BRAIN G-PROTEINS IN DRUG
DEPENDENCE: A POSTMORTEM STUDY

by

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A thesis submitted in conformity with the requirements
for the degree of Master of Science
Institute of Medical Sciences
University of Toronto

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0-612-45410-X
ABSTRACT

Substantial evidence implicates altered dopaminergic receptor and G protein-coupled cAMP signaling in the development of drug dependence. Chronic cocaine and morphine administration to rats reduces NACS Gαi levels, but the effects of these drugs on G protein levels in human brain have not been systematically studied. Accordingly, G-protein subunit immunolabelling was measured in NACS, putamen, amygdala, hippocampus, and occipital, temporal, frontal and cerebellar cortices from chronic cocaine (N=12), heroin (N=8), or methamphetamine (N=12) users using Western blotting.

Gαi₁, and Gαi₂ or Gαo were significantly lower (p<0.05) in NACS from heroin (Gαi₁, 47%; Gαi₂, 49%) and methamphetamine (Gαi₁, 32%; Gαo, 18%) users compared to controls. No significant differences were found in other regions or in brain from the cocaine group. Findings of reduced NACS Gαi immunolabelling in heroin and methamphetamine suggests that chronic use of these drugs may increase cAMP signaling through disinhibition of Gαi-regulated signal transduction.
ACKNOWLEDGEMENTS

My primary debts of gratitude go to my supervisors, Drs. Jerry Warsh and Stephen Kish, for their commitment to this work, insightful discussions, and for providing me with such an intellectually rewarding research project. Specifically, I would like to thank Dr. Warsh for his enduring support and counsel throughout the duration of this work. I am indebted to Dr. Kish for his enthusiasm and for encouraging me to speculate.

I would like to express my gratitude to Dr. Peter Li for his expertise in signal transduction research and prompt assistance. Thank you also to J.C. Cutz, David Sibony, Kin Po Siu, and Roula Andreopoulos for their technical advice and constructive suggestions. I am also grateful to Kathleen Shannak for her helpful managerial skills and assistance in dissection of the brain samples.

Thank you to my parents and sisters for their support and encouragement throughout this project. A special thank you to Connor for inspiring me. Finally, I am deeply grateful to Scott – thank you for your love and patience.
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<th>Description</th>
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<tbody>
<tr>
<td>AC</td>
<td>adenylyl cyclase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AP</td>
<td>activating protein</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine 5’-triphosphate</td>
</tr>
<tr>
<td>α</td>
<td>alpha subunit of G-protein</td>
</tr>
<tr>
<td>β</td>
<td>beta subunit of G-protein</td>
</tr>
<tr>
<td>βARK</td>
<td>beta-adrenergic receptor kinase</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>BZE</td>
<td>benzoylcyecgonine</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>calcium ion</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic 3’,5’adenosine monophosphate</td>
</tr>
<tr>
<td>°C</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>COMT</td>
<td>catechol-O-methyl transferase</td>
</tr>
<tr>
<td>CRE</td>
<td>cyclic AMP response element</td>
</tr>
<tr>
<td>CREB</td>
<td>cyclic AMP response element binding protein</td>
</tr>
<tr>
<td>CTX</td>
<td>cholera toxin</td>
</tr>
<tr>
<td>DA</td>
<td>dopamine</td>
</tr>
<tr>
<td>DAT</td>
<td>dopamine transporter</td>
</tr>
<tr>
<td>DOPAC</td>
<td>dihydroxyphenylacetic acid</td>
</tr>
<tr>
<td>ECL</td>
<td>enhanced chemiluminescence</td>
</tr>
<tr>
<td>EME</td>
<td>ecgonine methylester</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated protein kinase</td>
</tr>
<tr>
<td>FRA</td>
<td>fos related antigen</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>γ-G-protein</td>
<td>gamma subunit of G-protein</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine nucleotide-binding protein</td>
</tr>
<tr>
<td>HVA</td>
<td>homovanillic acid</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>LAD</td>
<td>L-aromatic amino acid decarboxylase</td>
</tr>
<tr>
<td>MAO</td>
<td>monoamine oxidase</td>
</tr>
<tr>
<td>MAP</td>
<td>mitogen activated protein</td>
</tr>
<tr>
<td>3-MT</td>
<td>3-methoxytyramine</td>
</tr>
<tr>
<td>NMDA</td>
<td>n-methyl-D-aspartate</td>
</tr>
<tr>
<td>NACs</td>
<td>nucleus accumbens</td>
</tr>
<tr>
<td>PDE</td>
<td>phosphodiesterase</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PLA</td>
<td>phospholipase A</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>PMI</td>
<td>post-mortem interval</td>
</tr>
<tr>
<td>PTX</td>
<td>pertussis toxin</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene difluoride</td>
</tr>
<tr>
<td>RMAR</td>
<td>release modulating autoreceptors</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulphate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SMAR</td>
<td>presynaptic synthesis modulating autoreceptors</td>
</tr>
<tr>
<td>PBST</td>
<td>tris-buffered saline (with 0.1% Tween-20)</td>
</tr>
<tr>
<td>TH</td>
<td>tyrosine hydroxylase</td>
</tr>
<tr>
<td>VMAT2</td>
<td>vesicular monoamine transporter</td>
</tr>
<tr>
<td>VTA</td>
<td>ventral tegmental area</td>
</tr>
</tbody>
</table>
CHAPTER 1: INTRODUCTION
1.1 WHAT IS DRUG ABUSE?

Drug abuse/addiction can be defined as the loss of control over the use of a drug (s) for non-medicinal purposes, despite adverse consequences (i.e., loss of effectiveness in society, behavioral psychopathology) for the individual (Altman, et al., 1996). The Diagnostics and Statistical Manual of Mental Disorders, Version IV of the American Psychiatric Association (American Psychiatric Association, 1994) uses the term substance dependence to define the behavioral syndromes in which the individual uses a drug in a maladaptive manner in a 12 month period leading to significant impairment in function or distress and accompanied by evidence of tolerance and withdrawal. Such behavioral correlates of repeated drug use are briefly described below and include sensitization, tolerance, craving, and withdrawal (Koob & Bloom, 1988; Self & Nestler, 1995).

