>conditioned place preference</td>
</tr>
<tr>
<td>D2IL2</td>
<td>D₂ dopamine receptor intracellular loop 2</td>
</tr>
<tr>
<td>D2R</td>
<td>D₂ dopamine receptor</td>
</tr>
<tr>
<td>DAMGO</td>
<td>D-Ala₂, N-MePhe₄ Gly-ol-enkephalin</td>
</tr>
<tr>
<td>DEA</td>
<td>Drug Enforcement Agency</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle's medium</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>DP</td>
<td>drug period</td>
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<tr>
<td>DSM-IV</td>
<td>Diagnostic and Statistical Manual, Fourth Edition</td>
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<tr>
<td>Dyn</td>
<td>dynamin</td>
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<tr>
<td>ECL</td>
<td>enhanced chemiluminescence</td>
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<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
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<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<td>FR</td>
<td>fixed ratio</td>
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<td>G</td>
<td>guanosine</td>
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<td>G protein</td>
<td>GTP-binding protein</td>
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<td>GABA</td>
<td>γ-aminobutyric acid</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>GIRK</td>
<td>G protein-coupled inwardly rectifying potassium channel</td>
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<td>Gly</td>
<td>glycine</td>
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<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
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<tr>
<td>GRK</td>
<td>G protein-coupled receptor kinase</td>
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<td>GTP</td>
<td>guanosine triphosphate</td>
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<td>HEK</td>
<td>human embryonic kidney</td>
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<td>HPA</td>
<td>hypothalamic pituitary adrenal</td>
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<td>horseradish peroxidase</td>
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<td>immunoglobulin</td>
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<td>IL</td>
<td>intracellular loop</td>
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<td>IRR</td>
<td>incidence rate ratio</td>
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<td>kD</td>
<td>kilodalton</td>
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<td>knockout</td>
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<td>LAAM</td>
<td>levo-alpha acetylmethadol</td>
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<td>lysergic acid diethylamide</td>
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<td>LTD</td>
<td>long-term depression</td>
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<tr>
<td>LTP</td>
<td>long-term potentiation</td>
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<td>M3G</td>
<td>morphine-3-glucuronide</td>
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<tr>
<td>M6G</td>
<td>morphine-6-glucuronide</td>
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<td>MOR</td>
<td>mu-opioid receptor</td>
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<td>MORIP</td>
<td>mu-opioid receptor interacting protein</td>
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<tr>
<td>MSP</td>
<td>major sperm protein</td>
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<td>MYTH</td>
<td>modified membrane yeast two-hybrid</td>
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<td>NAc</td>
<td>nucleus accumbens</td>
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<tr>
<td>NOP</td>
<td>nociceptin/orphanin FQ receptor</td>
</tr>
<tr>
<td>NSF</td>
<td>N-ethylmaleimide sensitive factor</td>
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<tr>
<td>PAG</td>
<td>periaqueductal grey</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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<td>PC12</td>
<td>pheochromocytoma 12</td>
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<td>Full Name</td>
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<tr>
<td>Pdyn</td>
<td>preprodynorphin</td>
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<tr>
<td>Penk</td>
<td>preproenkephalin</td>
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<td>PFC</td>
<td>prefrontal cortex</td>
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<tr>
<td>phe</td>
<td>phenalanine</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
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<tr>
<td>PKC</td>
<td>protein kinase C</td>
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<td>POMC</td>
<td>proopiomelanocortin</td>
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<td>PR</td>
<td>progressive ratio</td>
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<td>PVDF</td>
<td>polyvinylidine difluoride</td>
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<tr>
<td>REIN</td>
<td>reinstatement</td>
</tr>
<tr>
<td>SA</td>
<td>self-administration</td>
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<tr>
<td>SD</td>
<td>synthetic dropout</td>
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<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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<tr>
<td>SNA</td>
<td>signaled non-availability</td>
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<td>SNARE</td>
<td>soluble N-ethylmaleimide-sensitive component attachment protein receptor</td>
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<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
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<td>Spino</td>
<td>spinophilin</td>
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<td>timeout</td>
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<td>UGT</td>
<td>uridine 5'-diphosphate glucuronosyltransferase</td>
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<tr>
<td>VAMP</td>
<td>vesicle associated membrane protein</td>
</tr>
<tr>
<td>VAPA</td>
<td>VAMP-associated membrane protein</td>
</tr>
<tr>
<td>VTA</td>
<td>ventral tegmental area</td>
</tr>
<tr>
<td>WLS</td>
<td>wntless</td>
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Chapter 1

Literature Review

1.1 Opioid Abuse and Dependence

1.1.1 Scope of the Problem

Substance use disorders are extremely burdensome for individuals, their families, and society. No one initiates recreational drug use with the intention of becoming an addict. From the addict's point of view, "addiction may be an inadvertent, incidental, un-calculated, unanticipated, unforeseen, unintentional, and random consequence of drug use" (Madras, 2006).

An unprecedented rise in opioid abuse and dependence has emerged in the United States over the past two decades (Manchikanti et al., 2012). While the illicit use of prescription opioid drugs accounts for the dramatic increase in opioid abuse rates, data also indicate an increase in the number of current and first-time heroin users. Over 2 million people in the United States claim using non-medical opioid drugs for at least 100 days within the past year (Abuse, 2011). In 2007, the total societal costs of prescription opioid abuse were estimated at US$55.7 billion (Birnbaum et al., 2011). The adverse health consequences of non-medical use of prescription opioids are equally substantial—increased rates of opioid dependence and an increase in mortality and morbidity attributable to drug overdoses. Opioid use often leads to death as described in two recent New York Times articles: http://www.nytimes.com/2013/07/19/us/heroin-in-new-england-more-abundant-and-deadly.html?emc=eta1&_r=0; http://nyti.ms/16YVGnv. Since 1999, deaths due to drug overdose involving prescription opioids such as oxycodone, hydrocodone, fentanyl, and methadone have markedly increased. The rate of prescription opioid overdose deaths in the
The use of the illicit opioid, heroin, is also on the rise in the United States. Over 600,000 people in 2010 reported using heroin within the past year compared with an estimated 400,000 people in 2002. The number of past-month heroin users aged 12 or older also increased from approximately 150,000 in 2007 to over 200,000 in 2010 (Johnston et al., 2010; RTI, 2011). Like that associated with the abuse of opioid prescriptions, the economic burden of heroin abuse is substantial—in 1996, the cost of heroin addiction in the United States was an estimated US$21.9 billion. Overdose deaths, a major adverse health consequence associated with heroin use, have significantly increased since 2007 (White et al., 2005a; Cartwright, 2008; Birnbaum et al., 2006; Hansen et al., 2011).

Americans consume more narcotic medication, on a gram per gram basis, than any other nation. Data on the sales and distribution of opioids overwhelmingly suggest that an increased supply of opioids, high numbers of medical users, "doctor shoppers", and patients with co-morbid factors, play a contributory role in opioid-related fatalities. The sales of opioid analgesics quadrupled between 1997 and 2010 (an increase from 96 mg morphine equivalents per person in the United States in 1997 to 710 mg per person in 2010). Sales of oxycodone and methadone increased 866% and 1,293%, respectively, from 1997 to 2007. Additionally, the majority of opioids are prescribed outside of pain management settings. A study from Volkow et al., 2009 revealed that only a small percentage of prescriptions were issued from pain clinics or anesthesiologists specialized in pain management (Manchikanti et al., 2012). For opioid abusers, doses used for adequate pain control are equivalent to doses used by abusers that induce reinforcement. Both the analgesic and rewarding effects of opioids depend on the degree to which they bind to the mu-opioid receptor. In the pain management setting, the most potent
opioids must be prescribed. These prescriptions, however, also put the patient at risk for developing abuse or addiction (Compton and Volkow, 2006).

1.1.2 Disease Model of Opioid Dependence

Self-administration of a drug, by definition, precedes abuse or addiction. For some individuals, sporadic or intermittent drug use will proceed. Once an individual becomes addicted, targeted medications have shown to be more effective than abstinence-based approaches. Studies indicate that less than 20% of long-term heroin addicts are able to remain in a heroin- or illicit-opiate-free state on a permanent, long-term basis (Stafford, 2006). During their first year of drug abstinence, more than 80% of heroin addicts will relapse to opiate abuse and dependence (Madras, 2006).

The exact factors that cause the transition from a controlled use of a substance to the compulsive intake of addiction are not fully understood. It has been estimated that among all recreational users of drugs of abuse, approximately 8% to 32% of users will become addicts (Wagner and Anthony, 2001; Anthony and Petronis, 1995; Chen et al., 2005). When drug use transitions to the addictive stage, the substance of abuse becomes the primary source of euphoria or positive sensations. As addiction progresses, judgment deteriorates and adverse consequences for the drug user escalate. If abstinence from the drug occurs at this stage, the individual invariably manifests psychological and/or physiological withdrawal. Even following prolonged abstinence, the addicted brain can generate intense craving that may lead to compulsive drug-seeking. Craving tends to lead to relapse, and the addicted individual cycles through compulsive drug-seeking and use, withdrawal, and relapse. This cycle often occurs several times until the cycle can finally be broken (Madras, 2006).

Drug addiction can be viewed as a chronic, relapsing disease characterized by compulsive, uncontrollable use despite adverse consequences. This disease model of addiction offers a perspective that reduces stigmatization by professionals, helps to facilitate treatment
research, and focuses on problem solving. When utilizing the construct of the disease model, patients are urged to assume responsibility for their treatment. Thus, patient compliance is comparable to other chronic diseases such as asthma, hypertension, and diabetes (O'Brien, 2004; McLellan et al., 2000).

In medical settings, the terms drug abuse and drug dependence refer to a clinical syndrome. According to the fourth edition of the American Psychiatric Association's Diagnostic and Statistical Manual of Mental Disorders, "the essential feature of dependence is a cluster of cognitive, behavioral, and physiological symptoms indicating that the individual continues substance use despite significant substance-related problems." The syndrome of drug dependence has seven key signs and symptoms (Table 1.1). An individual must meet 3 of these in the same 12-month period to receive a diagnosis of dependence (DSM-IV-TR, 2000). The criteria for a diagnosis are the same regardless of the substance used (e.g., the criteria for opioid dependence are the same as those for nicotine dependence and cocaine dependence). The term drug dependence should only be used for individuals whose drug use is problematic. Physical dependence and tolerance are neither necessary, nor sufficient, for a diagnosis of drug dependence. If a patient is using legitimately prescribed medications as intended (e.g., opioids for chronic pain), and those medications are helping the patient to function better, that patient will not meet criteria for drug dependence, even if the patient has developed tolerance to the medication or becomes physically dependent.
Table 1.1: Diagnostic criteria for drug dependence and drug abuse

<table>
<thead>
<tr>
<th>DEPENDENCE (≥3 IN 12-MONTH PERIOD)</th>
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</thead>
<tbody>
<tr>
<td>1. Tolerance</td>
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<tr>
<td>2. Withdrawal</td>
<td></td>
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<tr>
<td>3. The substance is often taken in larger amounts for a longer period than intended</td>
<td></td>
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<tr>
<td>4. Unsuccessful efforts or a persistent desire to cut down or control substance use</td>
<td></td>
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<tr>
<td>5. A great deal of time is spent in activities necessary to obtain the substance or to recover from its effects</td>
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</tr>
<tr>
<td>6. Important social, occupational, or recreational activities given up or reduced because of substance use</td>
<td></td>
</tr>
<tr>
<td>7. Continued substance use despite knowledge of having had persistent or recurrent physical or psychological problems that are likely to be caused or exacerbated by the substance</td>
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<table>
<thead>
<tr>
<th>ABUSE (≥1 IN A 12-MONTH PERIOD)</th>
<th></th>
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<tbody>
<tr>
<td>1. Recurrent substance use resulting in failure to fulfill major role obligations at work, school, or home</td>
<td></td>
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<tr>
<td>2. Recurrent substance use in situations in which it is physically hazardous</td>
<td></td>
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<tr>
<td>3. Recurrent substance-related legal problems</td>
<td></td>
</tr>
<tr>
<td>4. Continued substance use despite having persistent or recurrent social or interpersonal problems caused or exacerbated by the effects of the substance and never met criteria for dependence</td>
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</tbody>
</table>

1.1.3 Pathobiology of Addiction

When environmental, individual, and drug-related risk factors converge in vulnerable individuals, drug use, abuse, and addiction can develop. It can be helpful to conceptualize the etiology of drug abuse by employing the public health model frequently cited when studying infectious disease, that is, as an interaction among the host (the potential drug user), the agent (a specific drug in this case), and the environment (the person's family life, peer group, and the social, cultural, and religious attitudes toward use of that substance) (Cecil et al., 2011).

The Host

Genetic disposition (Uhl et al., 2002; Uhl and Grow, 2004), psychiatric comorbidity (Volkow, 2001; Armstrong and Costello, 2002), personality disorders (Sher and Trull, 2002), poor school performance, inappropriate school behavior, and early drug use can all promote drug abuse
problems. Twin studies and adoption studies show that both genetic and environmental factors contribute to individual vulnerability, although the precise nature by which this occurs is still unknown and a subject of active research. One area of great research interest relates to whether people can be vulnerable to drug dependence in general, for example as a result of a risk-taking temperament or poor decision making, or whether they are at high risk to abuse particular substances perhaps due to a highly reinforcing response to a specific drug (Cecil et al., 2011).

A twin study designed to distinguish between the family environment and genetic factors as the source of observed family resemblance for substance use disorders examined twins in the Vietnam Era Twin Registry. This database was comprised of male twin pairs who served in the military between 1965 and 1975. Data was collected by phone interview for 3,372 pairs in which both twins participated. This study found a significant difference between concordance rates for monozygotic twins (26.2%) versus dizygotic twins (16.5%). Biometrical modeling indicated that genetic factors accounted for 34% of the variance on the individual's risk of developing a drug use disorder (Tsuang et al., 1996).

Psychiatric illness and personality disorders have also been shown to influence the likelihood of developing drug abuse. The presence of conduct disorder in childhood and adolescence and antisocial personality disorder in adulthood have both been shown to predispose individuals to subsequent drug abuse (Sher and Trull, 2002). Additionally, psychiatric disorders such as mood disorders are frequently found in people with drug abuse problems (Volkow, 2001). However, it is important to note that the presence of these disorders in an individual does not imply causation, even if one of the disorders manifests first. Severe emotional trauma, such as child abuse, death of a parent, and alcohol and drug-addicted parents are also risk factors that can lead adolescents or young adults into drug use. On the other hand, certain individual protective factors may reduce the likelihood of a substance abuse disorder. Individuals who have positive
familial relationships, academic success, and meaningful religious affiliation have a lower likelihood of developing drug addiction (Madras, 2006).

The Agent

Drugs of abuse exert "powerful but capricious" effects on the brain. The impact that a substance of abuse will have on an individual depends on several variables: dose, cost, accessibility, route of administration (intravenous, smoking, insufflation, oral), the chemical composition of the drug, and the often unpredictable nature of a user's response. Most drugs of abuse are naturally reinforcing; animals typically will self-administer commonly abused drugs. Not all drugs are equally reinforcing, however, and there is individual variation in drug preference. Some people like the stimulating effects of drugs such as cocaine and amphetamine, whereas others experience that level of stimulation as extremely uncomfortable. Some individuals enjoy the relaxation induced by drugs such as marijuana and sedative-hypnotics, whereas others feel "dead" and overly slowed down by these drugs. Although some individuals gravitate toward a particular drug of abuse because of its specific pharmacologic properties, others will use a variety of drugs indiscriminately based on availability. These individuals are primarily seeking to alter their current emotional state, regardless of the direction in which it is changed. The reinforcing properties of many drugs of abuse appear to be mediated through dopaminergic pathways, although other neurotransmitters, including \( \gamma \)-aminobutyric acid (GABA), serotonin, and norepinephrine are also involved in mediating drug-induced reinforcement (Cecil et al., 2011).

The Environment

Drug use does not occur in a vacuum. The environment is a major influence on whether adolescents and adults will recreationally experiment with drugs or develop an aversion to them. A key factor in the development, maintenance, and attenuation of drug abuse is the environment in which the drug use occurs. Societal factors, such as legal status, availability, price, perception
of dangerousness, social desirability, peer group, and religious beliefs influence behavior relating to substance use. Of these various factors, drug availability is known to be a substantial influence on the likelihood of substance use. A major factor that influences illicit drug use is the potential user's perception of the safety of the drug, the approval of one's social network, likelihood of incurring legal consequences, and peer group behavior. Treatment research and animal studies show that environmental influences can have a powerful effect on drug use (Puhl et al., 2012). Studies have shown, for example, that offering an alternative positive reward (e.g., a voucher that can be exchanged for desired goods and services such as movie tickets or clothes) in response to abstaining from drugs may help drug-dependent individuals overcome their severe craving and reduce their substance use. This type of treatment approach, based on the use of motivational incentives for abstinence, has been shown to be one of the most powerful treatment interventions available for the treatment of drug dependence (Grigson, 2009). The responsiveness of drug-dependent individuals to environmental contingencies demonstrates the importance of understanding the complexity of the interaction among the individual, the drug, and the environment in the determination of drug use (Madras, 2006).

*Neuroadaptations to Drugs of Abuse*

Understanding brain communication at the level of neurotransmission has been a major factor in elucidating how drugs of abuse trigger neuroadaptation. Drugs of abuse have structures that resemble endogenous neurotransmitters. For instance, heroin overlaps structurally with the endogenous peptides endorphins and enkephalins. The tightly regulated receptor mediated signaling and synaptic communication are geared for endogenous transmitters, not drugs of abuse. In general, neurons do not process or regulate drugs with the same robust precision involved in controlling endogenous compounds. Most psychoactive drugs, such as LSD, morphine, cocaine, and marijuana, are not substrates for transporters. Thus, these substances are not sequestered away from the synapse following a burst of receptor activity. Additionally, drugs
may activate receptors but may not necessarily trigger the receptor trafficking and processing that are needed to recycle active receptors. Morphine, for example, can activate the mu-opioid receptor, but it is far less effective in promoting receptor internalization than are endogenous peptides (Christie, 2008; Keith et al., 1998; Qiu et al., 2003). The internalization of the mu-opioid receptor is an important process in the recycling of a functional receptor. Different signaling cascades and patterns of gene induction are also triggered by drugs of abuse. These substances may trigger signals of anomalous strength and duration, and promote signaling cascades that ultimately translate into euphoria, hallucinations, delusions, anger, and a host of other behavioral manifestations (Madras, 2006).

Drugs may also induce mnemonic effects and drug-seeking behavior may be prompted by these memories (Dong et al., 2004). With repeated or chronic use, the brain may adapt to abnormal signals in several ways: altering the production of genes, protein levels, and signaling networks. The reversibility of many of these neuroadaptations remain unknown. At the psychological level, addiction is defined as a persistent compulsion to use a drug or the loss of control over drug use. Addiction is accompanied by a loss in life-sustaining activities and social pursuits, medical and legal adverse consequences (Cecil et al., 2011).

1.2 The Opioid System

1.2.1 Overview

The opioid system modulates diverse physiological functions including analgesia, reward, and stress responsivity (Koob and Kreek, 2007; Vaccarino and Kastin, 2001). The term opiate refers to compounds structurally related to plant alkaloids derived from opium. Opium, a word derived from opos, the Greek word for "juice," is aptly named; since natural opiates are derived from the resin of the opium poppy, Papaver somniferum. Opiates include the natural plant alkaloids—morphine, codeine, thebaine—and many semisynthetic derivatives. An opioid is
any agent that has the pharmacological and functional properties of an opiate, regardless of structure. Endogenous opioids are naturally occurring ligands for opioid receptors. Often used synonymously with endogenous opioid peptides, the term endorphin refers to a specific endogenous opioid, the β-endorphin. The term narcotic, which originally referred to any drug that induced narcosis or sleep, has become associated with opioids. It is often used in a legal context to refer to a variety of substances with abuse or addictive liability (Fox et al., 2011).

The opioid system consists of four receptors—mu (μ), kappa (κ), delta (δ), and the nociceptin/orphanin FQ receptor (NOP). The opioid receptors are activated by endogenous opioid peptides that are processed from three protein precursors—proopiomelanocortin, proenkephalin, and prodynorphin. Opioid receptors are recruited in response to natural rewarding stimuli and drugs of abuse. The mechanisms whereby activation and modifications of the opioid system contribute to drug craving and relapse remain unclear (Le Merrer et al., 2009).

Opium binding sites in the brain were established in 1973 and later referred to as mu, delta, and kappa opioid receptors (Terenius, 1973; Simon et al., 1973; Pert and Snyder, 1973). In 1975, met-enkephalin and leu-enkephalin were characterized as the first endogenous ligands for the opioid receptors. Enkephalins, dynorphins, and β-endorphin are produced by proteolytic cleavage of protein precursors known as preproenkephalin (Penk), preprodynorphin (Pdyn), and proopiomelanocortin (POMC), respectively. All of these opioid peptides share a NH$_2$-terminal Tyr-Gly-Gly-Phe signature sequence that interacts with opioid receptors (Akil et al., 1998).

The genes encoding the opioid peptide precursors were isolated in the late 1970s and early 1980s (Nakanishi et al., 1979; Comb et al., 1982; Kakidani et al., 1982), but receptor cloning was achieved much later. In 1992, the first opioid receptor gene was isolated by receptor cloning, followed by the identification of several homologous genes (Minami et al., 1993; Wang et al., 1994b; Thompson et al., 1993a; Thompson et al., 1993b; Yasuda et al., 1993; Kieffer et al., 1992; Meng et al., 1993; Evans et al., 1992). The opioid receptor gene family includes four
members encoding mu (Oprm1), delta (Oprd1), kappa (Oprk1), and the orphaninFQ/nociceptin (Oprl1) receptors. Opioid receptors are membrane receptors with a seven-transmembrane topology, belonging to the large G protein-coupled receptor superfamily (Le Merrer et al. 2009). Opioid receptors are Class A, rhodopsin-like G protein-coupled receptors (Christie, 2008).

Opioid peptides and receptors are broadly expressed in the peripheral and central nervous system. Opioid receptors are expressed primarily in the cortex, limbic system, and brain stem. Although binding sites for the different opioid receptors overlap, some brain structures exhibit higher expression of one receptor over the others. For example, the mu receptor is the most expressed opioid receptor in the amygdala, thalamus, mesencephalon, and some brain stem nuclei. Additionally, mu binding sites are the only receptor sites found in four thalamic nuclei, the sensory trigeminal nucleus, and the nucleus accumbens (Zöllner 2007;Dickenson and Kieffer, 2006).

Anatomical studies demonstrate that the opioid system is widespread. The sites of opioid receptor expression (mRNA) generally match the distribution of binding sites (protein). This suggests that many neurons synthesizing opioid receptors are local neurons. Opioid peptide immunoreactivity in projection fibers overlaps with the localization of opioid receptors. While Penk is the most abundant and widely distributed opioid precursor and is best described in the thalamus, where it overlaps with mu receptors, Pdyn is present in highest concentrations in the nucleus accumbens but nearly absent in the thalamus (Mathieu et al., 1996). POMC has a distribution restricted to mainly to brain stem subcortical regions (Leriche et al., 2007). Although selectivity factors do not exceed one-order of magnitude, pharmacological studies suggest that β-endorphin and enkephalin have the best affinities for mu and delta receptors, while there is preferred binding of dynorphins at kappa receptors (Le Merrer et al. 2009).

The opioid system plays a central role in nociception and analgesia (Akil et al., 1976). The opioid system also regulates responses to stress, respiration, gastrointestinal transit, as well as
endocrine and immune functions (Bailey and Connor, 2005). This system also functions in modulating mood and well-being, as well as addictive behaviors. The opioid system is hypothesized to have been developed in response to the evolutionary drive to survive. Goal-directed survival behaviors, such as foraging for food and water (Kelley et al., 2005; Glass et al., 1999; Berthoud, 2002; Berthoud, 2007), avoiding predators, seeking sexual partners, and caring for offspring (Nelson and Panksepp, 1998; Miranda-Paiva et al., 2003; Moles et al., 2004) must be flexible enough for the ability to constantly adapt to the environment. In this context, positive emotions and pleasure became associated with the ability to learn from experience and to increase the probability of the occurrence of a specific behavior—a phenomenon called positive reinforcement (Van Ree and de Wied, 1980). The mesolimbic dopaminergic projections that originate from the ventral tegmental area and project to different regions of the forebrain, with major input to the nucleus accumbens, play a major role in the reinforcement circuit (Le Merrer et al., 2009).

Opioid receptors and peptides are expressed throughout the reinforcement network. Systemic mu agonists are known to produce positive reinforcement (Spanagel et al., 1990), whereas kappa agonists produce aversion, hallucinations, and malaise (Shippenberg et al., 1996; Todtenkopf et al., 2004). Mu and delta antagonists suppress the reinforcing properties of natural rewards and opioid drugs (Shippenberg et al., 2009; Negus et al., 1993; Powell et al., 2002; Akil et al., 1976), while kappa antagonists facilitate rewarding effects (Jackson et al., 2010). The systemic effects of opioid drugs, however, reflect multiple actions of opioid compounds at several brain sites.

### 1.2.2 Opioid Receptors

There are four subtypes of opioid receptors: delta (\(\delta\)), kappa (\(\kappa\)), mu (\(\mu\)), and the nociceptin/orphanin FQ receptor (NOP). These receptors belong to the Class A (rhodopsin-like) family of G protein-coupled receptors (GPCRs). They share common structural features, such as
seven transmembrane spanning domains interspersed by three extracellular and intracellular loops. Following agonist binding to the opioid receptor, downstream effects are mediated by pertussis toxin-sensitive heterotrimeric G proteins (Wang et al., 1994a; Ueda et al., 1988). Thus, most measurable agonist-induced effects are pertussis toxin-sensitive (Corbett et al., 2006).

Cloning of the opioid receptors demonstrated that each receptor is encoded by a single gene in humans (Knapp et al., 1995; Wang et al., 1994a; Zhu et al., 1995). The opioid receptors are homologous with each other, as evidenced by amino acid sequence alignments. The µ and δ receptors share 60% identity; the µ and κ share 68% identity; δ and κ share 58% identity; NOP shares 50-60% identity with the µ, κ, and δ receptors. Variation between the receptors occurs mainly in the extracellular domains and the carboxyl-terminus (Waldhoer et al., 2004). It was found in studies utilizing recombinant chimeric receptors that the extracellular domains provide specificity to ligand binding (Zhu et al., 1995; Raynor et al., 1996; Kong et al., 1994; Minami et al., 1994).

The distribution of opioid receptors correlates with the functions of the opioid system (Waldhoer et al., 2004), however, differences in the synaptic location of different receptors may lead to opposing downstream effects. Activation of µ receptors in the periaqueductal grey (PAG) induces analgesia. Activation of NOPs in the PAG reduces the analgesic effects of µ agonists (Corbett et al., 2006). The difference in downstream effect can be attributed to the location of MORs on presynaptic inhibitory interneurons, which leads to a disinhibition of neuronal output from the PAG. NOPs, on the other hand, are located on postsynaptic neurons and their activation leads to inhibition of neuronal output from the PAG. Additionally, receptor-specific knockout studies in mice have revealed other important functional differences among the opioid receptors in the modulation of reward, pain, and behavior (Kieffer and Gavériaux-Ruff, 2002).
1.2.3 Regulation of the Mu-Opioid Receptor

Since MOR was found to be responsible for mediating the rewarding and analgesic properties of opioids, I will focus on the regulation of MOR in the following discussion of opioid receptor regulation. Like other G protein-coupled receptors, MOR is regulated by several different mechanisms, including receptor endocytosis, recycling, and degradation. These regulatory mechanisms are initiated by agonist binding and activation of the receptor (Bailey and Connor, 2005; Christie, 2008). Activated MORs are internalized by the process of endocytosis in response to most, but not all, opioid agonists (Petruzzi et al., 1997; Raehal and Bohn, 2005; Johnson et al., 2006b). In general, opioid agonist binding leads to downstream signaling events mediated by G\textsubscript{i}/G\textsubscript{o}-proteins. The activated receptor then becomes the target of G protein-coupled receptor kinases (GRKs), which phosphorylate receptors at specific serine and threonine residues. Phosphorylated receptors are subsequently bound by arrestins which also bind to clathrin and the clathrin-adaptor protein AP2 to promote the process of internalization (Gainetdinov et al., 2004). Following internalization, MORs are efficiently recycled back to the cell surface in a process termed resensitization (Tanowitz and von Zastrow, 2003).

Notably, the prototypical opioid agonist morphine does not cause endocytosis of the MOR in human embryonic kidney (HEK) 293 cells that have been stably transfected with the receptor (Keith et al., 1996). The efficacy of morphine to produce internalization is highly dependent on the neural system and brain region (Johnson et al., 2006b). Tissue samples from transgenic mice expressing epitope-tagged MOR in the neurons of the locus ceruleus did not exhibit morphine-induced internalization of MOR (Arttamangkul et al., 2008) nor did MOR heterologously expressed in hippocampal neurons (Bushell et al., 2002). On the other hand, morphine induced a rapid endocytosis of MOR in dissociated primary cultures of rat striatal neurons (Haberstock-Debic et al., 2005). These studies implicate that there may be cell-type specific differences in the regulatory mechanisms of MOR.
Although there are agonist-specific differences in MOR internalization, all agonists mediate similar physiologic responses short term (i.e., analgesia and euphoria) and long-term (i.e., tolerance and physical dependence) (Christie, 2008). The laboratory of Marshall Nirenberg assessed whether there is a correlation between the development of tolerance and physical dependence in an animal model and the specific effects that opioid agonists produce in cells. It was found that repeated administration of an opioid agonist on neurons diminished responsiveness to the drug, as evidenced by the progressive dampening of agonist-mediated cyclic adenosine monophosphate (cAMP) inhibition. Following removal of the agonist, the measured cAMP levels rebounded above baseline (Sharma et al., 1975; Sharma et al., 1977). This finding suggests that receptor desensitization and cellular compensatory mechanisms may underlie the development of tolerance and physical dependence. Although several other studies have examined the mechanisms of MOR regulation to try to understand the cellular milieu that accompanies tolerance, physical dependence, and addiction, a clear mechanistic picture remains to be elucidated (Bailey and Connor, 2005).