Sensitization

Sensitization can be operationally defined as a shift of a dose-response curve to the left following repeated drug administration (Altman et al., 1996). It is a progressive increase in an effect of a drug with repeated use or a hypersensitivity to an effect of the drug as a consequence of past exposure to the drug. Animal studies have shown that sensitization can occur to the rewarding and locomotor activating effects of cocaine, amphetamine, and morphine (for review see Robinson and Berridge, 1993).

Tolerance

Tolerance refers to a rightward shift of the dose response curve upon repeated drug use. Thus a higher dose of the drug is required to produce the same effect that was once experienced at a lower dose (Altman et al., 1996).
Craving

Craving can be considered a form of excessive wanting that is independent of a drug’s hedonic properties. It represents a motivational state marked by an uncontrollable urge to use drugs (Altman et al., 1996).

Withdrawal

Withdrawal syndrome is a cluster of symptoms that result from abstinence from the drug or from pharmacological precipitation. The syndrome may include somatic (autonomic) physical features as well as subjective (dysphoria) components (Altman et al., 1996).

1.2 EPIDEMIOLOGY OF COCAINE, METHAMPHETAMINE AND HEROIN USE

According to surveys conducted by the Addiction Research Foundation in Toronto (Ontario Profile - Alcohol and Other Drugs, 1996), the use of illicit drugs including cocaine, amphetamine, and heroin is prevalent among the general adult and youth populations in Ontario. In 1994, the lifetime prevalence of cocaine use was 5.7%, with the 30-39 age group having the highest rate at 11.6%. No data are available on the epidemiology of amphetamine use among adults. Lifetime heroin use among adults is 0.7%. Cocaine use among high school students has risen from 1.5% in 1993 to 2.4% in 1995. Overall, 4.6% of students reported using methamphetamine during 1994, up from 2.0% in 1993, and 1.1% reported using “ice”, a potent smokable form of methamphetamine. Heroin use in students has also increased, with 1.2% having reported using in 1993 and 2.0% reporting use in 1995 (reviewed in Ontario Profile - Alcohol and Other Drugs, 1996). Although most drugs of abuse are originally exploited for their pleasurable effects, including euphoria, a general sense of well-being and decreased anxiety, repeated drug use is associated
with several adverse effects, including psychosocial disruption, medical complications and increased mortality rates. The negative effects of drug use also extend beyond the user and to society as a whole, through both direct health costs and indirect costs in terms of crime and loss of earnings and productivity (Altman et al., 1996). Reducing the extent of drug abuse is a major goal of medicine and the elucidation of the neural pathophysiology underlying substance dependence should contribute its prevention and treatment.

1.3 THE PSYCHOSTIMULANTS: Cocaine and Methamphetamine

Cocaine and methamphetamine are classified as stimulants due to their ability to enhance mood, activate the central nervous system, increase alertness, and relieve fatigue, effects which are all believed to be the result of the release of central stores of the catecholamines dopamine and norepinephrine (Weiss et al., 1994). Central nervous stimulants can be divided into two categories based on their effects on catecholamine-containing neurons in the brain. Drugs in the amphetamine class inhibit re-uptake and promote release of catecholamines, whereas drugs in the non-amphetamine class (cocaine or methylphenidate) inhibit catecholamine re-uptake, but are limited in their releasing properties (Ritz & Kuhar, 1989).

1.3.1 Cocaine

Metabolism of cocaine

Benzoylecggonine (BZE) and ecgonine methyl ester (EME) are the major metabolites of cocaine in the absence of alcohol (see Figure 1.1). Approximately 30-50% of cocaine is converted to EME by hepatic esterases and plasma pseudocholinesterases, whereas spontaneous hydrolysis of cocaine into BZE accounts for 30-40%. The half-lives of cocaine, EME and BZE are 40 minutes, 4
hours and six hours, respectively. In the presence of ethanol, cocaethylene is formed in the liver via the transmethylation of the cocaine molecule and cocaethylene has been shown in animal studies to block dopamine reuptake and produce the same behavioral alterations as cocaine (Karch, 1993).

**Figure 1.1 Metabolic fate of cocaine**

![Diagram showing the metabolic fate of cocaine](image)

Legend to Figure 1.1: In the absence of ethanol, BZE and EME are the principle breakdown products of cocaine. Cocaine is converted to EME by hepatic esterases and plasma pseudocholinesterases and BZE forms spontaneously by non-enzymatic hydrolysis.

**Behavioral and pharmacological effects of cocaine**

Cocaine is a non-amphetamine class of stimulant as well as a local anesthetic with potent vasoconstrictive properties (Wood, 1996). It induces subjective feelings of intense euphoria and alertness, increased confidence and strength, heightened sexual feelings, and a general indifference to cares and concerns. Yet, these pleasurable sensations are rapidly replaced by despondency, anhedonia, and
despair (Gawin & Ellinwood, 1990). Furthermore, the rewarding effects of cocaine use are accompanied by the risk of death by cardiac arrhythmia, respiratory depression, and convulsions (Withers et al., 1995).

Leaf chewing, snorting (inhalation), smoking, and intravenous injection are all methods of cocaine administration. Addiction is a common complaint of some cocaine users, while others can use it intermittently for years without developing dependency. Stimulants tend to be used more irregularly than other drugs such as nicotine, alcohol, and opioids and binge use (terminating when the supply is exhausted) ranging from hours to days is common (Weiss et al., 1994).

Pharmacologically, cocaine increases monoamine availability at the synapse (Gropetti et al., 1973). It is a sympathomimetic drug that blocks the reuptake of noradrenaline, dopamine, and serotonin into synaptosomal preparations at the synaptic terminal, an effect abolished by reserpine pretreatment (Ross et al., 1967; Pifl et al., 1995). Cocaine also acts as a local anesthetic by inhibiting sodium currents and blocking peripheral nerve impulses (Ritchie & Greene, 1985). It is thought that the behavioral effects of cocaine are due to its ability to increase dopaminergic neurotransmission in specific brain regions by inhibiting the plasmalemmal dopamine transporter (Giros et al., 1996), (but see Rocha et al., 1998).