### 1.2.4 Opioid Receptor Knockout Mice Studies

The genes encoding the opioid receptors have been knocked-out both singularly and in combination in mice. The results of these studies have been thoroughly reviewed (Gaveriaux-Ruff and Kieffer, 2002; Kieffer and Gavériaux-Ruff, 2002; Kieffer, 1999). This section will focus on mu opioid receptor (MOR) knockout (KO) mice. The generation of MOR KO mice revealed the central importance of MOR in mediating the rewarding and analgesic properties of opioid drugs. Interestingly, these mice did not show any morphological abnormalities, and they did not differ in weight compared to their wild-type littermates. The MOR KO mice also bred normally and did not demonstrate any impairment of maternal behaviors (Matthes et al., 1996).

It was found, however, that MOR KO pups exhibit less attachment behavior when separated from their mothers. MOR KO pups are also less responsive to their own mother's cues.
MOR KO mice were insensitive to the analgesic effects of morphine and other agonists, as confirmed by tail immersion, tail flick, and hot plate tests (Matthes et al., 1996; Sora et al., 1997; Kitanaka et al., 1998). Other effects of opioid drugs such as respiratory depression, slowed gastrointestinal transit, and the stimulation of stress hormone release were abolished in MOR KO mice (Matthes et al., 1998; Roy et al., 2001; Roy et al., 1998; Dahan et al., 2001). MOR KO mice were insensitive to the rewarding effects of heroin and morphine in conditioned place preference studies (Contarino et al., 2002; Matthes et al., 1998; Sora et al., 2001). Additionally, the self-administration of morphine and naloxone-precipitated withdrawal following chronic morphine exposure were also abolished in MOR KO mice (Matthes et al., 1996; Sora et al., 2001; Becker et al., 2000).

1.2.5 Mu-Opioid Receptor Interacting Proteins

The mu-opioid receptor (MOR) belongs to the Class A (rhodopsin-like) family of G protein-coupled receptors (GPCRs). GPCR signaling is mediated by the activation of heterotrimeric guanosine triphosphate (GTP)-binding proteins (G proteins), which act as secondary messengers which activate or inhibit other proteins to create a cascade of events that produce a specific response in the cell (Hamm and Gilchrist, 1996). In the central nervous system, GPCRs serve as important targets of neuromodulatory drugs, such as antipsychotics and opioid analgesics (Hill, 2006). Evidence from several studies indicate that GPCRs bind to other proteins to form multiprotein signaling complexes or signalplexes (Kabbani et al., 2002; Kabbani and Levenson, 2007; Georgoussi et al., 2012; Milligan, 2005). Interacting proteins have the potential to mediate downstream effects or to induce alterations in the mechanisms of receptor signaling and regulation (Bockaert et al., 2010; Bockaert et al., 2004). A list of proteins that have been found to interact with the MOR is presented in Table 1.2.
<table>
<thead>
<tr>
<th>INTERACTING PROTEIN</th>
<th>FUNCTION</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha2A adrenergic receptor</td>
<td>Receptor signaling</td>
<td>(Jordan et al., 2003)</td>
</tr>
<tr>
<td>Beta-arrestin 1 Beta-arrestin 2</td>
<td>Receptor desensitization and endocytosis</td>
<td>(Bohn et al., 2000; Molinari et al., 2010)</td>
</tr>
<tr>
<td>Calmodulin</td>
<td>Interferes with activation of G proteins</td>
<td>(Wang et al., 1999)</td>
</tr>
<tr>
<td>Cannabinoid receptor (CB-1)</td>
<td>Inhibition of signaling</td>
<td>(Rios et al., 2006)</td>
</tr>
<tr>
<td>Delta opioid receptor</td>
<td>Modulation of signaling and ligand binding</td>
<td>(Gomes et al., 2000)</td>
</tr>
<tr>
<td>Filamin A</td>
<td>Regulation of receptor recycling and degradation</td>
<td>(Onoprishvili et al., 2003)</td>
</tr>
<tr>
<td>Gai2/Gai3</td>
<td>Receptor signaling</td>
<td>(Georgoussi et al., 1997)</td>
</tr>
<tr>
<td>Glycoprotein M6a</td>
<td>Endocytosis and recycling of receptor</td>
<td>(Wu et al., 2007)</td>
</tr>
<tr>
<td>G protein-regulated inducer of neurite outgrowth 1 (GRIN1)</td>
<td>Regulates receptor distribution in lipid rafts</td>
<td>(Ge et al., 2009)</td>
</tr>
<tr>
<td>Heat shock protein (HLJ1)</td>
<td>Function unknown</td>
<td>(Ancevska-Taneva et al., 2006)</td>
</tr>
<tr>
<td>Kappa opioid receptor</td>
<td>Sex-dependent antinociception</td>
<td>(Chakrabarti et al., 2010)</td>
</tr>
<tr>
<td>Opioid receptor-like 1 receptor (ORL1)</td>
<td>Inhibits receptor signaling</td>
<td>(Wang et al., 2005)</td>
</tr>
<tr>
<td>Periplakin</td>
<td>Interferes with activation of G proteins</td>
<td>(Feng et al., 2003)</td>
</tr>
<tr>
<td>Phospholipase D2</td>
<td>Regulates receptor endocytosis</td>
<td>(Koch et al., 2004)</td>
</tr>
<tr>
<td>Protein kinase Ci (PKCi)</td>
<td>Negatively regulates receptor desensitization</td>
<td>(Guang et al., 2004)</td>
</tr>
<tr>
<td>Ran binding protein 9 (RanBP9)</td>
<td>Negatively regulates receptor internalization</td>
<td>(Talbot et al., 2009)</td>
</tr>
<tr>
<td>Regulator of G protein signaling 4 (RGS4)</td>
<td>Signaling and scaffolding</td>
<td>(Georgoussi et al., 2006)</td>
</tr>
<tr>
<td>Regulator of G protein signaling 9-2 (RGS9-2)</td>
<td>Receptor signaling and endocytosis</td>
<td>(Garzón et al., 2005)</td>
</tr>
<tr>
<td>Somatostatin receptor</td>
<td>Internalization and desensitization</td>
<td>(Pfeiffer et al., 2002)</td>
</tr>
<tr>
<td>Spinophilin</td>
<td>Receptor signaling and endocytosis</td>
<td>(Charlton et al., 2008)</td>
</tr>
<tr>
<td>STAT5A</td>
<td>Regulates receptor-mediated transcription</td>
<td>(Mazarakou and Georgoussi, 2005)</td>
</tr>
</tbody>
</table>
Of these MOR interacting proteins (MORIPs), the discovery of the MOR-spinophilin interaction is particularly interesting. Spinophilin is a scaffolding protein that is enriched in dendritic spines. When spinophilin was overexpressed in pheochromocytoma 12 (PC12) cells, morphine could induce a rapid endocytosis of MOR. This effect was not observed in control PC12 cells that expressed low levels of spinophilin. Additionally, the loss of spinophilin expression in spinophilin knockout (KO) mice increased measures of MOR signaling by delaying receptor internalization. In behavioral studies, sphinophilin KO mice demonstrated both a higher propensity toward the rewarding properties of morphine and a greater degree of physical dependence (Charlton et al., 2008). This study provides evidence that the behavioral response to an opioid drug can be altered by changing the expression of a MORIP.

With the identification of novel MOR interactions and multiprotein complexes, it will be possible to discover new functional roles for MORIPs. Novel MORIPs have the potential to serve as new therapeutic targets and may enable more effective strategies for understanding the underlying mechanisms of tolerance, physical dependence, and addiction (Georgoussi et al., 2012). Additional research should be aimed at identifying and characterizing novel MORIPs to more fully understand MOR regulation, as well as the potential roles MORIPs play in the pathogenesis of complex neuropsychiatric diseases (Bockaert et al., 2010).

1.2.6 Mu-Opioid Receptor Polymorphisms and Genetic Variation

The human MOR gene, *OPRM1*, spans over 200 kilobases and consists of 11 exons that combine to yield splice variants (Xu et al., 2009; Xu et al., 2011; Pan et al., 2009). The most abundant transcript is MOR-1, which consists of exons 1, 2, 3, and 4 and encodes 400 amino
acids (Ide et al., 2005). The mouse homologue of MOR-1 shares 94% amino acid identity with human MOR-1. Over 28 splice variants from the mouse *OPRM1* gene have been isolated (Doyle et al., 2007a; Doyle et al., 2007b), and similar splicing patterns have been observed in rats (Pasternak et al., 2004) and humans (Pan et al., 2005). Although full-length genetic variants share a common binding pocket with the seven transmembrane domains conserved, there are differences in agonist-induced activation of the MOR (Bolan et al., 2004; Oldfield et al., 2008; Pan et al., 2005; Pasternak et al., 2004). Additionally, MOR variants have different distributions in the central nervous system and occupy different cell types (Abbadie et al., 2000c; Abbadie et al., 2000a; Abbadie et al., 2000b; Abbadie et al., 2001).

The dpSNP and NCBI databases of genetic variation contain over 700 single nucleotide polymorphisms (SNPs) for the *OPRM1* gene. Initial investigations into the human molecular genetics of the μ-opioid receptor defined common variants of the receptor that had uniquely distinct allelic frequencies in different ethic groups. The A118G variant is a very common genetic polymorphism of the μ-opioid receptor with significantly tighter binding of β-endorphin and greater signal transduction through G protein-coupled potassium inwardly rectifying channels with β-endophin activation of the receptor. The A118G variant of the human mu-opioid receptor gene (*OPRM1*) is in the coding region of the first exon and is an adenine to guanine transition at nucleotide position 118 of the coding sequence of the gene. This polymorphism codes for an asparagine to aspartic acid substitution at amino acid 40 in the amino terminus, thereby removing a potential extracellular glycosylation site (Kroslak et al., 2007). Utilizing a population of subjects with minimal ethnic admixture, central Sweden (where more than 70% of study subjects had Swedish ancestry), the Kreek laboratory demonstrated a highly significant association of the A118G variant and addiction to opioids (Bond et al., 1998). The attributable risk for opioid dependence in individuals with the A118G variant of the mu-opioid receptor was 18% in the
entire central Swedish population studied and 21% in Swedes with Swedish ancestry (Madras, 2006), as assessed in a genome-wide association study.

This study suggested that the A118G variant would alter normal physiology in systems under significant modulation by the μ-opioid receptor (Bond et al., 1998). It was hypothesized that stress responsivity would be altered in A118G variants; because it had already been demonstrated that the μ-opioid receptor agonist tonically inhibits the HPA axis. The functional significance of the A118G variant was initially investigated by comparing the relative binding of a variety of endogenous and exogenous opioids and opiates to the μ receptor. A three-fold greater binding affinity of β-endorphin to the A118G variant receptor was found. Molecular-cellular construct studies of the G protein-coupled inwardly rectifying potassium channels (GIRKs) revealed a similar three-fold increase in the activation of the variant when β-endorphin was the binding ligand (Bond et al. 1998). Interestingly, other endogenous or exogenous opioid ligands had similar binding affinity and functional properties with the A118G variant. It was hypothesized that individuals with one copy of this variant would show physiological differences in any system modulated by the μ-opioid receptor (Madras, 2006).

The A118G SNP has been shown to be associated with the analgesic and adverse effects of opioids. The consumption (Sia et al., 2008; Lötsc et al., 2002; Chou et al., 2006), as well as the dosage (Ginosar et al., 2009; Reyes-Gibby et al., 2007) of opioid drugs required to achieve analgesia in clinical settings has been shown to be greater in G-allele carriers compared to patients with the "wild-type" AA alleles. Other studies, which have been summarized in Table 1.3, have found an association between the G118 allele and heroin use, abuse, and dependence that is highly dependent on race and ethnicity (Mague, 2010).
Table 1.3: Association studies investigating OPRM1 A118G involvement in opioid dependence in humans

This table provides a summary of the association studies done with the A118G SNP and opioid dependence. Protection or risk is associated with the presence of at least one copy of the G118 allele. Overall, these studies show that the presence of the A118G SNP affects human populations differently depending on race or ethnicity. This table is adapted from a comprehensive review by Mague et al. 2010.

<table>
<thead>
<tr>
<th>FINDING</th>
<th>EFFECT</th>
<th>POPULATION (n)</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>G118 allele is associated with heroin dependence</td>
<td>Risk</td>
<td>Swedish (139 heroin-dependent; 149 control)</td>
<td>(Bart et al., 2004)</td>
</tr>
<tr>
<td>90% of G118 carriers were heroin users</td>
<td>Risk</td>
<td>European caucasians (118)</td>
<td>(Drakenberg, 2006)</td>
</tr>
<tr>
<td>Higher frequency of the G118 allele in opioid-dependent subjects</td>
<td>Risk</td>
<td>Indian (126 opioid-dependent; 156 control)</td>
<td>(Kapur et al., 2007)</td>
</tr>
<tr>
<td>G118 allele is associated with heroin dependence</td>
<td>Risk</td>
<td>Chinese males (200 heroin-dependent; 97 control)</td>
<td>(Szeto et al., 2001)</td>
</tr>
<tr>
<td>A118G SNP is associated with heroin dependence in Indian but not East Asian populations</td>
<td>Protective</td>
<td>Indian (20 dependent; 117 control); Malaysian (25 dependent; 131 control); Chinese (52 dependent; 78 control)</td>
<td>(Tan et al., 2003)</td>
</tr>
<tr>
<td>A118G SNP is associated with heroin dependence in Hispanics</td>
<td>Protective</td>
<td>African American (46 dependent; 16 controls); Caucasian (60 dependent; 44 control); Hispanic (116 dependent; 78 control)</td>
<td>(Bond et al., 1998)</td>
</tr>
<tr>
<td>None</td>
<td>No association</td>
<td>Chinese (48 heroin-dependent; 48 control)</td>
<td>(Shi et al., 2002)</td>
</tr>
</tbody>
</table>
1.3 Opioid Pharmacology

1.3.1 Opioid Therapeutics

Opioid drugs are mainly used to treat moderate to severe pain. In addition to reducing the sensation or perception of pain, opioids also induce a state of well-being or euphoria. Thus, opioids are often used illicitly to obtain relaxation or alter mood (Epstein et al., 2006). The potential of abuse that accompanies the use of opioids has generated much research aimed at separating the mechanism of analgesia from that of euphoria (Walwyn et al., 2010; Evans, 2004). The goal of eventually developing a potent analgesic that does not activate brain reward circuitry has become a "holy grail" in both pain management and addiction research. Although science has progressed in its understanding of the physiology of pain, the best medications for severe pain treatment remain derivatives of the opium poppy (Manchikanti et al., 2012).

Opioids are useful in both the treatment of chronic and acute pain. The subjective effects of opioids make this class of analgesics particularly useful in the management of acute pain. In high-anxiety situations, such as the crushing chest pain of a myocardial infarction, the relaxing, anxiolytic effects of opioids complement their analgesic effects. Patients taking opioids to treat chronic pain usually do not develop substance abuse or dependence (Fox et al., 2011).

Opioid analgesics are classified as strong, moderate, or weak agonists. The prototypical potent opioid agonists are morphine, hydromorphone, meperidine, methadone, and fentanyl. The designation as "strong" opioid agonists reflects not only their analgesic characteristics but also their abuse liability and their ability to cause respiratory depression. These opioid agonists are used to treat moderate to severe pain. Due to their abuse potential, most opioid agonists are classified as controlled substances by the United States Drug Enforcement Administration (Fox, Hawney and Kaye 2011).
1.3.2 Mechanism of Action

Opioids bind to the opioid receptors and activate G proteins. Opioid receptors are widespread in the central nervous system, the peripheral nervous system, and other tissues, including the immune system. Receptor sites in the central nervous system are involved with the processing of the affective and suffering aspects of pain perception. These receptor sites include the cortex, central gray medial thalamus, amygdala, limbic cortex, midbrain, and spinal cord. Presynaptic receptors are both excitatory and inhibitory, while postsynaptic receptors are only inhibitory (Crain and Shen, 1990). Opioids can bind to both presynaptic and postsynaptic receptors. Opioid binding to presynaptic opioid receptors decreases adenylate cyclase activity, inhibits calcium channels that are voltage-sensitive, and decreases the release of neurotransmitters such as glutamate, serotonin, norepinephrine, acetylcholine, and substance P. Opioid activation of postsynaptic receptors leads to an increase in the outward conductance of potassium, hyperpolarization, and a corresponding decrease in neural transmission (Fox et al., 2011).

1.3.3 Pharmacokinetics of Heroin and its Metabolites

During the synthesis of heroin, morphine molecules are acetylated in an excess of acetic anhydride at high temperatures. Morphine is a natural alkaloid harvested from the latex of Papaver somniferum poppies; opium latex may contain other alkaloids such as papaverine, codeine, noscapine, and thebaine. The chemical addition of the ester groups gives heroin an increased lipophilicity compared to morphine. Thus, heroin may pass through the blood-brain barrier much faster then morphine. The lipophilicity of heroin contributes to a more intense pharmacodynamic effect with a more immediate onset of heroin compared to morphine. Heroin shows a lower opioid receptor affinity than its metabolites that lack conjugates at the 3-hydroxyl group (i.e., 6-monoacetylmorphine, morphine, morphine-6-glucuronide). Heroin is therefore often considered a pro-drug that mainly acts through its metabolites. The ionization constant (pKa) of heroin is 7.6. An average of 40% of heroin will be in a non-ionized form at
physiological pH and will be accessible for membrane transport. Morphine, in comparison, has a pKa of 9.4. The binding capacities of heroin and morphine to serum albumin or erythrocytes are comparable at approximately 20-40% (Rook et al., 2006b).

The metabolism of heroin is shown in Figure 1.1. Heroin is rapidly hydrolyzed to 6-monoacetylmorphine in human plasma and finally into morphine. Subsequently, glucuronides are conjugated to the 3- and 6-positions of morphine. Morphine glucuronides are hydrophilic compounds that are mainly excreted in urine. Following intravenous administration, approximately 70% of the total heroin dose can be recovered in urine, mainly as conjugated morphine (55%). Different types of esterases catalyze the hydrolysis of heroin and 6-monoacetylmorphine. Esterases are present in tissues and in the circulation. Glucuronidation is catalyzed by uridine 5’-diphosphate-glucuronosyltransferases (UGTs). The N-demethylation of morphine into the minor metabolite normorphine is mediated by cytochrome P450 enzymes 3A4 and 2C8 (Rook et al., 2006b).
Figure 1.1: Metabolism of heroin
This figure shows the metabolism of heroin to 6-monoacetylmorphine, morphine, and morphine glucuronides. Enzymes and their tissue locations in this metabolic pathway are also presented.

After intravenous drug administration, heroin blood levels decline rapidly and become undetectable after approximately 10-40 minutes. Estimates of the volume of distribution of heroin range between 60-100 L. The half-life of heroin is about 1.3-7.8 minutes. Estimates of the mean heroin clearance range between 128-1939 L/hour, which far exceed the average renal and hepatic blood flow at 80 L/hour and 60 L/hour, respectively. This indicates that heroin is metabolized primarily in peripheral tissues and in the circulation. The high rate of clearance of heroin from plasma is mainly attributed to the rapid elimination by esterases and spontaneous hydrolysis of heroin in the basic environment of body fluid. Notably, heroin is virtually fully converted into its metabolites before excretion in urine (Rook et al., 2006b).

After the first 0.7-2.7 minutes following intravenous heroin administration, maximal concentrations of 6-monoacetylmorphine are reached. Having a higher receptor affinity than its
precursor heroin, 6-monoacetylmorphine is also very lipophilic. It is thought to be responsible for all of the acute effects following heroin administration. Levels of 6-monoacetylmorphine decline somewhat slower than heroin levels. The half-life is estimated to range between 5.4-52 minutes and the clearance ranged from 564-607 L/hour. 6-monoacetylmorphine can be detected in plasma for 1-3 hours, following heroin injection, and it is detectable in urine for approximately 1.2-4.3 hours following intravenous heroin administration (Rook et al., 2006b).

The formation of morphine following heroin administration occurs rapidly. Maximal concentrations of morphine can be measured between 3.6-8.0 minutes after a dose of heroin. Morphine, as a metabolite of heroin, has a half-life that ranges between 100-280 minutes. This half-life is comparable to that found when morphine is administered, suggesting that the formation of morphine from its precursor heroin is not the rate-limiting step in the metabolism of morphine after heroin administration. The morphine glucuronide, M6G, does not easily pass the blood-brain barrier, and pharmacodynamic effects from M6G are only evident after a significant accumulation in plasma. The other morphine glucuronide, M3G, lacks intrinsic opioid activity. Following heroin administration, the half-lives of the morphine glucuronides ranged between 0.7-5.1 hours. These estimates are comparable to the results of morphine pharmacokinetic studies. Enterohepatic cycling maintains a long circulation time for morphine and morphine glucuronides. Following excretion in bile, morphine glucuronides are hydrolyzed into morphine in the digestive tract by glucuronidase enzymes. It is thought that the contribution of enterohepatic cycling to the total bioavailability of morphine is considerable; following blocking of the digestive tract glucuronidase enzymes, the bioavailability of oral M6G declined by 65% (Rook et al., 2006b).

A study measuring the half-life of heroin and its metabolites in young, male Sprague-Dawley rats that had heroin administered versus non-heroin administered, control rats found high concentrations of heroin in the liver during the first 5 minutes post-injection in control rats,
whereas the level of heroin in rats with a history of heroin administration declined in the first 2 minutes following injection. In brain tissue, control rats that did not have a history of heroin administration showed heroin levels decreasing within 2 minutes. In the heroin administered rats, brain levels were 97-99% over the first 10 minutes. Thus, this study demonstrates that heroin metabolism differs significantly between heroin administered experimental rats and control rats. The tissues of experimental rats maintained a steady supply of heroin for the first 0-20 minutes post-injection. The pattern of metabolism in control rats indicated that approximately 5 minutes are required for the deacetylation of heroin in liver and brain tissue (Cohn, 1973).

### 1.4 Neural and Cellular Adaptations to Opioid Exposure

Several neuroadaptions develop in response to chronic opioid exposure. These neural and cellular adaptations are hypothesized to be critical for the expression of the major features of opioid addiction: tolerance, withdrawal, and processes that contribute to compulsive drug use and relapse. Potential cellular and neural mechanisms mediating opioid dependence have been thoroughly reviewed by Williams et al., 2001. This discussion will highlight some of the proposed mechanisms that may underlie the transition to opioid dependence. Cellular and neural adaptations occur at different levels in the nervous system including desensitization at the mu-opioid receptor (MOR) itself, cellular desensitization in opioid-sensitive neurons, and synaptic plasticity in opioid-sensitive nerve networks. Receptor desensitization is the waning responsiveness of the receptor in the face of persistent stimulation (Gainetdinov et al., 2004; Lefkowitz, 1998). Receptor desensitization involves the mechanisms of receptor regulation and internalization, whereas cellular desensitization to opioids includes the upregulation of cAMP/PKA and cAMP response element-binding signaling. The complexities of the neural networks or systems adaptations include some candidate neuropeptide systems that interact with MOR sensitive neurons. Also implicated in the neuropathology of opioid addiction are the
synaptic forms of learning, such as long-term potentiation and long-term depression (Christie, 2008).

Opioid tolerance and addiction are also hypothesized to be mediated by the activation of MORs in the mesolimbic dopaminergic reward pathway, such as the ventral tegmental area (VTA), the hippocampus, and the nucleus accumbens (NAc) (Le Merrer et al., 2009). Direct injection of endomorphins and morphine into the nucleus accumbens and hippocampus mediates both self-administration and conditioned place preference in animal models (Van Ree and de Wied, 1980; Zangen et al., 2002; Tang et al., 2005). Behavioral studies have further clarified the central role of MOR in mediating the rewarding effects of opioid drugs. Both wild-type and delta opioid receptor (DOR) knockout (KO) mice self-administered morphine injected into the VTA, but MOR KO mice did not (Chefer and Shippenberg, 2008; Contarino et al., 2002; Kieffer and Gavériaux-Ruff, 2002).

Chronic exposure to opioid agonists has been demonstrated to cause pronounced changes in the neuronal structure and synaptic organization of the brain (Robinson and Kolb, 2004). These lasting morphological and synaptic alterations may contribute to drug craving, compulsive drug use, and tolerance to the analgesic and rewarding properties of opioids (Christie, 2008). In dopaminergic neurons found in the VTA, chronic morphine administration reduced the overall size of neurons (Sklair-Tavron et al., 1996). Repeated opioid exposure also significantly led to less branching and fewer dendritic spines on medium spiny neurons in the shell of the nucleus accumbens and on pyramidal cells in both the prefrontal and parietal cortex (Robinson and Kolb, 1999). Morphological changes and the reorganization of synaptic connections in relevant neural circuits suggests that opioids may alter the processing of learning, memory, and other behavioral functions. Chronic exposure to opioid drugs might alter important neural connections that underlie drug-seeking behaviors. In the addiction-susceptible Lewis rat strain, morphine self-administration led to a decrease in the size and branching of dendrites in the pyramidal cells of
the motor cortex (Ballesteros-Yanez et al., 2007). In addiction-resistant Fischer 344 rats, however, the same morphological changes in neurons were not found (Ballesteros-Yanez et al., 2008). These studies, however, did find distinct differences in the neuronal morphology between the two strains of rats prior to the administration of morphine. Lewis rats demonstrated neurons with shorter dendrites containing fewer branches and spines than in Fischer 344 rats (Ballesteros-Yanez et al., 2008). These studies led to the hypothesis that significant changes to the dendritic structure of neurons prior to or following the response to long-term opioid agonist exposure may be responsible for the vulnerability to addiction in some individuals.

In addition to its inhibitory effects on dendritic structure, morphine has also been shown to decrease the number of newly formed neurons in the hippocampus of adult rats (Eisch et al., 2000). Morphine has also been demonstrated to inhibit long-term potentiation (LTP) of excitatory synapses on CA1 neurons in the hippocampus and γ-aminobutyric acid (GABA) releasing synapses in the VTA (Nugent et al., 2007). The hippocampus may play an important role in contextual conditioning, as well as the association of cues by which memories may be retrieved (Robbins et al., 2008). Electrophysiological and behavioral evidence suggest that the hippocampus mediates attention to contextual cues that are relevant to the formation of drug-related memories (Pennartz et al., 1994;Floresco et al., 2001). Recent evidence also suggests that the hippocampus may be important for the integration of the temporal aspect of memories (Deng et al., 2010). The inhibition of hippocampal neurogenesis by chronic morphine exposure may lead to deleterious effects on the functioning of this brain region. Interestingly, the role of MOR in mediating the effects of chronic opioids on the hippocampus was confirmed by experiments demonstrating that the maturation and neurogenesis of neurons in the granule cell layer were increased in MOR KO mice (Harburg et al., 2007).

The role of LTP is associated with the phenomenon of synaptic plasticity. Synaptic plasticity has been implicated as a potential cellular mechanism by which the brain can encode...
experiences and activity-dependent events (Cooke and Bliss, 2006). Substances of abuse, including opioids, influence synaptic plasticity that underlies both learning and memory in neural systems critical for the development of substance dependence (Hyman et al., 2006). Adaptations following chronic opioid use can influence LTP and long-term depression (Kauer and Malenka, 2007; Malenka and Bear, 2004). After chronic, intermittent morphine treatment, LTP at excitatory synapses onto CA1 hippocampal neurons was greatly inhibited (Williams et al., 2001), but this effect could be reversed by acute morphine treatment (Pu et al., 2002). This effect was suggested to be due to elevated cAMP signaling during withdrawal; because it could be blocked by PKA inhibitors.

1.5 Behavioral Models of Addiction

Although there is much cellular and anatomical evidence for the effects of chronic opioid administration on neural systems, behavioral models that mimic the symptoms of opioid dependence are needed. Addiction research is challenged by the limitations of animal models. It has recently become possible to model some aspects of addiction in animals (Deroche-Gamonet et al., 2004). However, it remains a great challenge to develop experimental models that mimic the compulsive drug-taking behaviors of addicted, social, and free-living human beings.

The phenomenon called "drug reinforcement" or "drug reward" has been shown to consist of a considerable number of clearly distinguishable components. Although the terms "reinforcer" and "reinforcement" were originally coined by Ivan Pavlov (Pavlov, 1941) to describe laboratory procedures used to reinvigorate the conditioned responses that had weakened upon repeated presentation of the conditioned stimulus alone, it was actually Burrhus Skinner who used the term "positive reinforcer" to describe a stimulus that increased or strengthened the behavior that led to its presentation (Skinner, 1953). In behavioral models, "reinforcement" either denotes the operation (i.e., the delivery of consequences when a response occurs). When
applying these definitions to drug consumption, it can be argued that drug injections technically function as positive reinforcers in typical drug self-administration studies because responding produces them (Catania, 2007). Interestingly, the term "reward" does not have a strict operational definition like that of "reinforcement," although it is often used to denote the same phenomenon. In contrast to the term "reinforcer," "reward" always carries a positive connotation and often refers to a stimulus that is considered good by the experimenter. The positive value of a reward is thus thought to produce positive reinforcement in operant conditioning paradigms. Additionally, sometimes "reward" is also used to denote the process of positive "reinforcement" (Catania 2007).

Beginning with the pioneering study Weeks, 1962, the field of drug addiction research has adopted operant conditioning approaches to assess the abuse liability of drugs. In such models, a laboratory animal is trained to associate an operant response (i.e., a lever press, lick or nose-poke at a spout) with the delivery of drug, usually by the intravenous route through an automated delivery system. Since delivery of a drug is made contingent upon the response of the animal, this type of drug administration is called "contingent administration" or "self-administration," in contrast to "non-contingent" administration during which the animal receives the drug passively by the experimenter without having to emit a response (i.e., without having to "work for drug"). Cardinal et al., 2002 identified six components that constitute reinforcement: 1) knowledge about the stimulus-response and action-outcome contingencies; 2) the incentive value or goal status of the reinforcer; 3) the hedonic value of the reinforcer as it is experienced directly; 4) the effects of any conditioned stimuli associated with the reinforcer to promote responding via a process variously called "Pavlovian-to-instrumental transfer (PIT)" or the "incentive salience of conditioned stimuli; 5) stimulus response habits, and 6) the effects of discriminative stimuli which can signal the instrumental contingency currently in force.
The modes of drug administration used to mimic chronic drug abuse are either intermittent and contingent (i.e., self-administered voluntary) or non-contingent (i.e., administered to the subject by experimenters). These modes of drug administration can influence measures of drug reinforcement in animals. Most researchers argue that, with respect to face validity, intermittent drug administration mimics human drug abuse patterns much better than continuous or experimenter-administered drug delivery. The most commonly used animal models of dependent drug consumption are operant conditioning experiments, in which the subject is given the opportunity to emit a response to obtain an intravenous infusion of drug. In such models, the rate of responding is the primary measure of the reinforcing effect and the abuse liability of the drug. Most often rats are used as the experimental subjects, but primates can also be employed. Researchers who have experience with both rats and monkeys often comment that high rates of response to the same drug of abuse are much harder to elicit from rats than from primates (Zernig, 2007). To increase the overall signal size of the dependent variable (i.e., response rate) researchers often give the animals only limited access to the drug, typically 1-3 hours per day (Zernig et al., 2007). Researchers also tend to increase the number of responses required for drug delivery, which brings operant response under the control of a schedule of reinforcement rather than the acute effects of drug, which enables the measurement of "drug-seeking" more than titration of drug levels by the animal. The response requirement for delivery of a drug can also be varied. At one end, each response can be followed by drug delivery (i.e., a fixed ratio (FR) 1 continuous reinforcement schedule. The FR1 schedule weights the contribution of acute, direct pharmacological effects of the drug as opposed to drug "reinforcement," which by definition requires multiple exposures and associative learning. It can be argued, however, that FR1 schedules, by requiring animals to give an "all-or-none" response, are particularly good for determining whether a drug is reinforcing or not. Thus, FR1 schedules are preferred to investigate whether within-session titration of drug levels occurs, but are much less useful when
trying to examine the true reinforcing effects of a drug (i.e., the incentive value of a drug or the incentive salience of drug-associated stimuli) (Ahmed and Koob, 2005; Crespo et al., 2006; Panlilio et al., 2003; Tsibulsky and Norman, 1999; Tsibulsky and Norman, 2005). In intermittent schedules of reinforcement, the subject must emit several responses to elicit a reinforcer. When FR schedules of reinforcement are utilized, rats are commonly trained to emit a maximum of only 5-10 responses to reach reinforcement (i.e., FR5 or FR10); whereas monkeys are capable of fulfilling response requirements of up to 30-100 (i.e., FR30-FR100) or even higher (Zernig et al., 2007). Rats, however, are also capable of completing increased response ratios (Salamone et al., 2005; Correa et al., 2003).

A special type of an intermittent schedule of reinforcement is the progressive ratio (PR) schedule. In a PR schedule, the subject must emit increasingly more responses to elicit each subsequent drug-delivery (i.e., 1 response for the first heroin infusion, 2 responses for the second heroin infusion, 4 to the third, 6 to the fourth, 9 to the fifth). Most currently used PR schedules use exponentially increasing response requirements. At some point in the schedule, the subject will stop responding to the drug stimulus. This point is known as the "breaking point" or "breakpoint" and is defined as the response requirement at which responding fails (Richardson and Roberts, 1996). Typically, breakpoint refers to the last completed response requirement or the the number of reinforcers (i.e., drug infusions) elicited during a session. PR schedules are much less vulnerable than FR schedules to acute rate-decreasing effects of the drug, such as a rate-decreasing effect due to impairment of motor output or a reflection of a self-titration process (Ahmed and Koob, 2005; Tsibulsky and Norman, 1999). For researchers, the PR schedule has good face validity with respect to the incentive value of the drug. PR schedules, however, may be susceptible to confounding pharmacological effects. Due to the continuously increasing inter-infusion intervals that are inherent in the PR schedule, at any constant unit dose the drug brain concentration at which the response requirement is fulfilled is constantly changing. Opioids at
higher doses, produce sedation, thus impairing motor output, which is important in PR schedules that depend on the subject's ability to sustain responses for increasingly longer durations (Zernig et al., 2007).

More sophisticated animal models have been developed to take into account the availability of alternative reinforcers in the environment. These models have been developed to try to mimic the human situation in which a number of reinforcers are available (Ahmed and Koob, 2005; Ahmed, 2005). The convention in the field is to refer to these other, non-drug reinforcers as "alternative" reinforcers. In its most extreme experimental form, a large number of non-drug reinforcers are introduced into the experimental setting. This altered environment has been given the term "environmental enrichment" (Diamond et al., 1972; Bennett et al., 1969).

The escalation of drug use is a hallmark of substance dependence and has traditionally been interpreted as reflecting the development of tolerance to the effects of a drug. On the basis of animal behavioral data, several studies suggest that an escalation in drug use may not be based on tolerance, but rather may be an indication of sensitization to the reinforcing effects of a drug, reward allostasis, an increase in the incentive salience of drug-associated stimuli, an increase in the reinforcing potency of the drug reinforcer relative to alternative reinforcers, or the formation of habitual drug-taking. Apparent discrepancies in animal experiments are that sensitization to reinforcement has been found more often for psychostimulants than for opioids, and that tolerance to the reinforcing effects of a drug has been observed more often for opioids than for cocaine. These seemingly contradictory outcomes may be resolved by the finding that cocaine levels are more tightly regulated at submaximal reinforcing levels than are opioid levels. As a consequence, animals self-administering opioids are more likely to have a drug intake at higher threshold doses than animals self-administering cocaine. Higher threshold doses may render the development of tolerance to opioids more likely than tolerance to psychostimulants (Zernig et al., 2007).
In terms of pharmacology, the development of sensitization in drug dependence models seems counterintuitive. Sensitization contradicts the well-known and extensively cited fact that upon repeated administration, most in vitro, in vivo, and ex vivo systems show tolerance to the effects of the tested drug, in particular to the effects of morphine or heroin (Cox, 1993; Cox and Werling, 1991; Sim et al., 1996; Sim-Selley et al., 2000). Significant tolerance to opioid effects can even develop after only a single administration of a high dose, and 100-fold rightward shifts in opioid dose-effect curves have been demonstrated in certain experimental conditions. Clinical evidence, however, suggests that escalation of drug use is predominantly based on an increase in the frequency of "intoxication" events rather than on an increase in the dose taken at each "intoxication" event (Zernig et al., 2007). Notably, MOR agonists have a much greater potency in tests of drug reinforcement than in analgesia, suggesting that in order to inhibit a MOR agonist's reinforcing effect, one needs to block a larger fraction of MOR than to inhibit its analgesic effect. As an example, alfentanil in rhesus monkeys has a high efficacy in drug reinforcement with only 0.3% of the MOR population needed to induce half-maximal effect, versus the 9% of the MOR population that was needed for half-maximal efficacy in a test of anti-nociception (50°C warm-water tail withdrawal assay). This suggests that reinforcement mediated by the MOR is vastly amplified through its own signaling cascades or other events downstream of the MOR (Zernig et al., 1997).

Although the development of tolerance is more likely with opioids than with stimulants, a recent study demonstrated that rats given extended access to heroin will escalate their intake over time. Specifically, rats given 12 and 23 hours of continuous heroin access on an FR1 schedule escalated their heroin intake, while rats given only 1 or 6 hours of heroin access did not escalate their intake of the drug. This study concluded that a continuous 12 hour period of heroin self-administration may be the optimal access time for producing escalation of heroin intake (Vendruscolo et al., 2011).
In addition to escalation of drug intake as a model of substance dependence, conditioned place preference (CPP) models have also been used to assess "addiction-like" behavior in animals. CPP experiments expose animals to a distinctive environment in the presence of drug. If a subject finds the substance of abuse positively reinforcing, the subject will show preference for that environment when later given a choice (Tzschentke, 2007). In a study by Bals-Kubik et al., 1993, unilateral microinjection of the MOR agonist D-Ala\textsubscript{2}, N-MePhe\textsubscript{4} Gly-ol-enkephalin (DAMGO) into the ventral tegmental area (VTA) of male Sprague-Dawley rats resulted in dose-dependent preferences for the drug-associated place. However, intracranial injections of DAMGO into the terminal projection sites of VTA dopaminergic neurons, the nucleus accumbens, and the medial prefrontal cortex did not have an effect on the rats' place preference. This study concluded that the rewarding effect of DAMGO is specifically associated with the activation of MOR in the VTA.

Another model of "addiction-like" behavior is the reinstatement model of drug relapse. Non-contingent priming injections either of a drug of abuse, or re-exposure to drug-paired cues, can reinstate the response behavior (i.e., lever presses, licks, or nose-pokes) following extinction of the drug-reinforced behavior (Shaham et al., 2003). In a study by Kuntz-Melcavage et al., 2009, behavioral data showed an increase in heroin-seeking and goal-directed behavior after a 14 day abstinence period when the rats were returned to the environment where they had previously received heroin. A subsequent genomic study revealed that several genes important for neuroplasticity were differentially regulated in these "reinstated" rats.

A recently developed rat model by Deroche-Gamonet et al., 2004 incorporates several hallmarks of substance dependence, including compulsive drug-seeking, willingness to work for drug, and persistence in drug-taking despite adverse consequences. These "addiction-like" behaviors emerge only after a prolonged period of self-administration of cocaine. Additionally, this study demonstrated differences in interindividual vulnerability, with only 17% of subjects
demonstrating "addiction-like" behaviors for all criteria measured (Deroche-Gamonet et al., 2004).

Although the Deroche-Gamonet et al., 2004 study demonstrated that "addiction-like" behaviors could be observed in rodents, the behavioral and pharmacological study of drugs of abuse remains a tremendous task that must combine diverse research disciplines such as psychology, pharmacology, behavioral science, and neurochemistry. Since drugs of abuse are pharmacologic agents, it is important to utilize the principles and models used in pharmacology. Many experimental approaches, however, do not take into account pharmacokinetic factors and the relative contribution of various components of apparent drug reinforcement. The lack of pharmacologic incorporation in behavioral studies often leads to data that are not interpretable from either a psychological or a pharmacological perspective (Zernig et al., 2007).

1.6 Rationale and Hypothesis

The objective of this dissertation is to develop a behavioral model to serve as a platform for investigating potential underlying neural substrates involved in the development of opioid addiction. My central hypothesis is that the transition from opioid use to opioid addiction is accompanied by differential regulation of mu-opioid receptor interacting proteins.

Criteria for "addiction-like" behavior in rats have been previously established for cocaine (Deroche-Gamonet et al., 2004). My working hypothesis for Chapter 2 of this dissertation is that similar behaviors can be observed in rats following the intake of opioid drugs.

The neurobiological mechanisms underlying opioid addiction remain unclear. In Chapter 3, I will test the hypothesis that mu-opioid receptor interacting proteins are differentially expressed in rats demonstrating high "addiction-like" behaviors for heroin. These proteomic changes may reflect molecular correlates of the development of opioid addiction.
This project is innovative; because it uses a novel model for opioid "addiction-like" behaviors in rats to enable proteomic analysis in key brain regions that mediate reward and addiction. This knowledge may be important for the generation of new opioid therapeutics that promote analgesia but minimize the development of addiction.
Chapter 2

Evidence of Addiction-like Behavior in Rats Self-administering Heroin

2.1 Abstract

Criteria for "addiction-like" behaviors in rats have been established for cocaine (Deroche-Gamonet et. al., 2004), and this study evaluated whether similar behaviors can be observed in rats following the intake of an opioid drug. I used a rat model of heroin self-administration that included key features of human addiction. Thus, responding for heroin was examined in 43 male Sprague-Dawley rats given the opportunity to intravenously self-administer heroin (0.06 mg/0.2 mL infusion) for 27 sessions. Criteria for addiction-like behaviors included greater drug seeking during periods of signaled non-availability, an increased willingness to work for drug when tested with a progressive ratio schedule of reinforcement, and greater lack of satisfaction and increased drug-seeking during timeout periods. My study revealed that although all rats self-administered approximately the same amount of heroin, only 9.3% of rats met all criteria for "addiction-like" behaviors. Additionally, behavior during the initial timeout and signaled non-availability periods (onset activity) was found to be an early predictor of the severity of "addiction-like" behavior that a rat would develop by the end of the study.

2.2 Introduction

Opioid addiction is a devastating chronic disorder characterized by compulsive drug seeking and loss of control over drug use. According to the 2010 National Survey on Drug Use and Health, there were 140,000 first-time heroin users aged 12 or older in the United States. In 2010, the number of persons who had heroin dependence or abuse increased from 214,000 in 2002 to 359,000. The estimated cost of heroin addiction in the United States in terms of medical care, lost productivity, crime, and social welfare was $21.9 billion at the end of the last decade.
(White et al., 2005b). Between 2002 and 2007, there was a 20% increase in the misuse of prescription pain medication (Manchikanti et al., 2012). The economic cost of non-medical use of prescription opioids in the United States totals more than $50 billion annually (Abuse, 2011).

A major goal of drug abuse research is to understand the underlying mechanisms of addiction. Previous research has examined the neurobiological effects of opioid use. These studies have identified changes in brain physiology and morphology that occur with exposure to heroin and other opioid drugs (Hyman et al., 2006;Connor et al., 2004;Christie, 2008). It remains unclear, however, which drug-induced modifications underlie the development of opioid addiction. Opioids activate the reward pathway by binding to the mu-opioid receptor (MOR). The MOR, a G protein-coupled receptor (GPCR), is part of a signaling complex in which protein-protein interactions regulate signal transduction following opioid exposure (Bailey and Connor, 2005;Christie, 2008). It is my hypothesis that identifying and characterizing novel MOR interacting proteins may help to elucidate the biological mechanisms involved in the development of opioid addiction.

Addiction, however, is defined not by just the taking of drugs, but by compulsive use of the drug despite negative consequences for the drug user. This disordered behavior occurs in only a small percentage (15-17%) of those exposed to drugs (Deroche-Gamonet et al., 2004). The transition from casual drug use to addiction in humans is complicated. One of the fundamental challenges of addiction research has been how to model this transition to addiction in animals (Hyman et al., 2006). According to the fourth edition of the American Psychiatric Association's Diagnostic and Statistical Manual of Mental Disorders, "the essential feature of dependence is a cluster of cognitive, behavioral, and physiological symptoms indicating that the individual continues substance use despite significant substance-related problems." Substance dependence affects many aspects of the addict's life and involves a failure to fulfill major obligations at work, school, or home. Substance dependence also involves a lack of participation in important social,
occupational, and recreational activities. In substance dependence, there is a high motivation for drug, as evidenced by a great deal of time spent obtaining or using the drug and recovering from drug-taking periods. There is also persistent desire for continued drug use, despite recurrent legal, social, physical, or psychological problems (DSM-IV-TR, 2000).

Until recently, animal models of addiction have focused on a single feature of substance dependence (i.e., tolerance, dependence, withdrawal, motivation for drug taking, or relapse). New models, however, have attempted to obtain more comprehensive evidence of an addiction-like state. In 2004, Deroche-Gamonet et al. demonstrated that "addiction-like" behaviors develop and can be studied in rats self-administering cocaine. "Addiction-like" behavior in this study was defined by three features: 1) Increased drug seeking in the absence of drug availability; 2) Increased motivation to work for procuring drug; 3) Persistent drug use despite adverse consequences in the form of punishment (Figure 2.1). Rats were assigned an addiction score which was determined on the basis of their performance on tests measuring these three "addiction-like" behavioral features.

Figure 2.1: Criteria for "addiction-like" behaviors in the Deroche-Gamonet et al. study
Rats were assessed for their willingness to work to procure drug, their drug-seeking during periods of signaled non-availability, and their persistence in drug-taking despite harm in the form of electric-shock punishment. If a rat scored in the 66th-99th percentile in a behavioral test (PR, SNA, or ES) were considered positive for that "addiction-like" behavior.
Following an extended period of cocaine access, a small percentage (17.2%) of rats demonstrated "addiction-like" behaviors. Interestingly, "addicted" and "non-addicted" rats did not show differences in total cocaine intake; thus enabling the identification of biological changes that occur specifically with the development of "addiction-like" behaviors in vulnerable individuals (Deroche-Gamonet et al., 2004).

Using this approach, I examined whether "addiction-like" behaviors would occur in rats following prolonged heroin self-administration. As of yet, no attempts have been made to use this model to assess the biological changes that occur following heroin self-administration. In this modified paradigm, "addiction-like" behavior was also defined by three features: 1) Increased drug seeking during signaled non-availability; 2) Increased motivation to work for drug when tested on a progressive ratio schedule of reinforcement; and 3) Lack of satisfaction with drug and increased heroin-seeking behavior during timeout periods (Figure 2.2). Following the paradigm of Deroche-Gamonet et al., 2004, 43 naive male Sprague-Dawley rats were allowed one daily 120 minute self-administration (SA) session for a total of 27 days (Figure 2.3 A).
Figure 2.2: Criteria for "addiction-like" behavior in heroin self-administering rats

Rats were assessed for their willingness to work to procure drug, their drug-seeking during periods of signaled non-availability, and their impulsivity of drug-seeking behavior. If a rat scored in the 75th-99th percentile in a behavioral test, the rat was considered positive for the behavior and received a point. Thus, rats could score a 0, 1, 2, or 3 out of the 3 "addiction-like" behaviors measured.

Each SA session (Figure 2.3 B) consisted of three 40 minute drug periods interspersed with two 15 minute periods of signaled non-availability (SNA). During each 40 minute drug period, the rats were allowed to self-administer heroin on a fixed ratio (FR) schedule of reinforcement, in which a set number of licks on the empty "active" spout—(either 1, 5, or 10 licks as varied across the SA training) elicited one 0.06 mg/infusion intravenous infusion of heroin.

Responding on the control empty "inactive" spout was without consequence. During the drug periods, the completion of the FR and delivery of one infusion of heroin was followed by a 34 second timeout (TO) period, in which additional licks at the "active" spout would not contribute to the completion of the required FR. Thus, a rat's licking at the "active" spout during timeout periods was used as a measure of impulsivity in heroin-seeking behavior.
Figure 2.3: Self-administration (SA) training schedule and SA session timing
A. Schematic overview of the SA training schedule which consisted of 27 sessions of a fixed ratio (FR) schedule of reinforcement, interspersed with 4 progressive-ratio (PR1-4) tests and 2 extinction/reinstatement tests (Rein1-2). B. Each SA session consisted of three 40 minute drug periods at a fixed ratio schedule, separated by two 15 minute periods of signaled non-availability. Each drug infusion was delivered intravenously over a period of 6 seconds, followed by a 34 second timeout period. During the timeout, additional licks would not elicit another infusion of heroin.

To measure motivation to work for procuring drug, I conducted four progressive ratio (PR) tests at different time intervals throughout the 27 sessions of SA training (Figure 2.3 A). During progressive ratio testing, rats were required to make progressively more responses on the empty "active" spout to obtain the next infusion.

As in the paradigm used by Deroche-Gamonet et al., 2004, rats were assigned an addiction score which was determined on the basis of their performance on tests measuring our three "addiction-like" behavioral features (Figure 2.2). I ultimately aimed to determine whether "addiction-like" behaviors emerged with heroin as occurred in the cocaine study. I also wanted to determine when, in the course of the study, "addiction-like" behaviors emerged, and if any
behavioral features predict which rats would ultimately show a higher number of "addiction-like" behaviors than others.

2.3 Experimental Procedures

2.3.1 Subjects

Naïve male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) 60 days of age at the beginning of the experiment were used. This study was conducted in 2 replications using a total of 48 subjects (n=24 for each experiment). Five subjects were eliminated during the course of the study due to loss of catheter patency or unexpected death. Data from the forty-three rats that completed the study in full are reported. Rats were housed individually in hanging wire mesh cages in a colony room with temperature, humidity, and ventilation controlled automatically. Subjects were allowed ad lib access to a nutritionally complete commercial laboratory rodent chow (Laboratory Rodent Diet 5001, PMI Feeds, Richmond, IN) and water, except where noted otherwise. The rats were maintained on a 12 hour light-dark cycle.

2.3.2 Catheter Construction and Implantation

Intrajugular catheters were custom-made in the Grigson laboratory as described previously (Twining et al., 2009). Rats were anesthetized using an intraperitoneal injection of ketamine/xylazine, and catheters were implanted into the jugular vein as described previously (Twining et al., 2009). After surgery, rats were allowed at least 7 days to recover. General maintenance of catheter patency involved daily examination and flushing of catheters with heparinized saline (0.2 mL of 30 IU/mL heparin). Catheter patency was verified, as needed, using 0.2 mL of propofol (Diprivan 1%) administered intravenously.

2.3.3 Intravenous Self-administration (SA) Apparatus

Each rat was trained in one of 12 identical operant chambers (MED Associates, St. Albans, VT) as described previously (Twining et al., 2009; Grigson and Twining, 2002; Puhl et al.,
Each chamber measured 30.5 cm in length, 24.0 cm in width, 29.0 cm in height, and was individually housed in a light- and sound-attenuated cubicle. The chambers consisted of a clear Plexiglas top, front, and back wall. The side walls were made of aluminum. Grid floors consisted of nineteen 4.8 mm stainless steel rods, spaced 1.6 cm apart (center to center). Each chamber was equipped with two retractable sipper spouts that entered through 1.3 cm diameter holes, spaced 16.4 cm apart (center to center). A stimulus light was located 6.0 cm above each tube. Each chamber was also equipped with a houselight (25 W), a tone generator (Sonalert Time Generator, 2900 Hz, Mallory, Indianapolis, IN), and a speaker for white noise (75 dB). Heroin reinforcement was controlled by a lickometer circuit that monitored empty spout licking to operate a syringe pump (Model A, Razel Scientific Instruments, Stamford, CT). A coupling assembly attached the syringe pump to the catheter assembly on the back of each rat and entered through a 5.0 cm diameter hole in the top of the chamber. This assembly consisted of a metal spring attached to a metal spacer with Tygon tubing inserted down the center, protecting passage of the tubing from rat interference. The tubing was attached to a counterbalanced swivel assembly (Instech, Plymouth Meeting, PA) that, in turn, was attached to the syringe pump.

Events in the chamber and collection of data were controlled online with a Pentium computer that used programs written in the Medstate notation language (MED Associates).

2.3.4 Drug Preparation

Heroin HCl was generously provided by the National Institute on Drug Abuse (Research Triangle Institute, Research Triangle Park, NC). Drug was dissolved in sterile, physiological (0.9%) saline at a concentration of 0.3 mg/mL. Each intravenous injection was a 0.06 mg/0.2 mL infusion dose delivered over 6 sec (Kuntz et al., 2008).

2.3.5 Procedure Timing

Habituation, self-administration training, and progressive-ratio testing were conducted during the light phase of the light-dark cycle (Puhl et al., 2011).


2.3.6 Habituation

Rats were water-deprived for approximately 16 hours and then were habituated to the operant chambers during two 15-minute sessions per day that occurred over the 2 days prior to the beginning of self-administration training. At the start of the habituation session, the houselight and white noise were turned on. During the habituation sessions, water was available in the right ("active") spout within the operant chamber, while the left ("inactive") spout was empty. Thereafter, rats were returned to ad libitum access to water for the duration of the study (Puhl et al., 2011).

2.3.7 Intravenous Self-administration (SA) Training Protocol

Self-administration training began immediately following the habituation phase. Each rat was trained during daily 150 minute sessions, as described previously (Belin et al., 2009; Deroche-Gamonet et al., 2004) for 27 sessions (Figure 2.4; see Appendix A for enlarged image).

![Timeline of self-administration (SA) behavioral training and experimental testing](image)

**Figure 2.4: Timeline of self-administration (SA) behavioral training and experimental testing**

SA behavioral training and experimental testing occurred over the course of 47 days, followed by live decapitation on day 48. After every 5 days of training/testing, 2 "off" days were used to allow rats to recover from heroin taking, especially following progressive-ratio testing. Abbreviations: HAB, habituation; SA, self-administration; FR, fixed ratio; PR, progressive-ratio; REIN, reinstatement, NA, drug non-availability; DECAP, decapitation.
Each 150 minute session consisted of three drug periods, separated by two signaled non-availability (SNA) periods (Figure 2.5; see Appendix A for enlarged image).

Rats were placed in the operant chambers in darkness. Immediately upon initiation of the 150 minute session, the white noise was turned on, the right and left empty spouts advanced into the chamber, and the cue light above the right spout was illuminated. Rats were then allowed to self-administer heroin (0.06 mg/0.2 mL infusion dose) for 40 minutes. The right spout was termed the "active" spout, while the left spout was termed the "inactive" spout. A fixed ratio (FR) 1 schedule of reinforcement was implemented initially (Sessions 1-3). During this time, completion of a single lick on the "active" empty spout was followed by a single intravenous infusion of heroin over 6 seconds. Drug delivery was signaled by offset of the stimulus light, retraction of the "active" spout, and onset of the houselight. The houselight remained on for a 34 second timeout (TO) period. During the TO periods, responses on the "active" spout could not elicit additional drug infusions. Responding on the "inactive" spout was without consequence throughout each 150 minute session, including the TO periods. The reinforcement schedule was increased to FR5 (Sessions 4-12) and then to FR10 (Sessions 13-27) to fully distinguish between active and inactive responding. A 15 minute SNA period followed the 40 minute drug period.

<p>| Table 2. Components of the self-administration (SA) training sessions |</p>
<table>
<thead>
<tr>
<th>Drug period 1</th>
<th>Signaled drug non-availability (SNA) period 1</th>
<th>Drug Period 2</th>
<th>SNA period 2</th>
<th>Drug Period 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infusion (INF) periods</td>
<td>Infusion (INF) periods</td>
<td>Infusion (INF) periods</td>
<td>Timeout (TO) periods</td>
<td>Infusion (INF) periods</td>
</tr>
<tr>
<td>6s</td>
<td>34s following each INF period</td>
<td>6s</td>
<td>34s following each INF period</td>
<td>6s</td>
</tr>
</tbody>
</table>

Figure 2.5: Components of the self-administration (SA) training sessions
Detailed schematic of SA training sessions showing the sequence of drug periods, signaled non-availability (SNA), and timeout (TO) periods.
during which time the cue light above the right spout was turned off, a light on the chamber wall opposite the spouts was illuminated, and the infusion pump was turned off. Responding on the "active" spout was without consequence during SNA periods. Following each self-administration training session, the rats were returned to their home cages. The number of infusions self-administered during drug periods was evaluated throughout self-administration training. In addition, responding during the early phases of FR training was compared to the final phase of FR training (i.e., Sessions 9, 13, 17 compared to Session 25). Responding was assessed similarly and independently during TO periods and across the intervening SNA periods.

2.3.8 Progressive Ratio Schedule

A progressive ratio (PR) schedule of reinforcement, as described previously (Belin et al., 2009; Deroche-Gamonet et al., 2004), was implemented periodically to test the rats' willingness to work for the drug. Thus, PR testing was conducted between the 9th and 10th, 13th and 14th, 17th and 18th, and 26th and 27th SA sessions.

<table>
<thead>
<tr>
<th>Progressive-ratio (PR) schedule of reinforcement</th>
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<tbody>
<tr>
<td>Testing schedule INF 1  INF 2  INF 3  INF 4  INF 5  INF 6  INF 7  INF 8  INF 9  INF 10</td>
</tr>
<tr>
<td>1</td>
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<td>2</td>
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<table>
<thead>
<tr>
<th>Testing schedule INF 11  INF 12  INF 13  INF 14  INF 15  INF 16  INF 17  INF 18  INF 19  INF 20</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>2</td>
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</tbody>
</table>

Figure 2.6: Progressive-ratio (PR) schedule of reinforcement

Two PR schedules of reinforcement were used in this study. Testing schedule 1 was used for PR tests 1-3, while testing schedule 2 was used for PR4. This schematic show the number of licks required to obtain each successive infusion (INF).

During PR testing, rats were placed in the operant chambers with conditions identical to those of self-administration training, except the ratio of responses required per infusion was increased after each infusion according to one of two PR testing schedules (Figure 2.6; see
Appendix A for enlarged image). For PR sessions conducted between the 9th and 10th, 13th and 14th, 17th and 18th SA sessions, testing schedule 1 was used in which the number of active responses required to receive the 1st infusion started at 10 and then progressively increased by a multiple of 10. Since responses for heroin were relatively low (e.g., compared to responding for cocaine), a different schedule was used for the last PR session. For this PR session, conducted between the 26th and 27th SA sessions, testing schedule 2 was used in which the number of active responses required to receive the 1st infusion started at 10 and then progressively increased by a multiple of 2 (Figure 2.6; see Appendix A for enlarged image).

During PR sessions, rats were allowed to self-administer heroin (0.06 mg/0.2 mL infusion) until a period of 30 minutes elapsed without receipt of an infusion. Terminal break point (i.e., the highest ratio completed during the final PR test, i.e., between the 26th and 27th SA sessions) was assessed.

2.3.9 Extinction/Reinstatement

Between the 21st and 22nd and 25th and 26th SA sessions, a 90 minute extinction-reinstatement (REIN) session was conducted in the same environment in which the rats had previously received heroin. During the first 45 minutes of the REIN session, 10 active responses caused the spouts to retract and a tone to sound for 20 seconds; however no drug was infused. A cue light above the active spout remained on until 10 active responses were completed. Following the initial 45 minutes, the subjects were primed with an infusion of heroin (0.06 mg/0.2 mL infusion dose). Following this "priming" dose of heroin, the spouts retracted, the cue light turned off, and tone sounded for 20 seconds. The final 45 minutes proceeded as before: 10 active responses caused the spouts to retract and a tone to sound for 20 seconds; however no drug was infused. A cue light above the active spout remained on until 10 active responses were completed. Responding during the initial 45 minutes and the 45 minutes following the heroin priming dose were assessed.
2.4 Data and Statistical Analysis

2.4.1 Persistence of Drug-Seeking in the Absence of Heroin

Persistence of drug-seeking in the absence of heroin was assessed daily by the responses at the active spout during the signaled non-availability periods of SA training. For analysis, active responses during SA sessions 9, 13, 17, and 25 were considered.

2.4.2 Willingness to Work for Heroin

The last ratio completed, or breakpoint, during a progressive ratio schedule of testing was considered as an index of motivation. The progressive ratio breakpoint was assessed during 4 independent testing sessions.

2.4.3 Lack of Satisfaction and Increased Heroin-Seeking Behavior

Impulsivity in heroin-eliciting behavior was assessed daily by measuring the responses at the active spout during the timeout periods of SA training. For analysis, active responses during timeout sessions 9, 13, 17, and 25 were considered.

2.4.4 Onset Activity

Onset activity was assessed as the sum of the normalized scores (Z-scores) for the active responses during the timeout periods of the initial drug period and the active responses during the initial signaled non-availability period during SA training. Onset activity for SA sessions 9, 13, 17, and 25 were considered.