1.3.2 Methamphetamine

Metabolism of methamphetamine

The main metabolites of methamphetamine are shown in figure 1.2. Methamphetamine is cleared from the body via multiple routes and has a half-life of approximately 12 hours (Karch, 1993). Approximately 45% of methamphetamine
remains unchanged in the urine, 20% is N-dealkylated to produce amphetamine, and 15% is converted to p-hydroxymethamphetamine. These compounds are further metabolized by a combination of deamination, p-hydroxylation, and conjugation (Karch, 1993).

**Figure 1.2: Metabolic fate of methamphetamine**

\[
\text{methamphetamine} \xrightarrow{\text{N-dealkylation}} \text{amphetamine} \xrightarrow{\text{β-hydroxylation}} \text{norephedrine} \xrightarrow{\text{deamination}} \text{4-hydroxyamphetamine} \quad \text{phenylacetone}
\]

Legend to Figure 1.2: Methamphetamine is dealkylated into amphetamine. Amphetamine is further metabolized into 4-hydroxyamphetamine, norephedrine and phenylacetone by a combination of hydroxylation and deamination.

*Behavioral and pharmacological effects of methamphetamine*

Central effects of amphetamine-like drugs include increased locomotor stimulation, alertness, euphoria and appetite suppression (Karch, 1993). The acute pleasurable effects of amphetamine are similar to those produced by cocaine and also like cocaine, these effects are generally replaced by depression, lack of motivation, paranoia and in some cases, psychosis. Amphetamine users also face increased mortality rates usually resulting from cardiovascular abnormalities due to
excess catecholamine levels (Karch, 1993). Amphetamine can be swallowed, injected, smoked or snorted (Gawin & Ellinwood, 1990; Karch 1993).

Like cocaine, amphetamine is an indirectly acting sympathomimetic which increases monoamine levels at the synapse (Gropetti et al., 1973). However, in addition to blocking re-uptake by interacting with the dopamine transporter at the plasmalemma, amphetamine also acts as a potent releaser of extravesicular newly synthesized pools of transmitter (Sulzer et al., 1995). The effects of amphetamine-type psychostimulants are not influenced by reserpine pretreatment (Cadoni et al. 1995; Sulzer et al., 1995). It is important to note that although different pools of dopamine may be involved in mediating the effects of cocaine and methamphetamine, both mechanisms lead to the increased dopaminergic transmission thought to be central in mediating the rewarding and locomotor activating effects of both drugs (reviewed in DiChiara, 1995).

1.4 HEROIN

Heroin is a pro-drug whose centrally active metabolite is morphine and thus, experimental studies with animals using morphine can be regarded as directly relevant to the mechanisms of heroin dependence in humans (DiChiara & North, 1992).

Metabolism of heroin

Once in the body, heroin is rapidly de-acetylated into 6-acetylmorphine within 10 to 15 minutes (Karch, 1993). A second step de-acetylation which converts 6-acetylmorphine into morphine is complete in a few hours. The physiological half-life of morphine in the system is under two hours and its main metabolites include
morphine-3-glucuronide, morphine-6-glucuronide, and nor-morphine (see Figure 1.3) (Karch 1993).

Behavioral and pharmacological effects of heroin

Heroin administration produces a variety of positive symptoms that can be described as warmth or high with intense pleasure (rush) compared to sexual orgasm (Karch, 1993). Heroin also has respiratory depressant, analgesic, sedative, and emetic properties (Karch, 1993). Repeated heroin users face increased mortality rates from three sources (1) direct effects of heroin or its active metabolites, (2) direct effects of adulterants or expedients injected along with the drug, and (3) infectious, mechanical, or lifestyle complications associated with the practices of drug use. Common methods of heroin administration are injection, smoking, and nasal inhalation (Karch 1993).

Figure 1.3: Metabolic fate of heroin

Legend to Figure 1.3: Heroin is rapidly de-acetylated into 6-acetylmorphine within 10 to 15 minutes. A second step de-acetylation which converts 6-acetylmorphine into morphine is complete in approximately 2 hours. The physiological half-life of morphine is under two hours and its main metabolites include morphine-3-glucuronide, morphine-6-glucuronide, and nor-morphine.
After intravenous injection, the effects begin in less than a minute. Heroin has high lipid solubility, crosses the blood brain barrier quickly, and is deacetylated into the active metabolites 6-acetylmorphine and morphine. The effects of heroin wear off in about 3 to 5 hours depending on the dose and experienced users may use heroin 2 to 4 times per day. The chronic heroin user is constantly oscillating between being “high” and feeling the negative effects of early withdrawal, leading to problems in homeostatic systems, including the hypothalamic-pituitary-gonadal axis and the hypothalamic-pituitary-adrenal axis, which are regulated in part by endogenous opioids (Karch 1993).

The body produces endogenous opioid peptides called enkephalins, endorphins and dynorphins which are widely distributed in the peripheral nervous system and brain and play important roles in modulating endocrine, cardiovascular, gastrointestinal, and immune functions (Karch, 1993). Their broad brain distribution suggests that they also function as neuromodulators and/or neurotransmitters (Reisine & Bell, 1993).

Pharmacological studies have identified at least three classes of opioid receptors, δ,κ, and μ, which differ in their affinity for various opioid ligands and in their distribution in the nervous system (Satoh & Minami, 1995; Lazarus et al., 1996; Standifer & Pasternak, 1997). The δ and μ receptors bind enkephalins and endorphins and the κ receptors potently bind dynorphins. Depending on which receptor is activated, the result may be analgesia, dysphoria, or respiratory depression (see Table 1.1). Morphine is a relatively selective agonist for μ-opioid receptors and this receptor is implicated in the reinforcing and withdrawal effects of morphine (Karch 1993; Matthes et al., 1996).
### Table 1.1: Opiate receptor subtypes

<table>
<thead>
<tr>
<th>Receptor Type</th>
<th>Result of Stimulation</th>
<th>Opiate Receptor Coupling</th>
</tr>
</thead>
<tbody>
<tr>
<td>μ</td>
<td>supraspinal analgesia, respiratory depression, sedation, brachycardia</td>
<td>adenyl cyclase inhibition, $K^+$ channel activation, $Ca^{2+}$ channel inhibition IP$_3$ inhibition</td>
</tr>
<tr>
<td>κ</td>
<td>spinal analgesia, sedation</td>
<td>adenyl cyclase inhibition, $K^+$ channel activation, $Ca^{2+}$ channel inhibition</td>
</tr>
<tr>
<td>δ</td>
<td>dysphoria, tachycardia</td>
<td>adenyl cyclase inhibition, $K^+$ channel activation, $Ca^{2+}$ channel inhibition PLC activation</td>
</tr>
</tbody>
</table>

$^{1}$Karch 1993; Uhl et al., 1994; Satoh & Minami, 1995; Tsu et al., 1995; Standifinder & Pasternak, 1997.

### 1.5 WHY USE DRUGS?

The two most widely accepted theories of addiction fall into two classes: negative reinforcement models, in which the drug is used to avoid the aversive symptoms of drug withdrawal and positive reinforcement models, in which the drug is misused due to its ability to induce pleasure/hedonia (for review see Robinson & Berridge, 1993). However, for several reasons, these theories do not adequately explain why people use addictive drugs.

First, studies have found that both humans and animals will both self-administer opioids in the absence of withdrawal symptoms (Ternes et al., 1985; Lamb et al., 1991). Second, maximal periods of drug self-administration often do not coincide with maximal periods of withdrawal (Wise & Bozarth, 1987). Third, many drugs used medically produce physical withdrawal symptoms, but are not typically self-administered including tricyclic antidepressants, anticholinergics and k-
opioid antagonists (Jaffe, 1992). Fourth, eliminating withdrawal in drug users is minimally effective in addiction treatment (Wise & Bozarth, 1987). Fifth, relapse is high after long periods of drug abstinence, long after withdrawal signs have subsided (Robinson & Berridge, 1993). And finally, animals self-administer drugs into brain regions that do not produce withdrawal signs (Wise & Hoffman, 1992).

There are two main shortcomings of positive reinforcement / euphoria models in explaining drug using behavior. This view of drug dependency does not explain drug craving or relapse caused by environmental stimuli associated with drug use (Robinson & Berridge, 1993). The strongest evidence against the notion that users are motivated by the pleasurable effects of drugs comes from investigations that have shown that humans will self administer low doses of morphine in the absence of subjective pleasure (Lamb et al., 1991).

Positive and negative reinforcement models cannot explain several core features of addiction, thus new theories such as the incentive sensitization theory of addiction have been proposed (Robinson & Berridge, 1993). It is well-established that repeated drug exposure can lead to neuronal alterations at the molecular level in brain regions which are hypothesized to mediate both withdrawal, reward, and sensitization (reviewed in Self & Nestler, 1995). Robinson and Berridge (1993) proposed that craving and relapse are due directly to drug-induced changes normally mediated by a neural system that undergoes sensitization-related neuroadaptations.

Addictive drugs share the ability to activate the mesolimbic dopamine system (Di Chiara & Imperato, 1988) and one function of this pathway is to attribute "incentive salience" perception and mental representation of events associated with activation of the pathway. Incentive salience is defined as a psychological process
that transforms the perception of stimuli, making them attractive or wanted (Robinson & Berridge, 1993). It is hypothesized that chronic drug use in some individuals leads to molecular changes in the mesolimbic dopamine system rendering the system hypersensitive to the drug and drug-associated stimuli (Robinson & Berridge, 1993). Associative learning gates the sensitization of the dopamine pathways, leading to excessive incentive salience to be attributed to drug taking. Thus, it is sensitization of incentive salience that transforms ordinary wanting into excessive drug craving producing compulsive drug seeking and drug taking (Robinson & Berridge, 1993). Although this theory can explain certain phenomena associated with chronic drug use, such as craving, it is still only a hypothetical model of drug dependence that will undoubtedly be amended as more information concerning drug use is discovered.

1.6 NEURAL PATHWAYS IMPLICATED IN DRUG DEPENDENCE

In order to understand fully the consequences of psychostimulant and heroin abuse, it is necessary to elucidate the impact on brain neurochemistry of these drugs. Chronic exposure to reinforcing drugs is hypothesized to induce long lasting adaptations (plasticity) in brain-reinforcement systems (reviewed in Self & Nestler, 1995; Hyman & Nestler, 1996). The positive reinforcing properties of drugs of abuse are believed to be related to their ability to induce neuroadaptations in specific brain pathways used by natural reinforcers such as sex, food and water. Thus, abused drugs can be regarded as biochemical surrogates of specific classes of motivational stimuli. Among the neuromodulators that participate in motivation, dopamine appears be key in mediating the locomotor and reinforcing effects of cocaine, methamphetamine and heroin (reviewed in DiChiara 1995).
1.6.1 Dopamine regulation in the brain

Figure 1.4 presents a schematic of a functioning dopaminergic nerve terminal.

Legend to Figure 1.4: Tyrosine is converted to L-DOPA by TH. L-DOPA is then converted to DA by LAD. Inside the nerve terminal DA can be stored in a cytoplasmic pool or in synaptic vesicles. DA is packaged into synaptic vesicles by vesicular monoamine transporter (VMAT2) and is released by exocytosis into the synapse where it can interact with postsynaptic DA receptors or presynaptic synthesis modulating autoreceptors (SMAR) and release modulating autoreceptors (RMAR). DA is metabolized in the synaptic cleft by COMT and MAO to form 3-MT and HVA. DA is converted to DOPAC by intraneurial MAO. The DAT removes DA from the synapse.