2.4.5 Addiction-like Criteria Classification

Subjects were scored for each addiction-like behavior (i.e., persistence of drug-seeking in the absence of heroin, willingness to work for heroin, and impulsivity in heroin-eliciting behavior) independently using mean "active" responses during SA sessions 23-25 and the breakpoint infusions during the 4th progressive ratio testing session. A rat was considered positive for an addiction-like behavior when its score was in the 75th-99th percentile. Four
groups of rats were then classified according to the number of positive criteria met (i.e., 0 criteria, 1 criterion, 2 criteria, 3 criteria).

2.4.6 Addiction-like Severity Score

Addiction severity scores were generated as the sum of normalized scores (Z-scores) for each of the 3 "addiction-like" behaviors assessed at the 9th, 13th, 17th, and 25th SA sessions and PR tests 1-4 (Belin et al., 2009). The Z-score for each behavior transforms the raw behavioral measurement into units of standard deviation above or below the mean with a mean of zero and a variance of one standard deviation. The addiction score is thus distributed along a scale from −7 to 7.

2.4.7 Statistical Analysis

All data were analyzed with Stata12 (StataCorp LP, College Station, TX). Three types of analyses were conducted: analysis of variance (ANOVA), nonparametric Spearman's correlation analysis, and linear regression. Newman-Keuls post hoc tests were conducted on significant ANOVAs with \( \alpha \) set at 0.05.

2.5 Results

For humans, a diagnosis of substance abuse or dependence is made when an individual meets a number of criteria outlined in fourth edition of the American Psychiatric Association's Diagnostic and Statistical Manual of Mental Disorders. The syndrome of drug dependence has seven key symptoms. An individual must meet 3 symptoms in the same 12-month period to receive a diagnosis of dependence. Using a similar approach, Deroche-Gamonet et al., 2004 scored rats that had self-administered cocaine for a pre-defined set of "addiction-like" behaviors. In my experiments, I adopted this approach to score rats that had self-administered heroin. For this analysis, I assessed rats (n=43) that completed 27 self-administration (SA) sessions and 4 progressive ratio (PR) testing periods. The approach used to study extinction/reinstatement
behavior, as mentioned in the experimental procedures section, failed to generate reliable, orderly reinstatement behavior, and reinstatement results will not be mentioned further here. An individual was considered positive for an "addiction-like" criterion when its score for one of the three "addiction-like" behaviors was in the 75th-99th percentile. I used the total score to separate our sample of rats into four groups according to the number of positive criteria met at SA session 25. The "addiction-like" behaviors examined were active licks during signaled non-availability as a measure of drug-seeking, the highest number of infusions, or breakpoint, that rats were willing to complete to receive the drug during progressive ratio testing, and the active licks during timeout periods as a measure of lack of satisfaction and increased drug-seeking behavior. The intensity of the three addiction-like behaviors examined (Figure 2.7) was proportional to the number of criteria met by the subjects. Results showed that seeking (i.e., the number of responses emitted during signaled non-availability) increased as a function of the number of criteria met. Thus, post-hoc tests of a significant main effect of criteria, $F(3,39)=18.69, p<0.0001$, revealed greater seeking behavior by rats scoring points on 1-3 criteria with $0<1<2=3$. Results showed that working (i.e., the number of infusions, or breakpoint, during progressive ratio testing) increased as a function of the number of criteria met. Post-hoc tests of a significant main effect of criteria, $F(3,39)=14.05, p<0.0001$, revealed greater working behavior by rats scoring points on 2-3 criteria with $0=1<2<3$. Results also showed that impulsivity of drug seeking (i.e., the number of responses emitted during timeout periods) increased as a function of the number of criteria met. Post-hoc tests of a significant main effect of criteria, $F(3,39)=48.37, p<0.0001$, revealed greater lack of satisfaction and increased seeking behavior by rats scoring points on 1-3 criteria with $0<1=2<3$. There was no main effect of criteria for the number of infusions that rats self-administered during the drug periods (DP), $F(3,39)=1.86, p=0.21$. 

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Figure 2.7: "Addiction-like" behaviors in heroin self-administering rats at SA session 25
Addiction-like behaviors in rats positive for 0, 1, 2, or 3 addiction-like criteria at SA session 25 (A-C). An individual rat was considered positive for an addiction-like criterion when it scored in the 75-99th percentile for each behavior. Persistence of drug seeking, as measured by the number of active licks (heroin-associated spout) during the signaled drug non-availability period (A). Motivation for the drug, as measured by the breakpoint (highest infusion number elicited) during the progressive ratio (PR) testing conducted between SA sessions 26 and 27 (C). Impulsivity of drug-eliciting behavior, as measured by the number of number of active licks during the timeout (TO) period (C). Total heroin infusions during the drug periods (DP) of the SA session (D). 0 criteria, n=24; 1 criteria, n=10; 2 criteria, n=5; 3 criteria, n=4. ***p<0.001, **p<0.01, *p<0.05 compared to zero criteria rats.
Overall, the group that met all three criteria represented 9.3% of the entire sample (n=4). The group that met two criteria comprised 11.63% of the sample (n=5), while the group that met 1 criterion made up 23.26% of the sample (n=10). A large portion of the sample, 55.81% (n=24) did not meet any of the "addiction-like" criteria (Figure 2.8).

Figure 2.8: Distribution of rats meeting 0, 1, 2, or 3 "addiction-like" criteria
Percentage of the total population (n=43) of rats positive for 0 (n=24), 1 (n=10), 2 (n=5), or 3 (n=4) criteria.
Additionally, despite the profound difference in "addiction-like" behavior scores, rats showing zero or three "addiction-like" behaviors when challenged did not differ on intake of heroin during FR responding across the entire SA period, $F(3, 78)=0.87, p=0.77$ (Figure 2.9).

![Graph showing heroin intake across self-administration (SA) sessions](image)

**Figure 2.9: Heroin intake across self-administration (SA) sessions**

There was no main effect of criteria or session for heroin intake over the 27 SA sessions of the study.

Unlike the findings for SA session 25, the intensity of all three "addiction-like" behaviors was not proportional to the number of criteria met at SA session 17 (Figure 2.10). A significant main effect of criteria was observed for PR breakpoint infusions, $F(3,39)=6.75, p<0.001$, and for TO active licks, $F(3,39)=5.41, p<0.01$, but the main effect of criteria was not statistically significant for SNA active licks, $F(3,39)=1.10, p=0.36$, or for the number of drug period infusions, $F(3, 39)<1$. By SA sessions 13 (Figure 2.11) or 9 (Figure 2.12), no main effect of criteria was evident for any of the "addiction-like" behaviors or for the number of drug period infusions. "Addiction-like" behavior, thus, develops with experience over time.
Figure 2.10: "Addiction-like" behaviors in heroin self-administering rats at SA session 17
Addiction-like behaviors in rats positive for 0, 1, 2, or 3 addiction-like criteria at SA session 17 (A-C). An individual rat was considered positive for an addiction-like criterion when it scored in the 75-99th percentile for each behavior. Persistence of drug seeking, as measured by the number of active licks (heroin-associated spout) during the signaled drug non-availability period (A). Motivation for the drug, as measured by the breakpoint (highest infusion number elicited) during the progressive ratio (PR) testing conducted between SA sessions 17 and 18 (C). Impulsivity of drug-eliciting behavior, as measured by the number of number of active licks during the timeout (TO) period (C). Total heroin infusions during the drug periods (DP) of the SA session (D).

**p<0.01, *p<0.05 compared to zero criteria rats.
Figure 2.11: "Addiction-like" behaviors in heroin self-administering rats at SA session 13

Addiction-like behaviors in rats positive for 0, 1, 2, or 3 addiction-like criteria at SA session 13 (A-C). An individual rat was considered positive for an addiction-like criterion when it scored in the 75-99th percentile for each behavior. Persistence of drug seeking, as measured by the number of active licks (heroin-associated spout) during the signaled drug non-availability period (A). Motivation for the drug, as measured by the breakpoint (highest infusion number elicited) during the progressive ratio (PR) testing conducted between SA sessions 13 and 14 (C). Impulsivity of drug-eliciting behavior, as measured by the number of active licks during the timeout (TO) period (C). Total heroin infusions during the drug periods (DP) of the SA session (D).

(A) SNA active licks (SA13) vs Criteria met
(B) Breakpoint infusions vs Criteria met
(C) TO active licks vs Criteria met
(D) DP infusions vs Criteria met
Figure 2.12: "Addiction-like" behaviors in heroin self-administering rats at SA session 9

Addiction-like behaviors in rats positive for 0, 1, 2, or 3 addiction-like criteria at SA session 9 (A-C). An individual rat was considered positive for an addiction-like criterion when it scored in the 75-99th percentile for each behavior. Persistence of drug seeking, as measured by the number of active licks (heroin-associated spout) during the signaled drug non-availability period (A). Motivation for the drug, as measured by the breakpoint (highest infusion number elicited) during the progressive ratio (PR) testing conducted between SA sessions 9 and 10 (C). Impulsivity of drug-eliciting behavior, as measured by the number of number of active licks during the timeout (TO) period (C). Total heroin infusions during the drug periods (DP) of the SA session (D).
I computed an addiction severity score (Belin et al., 2009) for the heroin self-administering rats that was normally distributed, centered on 0 with a variance of one standard deviation. As expected, the addiction severity score was highly correlated with each of the three "addiction-like" criteria (Figure 2.13; Spearman $R_s>0.7$, $p_s<0.001$). As such, a higher addiction severity score was highly correlated with greater seeking during signaled non-availability, increased willingness to work for drug during progressive ratio testing, and greater responding on the active operant during timeout periods. Using the addiction severity score, I assessed the relationship between the criteria met at SA session 25 (i.e. the time-point used to assign scores and group rats according to the number of criteria met) and the severity of heroin addiction-like behaviors exhibited at early and late SA sessions in the course of the study. Linear regression analysis (Figure 2.14) demonstrates that there was not a significant relationship between the number of criteria met for addiction-like criteria met at SA session 25 and the addiction severity score measured at SA session 9 or SA session 13 ($R^2s<0.1$). However, a significant relationship between addiction severity score determined at SA session 17 and the number of criteria met at the end of the study was evident ($R^2=0.31$, $p<0.001$). Again, this pattern of data reveals that "addiction" develops by experience over time, whereby 17 sessions of SA experience are required for performance on the addiction severity score to approach the terminal classification of low (0) to high (3) "addiction-like" behaviors for heroin.
Figure 2.13: Correlation between addiction severity score and "addiction-like" behaviors
Spearman rank order correlation showed that the addiction severity score at SA session 25 was highly correlated with the persistence of drug seeking during signaled non-availability (A), motivation, or the willingness to work for heroin infusions during progressive ratio testing (B), and impulsivity, or the inability to refrain from heroin seeking behavior during timeout periods (C).

Figure 2.14: Regression analysis of addiction severity score and number of criteria met
Regression analysis shows that there was not a significant relationship between the terminal number of criteria met by SA session 25 and the addiction severity score measured at SA session 9 or SA session 13 ($R^2$s $< 0.1$). By SA session 17, however, a significant relationship between addiction severity score at SA session 17 and number of criteria met at the end of the study was evident ($R^2=0.31, p<0.001$). ***$p<0.001$, **$p<0.01$, *$p<0.05$
Although "addiction-like" behaviors did not begin to emerge until SA session 17, I was interested in determining if any particular behaviors could predict which rats would meet three criteria versus zero criteria. Data was mined using correlational analysis to determine if any early behaviors were associated with the number of criteria met at the end of the study. There was a correlation with early active responses exhibited during the first drug period and first signaled non-availability period of the daily SA session and the number of criteria met. I defined this behavior as onset activity, which represents the sum of the normalized scores (Z-scores) for the active responses during the timeout periods of the initial drug period and the active responses during the initial signaled non-availability period during SA training. I measured the onset activity for SA sessions 9, 13, 17, and 25. These results showed that as early as SA session 13 (i.e., a time-point before any of the more typical "addiction-like" behaviors had emerged), there was significant difference in the onset activity of rats meeting zero and three criteria (Figure 2.15-A; ANOVAs and post-hoc Newman-Keuls tests). A correlational analysis demonstrated a relationship between onset activity at SA session 13 and number of criteria met (Spearman $R_s>0.4$, $p<0.01$) and this relationship became stronger and more highly significant by SA session 25 (Figure 2.15-B; Spearman $R_s>0.7$, $p<0.001$). While none of its components or the composite addiction severity score can predict terminal "addiction-like" behaviors, onset activity (i.e., persistence in drug seeking during the first drug period and first signaled non-availability period) can accurately predict the severity of terminal "addiction-like" behaviors in rats.
Heroin "addiction-like" behavior is specifically preceded by a combined increase in drug-seeking behavior and impulsivity of drug-eliciting behavior during the first drug active period of self-administration. Onset activity was defined as the sum of the normalized scores of active licks during the initial timeout and signaled drug non-availability periods. The 3 criteria rats (black circles) displayed a significantly higher level of onset activity at SA sessions 13 and 25 compared to SA session 9 (A). By SA session 13, 3 criteria rats also exhibited increased onset activity (A) compared to 0 criteria rats (white circles). At SA session 13 (B) and 25 (B), Spearman ranked order correlation showed a significant relationship between addiction severity score and onset activity. ***p<0.001, **p<0.01, *p<0.05 compared to 0 criteria.

Figure 2.15: Onset activity score predicts the severity of "addiction-like" behaviors
2.6 Discussion

This study demonstrates that after a prolonged period of heroin self-administration (SA), "addiction-like" behaviors can be observed in rats. This finding extends that of the Deroche-Gamonet et al. study (Deroche-Gamonet et al., 2004). In the current study, "addiction-like" behaviors were defined by: 1) a persistence in drug-seeking behavior despite the presence of signaled cue that drug was unavailable (i.e., active licks during signaled non-availability); 2) motivation for drug-taking as indicated by a willingness to work for each drug infusion (i.e., breakpoint infusions during progressive ratio testing); and 3) impulsivity of drug-seeking behavior (i.e., active licks during the timeout period following the delivery of an infusion of heroin). A rat was considered positive for an "addiction-like" behavior when its score was in the 75th-99th percentile for a behavior. Four groups of rats were then classified according to the number of positive criteria met (i.e., 0 criteria, 1 criterion, 2 criteria, 3 criteria). Although this scoring system follows the paradigm used in the Deroche-Gamonet et al., 2004 study, the cut-off for considering a rat positive for a behavior was narrowed from the 66th-99th percentile to the 75th-99th percentile. By considering rats performing at the top 1/4 of the sample in each behavior, I was able to achieve more stringent scores for each "addiction-like" behavior. In our study, only 9.3% of rats scored positive on all three measures of addiction. The Deroche-Gamonet et al., 2004 study reported 17.2% of rats meeting all three "addiction-like" criteria. This difference may correspond to the difference in cocaine and heroin or it may be attributed to the difference in the criteria used (i.e., punishment was used by Deroche-Gamonet et al., 2004 instead of impulsivity of drug seeking behavior used in this study).

The classification of rats revealed that the strength of the three addiction-like behaviors was correlated with the number of criteria met by the subjects at the terminal time-point in our study, SA session 25 (Figure 2.7). All three of the "addiction-like" behaviors, however, were not
The first "addiction-like" behaviors to emerge were motivation for drug-taking, as evidenced by the breakpoint infusions during the progressive ratio testing between SA sessions 17 and 18, and impulsivity of drug-seeking behavior, as evidenced by timeout active licks during SA session 17. Although the emergence of "addiction-like" behaviors occurred over a prolonged period of self-administration, the total number of heroin infusions self-administered by the rats remained relatively constant over the course of our study (Figure 2.9). Additionally, the number of heroin infusions was not proportional to the number of criteria met at any time-point in our experiment. This result mirrored that of the Deroche-Gamonet et al., 2004 study which observed no difference in cocaine intake in rats meeting zero or three criteria.

Although heroin intake was not significantly different across groups or SA sessions, I did find an early measure that seems to predict which rats will later evidence low (0) or high (3) "addiction-like" behavior. The onset activity score, which consisted of the sum of the active licks in the timeout periods during the first drug period and the active licks during the initial signaled non-availability period, was significantly different for 0 criteria versus 3 criteria rats as early as SA session 13. This suggests that the behavior (i.e., persistence in responding during the absence of drug) during the onset of each SA session may be an important marker of a rat's vulnerability or resistance to developing "addiction-like" behaviors. It should be noted that my use of timeout active licks as a criterion behavior is a novel index of "addiction-like" behavior. Activity during timeout periods, in a sense, is conceptually similar to activity during the signaled non-availability periods. In both cases, a cue signals to the rat that drug is unavailable. The major difference between activity during timeout periods and signaled non-availability periods is a time factor; the signaled non-availability periods are comprised of 15 minutes of a continuous signal that drug is unavailable, while timeout periods occur for a brief 34 seconds that follows the completion of a fixed ratio and delivery of one 6 second infusion of heroin. Thus, timeout periods are also
dependent on heroin intake. This factor may be pharmacologically relevant, as heroin is "on-board" during timeout periods but may be metabolized during the 15 minutes of a signaled non-availability period. The differences between the timeout and signaled non-availability periods strongly suggest that a rat's performance during these periods is indicative of separate and distinct behavioral endpoints, yet together, they predict later "addiction-like" behavior for drug.

Interestingly, the number of heroin infusions self-administered by the rats remained relatively constant over the course of the study. Further, and as mentioned, even though the amount of drug "on-board" was similar for all rats in this study, there were still differences in the severity of the "addiction-like" behaviors. Our data suggests that exposure to heroin itself does not lead to the development of "addiction-like" behaviors, but rather individual differences in a rat's vulnerability or resistance determine which subjects will demonstrate a higher degree of "addiction-like" behaviors.

This study, which used heroin self-administering rats, ultimately replicates the Deroche-Gamonet et al., 2004 paradigm, which used cocaine self-administering rats. This study demonstrates that using "addiction-like" behaviors as an index of addiction severity is a viable platform for separating "addicted" or 3 criteria rats from "non-addicted" or 0 criteria rats. This paradigm, then, is uniquely capable of distinguishing "addicted" and "non-addicted" rats regardless of the drug of abuse self-administered by subjects. Since cocaine, a stimulant, and heroin, a depressant, have different pharmacologic properties, it is significant that the same model would be able to separate rats based on similar "addiction-like" behaviors. This parallel suggests that "addiction-like" behaviors may be the same, regardless of the substance, and that this model is sensitive to the key features of "addiction."

The data presented in this chapter provide evidence that "addiction-like" behaviors can be observed in heroin self-administering rats. Additionally, the development of "addiction-like" behaviors in vulnerable subjects occurs independently of the total drug intake (i.e., both 0 criteria
and 3 criteria rats took approximately the same amount of drug throughout the study). Since heroin intake was not a factor in the classification of a rat as "addicted" or "resistant," a platform has now been established that can examine potential molecular mechanisms underlying the transition to addiction on a pharmacologically "level playing field" where all subjects received the same amount of the substance of abuse yet demonstrated different degrees of "addiction-like" behaviors. To gain an understanding of the proteomic changes that accompany the transition to "addiction-like" behaviors in rats, Chapter 3 will describe my experiments designed to measure the expression of several mu-opioid receptor interacting proteins, as well as the D₂ dopamine receptor, in key brain regions in the reward pathway.
Chapter 3

Candidate Gene Expression in Rats with "Addiction-like" Behaviors

3.1 Abstract

To gain an understanding of the molecular changes that accompany the development of behavioral criteria for "addiction-like" behavior, I analyzed the protein expression of known MOR interacting proteins and the D_2 dopamine receptor. Western blotting was used to assess protein expression in the prefrontal cortex, hippocampus, and nucleus accumbens from 14 rats from the heroin self-administration study. I found a differential expression of the D_2 dopamine receptor, spinophilin, and wntless that correlated with behavioral evidence of "addiction-like" behaviors. A decrease in the expression of the D_2 dopamine receptor in the hippocampus and nucleus accumbens was associated with a greater number of "addiction-like" criteria met. A decrease in spinophilin expression in the nucleus accumbens and a decrease in wntless expression in the prefrontal cortex were associated with a greater number of "addiction-like" criteria met. Decreased D_2 dopamine receptor expression in the hippocampus and in the nucleus accumbens was associated with greater drug-seeking during periods of signaled non-availability. Decreased D_2 dopamine receptor expression in the hippocampus was also associated with greater lack of satisfaction and increased drug-seeking during timeout periods. In the prefrontal cortex and the hippocampus, decreased D_2 dopamine receptor expression was associated with a higher progressive ratio breakpoint. Decreased spinophilin expression in the nucleus accumbens and decreased wntless expression in the prefrontal cortex were also associated with a higher progressive ratio breakpoint. These changes in protein expression may reflect molecular correlates of the development of opioid addiction.
3.2 Introduction

An estimated 11.7 million Americans use prescription opioid drugs illicitly (White et al., 2005a). The long-term effects of opioid use include tolerance and physical dependence, leading to compulsive drug seeking and abuse (Christie, 2008). Opioids activate the reward pathway by binding to the mu-opioid receptor (MOR). MOR is part of a signaling complex in which protein-protein interactions regulate signal transduction following opioid exposure (Bailey and Connor, 2005; Christie, 2008). The cellular and molecular mechanisms which underlie the transition to a state of addiction to opioids, however, remain unknown. Addiction is a state of compulsive drug use and loss over the ability to stop drug-taking despite treatment and other attempts to curb drug use. A fundamental challenge in studying opioid addiction is the limitation of animal models. Animal models have been helpful in increasing our understanding of relevant neural processes (i.e., reward-related learning); nonetheless, it is a great challenge to develop animal models that mimic the pathological drug-taking behaviors of addicted human beings (Hyman et al., 2006). Recently, it has become possible to model some aspects of cocaine addiction in animals; these aspects have been termed "addiction-like" behaviors (Deroche-Gamonet et al., 2004).

In the previous chapter, I presented the first evidence for the development of "addiction-like" behaviors in a similar rat model of heroin self-administration and behavioral testing. In this model, male Sprague-Dawley rats (n=43) intravenously self-administered heroin (0.06 mg/infusion) during 27 sessions. Rats were tested for greater drug seeking during signaled non-availability of drug, impulsivity of heroin seeking behavior during timeout periods, and for a greater willingness to work for drug during a progressive ratio schedule. A rat was considered positive for "addiction-like" behavior when it scored in the 75th to 99th percentile on one of the three behavioral tests. The data indicate that 9.3% of rats scored positive for all three "addiction-like" behaviors; 11.63% scored positive for two behaviors and 23.26% scored positive for only
one "addiction-like" behavior. The remaining 55.81% did not meet any criteria for "addiction-like" behavior. To gain an understanding of the proteomic changes that accompany the transition to "addiction-like" behaviors in our rats, I measured the expression of several mu-opioid receptor interacting proteins, as well as the D₂ dopamine receptor, in key brain regions in the reward pathway.

I focused my attention on the hippocampus, the nucleus accumbens, and the prefrontal cortex. The hippocampus is thought to play a role in contextual conditioning and the contextual association of cues by which memories may be retrieved (Robbins et al., 2008). Electrophysiological and behavioral evidence suggest that the hippocampus mediates attention to contextual cues (i.e., place memories) that are relevant to the formation of drug-related memories (Pennartz et al., 1994;Floresco et al., 2001). The nucleus accumbens has been shown to be important as an initial site of the reinforcing actions of drugs of abuse (Breiter et al., 1997). The blockade of mu-opioid receptors in the nucleus accumbens attenuates both heroin self-administration as well as the conditioned approach behavior that develops to cues associated with morphine administration (Shippenberg and Elmer, 1998; De Vries and Shippenberg, 2002). The integrity of the nucleus accumbens is necessary for opioid, as well as psychostimulant, self-administration (Zito et al., 1985). Additionally, heroin self-administration in rats is accompanied by both tonic and phasic increases of dopamine release into the nucleus accumbens (Kiyatkin et al., 1993). The nucleus accumbens core has primarily been implicated in motivated behavior and conditioning to particular cues, while the nucleus accumbens shell has been implicated in seeking behaviors (Robbins et al., 2008). The prefrontal cortex is important for decision making behavior (Schoenbaum and Shaham, 2008). The medial prefrontal cortex (mPFC) is important for enabling goal-directed behaviors (Hitchcott et al., 2007; George et al., 2007; George and Koob, 2010). Additionally, the infralimbic and ventral mPFC have been demonstrated to play roles in the expression of drug-seeking (Koya et al., 2009; Bossert et al., 2011; Peters et al., 2008). Gene
expression changes in the prefrontal cortex following extinction testing in a heroin behavioral incubation model were also recently found (Kuntz et al., 2008).

I analyzed the expression of the following MOR interacting proteins: dynamin, a MOR associated protein that has been implicated in MOR endocytosis (Whistler and von Zastrow, 1999; Whistler et al., 1999; Zhang et al., 1996); spinophilin, a scaffolding protein that modulates synaptic transmission (Charlton et al., 2008); VAPA, an ER/Golgi-resident membrane protein that mediates intracellular trafficking (Pennetta et al., 2002; Sengupta et al., 2010; Skehel et al., 2000); and WLS/GPR177, a Wnt-transport protein (Jin et al., 2010a; Jin et al., 2010b). Additionally, I used the expression of the D_2 dopamine receptor as a control for our study; since there is a well-characterized down-regulation of the D_2 dopamine receptor following exposure to opioids and other drugs of abuse (Volkow et al., 2001; Heinz et al., 2004; Maldonado et al., 1997; Johnson and Kenny, 2010; Volkow et al., 2004; Briand et al., 2008; Doehring et al., 2009; Volkow et al., 1993; Asensio et al., 2010). My results suggest that MOR interacting proteins may play a functional role during the behavioral transition to addiction. The animal model developed in Chapter 3 will ultimately enable the analysis of molecular changes that may underlie the development of substance dependence following prolonged drug use. Importantly, the use of this model allows such molecular changes to be linked to the severity of "addiction-like" behavior for heroin, rather than to the amount of drug exposure, per se.

3.3 Experimental Procedures

3.3.1 Sacrifice and Tissue Dissection

Twenty-four hours after the last self-administration session, rats were transported to a wet lab for sacrifice by decapitation without anesthesia. Brains were immediately removed and the prefrontal cortex, hippocampus, and nucleus accumbens were rapidly dissected on ice as
described previously (Heffner et al., 1980). Tissue samples were stored at −80°C until assayed via Western blotting.

3.3.2 Western Blotting

Brain tissue samples were solubilized on ice by dounce-homogenization in a detergent-based protein lysis buffer (100 mM NaCl, 20 mM HEPES, 1 mM EDTA, 1 mM dithiothreitol, 1.0% Tween20, 1 mM Na₃VO₄ with 1 Complete Mini EDTA-free Protease Inhibitor Cocktail Tablet (Roche Applied Science, Indianapolis, IN) for every 10 mL lysis buffer prepared, incubating with rocking for 15 minutes at 4°C, and centrifuging at 10,000 x g for 12 minutes at 4°C (VanGuilder et al., 2010). Supernatant protein concentrations were determined using the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL). After BCA assay quantitation, all protein samples were brought to a concentration of 2 µg/µL and diluted in lithium dodecyl sulfate sample buffer (BioRad, Hercules, CA) in volumes sufficient to load all gels from one preparation per sample to avoid aliquot-to-aliquot variation. A protein concentration of 8 µg of each subject's sample (n=14 per gel) were run in groups according to the brain region of interest (i.e., prefrontal cortex, hippocampus, or nucleus accumbens) and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 4-20% Mini-Protean TGX precast gels (BioRad). For immunoblotting, proteins were transferred using the iBlot dry transfer system (P3, 20 V for 4:50 min) to polyvinylidene difluoride (PVDF) membranes within the iBlot transfer stack (PVDF, Regular; Invitrogen, Carlsbad, CA). Membranes were stained with Ponceau S (Sigma, St. Louis, MO) as a loading control alternative to typical high-abundance single protein controls; since the expression of these controls is affected by prolonged heroin exposure. Following Ponceau S destain, membranes were blocked overnight in Tris-buffered saline with Tween20 (TBS-T; 20 mM Tris, pH 7.4; 275 mM NaCl, 3 mM KCl, 1.0% Tween20) containing 10.0% dry milk at 4°C while nutating. Membranes were incubated at room temperature, nutating, for 1 hour with the following antibodies diluted in TBS-T containing 5.0% dry milk: rabbit anti-
D₂ dopamine receptor (1:1,000, Millipore, Bedford, MA); mouse anti-dynamin (1:10,000, Oncogene Research Products, Cambridge, MA); rabbit anti-GPR177/WLS (1:20,000; Sigma); rabbit anti-VAP33/VAPA antisera (1:10,000, generous gift of Dr. Paul Skehel, Centre for Integrative Physiology, University of Edinburgh, Scotland). Membranes were rinsed in TBS-T, followed by three 10 minute washes in TBS-T while shaking. Proteins were visualized using HRP-conjugated goat anti-mouse or anti-rabbit secondary antibodies (1:20,000, Jackson ImmunoResearch, West Grove, PA) diluted in TBS-T containing 5.0% dry milk. Membranes were washed of secondary antibodies, as described above. Immunoreactivity was detected with enhanced chemiluminescence substrate using an ECL Plus kit (GE Healthcare, Piscataway, NJ), imaged on BioMax film (Kodak, Rochester, NY), and quantitated using the ImageJ software package (US National Institutes of Health, Bethesda, MD). Resultant immunoblot data for each sample/target protein were standardized to the corresponding densitometric volume of the total protein Ponceau S stain. Protein content for each sample/target protein was then expressed as an arbitrary unit (a.u.) relative to the total protein content.

### 3.3.3 Statistical Analysis

All data were analyzed with Stata12 (StataCorp LP, College Station, TX). Three types of analyses were conducted: nonparametric Spearman's correlation analysis, Poisson regression analysis, and negative binomial regression analysis, where appropriate. Bonferroni multiple test correction was used to adjust p-values, where appropriate.