1.6.2 The dopaminergic pathways

Two major dopaminergic systems originate in the ventral midbrain: the nigrostriatal dopamine system and the mesocorticolimbic dopamine system. The
nigrostriatal dopaminergic system originates in the substantia nigra pars compacta (area A9) and projects primarily to the putamen and caudate nucleus. Degeneration of this system contributes to the symptoms of Parkinson’s disease and this pathway is also implicated in the short-term extrapyramidal symptoms of anti-psychotics, including hand tremor and muscle rigidity, as well as in the more long term effects including tardive dyskinesia (Mayeux & Kandel, 1991)

The cell bodies of the mesocorticolimbic dopaminergic system originate in the ventral tegmental area (area A10) and project to the forebrain, largely the NACS, olfactory tubercle, frontal cortex, amygdala, hippocampus, and septal area (Ungerstedt, 1971). In the absence of drugs of abuse, the mesocorticolimbic dopamine system is hypothesized to act as a modulator or gating mechanism for signals from the limbic system which mediate basic biological drives and motivational variables (Mogenson et al., 1980). It is thought that these signals are ultimately translated into motor acts via the output of the extrapyramidal motor system. It is the mesocorticolimbic dopamine system that is implicated in mediating the behavioral effects of drugs of abuse (reviewed in Koob, 1992; Di Chiara, 1995).

1.6.3 Mechanisms of increased dopaminergic neurotransmission in the NACS

Cocaine is a potent blocker of exocytotically released DA reuptake (Ross & Benji, 1967), whereas amphetamine appears to increase DA from both a vesicular reserpine sensitive pool (Cadoni et al., 1995) and by promoting reverse operation of the DA transporter (Sulzer et al., 1995). Thus, both cocaine and amphetamine directly increase dopaminergic transmission in the NACS. Opiates, on the other hand, act indirectly to increase DA in the NACS by activating µ opioid receptors on
GABA inhibitory interneurons which project onto VTA dopaminergic fibres (Johnson et al., 1992). Released from inhibition, the VTA neurons increase dopaminergic transmission to the NACS (Leone et al., 1991).

1.6.4 Evidence implicating the mesocorticolimbic dopamine system in mediating the behavioral effects of cocaine, methamphetamine and heroin

Virtually all drugs which are reinforcing in humans are also reinforcing in laboratory animals (Koob, 1992; Robinson & Berridge, 1993). A substantial body of pharmacological and behavioral evidence has implicated the mesocorticolimbic dopamine system in mediating drug reinforcement. Several classes of abused drugs including psychostimulants, opioids, ethanol, and nicotine have been shown to increase dopaminergic neurotransmission in this region, in particular the NACS (DiChiara & Imperato, 1988; Carboni et al., 1989; Kuczenski et al., 1991).

Cocaine and Methamphetamine

Studies have shown that rats will self-administer amphetamine (Hoebel et al., 1983) and dopamine (Dworkin et al., 1985) directly into the NACS and the reinforcing effects of psychostimulants are mediated by an intact mesocorticolimbic dopamine system. Bilateral injections of selective dopamine receptor antagonists into the NACS (Maldonado et al., 1993), and lesions of neurons projecting from the ventral tegmental area to the NACS (Roberts & Koob, 1982) abolish the reinforcing properties of intravenously administered cocaine, whereas analogous destruction of noradrenergic and serotonergic neurons does not have a similar effect (Roberts et al., 1977). Dopamine uptake blockers, such as GBR 12909 and cocaine, produce decreases in brain self-stimulation thresholds, whereas both D₁ and D₂ like receptor antagonists produce increases in electrical self-stimulation thresholds (reviewed in
Kuhar et al., 1991). Furthermore, microdialysis studies have shown that cocaine and amphetamine administration lead to dose-dependent increases in extracellular dopamine levels in the nucleus accumbens (DiChiara & Imperato, 1988; DiCiano et al., 1995).

**Morphine**

Various behavioral studies have implicated the ventral tegmental area as an anatomical substrate of the rewarding effects of opiates. Intra-VTA injections of morphine lower brain self-stimulation thresholds (Broekkamp et al., 1979), induce conditioned place preferences (Phillips & LePiane, 1980) and support self-administration behavior (Bozarth & Wise, 1981). Morphine administered directly into the VTA, but not into the NACS, reinstates self-administration of heroin after the behavior has been extinguished (De Wit & Stewart, 1983; Stewart, 1984). Interestingly, amphetamine infused directly into the VTA will also reinstate heroin self-administration after it has been extinguished, and intra-VTA morphine will reinstate cocaine self-administration after it has been extinguished (De Wit & Stewart, 1983; Stewart, 1984). These latter results specifically implicate the dopaminergic projections from the VTA to the NACS in participating in the reinforcing actions of morphine, as both cocaine and amphetamine are known to directly potentiate dopaminergic neurotransmission (DiChiara & North, 1992). Moreover, lesions of dopaminergic neurons running from the VTA to the NACS or administration of D₁ or D₂ like receptor antagonists impair opiate reinforcement as determined by self-stimulation and place preference studies (Bozarth & Wise, 1981; Leone & DiChiara, 1987; Schippenberg & Herz, 1987; Spyraki et al., 1983).
Some studies, however, demonstrate that opiates may also act independently of dopamine neurons. Support for this notion comes from investigations showing that opiates are self-administered directly into the NACS (Olds, 1982) and that injections of opiate antagonists into this region (Vaccarino et al., 1985) or destroying NACS neurons with kainic acid (Zito et al., 1985) decrease reinforcement derived from intravenously injected opiates. Furthermore, Pettit et al., (1984) showed that 6-OHDA lesions in the NACS disrupt cocaine self-administration, but do not affect heroin self-administration in rats. It has been suggested that other dopamine independent mechanisms of reinforcement may exist, perhaps involving glutamatergic fibres, as phenylcyclidine and MK-801 are also self-administered into the NACS (Carlezon & Wise, 1993). Although other systems may contribute to opiate reinforcement, the bulk of available evidence does implicate a strong role for dopamine.

1.7 RECEPTORS IMPLICATED IN DRUG DEPENDENCE

1.7.1 Cocaine and Methamphetamine

Receptor binding studies originally suggested that the initial site of action leading to cocaine and amphetamine reinforcement is the dopamine transporter (Ritz et al., 1987). Recent gene manipulation studies have confirmed that the transporter is an obligatory target of both of these psychostimulants, as cocaine and amphetamine have no effect on locomotor activity or dopamine release and uptake in mice lacking the transporter (Giros et al., 1996). In wild type animals the increased levels of dopamine in the synapse after cocaine and amphetamine administration are then able to interact directly with both pre and post synaptic dopamine receptors.
Molecular cloning studies have revealed that dopamine is capable of acting through at least five different receptors, labeled D₁-D₅, with D₂ (Giros et al., 1989) and D₃ (Fishburn et al., 1993) receptors each exhibiting two splice variants. Pharmacologically these receptors can be grouped into a D₁-like family (D₁ and D₅) and a D₂-like family (D₂, D₃, and D₄) based on their affinities for various receptor ligands and their linkages to distinct signal transduction cascades (reviewed in Surmeier et al., 1993; Missale et al., 1998). Activation of D₁-like receptors leads to stimulation of adenylyl cyclase (AC), whereas activation of D₂-like receptors leads to AC inhibition (reviewed in Andersen et al., 1990). D₁ and D₅ receptors are coupled to the stimulatory G-protein, Gαs, whereas the effects of D₂, D₃, and D₄ receptors are mediated through the inhibitory G-proteins, Gαi and Gαo (reviewed in Gingrich & Caron, 1993). The D₂ receptor is also found pre-synaptically and functions as an autoreceptor, inhibiting synthesis and release of dopamine (reviewed in DiChiara, 1995). Gene transcripts of D₃, D₄, and D₅ receptors are much less abundant and are more discreetly expressed than those of D₁ and D₂ receptors, which are highly expressed in brain, including the caudate, putamen and NACS (Hall et al., 1994; reviewed in Sokoloff & Schwartz, 1995).

The exact roles of each of the D₁ and D₂ receptors in drug addiction and dependence remain to be elucidated. However, it appears that both receptor subtypes are involved in different aspects of psychostimulant addiction. It was originally thought that D₂ receptors mediate many effects of psychostimulants as D₂ receptor agonists have many cocaine-like behavioral effects in animals, including stimulation of locomotor activity, induction of stereotypical behavior, and positive
reinforcing effects. Furthermore, D₂ receptor antagonists block many of these behavioral effects (reviewed in Woolverton & Johnson, 1992).

Preliminary evidence suggested that D₁ receptors do not mediate the reinforcing effects of cocaine, as the D₁ agonist SKF38393 was not reinforcing in primates (Woolverton et al., 1984) and the D₁ antagonist SCH23390 did not reliably alter self-administration behavior (Woolverton, 1986). However, more recent studies have led researchers to believe that D₁ receptors do participate in mediating certain behavioral effects of cocaine. The highly lipophilic D₁ agonists (SKF82958 and SKF77434) do function as positive reinforcers in rats (Self & Stein, 1992), and this reinforcement was blocked by D₁, but not D₂ receptor antagonists, suggesting that D₁ receptor reinforcing mechanisms are independent of those mediated by D₂ receptors (Self et al., 1993). D₁-like antagonists can also prevent behavioral sensitization to amphetamine and block cocaine-like effects on locomotor activity and stereotypy, decreases in food intake and its effects on operant behavior (reviewed in Woolverton & Johnson, 1992; DiChiara, 1995).

Recent investigations with D₁ receptor knock-out mice have found that cocaine conditioned place preference is retained (Miner et al., 1995) and that psychomotor stimulant effects on locomotor behavior are disrupted (Miner et al., 1995; Moratalia et al., 1996). It seems as though the D₁ receptor may play a role in the locomotor stimulant effects of cocaine and amphetamine, whereas the D₂ receptor may be involved in the rewarding and reinforcing effects of these drugs (Miner et al., 1995). Moratalia et al., (1996) showed that cocaine and amphetamine can no longer stimulate cFos, BJun, or regulate dynorphin in the D₁ mutants. This study also demonstrated that D₂ dopamine receptors can function in the absence of
D₁ receptors, but that the D₁ receptor is essential for the locomotor effects of cocaine and amphetamine.

A recent study in rats elucidated an interesting dissociation between D₁ and D₂ like receptor processes in cocaine seeking behavior (Self et al., 1996). D₁ like and D₂ like dopamine receptor agonists were found to be reinforcing, yet they each mediate qualitatively different aspects of reinforcement. By examining whether pretreatment with D₁ or D₂ like agonists could modulate the priming effects of cocaine, it was found that the D₁ like agonist SKF 82958 blocked cocaine-induced responding, whereas the D₂ like agonist 7-OH-DPAT enhanced cocaine seeking behavior, showing a dissociation between dopamine receptor subtypes. In effect, it has been suggested that the D₂ dopamine receptors mediate reinforcement, whereas the D₁ dopamine receptors play a permissive role, allowing the expression of D₂ mediated behavior (Self et al., 1996).

Taken together, it is clear that both receptor types are involved in aspects of psychostimulant addiction. From the above discussion, it appears that the two receptors can have both synergistic and antagonistic effects on dopamine mediated behaviors yet their precise roles remain elusive.

1.7.2. Morphine

When considering the receptor subtypes involved in morphine addiction, it is important to highlight the fact that two distinct systems are potentially involved in mediating its behavioral effects: the indirect increase of dopaminergic transmission from the VTA to the NACS, which is mediated through dopamine receptors, and the direct action of morphine on μ opioid receptors located in the NACS (Nestler, 1993;
Satoh & Minami, 1995). The relative contribution of these two sites of opiate action to the reinforcing properties of opiates remains unknown.