### 3.4 Results

The hippocampus, nucleus accumbens, and prefrontal cortex were sampled from a subset of rats (n=14) that had self-administered heroin over 27 self-administration (SA) sessions. Due to limitations of the assay (i.e., the number of lanes available to load protein samples in the gel apparatus), I used a subset of 14 rats out of the 43 rats that had completed the study in Chapter 3.
The 14 rats in this study met a range of addiction-like behaviors, and this subset included 0 criteria, 1 criterion, 2 criteria, and 3 criteria rats. Western blots were run for each brain region, and blots were probed for our candidate genes: D_2 dopamine receptor, dynamin, spinophilin, and VAPA (Figures 3.1-3.4). Western blots using the prefrontal cortex samples were also probed for WLS/GPR177 (Figure 3.5). The number of heroin infusions self-administered during drug periods was not statistically significant for rats meeting 0, 1, 2, or 3 criteria. Thus, there was no main effect of criteria or session for heroin intake across the 27 sessions of the study, and all rats self-administered approximately the same amount of heroin (Figure 3.6). Tables of the subjects' performance on "addiction-like" behavioral criteria and corresponding levels of protein expression for each candidate gene in a brain region (i.e., hippocampus, nucleus accumbens, prefrontal cortex) were then constructed (Tables 3.1-3.3).
Figure 3.1: Western blots of D₂ dopamine receptor expression
Samples from 14 heroin self-administering rats were prepared from the hippocampus (HPC), nucleus accumbens (NAc), prefrontal cortex (PFC). Western blots were run for each brain region and probed for D₂ dopamine receptor. Subject identification numbers mark each lane. Bottom bands show Ponceau loading control.
Figure 3.2: Western blots of dynamin expression
Samples from 14 heroin self-administering rats were prepared from the hippocampus (HPC), nucleus accumbens (NAc), prefrontal cortex (PFC). Western blots were run for each brain region and probed for dynamin. Subject identification numbers mark each lane. Bottom bands show Ponceau loading control.
Figure 3.3: Western blots of spinophilin expression
Samples from 14 heroin self-administering rats were prepared from the hippocampus (HPC), nucleus accumbens (NAc), prefrontal cortex (PFC). Western blots were run for each brain region and probed for spinophilin. Subject identification numbers mark each lane. Bottom bands show Ponceau loading control.
Figure 3.4: Western blots of VAPA expression
Samples from 14 heroin self-administering rats were prepared from the hippocampus (HPC), nucleus accumbens (NAc), prefrontal cortex (PFC). Western blots were run for each brain region and probed for VAPA. Subject identification numbers mark each lane. Bottom bands show Ponceau loading control.
Figure 3.5: Western blot of WLS/GPR177 expression
Samples from 14 heroin self-administering rats were prepared from the prefrontal cortex (PFC). Western blots were run and probed for WLS/GPR177. Subject identification numbers mark each lane. Bottom bands show Ponceau loading control.

Figure 3.6: Heroin intake for rats sampled in protein study across all sessions
The number of heroin infusions self-administered during drug periods was not statistically significant for rats meeting 0, 1, 2, or 3 criteria. Thus, there was no main effect of criteria or session for heroin intake across the 27 self-administration sessions of the study.
Table 3.1: Subject data for hippocampus samples
This table provides the behavioral and protein expression data for the hippocampus samples from each subject in our study.

<table>
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<tr>
<th>Subject ID</th>
<th>Number of criteria met</th>
<th>Breakpoint infusions PR4</th>
<th>SNA active responses SA25</th>
<th>TO active responses SA25</th>
<th>DP infusions SA25</th>
<th>D2R level (%)</th>
<th>Dyn level (%)</th>
<th>Spino level (%)</th>
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Table 3.2: Subject data for nucleus accumbens samples
This table provides the behavioral and protein expression data for the nucleus accumbens samples from each subject in our study.

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<th>Subject ID</th>
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<th>Breakpoint infusions PR4</th>
<th>SNA active responses SA25</th>
<th>TO active responses SA25</th>
<th>DP infusions SA25</th>
<th>D2R level (%)</th>
<th>Dyn level (%)</th>
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Table 3.3: Subject data for prefrontal cortex samples
This table provides the behavioral and protein expression data for the prefrontal cortex samples from each subject in our study.

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<th>TO active responses SA25</th>
<th>DP infusions SA25</th>
<th>D2R level (%)</th>
<th>Dyn level (%)</th>
<th>Spino level (%)</th>
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Spearman rank order correlations were conducted to determine the relationship between each of the "addiction-like" criteria and the protein expression levels in each brain region (Tables 3.4-3.6). In the hippocampus, the number of criteria met by subjects was negatively correlated with the expression of D2 dopamine receptor ($r_s=-0.79, p<0.001$). The number of signaled non-availability active licks, a measure of drug seeking, was also negatively correlated with the expression of D2 dopamine receptor in the hippocampus ($r_s=-0.74, p<0.01$). In the nucleus accumbens, drug seeking as given by the number of signaled non-availability active licks was negatively correlated with the expression of D2 dopamine receptor ($r_s=-0.68, p<0.01$).

In the prefrontal cortex, the number of criteria met by subjects was negatively correlated with the expression of WLS/GPR177 ($r_s=-0.59, p<0.05$). The progressive ratio breakpoint was also negatively correlated with the expression of WLS/GPR177 in the prefrontal cortex ($r_s=-0.75, p<0.01$). Thus, lower expression of WLS/GPR177 within the prefrontal cortex was associated with a greater willingness to work for drug and a greater number of criteria met for "addiction-like" behavior. Also in the prefrontal cortex, the progressive ratio breakpoint was negatively
correlated with the expression of D₂ dopamine receptor \( r_s = -0.66, p<0.01 \). Thus, a greater willingness to work for heroin was associated with reduced expression of D₂ dopamine receptor in the prefrontal cortex.
### Table 3.4: Behavioral measures versus protein levels in the hippocampus

This table presents the results of Spearman rank order correlation analyses used to assess the relationship between the "addiction-like" behaviors and protein expression levels in the hippocampus. Abbreviations: D2R, D₃ dopamine receptor; Dyn, dynamin; PR, progressive ratio; SA, self-administration; SNA, signaled non-availability, Spino, spinophilin; TO, timeout.

<table>
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<th>Measure</th>
<th>Protein</th>
<th>Spearman R</th>
<th>p-value</th>
<th>Adjusted p-value</th>
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Table 3.5: Behavioral measures versus protein levels in the nucleus accumbens
This table presents the results of Spearman rank order correlation analyses used to assess the relationship between the "addiction-like" behaviors and protein expression levels in the nucleus accumbens. Abbreviations: D2R, D₂ dopamine receptor; Dyn, dynamin; PR, progressive ratio; SA, self-administration; SNA, signaled non-availability, Spino, spinophilin; TO, timeout.

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<th>Measure</th>
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<td>1.0000</td>
</tr>
<tr>
<td>TO active responses SA25</td>
<td>D2R</td>
<td>-0.1760</td>
<td>0.5472</td>
<td>1.0000</td>
</tr>
<tr>
<td>TO active responses SA25</td>
<td>Dyn</td>
<td>-0.3102</td>
<td>0.2804</td>
<td>1.0000</td>
</tr>
<tr>
<td>TO active responses SA25</td>
<td>Spino</td>
<td>-0.2420</td>
<td>0.4045</td>
<td>1.0000</td>
</tr>
<tr>
<td>TO active responses SA25</td>
<td>VAPA</td>
<td>-0.2506</td>
<td>0.3871</td>
<td>1.0000</td>
</tr>
</tbody>
</table>
Table 3.6: Behavioral measures versus protein levels in the prefrontal cortex
This table presents the results of Spearman rank order correlation analyses used to assess the relationship between the "addiction-like" behaviors and protein expression levels in the prefrontal cortex. Abbreviations: D2R, D2 dopamine receptor; Dyn, dynamin; PR, progressive ratio; SA, self-administration; SNA, signaled non-availability, Spino, spinophilin; TO, timeout.

<table>
<thead>
<tr>
<th>Measure</th>
<th>Protein</th>
<th>Spearman R</th>
<th>p-value</th>
<th>Adjusted p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of criteria met</td>
<td>D2R</td>
<td>-0.3332</td>
<td>0.2444</td>
<td>0.9776</td>
</tr>
<tr>
<td>Number of criteria met</td>
<td>Dyn</td>
<td>0.1023</td>
<td>0.7277</td>
<td>1.0000</td>
</tr>
<tr>
<td>Number of criteria met</td>
<td>Spino</td>
<td>-0.2975</td>
<td>0.3016</td>
<td>1.0000</td>
</tr>
<tr>
<td>Number of criteria met</td>
<td>VAPA</td>
<td>-0.0071</td>
<td>0.9807</td>
<td>1.0000</td>
</tr>
<tr>
<td>Number of criteria met</td>
<td>WLS</td>
<td>-0.5926</td>
<td>0.0255</td>
<td>0.1020</td>
</tr>
<tr>
<td>SNA active responses SA25</td>
<td>D2R</td>
<td>-0.1652</td>
<td>0.5725</td>
<td>1.0000</td>
</tr>
<tr>
<td>SNA active responses SA25</td>
<td>Dyn</td>
<td>-0.0330</td>
<td>0.9107</td>
<td>1.0000</td>
</tr>
<tr>
<td>SNA active responses SA25</td>
<td>Spino</td>
<td>-0.0110</td>
<td>0.9702</td>
<td>1.0000</td>
</tr>
<tr>
<td>SNA active responses SA25</td>
<td>VAPA</td>
<td>0.0022</td>
<td>0.9940</td>
<td>1.0000</td>
</tr>
<tr>
<td>SNA active responses SA25</td>
<td>WLS</td>
<td>-0.4780</td>
<td>0.0839</td>
<td>0.3356</td>
</tr>
<tr>
<td>Breakpoint infusions PR4</td>
<td>D2R</td>
<td>-0.6623</td>
<td>0.0099</td>
<td>0.0396</td>
</tr>
<tr>
<td>Breakpoint infusions PR4</td>
<td>Dyn</td>
<td>0.0557</td>
<td>0.8499</td>
<td>1.0000</td>
</tr>
<tr>
<td>Breakpoint infusions PR4</td>
<td>Spino</td>
<td>-0.3189</td>
<td>0.2665</td>
<td>1.0000</td>
</tr>
<tr>
<td>Breakpoint infusions PR4</td>
<td>VAPA</td>
<td>0.1717</td>
<td>0.5572</td>
<td>1.0000</td>
</tr>
<tr>
<td>Breakpoint infusions PR4</td>
<td>WLS</td>
<td>-0.7492</td>
<td>0.0020</td>
<td>0.0080</td>
</tr>
<tr>
<td>TO active responses SA25</td>
<td>D2R</td>
<td>-0.0836</td>
<td>0.7763</td>
<td>1.0000</td>
</tr>
<tr>
<td>TO active responses SA25</td>
<td>Dyn</td>
<td>0.0506</td>
<td>0.8636</td>
<td>1.0000</td>
</tr>
<tr>
<td>TO active responses SA25</td>
<td>Spino</td>
<td>-0.2222</td>
<td>0.4451</td>
<td>1.0000</td>
</tr>
<tr>
<td>TO active responses SA25</td>
<td>VAPA</td>
<td>0.0638</td>
<td>0.8284</td>
<td>1.0000</td>
</tr>
<tr>
<td>TO active responses SA25</td>
<td>WLS</td>
<td>-0.3410</td>
<td>0.2328</td>
<td>0.9312</td>
</tr>
</tbody>
</table>
Spearman rank order correlations also demonstrated relationships between the expression of different proteins in a brain region (Tables 3.7-3.9). In the hippocampus, the expression of spinophilin was positively correlated with the expression of VAPA ($r_s=0.54$, $p<0.05$). Dynamin levels in the nucleus accumbens showed a positive correlation with VAPA ($r_s=0.64$, $p<0.05$). Also in the nucleus accumbens, the expression of the D$_2$ dopamine receptor was positively correlated with spinophilin expression ($r_s=0.56$, $p<0.05$). In the prefrontal cortex, however, D$_2$ dopamine receptor levels positively correlated with the expression of WLS/GPR177 ($r_s=0.72$, $p<0.01$).

Table 3.7: Protein level associations in the hippocampus
This table presents the results of Spearman rank order correlation analyses used to assess the relationship between different proteins expressed in the hippocampus. Abbreviations: D2R, D$_2$ dopamine receptor; Dyn, dynamin; Spino, spinophilin.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Protein</th>
<th>Spearman R</th>
<th>p-value</th>
<th>Adjusted p-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>D2R</td>
<td>Dyn</td>
<td>0.2879</td>
<td>0.3182</td>
<td>1.0000</td>
</tr>
<tr>
<td>D2R</td>
<td>Spino</td>
<td>0.2791</td>
<td>0.3338</td>
<td>1.0000</td>
</tr>
<tr>
<td>D2R</td>
<td>VAPA</td>
<td>0.3231</td>
<td>0.2599</td>
<td>1.0000</td>
</tr>
<tr>
<td>Dyn</td>
<td>Spino</td>
<td>0.4857</td>
<td>0.0783</td>
<td>0.3132</td>
</tr>
<tr>
<td>Dyn</td>
<td>VAPA</td>
<td>0.3978</td>
<td>0.1590</td>
<td>0.6360</td>
</tr>
<tr>
<td>Spino</td>
<td>VAPA</td>
<td>0.5385</td>
<td>0.0470</td>
<td>0.1880</td>
</tr>
</tbody>
</table>
Table 3.8: Protein level associations in the nucleus accumbens
This table presents the results of Spearman rank order correlation analyses used to assess the relationship between different proteins expressed in the nucleus accumbens. Abbreviations: D2R, D₂ dopamine receptor; Dyn, dynamin; Spino, spinophilin.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Protein</th>
<th>Spearman R</th>
<th>p-value</th>
<th>Adjusted p-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>D2R</td>
<td>Dyn</td>
<td>0.3495</td>
<td>0.2207</td>
<td>0.8828</td>
</tr>
<tr>
<td>D2R</td>
<td>Spino</td>
<td>0.5560</td>
<td>0.0389</td>
<td>0.1556</td>
</tr>
<tr>
<td>D2R</td>
<td>VAPA</td>
<td>0.1297</td>
<td>0.6586</td>
<td>1.0000</td>
</tr>
<tr>
<td>Dyn</td>
<td>Spino</td>
<td>0.4945</td>
<td>0.0722</td>
<td>0.2888</td>
</tr>
<tr>
<td>Dyn</td>
<td>VAPA</td>
<td>0.6440</td>
<td>0.0129</td>
<td>0.0516</td>
</tr>
<tr>
<td>Spino</td>
<td>VAPA</td>
<td>0.2571</td>
<td>0.3748</td>
<td>1.0000</td>
</tr>
</tbody>
</table>
Table 3.9: Protein level associations in the prefrontal cortex
This table presents the results of Spearman rank order correlation analyses used to assess the relationship between different proteins expressed in the prefrontal cortex.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Protein</th>
<th>Spearman R</th>
<th>p-value</th>
<th>Adjusted p-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>D2R</td>
<td>Dyn</td>
<td>0.2659</td>
<td>0.3581</td>
<td>1.0000</td>
</tr>
<tr>
<td>D2R</td>
<td>Spino</td>
<td>0.0110</td>
<td>0.9703</td>
<td>1.0000</td>
</tr>
<tr>
<td>D2R</td>
<td>VAPA</td>
<td>-0.2967</td>
<td>0.3030</td>
<td>1.0000</td>
</tr>
<tr>
<td>D2R</td>
<td>WLS</td>
<td>0.7187</td>
<td>0.0038</td>
<td>0.0190</td>
</tr>
<tr>
<td>Dyn</td>
<td>Spino</td>
<td>-0.1956</td>
<td>0.5028</td>
<td>1.0000</td>
</tr>
<tr>
<td>Dyn</td>
<td>VAPA</td>
<td>0.4022</td>
<td>0.1540</td>
<td>0.7700</td>
</tr>
<tr>
<td>Dyn</td>
<td>WLS</td>
<td>0.1253</td>
<td>0.6696</td>
<td>1.0000</td>
</tr>
<tr>
<td>Spino</td>
<td>VAPA</td>
<td>0.2088</td>
<td>0.4738</td>
<td>1.0000</td>
</tr>
<tr>
<td>Spino</td>
<td>WLS</td>
<td>0.1385</td>
<td>0.6369</td>
<td>1.0000</td>
</tr>
<tr>
<td>VAPA</td>
<td>WLS</td>
<td>-0.3275</td>
<td>0.2531</td>
<td>1.0000</td>
</tr>
</tbody>
</table>
To evaluate the relative impact of the protein expression levels on the outcomes of each of the "addiction-like" behaviors, I used regression analysis. The count data analyzed did not fit a linear model, so I used Poisson regression and negative binomial regression for cases in which there was an overdispersion in the data (i.e., the variance was greater than the mean). Since the behavioral data (i.e., the response or outcome) were count data, I refer to the relationship of the behavioral measure to protein level (i.e., the risk factor) as an incidence rate ratio (IRR) (Hilbe, 2011).

I used a Poisson regression model to fit the number of "addiction-like" criteria met versus the protein expression levels in a given brain region (Table 3.10). In the hippocampus, Poisson regression showed a significant association between the number of "addiction-like" criteria met and the protein expression of the D2 dopamine receptor (Figures 3.7 A and 3.8 A). Thus, as the expression of the D2 dopamine receptor was reduced, there was a greater number of criteria met for "addiction-like" behaviors. In the nucleus accumbens, there were significant associations between the number of criteria met and the protein expressions of both the D2 dopamine receptor and spinophilin (Figures 3.7 B-C and 3.8 B-C). Thus, as the expression of the D2 dopamine receptor and spinophilin within the nucleus accumbens was reduced, there was a greater number of "addiction-like" criteria met. The number of criteria met and the protein expression of WLS/GPR177 was significantly associated in the prefrontal cortex (Figures 3.7 D and 3.8 D). Thus, as the expression of WLS/GPR177 was reduced within the prefrontal cortex, there was a greater number of "addiction-like" criteria met.
Table 3.10: Poisson regression results of the association of number of criteria met with protein expression

This table presents the results of a Poisson regression analysis of the association of the number of "addiction-like" criteria met with protein expression. Abbreviations: CI, confidence interval; D2R, D2 dopamine receptor; Dyn, dynamin; HPC, hippocampus; IRR, incidence rate ratio; NAc, nucleus accumbens, PFC, prefrontal cortex; Spino, spinophilin.

<table>
<thead>
<tr>
<th>Region</th>
<th>Protein</th>
<th>IRR</th>
<th>95% CI</th>
<th>p</th>
<th>Adjusted p-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPC</td>
<td>D2R</td>
<td>0.412</td>
<td>0.238 - 0.711</td>
<td>0.001</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td>Dyn</td>
<td>0.893</td>
<td>0.642 - 1.243</td>
<td>0.503</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td>Spino</td>
<td>0.960</td>
<td>0.834 - 1.106</td>
<td>0.575</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td>VAPA</td>
<td>0.832</td>
<td>0.669 - 1.035</td>
<td>0.099</td>
<td>0.396</td>
</tr>
<tr>
<td>NAc</td>
<td>D2R</td>
<td>0.518</td>
<td>0.276 - 0.972</td>
<td>0.041</td>
<td>0.164</td>
</tr>
<tr>
<td></td>
<td>Dyn</td>
<td>0.704</td>
<td>0.393 - 1.261</td>
<td>0.238</td>
<td>0.952</td>
</tr>
<tr>
<td></td>
<td>Spino</td>
<td>0.759</td>
<td>0.577 - 0.999</td>
<td>0.049</td>
<td>0.196</td>
</tr>
<tr>
<td></td>
<td>VAPA</td>
<td>0.949</td>
<td>0.784 - 1.148</td>
<td>0.591</td>
<td>1.000</td>
</tr>
<tr>
<td>PFC</td>
<td>D2R</td>
<td>0.773</td>
<td>0.500 - 1.194</td>
<td>0.245</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td>Dyn</td>
<td>0.993</td>
<td>0.796 - 1.240</td>
<td>0.954</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td>Spino</td>
<td>0.952</td>
<td>0.828 - 1.095</td>
<td>0.493</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td>VAPA</td>
<td>0.982</td>
<td>0.786 - 1.225</td>
<td>0.869</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td>WLS</td>
<td>0.830</td>
<td>0.691 - 0.997</td>
<td>0.046</td>
<td>0.23</td>
</tr>
</tbody>
</table>
Figure 3.7: Protein expression by the number of criteria met for significant associations
Bar graphs present a visual representation of the protein expression by the number of criteria met for significant associations from Poisson regression. The expression of the D_2 dopamine receptor in the hippocampus (A) and the nucleus accumbens (B) was significantly associated with the number of criteria met. Spinophilin expression in the nucleus accumbens (C) and wntless expression in the prefrontal cortex (D) was significantly associated with the number of criteria met.
Figure 3.8: Significant results from the Poisson regression of the association between the number of criteria met and protein expression levels
Graphical representation of the Poisson regression curve fitting for the association of the number of “addiction-like” criteria met and expression levels of (A) D₂ dopamine receptor (D₂R) in the hippocampus (HPC), (B) D₂ dopamine receptor (D₂R) in the nucleus accumbens (NAc), (C) spinophilin (Spino) levels in the nucleus accumbens (NAc), and (D) WLS levels in the prefrontal cortex (PFC).
Negative binomial regression models were used to fit the association of each of the "addiction-like" criteria with the protein expression levels in a given brain region (see Table 3.11-3.13). The negative binomial regression analysis demonstrated several significant associations between "addiction-like" behaviors and protein expression levels. For signaled non-availability behavior, in the hippocampus and nucleus accumbens, there was a significant association of drug seeking with the expression of the D$_2$ dopamine receptor (Figures 3.8 A-B and 3.9 A-B). Additionally, in the hippocampus, there was a significant association of timeout active licks, or impulsivity of drug seeking, with D$_2$ dopamine receptor levels (Figures 3.8 C and 3.9 C).

Negative binomial regression also revealed significant association of progressive ratio breakpoint, or a rat's willingness to work for drug, with the expression of the D$_2$ dopamine receptor in both the hippocampus (Figures 3.10 A and 3.11 A) and prefrontal cortex (Figure 3.10 C and 3.11 C). In the hippocampus, there was a significant association of progressive ratio breakpoint with spinophilin levels (Figures 3.10 B and 3.11 B). Additionally, we found a significant association of progressive ratio breakpoint with WLS expression in the prefrontal cortex (Figures 3.10 D and 3.11 D). A summary of the significant associations between the behavioral measures and protein expression data is provided in Table 3.14.
Table 3.11: Negative binomial regression results of the association of signaled non-availability active licks with protein expression

This table presents the results of a negative binomial regression analysis of the association of signaled non-availability (SNA) active licks with protein expression. Abbreviations: CI, confidence interval; D2R, D2 dopamine receptor; Dyn, dynamin; HPC, hippocampus; IRR, incidence rate ratio; NAc, nucleus accumbens, PFC, prefrontal cortex; Spino, spinophilin.

<table>
<thead>
<tr>
<th>Region</th>
<th>Protein</th>
<th>IRR</th>
<th>95% CI</th>
<th>p</th>
<th>Adjusted p-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPC</td>
<td>D2R</td>
<td>0.684</td>
<td>0.508</td>
<td>0.920</td>
<td>0.012</td>
</tr>
<tr>
<td></td>
<td>Dyn</td>
<td>0.829</td>
<td>0.591</td>
<td>1.162</td>
<td>0.276</td>
</tr>
<tr>
<td></td>
<td>Spino</td>
<td>0.975</td>
<td>0.847</td>
<td>1.122</td>
<td>0.723</td>
</tr>
<tr>
<td></td>
<td>VAPA</td>
<td>0.914</td>
<td>0.789</td>
<td>1.059</td>
<td>0.230</td>
</tr>
<tr>
<td>NAc</td>
<td>D2R</td>
<td>0.602</td>
<td>0.369</td>
<td>0.983</td>
<td>0.043</td>
</tr>
<tr>
<td></td>
<td>Dyn</td>
<td>0.680</td>
<td>0.394</td>
<td>1.175</td>
<td>0.167</td>
</tr>
<tr>
<td></td>
<td>Spino</td>
<td>0.899</td>
<td>0.767</td>
<td>1.055</td>
<td>0.192</td>
</tr>
<tr>
<td></td>
<td>VAPA</td>
<td>0.999</td>
<td>0.820</td>
<td>1.217</td>
<td>0.990</td>
</tr>
<tr>
<td>PFC</td>
<td>D2R</td>
<td>0.916</td>
<td>0.703</td>
<td>1.914</td>
<td>0.517</td>
</tr>
<tr>
<td></td>
<td>Dyn</td>
<td>0.993</td>
<td>0.755</td>
<td>1.307</td>
<td>0.961</td>
</tr>
<tr>
<td></td>
<td>Spino</td>
<td>0.960</td>
<td>0.853</td>
<td>1.080</td>
<td>0.494</td>
</tr>
<tr>
<td></td>
<td>VAPA</td>
<td>0.979</td>
<td>0.749</td>
<td>1.280</td>
<td>0.877</td>
</tr>
<tr>
<td></td>
<td>WLS</td>
<td>0.890</td>
<td>0.780</td>
<td>1.016</td>
<td>0.085</td>
</tr>
</tbody>
</table>
Table 3.12: Negative binomial regression results of the association of progressive ratio breakpoint with protein expression

This table presents the results of a negative binomial regression analysis of the association of progressive ratio (PR) breakpoint with protein expression. Abbreviations: CI, confidence interval; D2R, D$_2$ dopamine receptor; Dyn, dynamin; HPC, hippocampus; IRR, incidence rate ratio; NAc, nucleus accumbens, PFC, prefrontal cortex; Spino, spinophilin.

<table>
<thead>
<tr>
<th>Region</th>
<th>Protein</th>
<th>IRR</th>
<th>95% CI</th>
<th>p</th>
<th>Adjusted p-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPC</td>
<td>D2R</td>
<td>0.729</td>
<td>0.536 – 0.993</td>
<td>0.045</td>
<td>0.180</td>
</tr>
<tr>
<td></td>
<td>Dyn</td>
<td>1.027</td>
<td>0.738 – 1.429</td>
<td>0.873</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td>Spino</td>
<td>1.014</td>
<td>0.880 – 1.168</td>
<td>0.849</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td>VAPA</td>
<td>0.874</td>
<td>0.738 – 1.035</td>
<td>0.119</td>
<td>0.476</td>
</tr>
<tr>
<td>NAc</td>
<td>D2R</td>
<td>0.760</td>
<td>0.465 – 1.242</td>
<td>0.273</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td>Dyn</td>
<td>0.927</td>
<td>0.563 – 1.524</td>
<td>0.764</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td>Spino</td>
<td>0.776</td>
<td>0.622 – 0.969</td>
<td>0.025</td>
<td>0.100</td>
</tr>
<tr>
<td></td>
<td>VAPA</td>
<td>0.911</td>
<td>0.728 – 1.141</td>
<td>0.418</td>
<td>1.000</td>
</tr>
<tr>
<td>PFC</td>
<td>D2R</td>
<td>0.710</td>
<td>0.505 – 0.998</td>
<td>0.049</td>
<td>0.245</td>
</tr>
<tr>
<td></td>
<td>Dyn</td>
<td>0.993</td>
<td>0.772 – 1.277</td>
<td>0.954</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td>Spino</td>
<td>0.908</td>
<td>0.792 – 1.042</td>
<td>0.169</td>
<td>0.845</td>
</tr>
<tr>
<td></td>
<td>VAPA</td>
<td>1.043</td>
<td>0.819 – 1.328</td>
<td>0.732</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td>WLS</td>
<td>0.846</td>
<td>0.737 – 0.972</td>
<td>0.018</td>
<td>0.090</td>
</tr>
</tbody>
</table>
Table 3.13: Negative binomial regression results of the association of timeout active licks with protein expression

This table presents the results of a negative binomial regression analysis of the association of timeout (TO) period active licks with protein expression. Abbreviations: CI, confidence interval; D2R, D2 dopamine receptor; Dyn, dynamin; HPC, hippocampus; IRR, incidence rate ratio; NAc, nucleus accumbens, PFC, prefrontal cortex; Spino, spinophilin.

<table>
<thead>
<tr>
<th>Region</th>
<th>Protein</th>
<th>IRR</th>
<th>95% CI</th>
<th>p</th>
<th>Adjusted p-values</th>
</tr>
</thead>
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<tr>
<td>HPC</td>
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<td>0.601</td>
<td>0.972</td>
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<tr>
<td></td>
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<td>0.668</td>
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<tr>
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<td>0.898</td>
<td>1.178</td>
<td>0.685</td>
</tr>
<tr>
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<td>0.902</td>
<td>0.791</td>
<td>1.028</td>
<td>0.123</td>
</tr>
<tr>
<td>NAc</td>
<td>D2R</td>
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<td>0.510</td>
<td>1.188</td>
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</tr>
<tr>
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<td>0.726</td>
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<tr>
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<td>VAPA</td>
<td>0.894</td>
<td>0.752</td>
<td>1.064</td>
<td>0.208</td>
</tr>
<tr>
<td>PFC</td>
<td>D2R</td>
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<td>0.667</td>
<td>1.131</td>
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<td>1.178</td>
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</tr>
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<td>0.729</td>
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</tr>
<tr>
<td></td>
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<td>0.818</td>
<td>1.043</td>
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</table>
Figure 3.9: Protein expression by SNA and timeout responses for significant associations
Bar graphs present a visual representation of the protein expression by SNA and timeout active responses for significant associations from negative binomial regression. Expression of the D2 dopamine receptor in the hippocampus (A) and nucleus accumbens (B) was associated with the number of SNA active responses in a negative binomial regression. Expression of the D2 dopamine receptor in the hippocampus was associated with timeout active responses.
Figure 3.10: Significant results from the negative binomial regression of the association between signaled non-availability and timeout active licks and protein expression levels
Graphical representation of the negative binomial regression curve fitting for the association of signaled non-availability (SNA) active licks and expression levels of (A) D_2 dopamine receptor (D_2R) in the hippocampus (HPC) and (B) D_2 dopamine receptor (D_2R) in the nucleus accumbens (NAc). A significant association of timeout (TO) period active licks with (C) D_2 dopamine receptor (D_2R) in the hippocampus (HPC) was also found.
Figure 3.11: Protein expression by the PR breakpoint infusions for significant associations
Bar graphs present a visual representation of the protein expression by the number of breakpoint infusions during progressive ratio testing for significant associations from negative binomial regression. The expression of the D₃ dopamine receptor in the hippocampus (A) and prefrontal cortex (C) was associated with PR breakpoint infusions. The expression of spinophilin in the nucleus accumbens and the expression of wntless in the prefrontal cortex were associated with the number of breakpoint infusions during progressive ratio.
Figure 3.12: Significant results from the negative binomial regression of the association between progressive ratio breakpoint and protein expression levels

Graphical representation of the negative binomial regression curve fitting for the association of progressive ratio (PR) breakpoint with expression levels of (A) D$_2$ dopamine receptor (D$_2$R) in the hippocampus (HPC), (B) spinophilin (Spino) in the nucleus accumbens (NAc), (C) D$_2$ dopamine receptor (D$_2$R) in the prefrontal cortex (PFC), and (D) WLS in the PFC.
Table 3.14: Summary of significant associations between behavioral measures and protein expression

This table presents an overview of the statistically significant associations between the behavioral measures and protein expression in a brain region. The plus signs (+) indicate a significant association. Greater "addiction-like" behavior was associated with reduced D_2 dopamine receptor expression in the hippocampus and nucleus accumbens. Additionally, greater "addiction-like" behavior was associated with reduced expression of spinophilin in the nucleus accumbens and with reduced expression of WLS in the prefrontal cortex.