As previously discussed, lesioning dopaminergic neurons or administering dopamine receptor D$_1$ or D$_2$ like antagonists impair opiate reward (Bozarth & Wise, 1981; Leone & DiChiara, 1987; Shippenberg & Herz, 1987; Spyraki et al., 1983). Also, drugs that impair dopamine transmission also reduce opiate-induced locomotion. D$_1$ receptor antagonists block morphine-induced hypermotility in mice (Longoni et al., 1987), and stereotyped behavior and opiate behavioral sensitization in rats (Pollock & Kornetsky, 1989). D$_2$ receptor antagonists in mice (Carroll & Sharp, 1972) and rats (Ayhan & Randrup, 1973) also strongly reduce morphine-induced locomotion and behavioral stimulation. Furthermore, a recent study of D$_2$ knockout mice found a total suppression of morphine's rewarding effects in a place-preference test (Maldonado et al., 1997). In all, it seems that reinforcement, hypermotility (locomotor activation) and behavioral sensitization elicited by systemic opiates are at least in part due to potentiation of dopaminergic transmission in the NACS.

However, the effects of opiates may also be mediated by a dopamine independent, $\mu$-receptor mediated mechanism as discussed above. Stimulation of opioid $\mu$ receptors leads to neuronal inhibition by increasing $K^+$ channel conductance and decreasing Na$^+$-dependent inward currents. AC activity and the downstream target of cAMP, cAMP dependent protein kinase (PKA) activity, is also reduced (reviewed in Satoh & Minami, 1995). Strong evidence supporting a role for the $\mu$ opioid receptor as being a mandatory component of the opioid system comes from a knock-out study which found that the $\mu$-opioid gene product is necessary for the
expression of morphine’s rewarding, withdrawal and analgesic properties, whereas a lack of \( \mu \) receptors did not influence morphine-induced locomotor activity in mice (Matthes et al., 1996).

The relative contributions of dopamine and \( \mu \) receptors in the behavioral effects of morphine are unclear. From the gene knock-out studies, it appears that both D\(_2\) dopamine receptors and \( \mu \) opioid receptors may mediate at least opiate reinforcement. Perhaps such reward is mediated through cooperativity between receptor subtypes, which may occur through receptor allosteric interactions, second messenger pathways, or at a functional level on separate neuronal populations (Traynor & Elliot, 1993; Matthes et al., 1996). Investigations into such questions will undoubtedly tease apart the roles of dopamine receptors and opioid \( \mu \) receptors in opiate dependence.

1.8 THE ROLE OF GLUTAMATERIC PATHWAYS IN DRUG DEPENDENCE

N-methyl-D-aspartate (NMDA) receptors have been implicated in several types of neural and behavioral plasticity, including changes seen in response to administration of drugs of abuse. NMDA receptors are a class of excitatory amino acid receptor that are under complex regulation. Ultimately, NMDA receptor stimulation leads to \( \text{Ca}^{2+} \) and \( \text{Na}^+ \) influx, and \( \text{K}^+ \) efflux from the neuron. Drugs such as PCP and MK-801 block the ion channel, thereby antagonizing NMDA receptor activation (reviewed in Trujillo & Akil, 1995).

Several studies have found that the antagonist MK-801 blocks the development of sensitization to the convulsant, stereotypic and locomotor effects of cocaine, the stereotypic and locomotor effects of amphetamine (Karler et al., 1989), and the locomotor effects of morphine (Wolf & Jeziorski, 1993). Interestingly, MK-
801 inhibits the development, but not the expression of amphetamine sensitization once it is established, indicating that glutamatergic pathways may be contributing to neural plasticity underlying sensitization, and not to the acute effects of amphetamine (Karler et al., 1990). However, MK-801 also prevents the ability of amphetamine pre-exposure to increase the acquisition of cocaine self-administration, providing evidence that NMDA receptors may also be involved in sensitization to the reinforcing effects of psychostimulants (Schenk et al., 1992). NMDA receptors also appear to be involved in the development of tolerance, sensitization, and potentially in physical dependence to morphine, as MK-801 interferes with the development, but not the expression of these behaviors (reviewed in Trujillo & Akil, 1995). The potential involvement of NMDA receptors in the development of physical behaviors to chronic drug use suggests the involvement of neuroplastic changes in these behaviors, as NMDA receptor activation also appears to modulate other forms of neural plasticity, ranging from neuronal development to induction of long-term potentiation (learning) (Trujillo & Akil, 1995).

1.9 CELLULAR SITES OF DRUG ACTION

The discovery of endogenous opiate receptors and that psychostimulants affect catecholamine re-uptake and/or release mechanisms raised the possibility that these drug classes exert their chronic effects through modulation of opiate and/or dopamine receptors, respectively. However, despite intense investigation, studies have failed to consistently account for opiate and psychostimulant dependence in terms of regulation of neurotransmitters and receptors, thus attention has shifted to postreceptor mechanisms and there is strong evidence suggesting that
heterotrimeric G-proteins may participate in the pathophysiology seen in addiction. (reviewed in Nestler et al., 1992).

1.10 WHAT ARE HETEROTRIMERIC G-PROTEINS?

Guanine-nucleotide binding proteins (G proteins) are members of a superfamily of GTPase hydrolyzing proteins that act as molecular switches which can be subdivided into monomeric GTPases and heterotrimeric G-proteins. Monomeric GTPases, currently categorized into five families, namely Ras, Rab, ARF, Ran, and Rho, are found in the cytoplasm and in membrane associated pools, and are involved in a plethora of cellular events such as transmission of hormonal signals, modulation of cellular growth and development, protein transport, cytoskeleton architecture, and exocytosis. Heterotrimeric G-proteins are membrane-associated transducers mediating extracellular hormonal and neurotransmitter signals to the cell's interior (reviewed in Neer, 1995). Stimulation of G-proteins via activation of heptahelical receptors by hormonal, or neurotransmitter signals results in the regulation of a multitude of effector enzymes (Birbaumer et al., (1990), activation of ion channels (Wickman & Clapham, 1995), and vesicular transport (Helms, 1995).