3.5 Discussion

The results suggest that MOR interacting proteins and the D_2 dopamine receptor are differentially regulated in heroin self-administering rats as a function of the number of "addiction-like" behaviors met. Spearman rank order correlation demonstrated significant associations between behavioral measures and protein expression levels in all brain regions analyzed. These correlations show that, as the number of "addiction-like" criteria met by subjects increases (i.e., 0 criteria to 3 criteria), the expression of the D_2 dopamine receptor decreases in the hippocampus. I also found that the expression of the D_2 dopamine receptor in both the hippocampus and the nucleus accumbens decreases as the number of signaled non-availability (SNA) active licks increases. Since the nucleus accumbens is an important mediator of cues and contextual behavior, it was not surprising to find a decrease in D_2 dopamine receptor levels in the nucleus accumbens. There was also a decrease in D_2 dopamine receptor expression in the hippocampus. It is known that the chronic abuse of opioids leads to severe alteration of hippocampal long-term
potentiation and modulation of hippocampal synaptic plasticity (Bao et al., 2007; Pu et al., 2002). This finding suggests that decreases in the expression of the D₂ dopamine receptor in learning and memory pathways may play a greater role in the transition to "addiction-like" behaviors than had been anticipated.

Additionally, I found a decrease in the expression of WLS/GPR177 in the prefrontal cortex as the number of criteria met by subjects increases. Since WLS mediates the secretion of Wnt proteins from Wnt-producing cells for neuronal development and adult neurogenesis, it has been recently hypothesized that WLS plays a crucial role in the cellular response to opioids. It has been shown that binding of an opioid to MOR enhances the MOR-WLS interaction and serves to "trap" WLS at the cell surface. This results in the inhibition of Wnt protein secretion, which is necessary to maintain dendritic spines and promote hippocampal neurogenesis (Jin et al., 2010a; Jin et al., 2010b). If there is a down-regulation of WLS that accompanies the transition to "addiction-like" behaviors, there will also be an inhibition of Wnt protein secretion, further damaging the cell's ability to maintain dendritic spines.

Another interesting finding was a decrease in both D₂ dopamine receptor expression and WLS expression in the prefrontal cortex that correlated with an increase in the progressive ratio breakpoint. The prefrontal cortex is important for decision making (Schoenbaum and Shaham, 2008) and goal-directed behavior (Hitchcott et al., 2007; George et al., 2007; George and Koob, 2010). In this study, the progressive ratio breakpoint served as an important indicator of a rat's motivation or willingness to work for an infusion of heroin. It might be hypothesized that lower levels of the D₂ dopamine receptor and WLS in the prefrontal cortex lead to an increased motivation for drug. Alternatively, higher levels of D₂ dopamine receptor and WLS in the prefrontal cortex may protect a rat from developing an "addiction-like" behavior in the form of a willingness to work for drug. Rats that scored a 0 in "addiction-like" behavioral criteria are predicted to have increased protein expression levels of the D₂ dopamine receptor and WLS.
Spearman correlational analysis was also used to determine if there were any relationships between the expression of the different candidate genes. In the hippocampus, as spinophilin levels increased, VAPA levels also increased. Both spinophilin (Charlton et al., 2008) and VAPA (Sengupta et al., 2010; Weir et al., 1998; Weir et al., 2001; Pennetta et al., 2002; Lev et al., 2008) can be considered as dendritic scaffolding proteins. Therefore, there may be a functional relationship between VAPA and spinophilin in the hippocampus, even though spinophilin and VAPA levels in the hippocampus were not correlated with overall "addiction-like" behaviors. In the nucleus accumbens, there was a positive correlation between D\textsubscript{2} dopamine receptor levels and spinophilin levels, each of which was negatively correlated with greater "addiction-like" behaviors. The third intracellular loop of the D\textsubscript{2} dopamine receptor interacts with spinophilin. It has been hypothesized that spinophilin is crucial for establishing a signaling complex for dopaminergic neurotransmission through D\textsubscript{2} receptors by linking the receptors to the actin cytoskeleton and downstream signaling molecules (Smith et al., 1999). Thus, a relationship between spinophilin and D\textsubscript{2} receptor expression in the hippocampus and nucleus accumbens may also suggest a functional role for the proteins in protecting against the transition to "addiction-like" behaviors. Other protein-protein correlations included a positive correlation between dynamin and VAPA levels in the nucleus accumbens and a positive correlation between the D\textsubscript{2} dopamine receptor and WLS in the prefrontal cortex.

To further assess the association between the behavioral measures and protein expression of the candidate genes, I conducted regression analyses. Since the behavioral outcomes could be considered count data which were not normally distributed, I used Poisson regression analysis. I also used negative binomial regression analysis for cases in which there was overdispersion of an otherwise Poisson model. These regression models take into account the distribution of the experimental data to generate a model that achieved the best statistical fit.
Since the "addiction-like" criteria met variable was not overdispersed, I used Poisson regression to model how protein expression changes influence the number of criteria met by subjects. I found in both the hippocampus and the nucleus accumbens that lower levels of the D₂ dopamine receptor predict a higher number of criteria met. This suggests that higher levels of the D₂ dopamine receptor protect rats from the developing "addiction-like" behaviors, while lower levels of the D₂ dopamine receptor may put an animal at risk. In the nucleus accumbens, lower spinophilin levels predicted a higher number of criteria met. Likewise, in the prefrontal cortex, a decreased expression of WLS predicted a higher number of criteria met.

I next used negative binomial regression analysis to determine the association of individual "addiction-like" behaviors and protein level expression. In both the hippocampus and nucleus accumbens, lower levels of the D₂ dopamine receptor predicted a higher level of drug-seeking, as indicated by active licks during signaled non-availability periods. Additionally, in the hippocampus, lower levels of the D₂ dopamine receptor predicted an increase in impulsivity of drug-eliciting behavior, as indicated by active licks during timeout periods.

Several significant associations were found for a rat's motivation or willingness to work for drug, as indicated by progressive ratio breakpoint, and protein expression. In the hippocampus, lower levels of the D₂ dopamine receptor predicted an increase in a rat's willingness to work for drug. Decreased expression of spinophilin in the nucleus accumbens was associated with an increase in motivation for drug. Additionally, in prefrontal cortex, lower levels of the D₂ dopamine receptor and WLS predicted an increase in a rat's willingness to work for drug.

Based on these results, it can be hypothesized that lower levels of the D₂ dopamine receptor and MOR interacting proteins may increase an individual's vulnerability to developing "addiction-like" behaviors. Alternatively, higher levels of the D₂ dopamine receptor and MOR interacting proteins may be protective against the development of "addiction-like" behaviors. It
remains unknown if the individual male Sprague-Dawley rats used in our study were genetically predisposed to express lower levels of the D<sub>2</sub> dopamine receptor and MOR interacting proteins or if the presence of heroin elicited proteomic changes in vulnerable individuals.

It is clear from the Western blots in our study that there is a great deal of inter-animal variability in protein expression. Thus, it is noteworthy that significant associations were found for the "addiction-like" behaviors and our small subset of candidate proteins. The results suggest that there may be a functional significance for our candidate genes in the transition from use to abuse and the onset of "addiction-like" behaviors. However, a cause-effect relationship between protein expression and "addiction-like" behaviors cannot be deduced from this study. Future experiments should be directed at altering the expression of candidate genes, either pharmacologically or genetically, and determining if there is, in turn, a significant change in the development or absence of these "addiction-like" behaviors.
Chapter 4

Closing Discussion

The overall goal of this thesis was to understand how mu-opioid receptor (MOR) interacting proteins contribute to the molecular adaptations induced by opioid exposure and addiction. To achieve this goal, I developed a rodent model for "addiction-like" behaviors for heroin that served as a platform for investigating potential underlying neural substrates involved in the development of opioid addiction. My central hypothesis was that the transition from opioid use to opioid addiction is accompanied by differential regulation of MOR interacting proteins. This hypothesis was developed based on evidence that the MOR is the key opioid receptor mediating both the analgesic and rewarding properties of opioid agonists (Matthes et al., 1996), the growing body of research which suggests G protein-coupled receptors are part of larger multiprotein signaling complexes which regulate the function of the receptor (Kabbani and Levenson, 2007; Georgoussi et al., 2012), and the gap in knowledge of the molecular underpinnings of opioid addiction. My central hypothesis was validated by protein expression studies that revealed a differential expression of the MOR interacting proteins, wntless and spinophilin, in heroin self-administering rats showing "addiction-like" behaviors.

4.1 Summary of Experiments

The identification and characterization of MOR interacting proteins is a critical step for understanding the regulation of the MOR life-cycle, as well as how MOR signaling and trafficking may be altered in response to drugs of abuse. For example, the previously found interaction between spinophilin and MOR has been shown to alter the trafficking of the receptor. The overexpression of spinophilin in rat pheochromocytoma 12 cells enabled morphine-induced endocytosis of MOR. Additionally, spinophilin knock-out mice (KO) showed an increase in
MOR signaling due to delaying of receptor internalization. In behavioral studies, spinophilin KO mice demonstrated greater sensitivity to the rewarding properties of morphine. Spinophilin KO mice also developed a higher degree of physical dependence compared to their wild-type littermates (Charlton et al., 2008). Thus, spinophilin is a prime example of the important role MOR interacting proteins play in both receptor regulation and behavioral phenotypes. Identifying and characterizing novel MOR interacting proteins will provide new understanding of the cellular and molecular mechanisms that underlie the development of opioid addiction.

Utilization of a modified membrane yeast two-hybrid (MYTH) screen identified 10 novel components of the MOR signaling complex (Petko et al., 2013). The novel MOR interacting proteins had a variety of cellular functions, including the transport of ions to modulate electrical signaling, formation of intracellular channels, the efficient exporting of proteins in the endocytic pathway, and the secretion of wnt proteins which is required for the maintenance of dendritic spines and adult neurogenesis.

Of particular interest, the MYTH screen identified wntless (WLS), also known as GPR177, as a novel MOR interacting protein. WLS is the mammalian ortholog of Drosophila Wntless/Evi/Sprinter. Wntless is an evolutionarily conserved protein that mediates the secretion of Wnt proteins from Wnt-producing cells. This protein is of particular interest; since the Wnt pathway is important in neuronal development and adult neurogenesis. Recently WLS has also been shown to play a crucial role in the cellular response to opioids. The binding of an opioid to MOR enhances the MOR-WLS interaction and serves to "trap" WLS at the cell surface. This results in the inhibition of Wnt protein secretion, which is necessary to maintain dendritic spines and promote hippocampal neurogenesis (Jin et al., 2010a; Jin et al., 2010b).

The MYTH screen also identified an interaction between MOR and VAPA, a protein involved in trafficking and signaling modulation. Due to the potential function of this protein in regulating MOR endocytic trafficking, I decided to further characterize the VAPA-MOR
interaction. Alterations in the intracellular trafficking of MOR in response to opioids are poorly understood. It is thought that MOR internalization is a key step for inducing receptor desensitization to opioid drugs (Christie, 2008). However, newly synthesized MOR undergoes multiple maturation steps in the endoplasmic reticulum and Golgi apparatus (Leskelä et al., 2007; Markkanen and Petäjä-Repo, 2008; Vukojević et al., 2008). VAPA has been shown to contribute to the regulation of intracellular trafficking (Soussan et al., 1999; Prosser et al., 2008). Thus endoplasmic reticulum/Golgi transport may represent a regulatory point in MOR transport. My subsequent experiments confirmed the VAPA-MOR interaction in mammalian cells and whole mouse brain lysates and localized the interaction to the endoplasmic reticulum.

To identify possible roles for MOR interacting proteins in addiction and analgesia phenotypes, I measured the protein expression levels of VAPA, spinophilin, dynamin, and GPR177/WLS in the cerebral cortex and hippocampus of morphine-treated OPRM1 A112G knock-in mice. I found that spinophilin, WLS, and VAPA were differently regulated in the cerebral cortex of morphine-treated G/G mice, whereas in the hippocampus, spinophilin and VAPA were differentially expressed in morphine-treated G/G mice.

My experiments suggested that MOR interacting proteins demonstrate altered protein expression in a non-contingent mouse model of opioid dependence. However, I could not conclude that MOR interacting proteins are altered in a behavioral model of opioid addiction. To achieve this end, I next developed a model of heroin "addiction-like" behaviors in rats based on the paradigm described previously for cocaine (Deroche-Gamonet et al., 2004). My study demonstrated that after a prolonged period of heroin self-administration, "addiction-like" behaviors can be observed in rats. In my study "addiction-like" behaviors were defined by: 1) a persistence in drug-seeking behavior despite the presence of a signaled cue that drug was unavailable; 2) a high motivation for drug-taking as indicated by a willingness to work for each drug infusion; and 3) a high impulsivity of drug-seeking behavior. Only 9.3% of rats
demonstrated evidence for all three "addiction-like" behaviors, despite the fact that all rats in my study self-administered approximately the same amount of heroin. Although heroin intake was not statistically different across groups or self-administration sessions, I found that the onset activity score, which consisted of the sum of the active responses during the initial timeout and signaled non-availability periods, was an early predictor (i.e., session 13) of which rats would be "addicted" or "non-addicted" at the end of my study. Since heroin intake was not a factor in the classification of rats as "addicted" or "non-addicted," I next examined the expression of MOR interacting proteins and the D₂ dopamine receptor to determine the proteomic changes that accompany the transition to addiction, not merely opioid exposure, in key brain regions in the reward pathway.

The hippocampus, nucleus accumbens, and prefrontal cortex were sampled from a subset of rats (n=14) that self-administered heroin over 27 sessions, and the protein expression levels of the D₂ dopamine receptor, dynamin, spinophilin, VAPA, and WLS were analyzed. Spearman rank order correlations and Poisson/negative binomial regression analyses were conducted to determine the relationship between each of the "addiction-like" behaviors and the protein expression levels in each brain region. Several significant relationships between protein expression levels and "addiction-like" behaviors were identified (Table 4.1).
The "addiction-like" behaviors have been operationally defined by the endpoint behavior measured; for more detailed text for the "addiction-like" behaviors see Table 4.14. The plus signs (+) indicate a significant association. Greater "addiction-like" behavior was associated with reduced D₂ dopamine receptor expression in the hippocampus and nucleus accumbens. Additionally, greater "addiction-like" behavior was associated with reduced expression of spinophilin in the nucleus accumbens and with reduced expression of WLS in the prefrontal cortex.

These results show that the D₂ dopamine receptor and the MOR interacting proteins, spinophilin and WLS, are differentially expressed in rats demonstrating evidence of "addiction-like" behaviors. Greater "addiction-like" behavior was associated with reduced D₂ dopamine receptor expression in the hippocampus and nucleus accumbens. Additionally, greater "addiction-like" behavior was associated with reduced expression of spinophilin in the nucleus accumbens and with reduced expression of WLS in the prefrontal cortex. The results suggest that there may be a functional significance for MOR interacting proteins as candidate genes in the transition from opioid use to opioid dependence and the onset of "addiction-like" behaviors. However, a cause and effect relationship between protein expression and "addiction-like" behaviors cannot be deduced from my study. Future work should be aimed at altering the expression or the interaction of WLS and spinophilin with MOR, either pharmacologically or genetically, and determining if there is a significant change in the development or absence of these "addiction-like" behaviors.
4.2 Strengths and Limitations of the Behavioral Model

The behavioral model used in my studies modifies and extends the work pioneered by Deroche-Gamonet et al., 2004 which was carried out with cocaine self-administering rats. To my knowledge, my study was the first to determine if "addiction-like" behaviors would occur in heroin self-administering rats. An important strength of this self-administration paradigm is the use of multiple behavioral endpoints to measure the severity of "addiction-like" behaviors. In this model, a rat scores positive for an "addiction-like" behavior when the rat's performance is in the top percentile for that behavior. Each positive score for a behavior is added to achieve the number of "addiction-like" criteria met. As noted by Deroche-Gamonet et al., 2004 this method of scoring rats is similar to the way humans are clinically diagnosed with drug dependence. According to the DSM-IV, an individual must meet 3 of the 7 symptoms of drug dependence in the same 12-month period to receive a diagnosis of dependence (DSM-IV-TR, 2000). Thus, in both clinical settings and the behavioral model used in my study, drug dependence is treated as a syndrome defined by a cluster of different symptoms. Another strength of the behavioral model used in my study is that it takes into account the hallmarks of drug addiction, that is, "compulsivity, interindividual vulnerability, and its temporal dimension" (Belin et al., 2009). In my model, heroin "addiction-like" behavior developed with experience over an extended period of time, as does addiction in humans. Additionally, a small percentage of rats (9.3%) had a high score for all three "addiction-like" behaviors. This percentage is similar to the 17% of cocaine self-administering rats that showed a high score for all three "addiction-like" criteria (Deroche-Gamonet et al., 2004). As pointed out by Deroche-Gamonet et al., this percentage is similar to the 15% of cocaine users who actually become diagnosed as addicts (Wagner and Anthony, 2001; Anthony and Petronis, 1995; O'Brien and Anthony, 2005). The difference in percentage from my study (9.3%) compared to the Deroche-Gamonet et al. study (17%) can be attributed to scoring rats positive for an "addiction-like" behavior when performance for a behavior was in the
top 25% of the distribution, instead of the top 33% of the distribution, as used by Deroche-Gamonet et al., 2004. Additionally, the difference in percentage of "addicted" rats may be partly attributed to the different criteria used for "addiction-like" behaviors. My study did not assess punished responding for drug; it assessed persistence in drug-seeking behavior in spite of having received a drug reinforcement. Another obvious difference between my study and that of Deroche-Gamonet et al., 2004 was the drug that was self-administered. The Deroche-Gamonet et al., 2004 study assessed the development of "addiction-like" behaviors following cocaine exposure, while my study assessed heroin-induced "addiction-like" behaviors.

Although there are several strengths of the behavioral model, the extended period of time required for "addiction-like" behaviors to emerge and the large cohort of rats needed to achieve statistically meaningful data are some limitations. My study was conducted in two replications with each replication occurring over 48 days with a sample size of 24 rats. Due to loss of catheter patency or unexpected death, a total of 43 rats completed the study. Of these 43 rats, only 4 rats had a high score for all 3 "addiction-like" behaviors. Ideally, for the protein expression studies, I would have liked to compare "addicted" rats that met all three criteria with "non-addicted" rats that did not meet any of the "addiction-like" criteria. However, this would have required an estimated cohort size of 76-80 rats (if 9.3% remained the percentage of rats that met all 3 "addiction-like" criteria).

Another limitation of the model was the extinction/reinstatement procedure utilized in my experiments. The extinction/reinstatement procedure was conducted between the 21\textsuperscript{st} and 22\textsuperscript{nd} and 25\textsuperscript{th} and 26\textsuperscript{th} self-administration sessions. In this procedure, rats were placed in the same self-administration chambers as in the normal self-administration session, however, drug was not available for a period of 45 minutes. Following this 45 minutes of "extinction," a priming infusion of heroin (0.06 mg/0.2 mL infusion dose) was delivered. Following the priming infusion, rats were monitored for responding during an additional 45 minutes of drug non-
availability. As mentioned previously, this approach used to study extinction/reinstatement behavior failed to generate reliable, orderly seeking behavior. In future studies, an alternative approach should be used to generate extinction/reinstatement data. An alternative approach is to have the last self-administration session immediately followed by a 5- or 30-day period of withdrawal. Following the withdrawal period, rats can be exposed to stimuli known to induce relapse in humans, such as a small amount of heroin or a conditioned stimulus associated with drug-taking (Deroche-Gamonet et al., 2004). An another alternative approach, previously used by the Grigson laboratory, is to have a 14 day period of drug-free enforced abstinence immediately following the final self-administration session. After the extended period of abstinence, responding during a drug-free 90 minute extinction session (in the same environment in which rats had previously self-administered heroin) can be monitored (Kuntz-Melcavage et al., 2009; Kuntz et al., 2008). The Grigson laboratory has recently begun to examine seeking behavior over a 6 hour extinction session. Thereafter, the rats receive one experimenter-delivered intravenous drug challenge, and drug-induced reinstatement is assessed over 1 hour. This approach has yielded a marked and reliable effect.

Despite these limitations, my behavioral model is important because it uses a set of criteria to model the development of "addiction-like" behaviors in heroin self-administering rats. The greatest strength of this behavioral model is the fact that all rats, regardless of demonstrating "addiction-like" behaviors, took approximately the same amount of drug throughout the 27 self-administration sessions. The similar heroin intake across all rats and all sessions of the study suggests that drug exposure alone does not account for the development of "addiction-like" behaviors. There must be some other factor or factors in addition to drug exposure that accounts for a rat's vulnerability or resistance to developing "addiction-like" behaviors. The molecular effects of drug exposure had been previously studied in non-contingent (experimenter-delivered drug) models, in which the pharmacologic effects of the drug could be studied in animals that
received the same amount of drug. A major pitfall of non-contingent models, however, was the fact that interindividual vulnerability or resistance to developing behavioral evidence of drug dependence was not taken into account. Although non-contingent models revealed genomic and proteomic changes induced by drugs of abuse, correlations to behavior could not be made. The behavioral model developed in this thesis provides a platform for studying the underlying molecular mechanisms that accompany the transition from drug exposure to drug dependence in a model in which all rats self-administer approximately the same amount of drug and, thus, are pharmacologically equivalent. In this thesis, I utilized this strength of the behavioral model to investigate the protein expression of MOR interacting proteins as candidate genes which play a role in the transition to addiction.

4.3 Alternative Behavioral Models

A major goal of drug addiction research is to understand the differences between controlled and compulsive drug use. It has been suggested that the transition from controlled drug use to compulsive drug seeking and taking can be effectively modeled in rats using an escalation paradigm. Monitoring the escalation of drug intake is an alternative to the "addiction-like" criteria model of addiction in animals (Negus, 2006; Deneau et al., 1969). In a study by Lenoir and Ahmed, rats with a 6 hour access to intravenous heroin self-administration increased their heroin access over time to become excessive compared to the heroin intake of rats given 1 hour of heroin access. After escalation of heroin intake, rats with 6 hours of heroin access responded to the priming effects of heroin, as measured by the ability of heroin to induce reinstatement of extinguished drug-seeking behavior. This effect was not observed in rats given only 1 hour of heroin access (Lenoir and Ahmed, 2006).

In a recent study by the Koob laboratory, the pattern of heroin intake in rats with varying periods of heroin access were compared. Rats were allowed to lever press on a fixed ratio 1
(FR1) schedule of reinforcement to receive intravenous infusions of heroin for 1, 6, 12, or 23 hours per day for 14 sessions. It was found that heroin intake for rats in the 12 and 23 hour per day groups increased over time, whereas heroin intake in the 1 hour group remained stable and in the 6 hour group only modestly escalated. In this study, total heroin intake was approximately the same in the 12 and 23 hour groups, but the rate of heroin self-administration was two-fold higher in the 12 hour group compared to the 23 hour group. During the first hour of heroin self-administration, rats in the 23 hour group did not "binge" on heroin, but rather titrated their heroin intake throughout the entire time of the session. The rate of heroin self-administration in the 12 hour group was much higher than in the 23 hour group. Additionally, rats in the 12 hour group had repeated cycles of intoxication and abstinence, whereas the 23 hour group had minimal periods of abstinence. It was concluded that 12 hours of heroin access per day is the optimal access time for producing an escalation of heroin intake. It was suggested that the repeated, persistent abstinence pattern in the 12 hour group was an essential driving factor in the escalation of heroin intake. Since the pattern of heroin intake in the 12 hour group appeared to be more closely related to the human condition, it was concluded that rats given 12 hours of heroin access may be a more representative, face-valid model of heroin dependence (Vendruscolo et al., 2011).

Other behavioral models of heroin self-administration have explored motivational withdrawal, or psychological dependence. In a study by Kenny et al., 2006 rats were grouped as control (0 hours of heroin access), non-dependent (1 hour of heroin access), or dependent (23 hours of heroin access) and had daily intra-cranial self-stimulation threshold assessed. For non-dependent rats, heroin self-administration induced a brief activation of reward systems and a lowering of the intra-cranial self-stimulation thresholds. In dependent rats, however, an escalation of heroin intake across sessions occurred and was associated with a gradual decrease in reward sensitivity, as evidenced by progressively elevated intra-cranial self-stimulation thresholds. It was concluded that as heroin dependence develops, drug is consumed for both its
acutely rewarding effects and to counter persistent deficits in reward sensitivity. Precipitated heroin withdrawal with the opioid receptor antagonist naloxone increased heroin intake and reversed heroin-induced lowering of intra-cranial self-stimulation thresholds in non-dependent rats. In dependent rats, however, naloxone increased heroin intake and elevated intra-cranial self-stimulation thresholds above their already elevated baseline levels. Additionally, stimuli that had been repeatedly paired with naloxone-precipitated withdrawal induced heroin consumption and elevated intra-cranial self-stimulation in dependent rats. It was ultimately concluded that the conditioned stimuli predicting the onset of heroin withdrawal and reward deficit may play a role in provoking craving and relapse in human opioid addicts (Kenny et al., 2006).

Another avenue for exploring addiction in animals is to assess the de-valueation of natural rewards that occurs following exposure to drugs of abuse (Grigson, 2009). Early experiments carried out in the Grigson laboratory resulted in the first animal model for the systematic study of drug-induced devaluation of natural rewards (Grigson, 1997; Grigson and Twining, 2002). The incorporation of drug self-administration into the model makes it possible to study how drugs can devalue natural rewards and how natural reward taste cues might provoke drug-seeking and relapse (Grigson et al., 2008; Wheeler et al., 2008). Recent data suggests that the onset of aversive taste reactivity behavior (gapes) to a drug-paired taste cue can predict later drug-seeking and drug-taking behavior. The strength of this model is that it is possible to identify vulnerability for risk or resilience early in experience, thus shortening the timeframe of the study (Colechio and Grigson, in preparation). Since the "addiction-like" behavior model required approximately 7 weeks of experimentation, the devaluation of natural rewards model is a viable alternative for determining subjects that are at risk for the development of addiction.

Another alternative behavioral approach that must be considered is to examine mice instead of rats in the current paradigm. Mice have been previously shown to intravenously self-administer both heroin and morphine (Roberts et al., 1997; Salmon et al., 2001; Kuzmin et al., 2011).
While it remains to be demonstrated that heroin self-administering mice develop "addiction-like" behaviors, mice present the opportunity for genetic manipulation that is not currently available for rats. Since a decreased level of spinophilin in the nucleus accumbens was associated with greater "addiction-like" behaviors, spinophilin knock-out mice can be tested in the current paradigm to determine if there is a concomitant increase in the degree of "addiction-like" behaviors compared to wild-type littermates. The ability to genetically alter mice presents an opportunity to demonstrate a direct cause and effect relationship between candidate gene expression and the development of "addiction-like" behaviors.

4.4 Strengths and Limitations of the Candidate Gene Study

An overarching hypothesis of my thesis is that the identification and characterization of mu-opioid receptor (MOR) interacting proteins will provide new insights into the cellular and molecular mechanisms that underlie the development of opioid addiction. Therefore in my protein expression studies, MOR interacting proteins were the candidate genes evaluated for potential roles in mediating opioid addiction. A major strength of using this candidate gene approach is that it is hypothesis-driven, rather than hypothesis-generating as with high-throughput proteomic studies. My study was made more feasible by selecting only a few candidate genes to assess by Western blotting.

Although my candidate gene approach suggested potential roles for spinophilin, WLS, and the D2 dopamine receptor in mediating "addiction-like" behaviors, a cause and effect relationship cannot be deduced from my experiments. The lack of direct causality is a major limitation for my study. Future research that alters the expression of the candidate genes, either pharmacologically or genetically, is necessary for proving causality between the expression of spinophilin and WLS and behavioral signs of addiction.
Another limitation in my protein study is the small subset of rats that could be sampled with Western blotting. Due to limitations in my assay (14 lanes per gel), I could only assess the protein expression in a single brain region and from 14 subjects at the same time. A potential solution to this problem is to run a pooled sample lane in multiple gels and standardize to this pooled sample. While this seems like a viable alternative, this would require running a minimum of 4 gels per brain region to ensure that all 43 rats from my study were sampled. This requires a large number of gels and introduces gel-to-gel variation as a source of experimental error.

One of the obvious drawbacks to Western blotting is the need for antibody specificity. I was able to use specific antibodies for the D2 dopamine receptor, dynamin, spinophilin, VAPA, and WLS in my experiments. Unfortunately, I was unable to find a specific antibody for MOR and was unable to generate data on expression of this essential receptor. The major limitation of utilizing Western blotting, however, is the fact that the method is only semi-quantitative.

Despite these limitations, the Western blots in my study provided insight into novel associations of MOR interacting proteins with specific "addiction-like" behaviors. I found that "addiction-like" behavior was associated with the protein expression of both spinophilin and wntless (WLS), two MOR interacting proteins which serve as key regulators of MOR. As the protein expression of both spinophilin and WLS decreased, there was an associated increase in the severity of "addiction-like" behaviors. A decrease in spinophilin expression in the nucleus accumbens was associated with an increase in drug-seeking behavior, whereas in both the hippocampus and prefrontal cortex, a decrease in spinophilin expression was associated with an increased willingness to work for drug. In the prefrontal cortex, a decrease in WLS expression was associated with both an increase in the number of "addiction-like" criteria met and an increase in a rat's willingness to work for heroin. A critical strength of my protein study is that it provides evidence that WLS and spinophilin are differentially regulated, not only with opioid
exposure, but with the development of "addiction-like" behaviors following heroin self-administration.

Additionally, my study evaluated the expression of the D2 dopamine receptor as a control for my experiments. Since there is a well-characterized down-regulation of the D2 dopamine receptor following exposure to drugs of abuse in rodents and man (Volkow et al., 2001; Heinz et al., 2004; Maldonado et al., 1997; Johnson and Kenny, 2010; Volkow et al., 2004; Volkow et al., 1999; Volkow et al., 1993; Briand et al., 2008; Doehring et al., 2009; Asensio et al., 2010), it was expected that a decrease in the expression of the D2 dopamine receptor would accompany the development of "addiction-like" behaviors. In the hippocampus and the nucleus accumbens, a decrease in the expression of the D2 dopamine receptor was associated with an increase in the overall number of "addiction-like" criteria met. Interestingly, in the hippocampus and nucleus accumbens, a decrease in D2 dopamine receptor expression was associated with higher drug-seeking. In the hippocampus and prefrontal cortex, a decrease in D2 dopamine receptor expression was associated with a greater willingness to work for heroin. To my knowledge, this is the first study to find an association with the protein expression of the D2 dopamine receptor and specific "addiction-like" behaviors. My experiments suggest that the D2 dopamine receptor may mediate different aspects of addiction in different brain regions. This is the first time that "addiction-like" behaviors in an animal model have been linked to the differential expression of the D2 dopamine receptor. Overall, my study demonstrates that combining a candidate gene approach with a robust behavioral model will open new avenues for understanding the molecular and genetic underpinnings of addiction.

4.5 Alternatives to a Candidate Gene Approach

An alternative to the candidate gene approach is to use high-throughput genomic and proteomic techniques to catalogue the genes and proteins that are differentially regulated with the
development of "addiction-like" behaviors. As Freeman and Vrana point out in book chapter titled, "Quantitative Functional Genomics and Proteomics of Drug Abuse," both functional genomics and proteomics have emerged as important tools for characterizing the underlying biology of substance abuse. The advantage of using a genomic (DNA) or transcriptomic (mRNA) approach is that analysis of the complete genome or transcriptome can be carried out, as opposed to the 1-10% of the proteome of a cell or tissue. Although only 1-10% of the proteome can be assessed, this is still a major innovation compared to the five proteins analyzed with my candidate gene approach (Madras, 2006).

It should be noted, however, that changes in RNA in the transcriptome do not necessarily reflect changes in protein expression. There are also several post-translational processing events in the life-cycle of proteins that may be missed with a transcriptomic approach. Additionally, RNA is not a functional product, as is protein. Despite these limitations of the genomic and transcriptomic approach, DNA and RNA microarray assays have developed to the point where, in a single experiment, the expression of essentially every gene in the human genome can be examined, as well as the mRNA expression of essentially all genes in an RNA sample. Taken together, both transcriptomics and proteomics serve complementary roles based on the underlying biological features to be probed (Madras, 2006). A combination of genomic, transcriptomic, and proteomic approaches will certainly be important for demonstrating new candidate genes and for the generation of novel hypotheses for the underlying molecular mechanisms of addiction.

4.6 Future Directions

The "holy grail" of opioid research is to develop an effective treatment for moderate to severe pain that does not have abuse liability. The research generated in this thesis suggests that the MOR interacting proteins, spinophilin and WLS, may be important neural substrates mediating the transition from opioid use to opioid addiction. To test this hypothesis, the
interaction of spinophilin and WLS with MOR can be blocked with specifically engineered inhibitory peptides or induced with an enhancer peptide administered via a viral vector. With the peptide administered, the behavioral model can be carried out to test whether blocking or inducing the interaction of spinophilin and WLS with MOR can prevent, or alternatively increase, the development of "addiction-like" behaviors.

To carry out such an experiment, lentiviral vectors expressing specific peptides capable of blocking or inducing the interaction between spinophilin or WLS are needed. The ability to shuttle large genetic payloads and maintain stable, long-term transgene expression make lentiviral vectors an excellent delivery vehicle for the behavioral experiment. Lentivirus vectors are capable of infecting both dividing and non-dividing cells and can induce the long-term gene expression in vivo that is required for running a lengthy behavioral paradigm. Lentiviral vectors also do not induce cellular or humoral immune responses when the virus is injected into the brain (Cockrell and Kafri, 2007). In a recent study by Zheng et al., a lentivirus vector approach was used successfully to analyze the role of neurogenic differentiation 1 (NeuroD) in opioid agonist-selective regulation of adult neurogenesis and contextual memory extinction in a conditioned place preference paradigm. This study demonstrated that a single NeuroD lentivirus injection at the dentate gyrus was capable of altering conditioned place preference behaviors to morphine and fentanyl for 100 days of experimentation (Zheng et al., 2013).

To determine the effect of spinophilin on the development of "addiction-like" behaviors, a peptide antagonist can be delivered by a lentivirus vector into the nucleus accumbens. The nucleus accumbens was the brain region that had decreased spinophilin levels associated with greater "addiction-like" criteria met, greater drug-seeking during signaled non-availability, and greater willingness to work for drug. A spinophilin peptide antagonist that is capable of blocking the MOR-spinophilin interaction is predicted to increase the degree of "addiction-like" behaviors exhibited by the rats. Similarly, to determine the activity of WLS on the development of
"addiction-like" behaviors, a WLS peptide antagonist in a lentiviral vector can be injected into the prefrontal cortex. The prefrontal cortex was the brain region that had decreased WLS levels associated with greater "addiction-like" criteria met and greater willingness to work for drug. A WLS peptide antagonist that is capable of blocking the MOR-WLS interaction is predicted to increase the degree of "addiction-like" behaviors exhibited by the rats. During the behavioral study, the onset activity score can serve as an early indication that the peptide is altering the behavior of the rats; since the onset activity score was found in my study to be an early predictor of which rats would show a high degree of "addiction-like" behavior.

If such an experiment is successful, the results will prove a cause and effect relationship between the protein associations with "addiction-like" behaviors from my study and also reveal potential biomarkers of vulnerability for opioid addiction. By utilizing a peptide antagonist, such experiments would additionally provide proof-of-concept for the future development of small molecules capable of blocking or reversing "addiction-like" behaviors by targeting interacting proteins.

Ultimately, the development of a potential therapeutic agent intended to block the interaction between MOR and spinophilin or WLS requires careful characterization of the interactions through biophysical methods. One approach is to use fluorescence polarization assays based on the anisotropy measurements of a small fluorescently-tagged MOR peptide and its binding to the protein partners, spinophilin and WLS. This approach requires knowledge of the specific region of interaction between the two binding partners. Such an approach has been successfully applied to the interaction of the D₂ dopamine receptor with NCS-1 in a collaboration between the Levenson and Flanagan laboratories (Woll et al., 2011). In the case of the MOR, the relatively high degree of sequence homology among opioid receptors may allow for the interaction of spinophilin or WLS with other opioid receptor subtypes, and thus provide information about specific amino acid sequences of the receptors required for the binding of the
partner protein. An interaction between spinophilin and WLS with the kappa- or delta-opioid receptor may provide further insight into the role of MOR interacting proteins in response to opioid drugs.

These future studies will elucidate new avenues for the development of novel pharmacotherapeutics for the treatment of opioid addiction. Using such approaches, it may be ultimately possible to generate a novel opioid analgesic that does not carry the risk of addiction. This is critical for the ability to clinically manage chronic, moderate to severe pain without the potential for abuse liability.
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Appendix B

Biochemical and Genetic Analysis of MOR Interacting Proteins

B.1 Introduction

The therapeutic use of opioids for analgesia and management of drug addiction is limited by their tendency to induce drug tolerance. Tolerance is defined as the progressive attenuation of responsiveness to a drug, following repeated exposure (Johnson et al., 2006a). Although opioids are currently the most effective analgesic available for treating moderate to severe pain (Koch and Höllt, 2008), the development of tolerance poses the risk of physical dependence and addiction (Bailey and Connor, 2005). Additionally, it is estimated that 11.7 million Americans (White et al., 2005b) illegally abuse prescription opioid drugs for their rewarding effects—feelings of euphoria, relaxation, and well-being (Christie, 2008). As with other drugs of abuse, prolonged exposure to opioids results in the need to use increasingly higher doses of the drug to experience the desired effect (Contet et al., 2004; Christie, 2008; Koch et al., 2005). The molecular adaptations that occur throughout the central nervous system in response to chronic opioid use remain unclear (Goodman et al., 1996; Bailey and Connor, 2005; Christie, 2008; Martini and Whistler, 2007).

It is well understood that cellular desensitization to opioids develops in response to prolonged activation of the mu-opioid receptor (MOR). Opioid receptors belong to the Class A (rhodopsin-like) family of seven transmembrane, G protein-coupled receptors (Minami et al., 1993; Wang et al., 1994a; Thompson et al., 1993a, 1993b; Yasuda et al., 1993; Kieffer et al., 1992; Meng et al., 1993; Evans et al., 1992). There are four opioid receptor subtypes—mu, delta, kappa, and nociceptin/orphanin FQ receptor. Transgenic mice lacking MOR fail to respond to the analgesic and addictive effects of morphine. This attenuation of reward and analgesia was not
observed in mice lacking either the delta or the kappa opioid receptors. These findings unambiguously demonstrated that MOR mediates the pain-relieving and rewarding properties of opioids (Matthes et al., 1996). Since MOR must be present at the cell surface to be activated, it is implied that rapid MOR internalization enhances the development of cellular desensitization and physiologic tolerance. MOR desensitization is modeled after the thoroughly studied $\beta_2$-adrenergic receptor (Goodman et al., 1996; Christie, 2008; Connor et al., 2004).

Figure B.1: Opioid receptor activation and internalization
Growing evidence suggests that receptor desensitization involves enhancement or acceleration of the internalization process. Opioid receptor activation, internalization, and desensitization are well established, but the precise mechanisms are not clearly known. Desensitization of the receptor precedes internalization; however it is unknown whether this process is dependent on GRK association and phosphorylation. The major initial signaling steps (the release of G protein $\alpha$ and $\beta\gamma$ subunits) are attenuated by the enhanced desensitization or internalization. MOR interacting proteins, such as synaptophysin, play an important role in the regulation of MOR trafficking.
In this model (Figure B.1), agonist binding causes MOR to couple with $G_i / G_o$, pertussis toxin-sensitive GTP-binding protein. When G protein $\alpha$ and $\beta\gamma$ subunits dissociate, signal transduction inhibits intracellular cAMP production, blocks voltage gated Ca$^{2+}$ channels (Koch and Höllt, 2008), and stimulates inwardly rectifying K$^+$ channels (Law et al., 2000). G protein-coupled receptor kinase 2 (GRK2) subsequently phosphorylates MOR, which recruits arrestin 3. Arrestin 3 uncouples the receptor from G protein signaling. Uncoupling desensitizes the receptor and induces receptor internalization via clathrin-coated pits associated with dynamin and synaptophysin (Liang et al., 2007). Following internalization, MOR can be recycled to the cell surface, resensitizing the receptor to opioid agonists (Connor et al., 2004; Tanowitz and von Zastrow, 2003).

Despite the validation of this model, it remains unclear why different agonists exhibit variable MOR desensitization and internalization efficiencies. For example, D-Ala$_2$, N-MePhe$_4$, Gly-ol-enkephalin (DAMGO) and methadone induce MOR endocytosis throughout the CNS, but morphine does not (Arttamangkul et al., 2008; Arttamangkul et al., 2006; Christie, 2008; Koch and Höllt, 2008; Dang and Christie, 2012; Dang et al., 2009). Additional mechanisms of regulation must be involved.

A growing body of research shows that G protein-coupled receptor (GPCR) signaling is modulated by proteins that interact with the GPCR to form multi-protein signaling complexes. Several proteins that interact with MOR have been identified. These MOR interacting proteins, or MORIPs, have been linked to the processes of MOR biogenesis, trafficking, and signaling (Georgoussi et al., 2012). An important research goal, therefore, is to identify and characterize novel MORIPs that may help elucidate the molecular mechanisms underlying opioid addiction.

To obtain a global view of MOR-mediated signaling and to identify novel components of this pathway, a modified membrane yeast two-hybrid (MYTH) approach was used to generate an
interactome of the full-length, human, ligand-unoccupied MOR in its native membrane environment.

The MYTH screen identified 10 putative MOR interacting proteins. Of particular interest, the MYTH screen found an interaction between MOR and vesicle-associated membrane protein (VAMP)-associated protein (VAPA), a protein involved in trafficking of proteins and in signaling modulation by neurotransmitter release. Due to this protein’s potential function in regulating MOR endocytic trafficking, I decided to further characterize the VAPA-MOR interaction.

Alterations in the intracellular trafficking of MOR in response to opioids are poorly understood. It is thought that MOR internalization is a key step for inducing receptor desensitization to opioid drugs (Christie, 2008). However, newly synthesized MOR undergoes multiple maturation steps in the endoplasmic reticulum and Golgi apparatus (Leskelä et al., 2007; Markkanen and Petäjä-Repo, 2008; Vukojević et al., 2008). VAPA has been shown to contribute to the regulation of intracellular trafficking (Soussan et al., 1999; Prosser et al., 2008). Thus endoplasmic reticulum/Golgi transport may represent a regulatory point in MOR transport. VAPA is highly expressed in the endoplasmic reticulum and Golgi apparatus (Soussan et al., 1999; Foster et al., 2000; Nishimura et al., 1999) to function in vesicular docking and exocytosis (Skehel et al., 2000; Laurent et al., 2000). VAPA binding to MOR may regulate trafficking of MOR from the endoplasmic reticulum to the plasma membrane. Changes in endoplasmic reticulum/Golgi transport could therefore modulate receptor synthesis and turnover (Liang et al., 2007; Puthenveedu et al., 2007; von Zastrow and Sorkin, 2007; Sorkin and von Zastrow, 2009). The VAPA-MOR interaction may represent a novel control point in MOR trafficking and in the development of MOR desensitization.

To confirm the VAPA-MOR interaction identified in the MYTH screen, the colocalization of MOR and VAPA was assayed in vivo in stably transfected MOR HEK293 cells.
using confocal microscopy and in mouse striatal neurons using immunoelectron microscopy. The co-expression in the mouse striatum was also confirmed by electron photomicrographs showing dual immunogold-silver labeling of VAPA and MOR in dendrites and soma. Additionally, co-immunoprecipitation studies with MOR stably transfected in HEK293 cells and with mouse whole brain lysates were also used to confirm the interaction of MOR with VAPA. A conventional yeast-two hybrid (Y2H) screen was conducted to map the MOR-VAPA interaction. Preliminary experiments were also conducted to determine if the protein is differentially expressed in mice exposed to morphine.

Other MOR interacting proteins of interest in my study were GPR177/WLS, spinophilin, and dynamin. Wntless (WLS), also known as GPR177, was another MOR interacting protein found in the MYTH screen. WLS is the mammalian ortholog of *Drosophila* Wntless/Evi/Sprinter. Wntless is an evolutionarily conserved protein that mediates the secretion of Wnt proteins from Wnt-producing cells. This protein is of particular interest; since the Wnt pathway is important in neuronal development and adult neurogenesis. Recently WLS has also been shown to play a crucial role in the cellular response to opioids. The binding of an opioid to MOR enhances the MOR-WLS interaction and serves to "trap" WLS at the cell surface. This results in the inhibition of Wnt protein secretion, which is necessary to maintain dendritic spines and promote hippocampal neurogenesis (Jin et al., 2010a; Jin et al., 2010b).

Spinophilin is another MOR interacting protein of interest. Spinophilin is a scaffolding protein that is enriched in dendritic spines. The overexpression of spinophilin in rat pheochromocytoma 12 cells enabled morphine-induced endocytosis of MOR. Spinophilin knock-out mice showed an increase in MOR signaling due to delaying of receptor internalization. In behavioral studies, spinophilin KO mice demonstrated greater sensitivity to the rewarding properties of morphine. Spinophilin KO mice also developed a higher degree of physical dependence compared to their wild-type littermates. The extensive study by Charlton et al.
demonstrated that altering the expression of a MOR interacting protein could affect the behavioral response of an animal to opioids (Charlton et al., 2008).

Since MOR endocytosis has been proposed as a potential mechanism that mediates receptor desensitization, I was also interested in dynamin, a previously identified MOR associated protein. Dynamin is a cytoplasmic GTPase that mediates MOR endocytosis via clathrin-coated pits and caveoli. Although endocytosis of MOR occurs via a dynamin-dependent manner, dynamin can also function to regulate mitogenic signaling by the receptor (Whistler and von Zastrow, 1999; Whistler et al., 1999). Alterations in dynamin-mediated endocytosis or signaling following chronic opioid drugs have been studied in cellular models (Keith et al., 1996; Keith et al., 1998), but the role dynamin may play in opioid dependence has only been recently studied. A study by Pawar et al., 2007 found that high efficacy opioid agonists were associated with MOR downregulation and dynamin-2 upregulation. In contrast, lower efficacy agonists produced more tolerance to the analgesic effects of the drugs in mice, but did not regulate either MOR density or dynamin-2 abundance. It was concluded that agonist efficacy plays a central role in the development of tolerance (Pawar et al., 2007). It remains unknown if there is a differential expression of dynamin in behavioral models of opioid dependence and addiction.

To identify possible roles for MOR interacting proteins in addiction and analgesia phenotypes, I measured the protein expression levels of VAPA, spinophilin, dynamin, and GPR177/WLS in the cerebral cortex and hippocampus of morphine-treated OPRM1 A112G knock-in mice. This mouse model mimics the equivalent single nucleotide polymorphism in the human receptor, A118G (Mague, 2010; Wang et al., 2012). Many single nucleotide polymorphisms in the OPRM1 gene occur; however the A118G SNP is the most thoroughly studied in terms of its effects on sensitivity to the rewarding and analgesic properties of opioids. The consumption (Sia et al., 2008; Lötsch et al., 2002; Chou et al., 2006) and dosage (Ginosar et al., 2009; Reyes-Gibby et al., 2007) of opioid drugs required to achieve analgesia in clinical
settings has been shown to be greater in G-allele carriers compared to patients with the "wild-type" AA alleles. Other studies have found an association between the G118 allele and heroin use, abuse, and dependence (Mague, 2010).

B.2 Experimental Procedures

B.2.1 Membrane yeast two-hybrid screen

A modified split-ubiquitin yeast two-hybrid (MYTH) screen was performed in collaboration with Igor Stagljar (University of Toronto) as described previously (Paumi et al., 2007a; Paumi et al., 2007b; Kittanakom, 2009). Briefly, the full-length human MOR (transcript MOR-1) cDNA was cloned into the bait vector pCCW-STE (Dualsystems Biotech AG, Switzerland) and the human fetal brain cDNA library was cloned into the prey vector pRP3-N (Dualsystems). These constructs were then sequentially transformed into S. cerevisiae reporter strain THY.AP4. This transformation yielded 6 x 10^6 transformants/µg DNA on synthetic dropout (SD) agar plates (-Trp/-Leu/-His/-Ade; Clontech, Palo Alto, CA) containing 3-amino-1,2,4-triazole (3AT). The identity of the putative MORIPs was determined by recovering the prey plasmid from the transformed yeast colonies. The cDNA clone in the plasmid was sequenced using primers designed against the regions flanking the cloning site and sequences were then analyzed using Basic Local Alignment Search Tool (BLAST) to determine the identity of the clone.

B.2.2 Mapping of VAPA binding region

To map sites of interaction between the MOR and the newly hypothesized MORIPs, each MOR intracellular loop (IL) was tested for interaction with individual MORIPs using the traditional yeast two-hybrid method. MOR IL domains (IL1, amino acids 97-102; IL2, amino acids 166-187; IL3, amino acids 259-282; and C-tail residues 361-420) were separately ligated into pAS2-1 (Clontech) and assayed for interaction with candidate MORIP cDNA clones in

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pACT2. Bait and prey plasmids were simultaneously co-transformed into *S. cerevisiae* strain MaV103. Interactions were assayed for β-galactosidase (β-gal) activity via the nitrocellulose lift method (Binda et al., 2002).

**B.2.3 Cell culture co-immunoprecipitation**

Human embryonic kidney (HEK) 293 cells stably transfected with FLAG-tagged mu-opioid receptor were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 400 µg/mL G418. HEK-MOR cells were generously provided by Dr. Mark van Zastrow, University of California, San Francisco. HEK-MOR were transfected with a construct encoding full-length VAPA sub-cloned in the pCMV-Tag3B expression vector (Stratagene, La Jolla, CA) containing a myc-tag. Transfections were carried out using Effectene transfection reagent (Qiagen, Valencia, CA). Cells were cultured for 24 hours in DMEM and then lysed in 1X lysis buffer (20 mM Tris-HCl pH7.5, 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na₃VO₄, 1% Triton X-100 and 1mg/mL leupeptin) supplemented with protease inhibitor cocktail (Pierce, Rockford, IL). MOR was immunoprecipitated from total cell lysates using a polyclonal rabbit anti-FLAG antibody (Sigma, St. Louis, MO) coupled to Protein-G Dynabeads (Invitrogen). Western blot analysis of immunoprecipitated complexes was performed by using a monoclonal mouse anti-myc antibody (1:5000 dilution; Millipore, Billerica, MA) and a rabbit anti-mouse HRP-conjugated secondary antibody (1:20,000 dilution; Jackson ImmunoResearch, West Grove, PA). Immunoreactivity was detected by ECL Plus.

**B.2.4 Total mouse brain co-immunoprecipitation**

Lysates were prepared from total mouse brain and immunoprecipitated with anti-MOR antibody (1:2,000, AB1580, Millipore, Bedford, MA). Immunocomplexes were separated by SDS-PAGE, transferred to a PVDF filter, and probed for the presence of VAPA using rabbit anti-
VAPA antisera (1:10,000, generous gift of Dr. Paul Skehel, Centre for Integrative Physiology, University of Edinburgh, Scotland).

**B.2.5 Confocal microscopy**

The in vivo interaction of MOR and VAPA was subsequently verified in HEK-MOR cells using confocal microscopy. Immunohistochemistry was performed by transfecting cells transiently with myc-tagged VAPA. Cells were then split onto collagen coated coverslips (BD Biosciences) and allowed to grow overnight. After 18 hours, cells were fixed with 4% paraformaldehyde and 5% sucrose. The cells were immunostained with monoclonal mouse anti-myc antibodies (1:1000 dilution; Millipore) and rabbit anti-MOR antibodies (1:500; AB5511, Millipore). After this initial staining, the cells were washed with 1X PBS, and exposed to FITC conjugated donkey anti-rabbit antibodies (1:500 dilution; Jackson ImmunoResearch) and rhodamine conjugated donkey anti-mouse antibodies (1:500 dilution; Jackson ImmunoResearch). Images were obtained with a confocal microscope (Zeiss LSM 510 Meta, Carl Zeiss Inc., Thornwood, NY), and digital images were captured and imported with the LSM 5 image browser (Carl Zeiss, Inc.).

**B.2.6 Immunoelectron microscopy**

Mice were perfused with (1) 10 mL heparinized saline, (2) 50 mL of 4% formaldehyde in 0.1 M PBS, pH 7.4. Immediately after perfusion, brains were removed, sectioned into coronal slices and postfixed overnight at 4°C in the same fixative. Alternate 40 µm thick sections through the rostrocaudal extent of the striatum were incubated with a cocktail of mouse anti-VAPA (1:1,000 dilution, Abnova, Atlanta, GA) and rabbit anti-MOR (1:2,000 dilution) antibodies. Immunoperoxidase labeling was used to identify MOR immunoreactivity while immunogold-silver labeling was used to identify VAPA. Primary antibodies were complexed with a mixture of biotinylated donkey and anti-rabbit (1:400 dilution; Jackson ImmunoResearch) and ultra small (< 1 nm) gold-coupled goat anti-mouse (1:100 dilution; Electron Microscopy Sciences, Fort
Washington, PA) secondary antibodies. MOR was detected by incubation with avidin-biotin complex (Vector Laboratories, Burlingame, CA) and visualized by DAB (3,3-diaminobenzidine; Sigma). To visualize immunogold-labeled VAPA, silver enhancement of the gold particles was performed using a silver enhancement kit (Amersham Bioscience, Piscataway, NJ). Sections were flat embedded in Epon 812 (Electron Microscopy Sciences). Thin sections (50-100 nm) were collected on copper mesh grids and examined with an electron microscope (Morgagni Fei Company, Hillsboro, OR). Digital images were captured using an AMT advantage HR/HR-B CCD camera system (Advance Microscopy Techniques Corp., Danvers, MA).

For dual immunogold labeling, sections through the rostrocaudal extent were incubated in a cocktail containing mouse anti-VAPA (1:1,000 dilution) and rabbit anti-MOR (1:2,000 dilution) primary antibodies. Sections were incubated with ultra small gold-coupled goat anti-rabbit (1:100 dilution; Amersham Bioscience) secondary antibody, followed by the first silver enhancement (300 µl R-Gent SE-EM enhancement mixture; Amersham Bioscience). Sections were then incubated with ultra small gold-coupled goat anti-mouse (1:100 dilution; Amersham Bioscience) secondary antibody, followed by the second silver enhancement (300 µl R-Gent SE-EM enhancement mixture; Amersham Bioscience). Immunogold-silver particles measuring less than 0.05 µm in cross-sectional diameter were scored as small, while immunogold-silver particles measuring greater than 0.051 µm were scored as large. Tissues sections were incubated in 2% osmium tetroxide (Electron Microscopy Sciences) and flat embedded in Epon 812. Digital image capturing was as described above.

**B.2.7 Western blotting of OPRM1 A112G mice**

Brain tissue samples of control and morphine treated OPRM1 A112 +/+ or G112 +/+ mice were a generous gift of Julie Blendy, University of Pennsylvania, PA. The A112G MOR knock-in mouse line was generated in the C57Bl/6 background and propagated by heterozygote matings. For a more detailed description of the generation of OPRM1 knock-in mice (see Mague 136
Morphine dependence was achieved by subcutaneously implanting a single placebo (cellulose) or morphine (25 mg morphine base) pellet into the dorsal surface of mice under general isoflurane anesthesia for 3 days before sacrifice. Three days of morphine pellet implantation models chronic opioid exposure in mice.

Frozen prefrontal cortex or hippocampus samples were homogenized on ice by Dounce-homogenization in a detergent-based protein lysis buffer (100 mM NaCl, 20 mM HEPES, 1 mM EDTA, 1 mM dithiothreitol, 1.0% Tween20, 1 mM Na$_3$VO$_4$ with 1 Complete Mini EDTA-free Protease Inhibitor Cocktail Tablet (Roche Applied Science, Indianapolis, IN) for every 10 mL lysis buffer prepared, incubating with rocking for 15 minutes at 4°C, and centrifuging at 10,000 xg for 12 min at 4°C (VanGuilder et al., 2010). Supernatant protein concentrations were determined using the bicinchoninic acid protein (BCA) assay (Pierce, Rockford, IL), after which all protein samples were brought to a concentration of 2 µg/µL and diluted in lithium dodecyl sulfate sample buffer (BioRad, Hercules, CA). A protein concentration of 8 µg of each subject's sample (n=14 per gel) were run in groups according to the brain region of interest (i.e., prefrontal cortex or hippocampus) and genotype/drug treatment (i.e., A/A control, n=4; A/A morphine, n=4; G/G control, n=4; G/G morphine, n=2) then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 4-20% Mini-Protean TGX precast gels (BioRad).

For immunoblotting, proteins were transferred using the iBlot dry transfer system (P3, 20 V for 4:50 min) to polyvinylidene difluoride (PVDF) membranes within the iBlot transfer stack (PVDF, Regular; Invitrogen, Carlsbad, CA). Membranes were stained with Ponceau S (Sigma, St. Louis, MO) as a loading control alternative to typical high-abundance single protein controls; since the expression of these controls is affected by prolonged heroin exposure (Neuroscience Information Framework databases, www.neuinfo.org). Following Ponceau S destain, membranes were blocked overnight in Tris-buffered saline with Tween20 (TBS-T; 20 mM Tris, pH 7.4; 275 mM NaCl, 3 mM KCl, 1.0% Tween20) containing 10.0% dry milk at 4°C while nutating.
Membranes were incubated at room temperature, nutating, for 1 hour with the following antibodies diluted in TBS-T containing 5.0% dry milk: rabbit anti-D_2 dopamine receptor (1:1,000, Millipore, Bedford, MA); mouse anti-dynamin (1:10,000, Oncogene Research Products, Cambridge, MA); rabbit anti-GPR177/WLS (1:20,000, Sigma); rabbit anti-VAP33/VAPA antisera (1:10,000, generous gift of Dr. Paul Skehel, Centre for Integrative Physiology, University of Edinburgh, Scotland). Membranes were rinsed in TBS-T, followed by three 10 minute washes in TBS-T while shaking.

Proteins were visualized using HRP-conjugated goat anti-mouse or anti-rabbit secondary antibodies (1:20,000, Jackson ImmunoResearch, West Grove, PA) diluted in TBS-T containing 5.0% dry milk. Membranes were washed of secondary antibodies, as described above.

Immunoreactivity was detected with enhanced chemiluminescence substrate using an ECL Plus kit (GE Healthcare, Piscataway, NJ), imaged on BioMax film (Kodak, Rochester, NY), and quantified using the ImageJ software package, Version 1.47 for Mac (US National Institutes of Health, Bethesda, MD). Resultant immunoblot data for each sample/target protein were standardized to the corresponding densitometric volume of the total protein Ponceau S stain. Protein content for each sample/target protein was then expressed as a relative unit with 1.0 given as the total level of expression of the control OPRM1 A/A mice and reported as relative densitometric units (OD). Data was analyzed using ANOVA and Bonferroni post-hoc tests or a student's t-test for G/G placebo vs. G/G morphine-treated mice.

**B.3 Results**

The modified split-ubiquitin yeast two-hybrid (MYTH) screen yielded 104 positive clones from 6x10^6 transformants screened. Several hypothesized MOR interacting proteins were identified (Table B.1).
Table B.1: Hypothesized MOR interacting proteins identified in MYTH screen

<table>
<thead>
<tr>
<th>Protein</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADD3</td>
<td>Adducin gamma subunit</td>
</tr>
<tr>
<td>GJA4</td>
<td>Gap junction protein, alpha 4</td>
</tr>
<tr>
<td>GPR177/WLS</td>
<td>Orphan G protein-coupled receptor/Wntless</td>
</tr>
<tr>
<td>KCNF1</td>
<td>Potassium channel subunit</td>
</tr>
<tr>
<td>SLC31A2</td>
<td>Putative copper transporter</td>
</tr>
<tr>
<td>SLC9A9</td>
<td>Putative Na⁺/H⁺ exchanger</td>
</tr>
<tr>
<td>VAPA</td>
<td>VAMP-associated protein A</td>
</tr>
<tr>
<td>WHSC2</td>
<td>Wolf-Hirschhorn syndrome candidate 2</td>
</tr>
<tr>
<td>SLC39A13</td>
<td>Putative zinc transporter</td>
</tr>
<tr>
<td>YIPF3</td>
<td>YIPL domain family member 3</td>
</tr>
</tbody>
</table>

Of these hypothesized MOR interacting proteins, adducins (ADD3) are cytoskeletal proteins found in junctional complexes within the cell. Adducins bind and regulate actin and actin-spectrin complexes. Adducins are expressed at high levels in the brain where they serve as components of synaptic structures, such as dendritic spines and growth cones of neurons. Adducins are capable of inducing changes in dendritic spines as a result of activity-dependent synaptic plasticity processes associated with learning and memory (Porro et al., 2010).

Gap junction proteins (GJA4) consist of oligomerized connexin proteins that form intercellular channels to allow the exchange of ions, second messengers, and metabolites. The communication among neighboring cells is thought to be important for the propagation of signals along electrically excitable cells (Anzini et al., 1997).

Wntless (WLS), also known as GPR177, is a putative orphan GPCR. WLS is the mammalian ortholog of *Drosophila* Wntless/Evi/Sprinter. Wntless is an evolutionarily conserved protein that mediates the secretion of Wnt proteins from Wnt-producing cells. The Wnt pathway is important in neuronal development and adult neurogenesis. Recently WLS has also been shown to play a crucial role in the cellular response to opioids. The binding of an opioid to MOR enhances the MOR-WLS interaction and serves to "trap" WLS at the cell surface. This results in
the inhibition of Wnt protein secretion, which is necessary to maintain dendritic spines and promote hippocampal neurogenesis (Jin et al., 2010a; Jin et al., 2010b).

The transport of ions in neurons serves to regulate the depolarization and repolarization of the cell following the generation of an action potential. Alterations in electrical signaling in neurons can contribute to synaptic plasticity by long-term potentiation, or alternatively, by long-term depression (Dacher and Nugent, 2011). Thus, the MOR interacting proteins identified in the MYTH screen involved in ion exchange and transport (KCNF1, SLC31A2, SLC39A13, and SLC9A9) may serve as important mediators of the synaptic plasticity which occurs in opioid addiction.

Wolf-Hirschhorn syndrom candidate 2 (WHSC2) is expressed ubiquitously and the encoded protein includes functional nuclear localization signals suggesting a transcriptional role for the gene. Wolf-Hirschhorn syndrome (WHS) is a genetic disease characterized by epilepsy, overt mental retardation, a mild facial phenotype, and modest growth delay. The role of WHSC2 in the pathogenesis of WHS, however, remains to be elucidated.

The YIPL domain (YIPF3) is a Golgi apparatus export signal domain. Thus, YIPF3 is implicated in intracellular quality control mechanisms and trafficking of proteins to the cell surface (Steele and Fedida, 2012).

Vesicle-associated membrane protein (VAMP) associated protein A (VAPA), a member of the SNARE (soluble N-ethylmaleimide-sensitive component attachment protein receptor) family, is involved in vesicular docking and exocytosis (Soussan et al., 1999; Skehel et al., 2000; Laurent et al., 2000). VAPA is highly expressed in the ER and on perinuclear microtubules where it likely mediates vesicular transport (Skehel et al., 2000; Weir et al., 2001).

Data from a split-ubiquitin yeast-two-hybrid screen, conducted in collaboration with Dr. Igor Stagljar (University of Toronto), revealed that a truncated VAPA protein (D86-E237) interacted with MOR. This VAPA fragment contains the central coiled-coil region and C-terminal
transmembrane domain. Based on this interaction, a directed yeast-two-hybrid screen was completed to determine which MOR intracellular loop interacts with VAPA. For this experiment, all three MOR intracellular loops and the C-terminal tail were separately subcloned into a pAS2.1 vector. This vector expressed the binding domain of the yeast GAL4 transcription factor. The truncated VAPA construct was subcloned into a pACT2 vector, which expressed the GAL4 activating domain. The constructs were transformed into MAV103 yeast and incubated on nutrient-depleted dropout plates for 5-7 days. Resulting colonies that expressed the β-galactosidase reporter gene, as evidenced by blue coloration of the colony following X-gal treatment, confirmed a VAPA-MOR interaction and mapped the binding site to the second intracellular loop (IL2) of MOR (Figure B.2).

**Figure B.2: Mapping of the VAPA-MOR binding region**

MOR intracellular loops (IL1, IL2, IL3) and C-terminal tail (C-tail) were subcloned into the pAS2 vector, which expresses the GAL4 binding domain (bait construct). Truncated VAPA (amino acids 86-237) was subcloned into the pACT2 vector, which expresses the GAL4 activating domain (prey construct). Empty pACT2 vector was included as a negative control. An interaction between known binding partners, the calcium-dependent activator protein for secretion (CAPS) and the second intracellular loop of human D2 dopamine receptor (D2IL2) was tested as a positive control.
To confirm the interaction of VAPA with full-length MOR, co-immunoprecipitation (co-IP) experiments were conducted in cultured mammalian cells. An interaction was verified in HEK 293 cells stably transfected with FLAG-tagged MOR and transiently transfected with myc-tagged VAPA. MOR was immunoprecipitated from cells using anti-FLAG antibody and Western blots of the the immunoprecipitated lysates were probed for VAPA with anti-myc antibody. Anti-myc antibody detected immunoreactive bands at ~33 kD in the total lysate and co-IP experimental lanes, but not in the mock lane (Figure B.3). The mock lane represented a co-IP experiment with no anti-FLAG antibody; instead a non-specific IgG antibody was added for a negative control.

![Figure B.3: Co-immunoprecipitation of VAPA-MOR in cell culture](image)

MOR was immunoprecipitated from FLAG-tagged MOR-HEK293 cells overexpressing myc-tagged VAPA using anti-FLAG antibody. Immunocomplexes were probed for the presence of VAPA using anti-myc antibody. An immunoreactive band of approximately 33 kDa was detected in the lysate and co-immunoprecipitation (Co-IP) lanes but not in immunocomplexes from FLAG-tagged MOR cells that were not transfected with VAPA (-VAPA) or in a control in which non-specific IgG antibody was added (Mock). Data shown is representative of experiments conducted in triplicate.

To further confirm the interaction of VAPA with full-length, endogenous MOR, co-immunoprecipitation (co-IP) experiments were conducted using total brain lysates from mice. MOR was immunoprecipitated from cells using anti-MOR antibody and Western blots of the immunoprecipitated lysates were probed for VAPA with anti-VAPA antibody. Anti-VAPA antibody detected immunoreactive bands at ~33 kD in the total lysate and co-IP experimental
lanes, but not in the mock lane (Figure B.4). The mock lane represented a co-IP experiment with no anti-MOR antibody; instead non-specific IgG antibody was added for a negative control.

**Figure B.4: Co-immunoprecipitation of VAPA-MOR in total brain lysate**

Lysates were prepared from total mouse brain and immunoprecipitated with anti-MOR antibody. Immunocomplexes were separated by SDS-PAGE, transferred to a PVDF filter, and filters probed with anti-VAPA antisera. An immunoreactive band of ~33 kDa was detected in the lysate input lanes and Co-IP lane, but not in an IP in which non-specific IgG antibody was added (Mock lane). Data shown is representative of experiments done in triplicate.

To determine the cellular distribution of the VAP33-MOR interaction, we used confocal microscopy to conduct co-localization studies using HEK293 cells that stably express FLAG-MOR. VAPA has been shown to be a resident protein of the ER and Golgi apparatus (Skehel et al., 2000; Laurent et al., 2000; Weir et al., 1998; Peretti et al., 2008), and VAPA-MOR co-localization was found in a perinuclear distribution as expected (Figure B.5). The co-localization observed in this type of experiment provides strong evidence that the two proteins are localized to the same cellular location in vivo.
Immunohistochemistry was performed by transfecting HEK293 cells stably transfected with myc-tagged MOR. The cells were immunostained with monoclonal mouse anti-myc antibodies and rabbit anti-MOR antibodies. After this initial staining, the cells were washed with 1X PBS, and exposed to FITC conjugated donkey anti-rabbit (MOR) antibodies and rhodamine-conjugated donkey anti-mouse (myc) antibodies. Overlay shows the merging of labels (yellow color) in the confocal image.

Dual immunogold labeling was used to determine the sub-cellular localization of the VAPA-MOR interaction. Sections through the rostrocaudal extent of mouse striatum were incubated in a cocktail containing mouse anti-VAPA and rabbit anti-MOR primary antibodies. Sections were incubated with ultra small gold-coupled goat anti-rabbit secondary antibody and ultra small gold-coupled goat anti-mouse secondary antibody. Immunogold-silver particles measuring less than 0.05 µm in cross-sectional diameter were scored as small (MOR), while immunogold-silver particles measuring greater than 0.051 µm were scored as large (VAPA). The VAPA-MOR interaction localized to the endoplasmic reticulum (Figure B.6).
To more precisely identify possible roles of MOR interacting proteins in addiction and analgesia phenotypes, we compared and quantified MOR interacting protein expression levels in the cerebral cortex and hippocampus of placebo- and morphine-treated OPRM1 A112G knock-in mice. This mouse model mimics the equivalent single nucleotide polymorphism in the human receptor (A118G). In the cerebral cortex, no significant differences in the protein expression of either the D₂ dopamine receptor (Figure B.7) or dynamin (Figure B.8) were observed for placebo vs. morphine-treated A/A or G/G mice. Spinophilin protein expression in morphine-treated G/G mice in the cerebral cortex was significantly increased compared to both placebo- and morphine treated A/A mice ($p<0.01$), as well as placebo-treated G/G mice ($p<0.05$) (Figure B.9). GPR177/WLS protein expression in morphine-treated G/G mice in the cerebral cortex showed a decrease in expression compared to placebo-treated G/G mice ($p<0.05$) (Figure B.10). Morphine-treated
A/A mice, on the other hand, showed an increase in GPR177/WLS expression compared to placebo-treated A/A mice ($p<0.05$). MOR protein expression in morphine-treated A/A mice in the cerebral cortex was significantly decreased compared to placebo-treated A/A mice ($p<0.01$), suggesting that morphine causes a down-regulation in MOR (Figure B.11). In G/G mice that were treated with morphine, however, no difference in MOR expression compared to placebo-treated G/G mice was observed. In the cerebral cortex, VAPA expression in placebo-treated G/G mice was increased in comparison to both placebo- and morphine-treated A/A mice ($p<0.05$) (Figure B.12). Additionally, morphine treatment of G/G mice resulted in an increase in VAPA protein levels compared to placebo G/G mice ($p<0.05$).
Figure B.7: D2 dopamine receptor protein expression levels in the cerebral cortex of placebo- and morphine-treated OPRM1 wild-type (A/A) and A112G knock-in (G/G) mice

Western blot of D2 dopamine receptor protein levels expressed in the cerebral cortex and Ponceau total protein stain which served as experimental loading control. AA Plac: Placebo-treated OPRM1 wild-type mice (A/A); AA Morph: Morphine-treated OPRM1 wild-type mice (A/A); GG Plac: Placebo-treated OPRM1 A112G knock-in (G/G) mice; GG Morph: Morphine-treated OPRM1 A112G knock-in (G/G) mice.
Figure B.8: Dynamin protein expression levels in the cerebral cortex of placebo- and morphine-treated OPRM1 wild-type (A/A) and A112G knock-in (G/G) mice

Western blot of dynamin protein levels expressed in the cerebral cortex and Ponceau total protein stain which served as experimental loading control. AA Plac: Placebo-treated OPRM1 wild-type mice (A/A); AA Morph: Morphine-treated OPRM1 wild-type mice (A/A); GG Plac: Placebo-treated OPRM1 A112G knock-in (G/G) mice; GG Morph: Morphine-treated OPRM1 A112G knock-in (G/G) mice.
Figure B.9: Spinophilin protein expression levels in the cerebral cortex of placebo- and morphine-treated OPRM1 wild-type (A/A) and A112G knock-in (G/G) mice
Western blot of spinophilin protein levels expressed in the cerebral cortex and Ponceau total protein stain which served as experimental loading control. Results from ANOVA and a post-hoc Bonferroni test show an increase spinophilin expression in GG morphine mice compared to AA mice (p<0.01) and GG placebo mice (p=0.03). AA Plac: Placebo-treated OPRM1 wild-type mice (A/A); AA Morph: Morphine-treated OPRM1 wild-type mice (A/A); GG Plac: Placebo-treated OPRM1 A112G knock-in (G/G) mice; GG Morph: Morphine-treated OPRM1 A112G knock-in (G/G) mice.
Figure B.10: GPR177/WLS protein expression levels in the cerebral cortex of placebo- and morphine-treated OPRM1 wild-type (A/A) and A112G knock-in (G/G) mice
Western blot of GPR177/WLS protein levels expressed in the cerebral cortex and Ponceau total protein stain which served as experimental loading control. Results from ANOVA and post-hoc Bonferroni test show an increase in GPR177 expression in AA morphine-treated mice compared to AA placebo mice (p<0.05) and a decrease in GPR177 in GG morphine treated mice compared to GG placebo mice (<0.05). Plac: Placebo-treated OPRM1 wild-type mice (A/A); AA Morph: Morphine-treated OPRM1 wild-type mice (A/A); GG Plac: Placebo-treated OPRM1 A112G knock-in (G/G) mice; GG Morph: Morphine-treated OPRM1 A112G knock-in (G/G) mice.
Figure B.11: MOR protein expression levels in the cerebral cortex of placebo- and morphine-treated OPRM1 wild-type (A/A) and A112G knock-in (G/G) mice
Western blot of MOR protein levels expressed in the cerebral cortex and Ponceau total protein stain which served as experimental loading control. Results from an ANOVA and post-hoc Bonferroni test show a decrease in MOR expression in AA morphine-treated mice compared to AA placebo mice (p<0.01). AA Plac: Placebo-treated OPRM1 wild-type mice (A/A); AA Morph: Morphine-treated OPRM1 wild-type mice (A/A); GG Plac: Placebo-treated OPRM1 A112G knock-in (G/G) mice; GG Morph: Morphine-treated OPRM1 A112G knock-in (G/G) mice.
Figure B.12: VAPA protein expression levels in the cerebral cortex of placebo- and morphine-treated OPRM1 wild-type (A/A) and A112G knock-in (G/G) mice
Western blot of VAPA protein levels expressed in the cerebral cortex and Ponceau total protein stain which served as experimental loading control. Results from ANOVA and post-hoc Bonferroni test show an increase in VAPA expression in GG placebo mice compared to both AA placebo and AA morphine treated mice (p<0.05). There was also a significant increase in VAPA expression in GG morphine-treated mice compared to both AA placebo and AA morphine treated mice (p<0.01). A student's t test shows a significant increase in VAPA expression in GG morphine-treated mice compared to GG placebo mice (p<0.05). AA Plac: Placebo-treated OPRM1 wild-type mice (A/A); AA Morph: Morphine-treated OPRM1 wild-type mice (A/A); GG Plac: Placebo-treated OPRM1 A112G knock-in (G/G) mice; GG Morph: Morphine-treated OPRM1 A112G knock-in (G/G) mice.
As with the results obtained in the cerebral cortex samples, no significant differences in the protein expression of either the D_2 dopamine receptor (Figure B.13) or dynamin (Figure B.14) were observed for placebo vs. morphine-treated A/A or G/G mice in the hippocampus. Noteworthy, however, is the inter-animal variability in the protein expression levels for both D_2 dopamine receptor and dynamin. In the hippocampus, morphine-treated G/G mice showed a decrease in spinophilin protein levels compared to both wild-type (A/A) placebo- and morphine-treated mice \((p<0.01)\) (Figure B.15). Additionally, in comparison to placebo-treated G/G mice, the morphine-treated G/G mice showed a significantly decreased expression of spinophilin \((p<0.05)\). In the hippocampus, both placebo- and morphine-treated G/G mice had increased VAPA protein levels compared to either placebo- or morphine-treated wild-type (A/A) mice (Figure B.16). These results demonstrate that the G/G phenotype of OPRM1 has increased VAPA levels compared to the A/A phenotype. The increased VAPA levels in G/G mice appears to be independent of opioid treatment, as morphine-treated G/G mice showed similar VAPA expression levels as placebo G/G mice.
Figure B.13: D2 dopamine receptor protein expression levels in the hippocampus of placebo- and morphine-treated OPRM1 wild-type (A/A) and A112G knock-in (G/G) mice

Western blot of D₂ dopamine receptor protein levels expressed in the hippocampus and Ponceau total protein stain which served as experimental loading control. AA Plac: Placebo-treated OPRM1 wild-type mice (A/A); AA Morph: Morphine-treated OPRM1 wild-type mice (A/A); GG Plac: Placebo-treated OPRM1 A112G knock-in (G/G) mice; GG Morph: Morphine-treated OPRM1 A112G knock-in (G/G) mice.
Figure B.14: Dynamin protein expression levels in the hippocampus of placebo- and morphine-treated OPRM1 wild-type (A/A) and A112G knock-in (G/G) mice
Western blot of dynamin protein levels expressed in the hippocampus and Ponceau total protein stain which served as experimental loading control. AA Plac: Placebo-treated OPRM1 wild-type mice (A/A); AA Morph: Morphine-treated OPRM1 wild-type mice (A/A); GG Plac: Placebo-treated OPRM1 A112G knock-in (G/G) mice; GG Morph: Morphine-treated OPRM1 A112G knock-in (G/G) mice.
Figure B.15: Spinophilin protein expression levels in the hippocampus of placebo- and morphine-treated OPRM1 wild-type (A/A) and A112G knock-in (G/G) mice

Western blot of spinophilin protein levels expressed in the hippocampus and Ponceau total protein stain which served as experimental loading control. ANOVA and post-hoc Bonferroni test show a decrease in spinophilin expression in GG morphine-treated mice compared to AA placebo and AA morphine-treated mice (p<0.01) and compared to GG placebo mice (p<0.05). A student's t-test shows a decrease in spinophilin expression in GG morphine-treated mice compared to GG placebo mice (p<0.05). AA Plac: Placebo-treated OPRM1 wild-type (A/A); AA Morph: Morphine-treated OPRM1 wild-type mice (A/A); GG Plac: Placebo-treated OPRM1 A112G knock-in (G/G) mice; GG Morph: Morphine-treated OPRM1 A112G knock-in (G/G) mice.
Figure B.16: VAPA protein expression levels in the hippocampus of placebo- and morphine-treated OPRM1 wild-type (A/A) and A112G knock-in (G/G) mice

Western blot of VAPA protein levels expressed in the hippocampus and Ponceau total protein stain which served as experimental loading control. Results from ANOVA and post-hoc Bonferroni test show an increase in VAPA expression in GG placebo mice compared to AA placebo mice (p<0.05) and an increase in VAPA expression in GG morphine-treated mice compared to both AA placebo mice and AA morphine treated mice (p<0.05). AA Plac: Placebo-treated OPRM1 wild-type mice (A/A); AA Morph: Morphine-treated OPRM1 wild-type mice (A/A); GG Plac: Placebo-treated OPRM1 A112G knock-in (G/G) mice; GG Morph: Morphine-treated OPRM1 A112G knock-in (G/G) mice.
B.4 Discussion

G protein-coupled receptors (GPCRs) are the largest family of transmembrane receptors with key roles in regulating signaling pathways targeted by therapeutics. The mu-opioid receptor (MOR) belongs to the Class A rhodopsin-like family of G protein-coupled receptors (GPCRs). To obtain a better understanding of MOR-mediated signaling and to identify novel components of the MOR pathway, a modified membrane yeast two-hybrid (MYTH) approach was used to generate an interactome of the full-length human ligand-unoccupied MOR. The resulting MOR interactome connects 10 proteins to MOR with functions that range from cellular trafficking to regulation of electrical potentials of neurons. Of the 10 hypothesized interacting proteins identified in our MYTH screen, 4 interactors support the transport of ions in neurons to modulate electrical signaling and ultimately synaptic plasticity. These interactors include a subunit of the potassium channel, putative copper and zinc transporters, and a Na\(^+/H^+\) exchanger. Another identified interactor, gap junction protein alpha 4, may also modulate electrical signaling by enabling the cell to form intercellular channels to allow the exchange of ions and metabolites. The roles of Wolf-Hirschhorn syndrome candidate 2 and YIPL domain family member 3 have not been thoroughly studied. However, YIPL may serve as a Golgi export signal, allowing efficient intracellular trafficking of MOR. Another identified interactor, GPR177/WLS has been well characterized by our laboratory (Jin et al., 2010a; Jin et al., 2010b). It was discovered that the MOR-WLS interaction sequesters WLS at the cell surface resulting in the inhibition of Wnt protein secretion which is required for the maintenance of dendritic spines and the promotion of hippocampal neurogenesis.

The MYTH screen also identified an interaction between MOR and vesicle-associated membrane protein (VAMP)-associated protein (VAPA), a protein involved in trafficking and signaling modulation. VAPA, a member of the SNARE (soluble N-ethylmaleimide-sensitive component attachment protein receptor) family, is involved in vesicular docking and exocytosis.
VAPA is highly expressed in the ER and on perinuclear microtubules where it likely mediates vesicular transport (Skehel et al., 2000; Weir et al., 2001; Lotz et al., 2008). VAPA binding to MOR could modulate trafficking of MOR from the ER to the plasma membrane. Several structural features of VAPA support this idea (Figure B.17).

**Figure B.17: VAPA protein functional domains**

VAPA consists of a major sperm protein domain (amino acids 1-124), a coiled-coil domain (amino acids 159-196), and a transmembrane (TM) domain (amino acids 223-242).

VAPA exhibits strong homology across different species, containing highly conserved N- and C-termini and a central coiled-coil domain (Weir et al., 1998; Laurent et al., 2000; Skehel et al., 2000; Lotz et al., 2008; Pennetta et al., 2002). At the C-terminus, VAPA contains a short hydrophobic, transmembrane domain. This domain anchors VAPA to intracellular membranes via hydrophobic interactions. The centrally located alpha-helical, coiled-coil domain gives VAPA SNARE-like properties. The stretch of coiled-coils mediates VAPA interactions with vesicle associated membrane proteins, such as synaptobrevin and N-ethylmaleimide sensitive factor (NSF) protein. VAPA also self-dimerizes (Weir et al., 1998; Nishimura et al., 1999) and has been shown to bind promiscuously to many proteins in the SNARE family, including VAMP, syntaxin 1A, rbet, and rsec22 (Weir et al., 2001). At the N-terminus, VAPA consists of a 100-amino acid stretch, the major sperm protein (MSP) domain, which functions similarly to the actin cytoskeleton at the leading edge of migrating cells (Laurent et al., 2000). During synaptic bouton budding at the neuromuscular junction in *Drosophila*, VAPA associates with microtubules via its MSP domain.
VAPA is thought to stabilize microtubules into a bundled array of fibers that is necessary for bouton division and branching (Pennetta et al., 2002). An increase in dendritic arborization has been observed with chronic opioid administration in rat studies (Abbadie et al., 2000a; Eckert et al., 2003). This implies that VAPA could contribute to neural adaptations by interacting with MOR at the MSP domain. My overall goal was to map the binding site of VAPA to MOR and to characterize the both the cellular and subcellular localization of the protein interaction.

My experiments successfully confirmed the interaction of the full-length bait and prey in mammalian cells, localized the VAPA-MOR interaction to the endoplasmic reticulum, and demonstrated the effects of agonist on VAPA protein levels. To measure the effects of opioids on VAPA protein levels, we analyzed knock-in *OPRM1* A112G mice treated with morphine. Compared to wild-type A/A, the levels of VAPA protein increased in G/G mice in response to morphine. From this data, it can be hypothesized that morphine induces an upregulation of VAPA in certain individuals that may mediate the rewarding effect of the opioid. This would suggest that the novel MOR interacting protein VAPA plays a role beyond that of a cargo carrier.

Additional studies aimed at revealing how VAPA regulates MOR trafficking in the presence of opioid agonist are needed to address the cellular mechanisms underlying the upregulation of VAPA in the presence of opioids. Based on its localization to the endoplasmic reticulum and increased expression in the presence of morphine in mice, VAPA may be involved in the trafficking of newly synthesized MOR from the ER/Golgi to the plasma membrane (Figure B.18).
Figure B.18: Hypothetical role for VAPA in mediating MOR trafficking in the vesicular transport pathway

VAPA as a MOR interacting protein may regulate the trafficking of MOR through the endoplasmic reticulum (ER) and Golgi apparatus in the vesicular transport pathway.

Intracellular trafficking of MOR through the ER and Golgi represents an unanticipated control point in MOR transport and recycling. Knowledge about opioid receptor trafficking at this step in the transport pathway has the potential to conceptually challenge our current understanding of the development of tolerance and addiction to opioid drugs.

In addition to studying the biochemical and protein expression of VAPA, I also assessed whether mice expressing a common single nucleotide polymorphism of MOR would differentially express MOR interacting proteins as well as GPCRs, the D₂ dopamine receptor and MOR. Several OPRM1 gene splicing variants have been identified and analyzed for their associations with pain sensitivity and susceptibility for developing opioid dependence. Some studies suggest that there are significant associations between genetic variations in OPRM1 and the frequency of opioid dependence or vulnerability for opioid tolerance (Mague, 2010). Other studies, including a recent meta-analysis, have concluded that there is a lack of association between the A118G polymorphism and opioid dependence (Coller et al., 2009). Another study
found sex-specific reductions in the rewarding properties of morphine and the aversive components of naloxone-precipitated morphine withdrawal in A112G mice (Mague et al., 2009). Other studies have found that the A118G allele can act as either a risk or protective factor depending on the race or ethnicity carrying the G allele (Mague, 2010). In our study, protein expression was measured in a mixture of male and female A112G mice. The functional interpretation of any protein expression changes we observed in our A112G mice is therefore highly debatable. Summary tables of protein expression in the cerebral cortex (Table B.2) and hippocampus (Table B.3) show significant protein expression changes in comparison to A/A placebo mice.

Table B.2: Summary of significant protein expression changes in the cortex

<table>
<thead>
<tr>
<th>Cortex</th>
<th>AA Placebo</th>
<th>AA Morphine</th>
<th>GG Placebo</th>
<th>GG Morphine</th>
</tr>
</thead>
<tbody>
<tr>
<td>D2R</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dynamin</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Spinophilin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GPR177</td>
<td>↑</td>
<td>↑</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MOR</td>
<td>↓↓</td>
<td>↑</td>
<td>↑</td>
<td></td>
</tr>
<tr>
<td>VAPA</td>
<td></td>
<td>↑</td>
<td>↑↑</td>
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</tr>
</tbody>
</table>
Table B.2: Summary of significant protein expression changes in the hippocampus

<table>
<thead>
<tr>
<th>Hippocampus</th>
<th>AA Placebo</th>
<th>AA Morphine</th>
<th>GG Placebo</th>
<th>GG Morphine</th>
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<tbody>
<tr>
<td>D2R</td>
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<tr>
<td>Dynamin</td>
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<td>Spinophilin</td>
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<tr>
<td>VAPA</td>
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</table>

VAPA expression in both the cerebral cortex and hippocampus of G/G mice was increased in comparison to wild-type A/A mice. Another significant protein difference in the A/A versus G/G mice was observed with spinophilin. In the cortex, morphine-treated G/G mice showed increased expression levels compared to placebo-treated G/G mice; however in A/A mice that were treated with morphine there was no change in spinophilin compared to the placebo control. Spinophilin is a dendritic spine-enriched scaffold protein that modulates synaptic transmission. In spinophilin knockout (KO) mice, there is a reduced sensitivity to the analgesic effects of morphine and an early development of tolerance. Spinophilin KO mice also showed a higher degree of physical dependence and increased sensitivity to the rewarding actions of the drug (Charlton et al., 2008). The increase in spinophilin expression following morphine in G/G mice may serve a protective role by preventing an increased vulnerability to the rewarding aspects of morphine that would be predicted with low levels of spinophilin. This would also align with evidence for the protective effect of the G allele against heroin dependence in some populations (Mague, 2010).

On the other hand, the reciprocal effect was observed with spinophilin expression in the hippocampus, where G/G mice treated with morphine demonstrated decreased levels of
spinophilin compared to placebo A/A mice. Spinophilin KO mice show increased sensitivity to 
the rewarding effects of morphine and in some populations carriers of the G allele may be at 
increased risk of developing opioid dependence. The observed decrease in spinophilin in the G/G 
mice following morphine treatment suggests that G/G mice cannot induce an overexpression of 
spinophilin in the hippocampus following morphine treatment, and the low spinophilin levels in 
G/G mice make them particularly vulnerable to the rewarding effects of opioids.

The experiments in Appendix B primarily used cellular models and a non-contingent 
(experimenter-delivered drug) model of morphine dependence. Although these experiments 
confirmed VAPA as a novel MOR interacting protein that is differentially expressed following 
morphine exposure in mice, it cannot be concluded that VAPA or other MOR interacting proteins 
are differentially expressed in a behavioral model of addiction. Since the goal of my research is 
to determine if MOR interacting proteins are candidate genes that show altered expression in a 
state of opioid dependence and addiction, I need a behavioral paradigm that can differentiate 
between "addicted" and "non-addicted" subjects. To achieve this end, I modified an animal 
model of cocaine self-administration that demonstrated evidence of "addiction-like" behaviors in 
rats (Deroche-Gamonet et al., 2004). In Chapter 2, I present evidence that "addiction-like" 
behaviors develop with experience over time in rats that had self-administered heroin. In Chapter 
3, I present data showing that several MOR interacting proteins are differentially expressed in rats 
demonstrating "addiction-like" behaviors.
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