Heterotrimeric G-proteins are composed of three distinct polypeptide subunits: an \( \alpha \) subunit that binds and hydrolyzes GTP and a \( \beta \gamma \) dimer that serves as a functional monomer (Birbaumer, 1992). G-proteins couple the activation of heptahelical membrane receptors by extracellular signals to appropriate intracellular effectors. In its resting state, the GDP liganded form of the \( \alpha \) subunit forms a high affinity complex with the \( \beta \gamma \) dimer. The binding of the heterotrimer to an agonist-activated receptor results in a conformational change in the \( \alpha \) subunit that decreases its affinity for GDP. The release of GDP and subsequent GTP binding cause the \( \alpha \)
subunit to assume its active conformation and dissociate from both the receptor and from the $\beta \gamma$ subunit. Both the free $\alpha$ subunit and tight noncovalent bound $\beta \gamma$ dimer are capable of effector regulation. The activated state of the $\alpha$ subunit is terminated by the hydrolysis of GTP to GDP by the intrinsic GTPase activity characteristic of all $\alpha$ subunits (see Table 1.2).

All isoforms of $\alpha$ subunits are GTPases and the rates of hydrolysis depend on the particular $\alpha$ subunit. The rate of GTP hydrolysis influences the duration of the active states of both the $\alpha$ and $\beta \gamma$ subunits. Reassociation of the subunits ends the cycle, with the system returning to its reconstituted inactive state ready to respond to receptor activation (reviewed in Gilman, 1987; Neer, 1995; Hamm, 1998). Figure 1.5 depicts the recursive cycle of activation by receptors and termination by GTPase hydrolysis of these molecular switches.

**Table 1.2: Examples of effectors regulated by G-protein subunits**

<table>
<thead>
<tr>
<th>Effector</th>
<th>Regulator</th>
<th>Type of G$<em>\alpha$/G$</em>\beta \gamma$ interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K^+$ channel (I$_{K\text{ACM}}$)</td>
<td>+</td>
<td>$G_{\beta \gamma}$</td>
</tr>
<tr>
<td>$K^+$ channel (I$_{K\text{ATP}}$)</td>
<td>+</td>
<td>$-$</td>
</tr>
<tr>
<td>PLA$_\gamma$</td>
<td>?</td>
<td>+</td>
</tr>
<tr>
<td>AC I</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>AC II (IV)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>AC III</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>PLC$_{B1-3}$</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ca$^{2+}$ channels</td>
<td>+</td>
<td>unknown</td>
</tr>
<tr>
<td>$\beta$ARK</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>cGMP</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>cGMP</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>muscarinic</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

$'+$' indicates an interaction, whether it be stimulatory or inhibitory to effector function; $'-$' indicates no demonstrated effect on effector function; 'independent' refers to the activation of either the $\alpha$ or $\beta$ subunits independent of the other; 1) adapted from Clapham & Neer, 1993.
Figure 1.5: Mechanism of G-protein activation and signaling.

1.10.1 Gα subunit structure and function

To date, twenty different α subunits (Birnbaumer et al., 1991), five β subunits (Gao et al., 1987), and 12 γ (Cali et al., 1992) subunits have been cloned from both vertebrates and invertebrates. Although random association of subunits would generate hundreds of potential combinations, it appears that there are preferred combinations of subunits that form a limited number of functional complexes (Pronin & Gautum, 1992; reviewed in Hamm, 1998). The Gα subunits range in size from 39-52 kDa and have been divided into four different classes based on sequence homology which ranges from 56%-95%, and functionality: Gαs (Gαs and Gαolf), Gαι (Gαι, Gαt, Gαo, Gαgust, and Gαz), Gαq (Gαq, Gα11, Gα14, Gα15, and Gα16) and Gα12 (Gα12 and Gα13) (reviewed in Neer, 1995). However, G-proteins were first classified according to functional criteria. The term Gs (or stimulatory) was used to designate G-proteins which stimulated AC, whereas Gi (or inhibitory) was so
classified based on their ability to inhibit AC. The protein that couples photoreceptors to cGMP phosphodiesterase is Ga\textsubscript{t} or transducin, while G-proteins originally isolated from brain with no known function were termed G\textalpha\text{other} (Spiegal et al., 1992). Although no longer considered adequate for differentiating G-protein subtypes, this nomenclature is often still used.

Several G\textalpha subunits are subject to post-translational modifications. All \textalpha subunits are modified at or near their N-terminal end by the addition of myristate and/or palmitate. \textalpha subunits of the \textalpha\text{family} are myristoylated and all \textalpha subunits except \textalpha\text{i} contain palmitate (Wedegaertner et al., 1995). Both myristoylation and palmitoylation contribute to the membrane attachment of the G\textalpha subunit (Milligan et al., 1995). Myristoylation also increases the affinity of the \textalpha\text{family} for \beta\gamma (Degtgarev et al., 1994) and myristoylation of \textalpha\text{i} is required for its inhibition of AC (Taussig et al., 1993). A number of G\textalpha subunits are also targets for covalent ADP-ribosylation by cholera (CTX) and pertussis (PTX) toxins. CTX irreversibly activates the \textalpha subunit of G\textalpha\text{s} and Ga\textsubscript{t} by inhibiting the intrinsic GTPase activity (Gill & Meren, 1978), while PTX irreversibly uncouples Ga\textsubscript{i}, Ga\textsubscript{t}, and G\textalpha\text{o} from their receptors and stabilizes the G-protein in its \alpha\beta\gamma undissociated conformation, thereby rendering the \textalpha subunit inactive (Murayama & Ui, 1983). Both CTX and PTX are useful tools to functionally define and identify subsets of G-proteins.

The \textalpha subunit consists of three distinct domains: a GTPase domain that consists of a 6 stranded \beta sheet surrounded by 6 helices, an \alpha-helical domain consisting of a long central helix surrounded by 5 shorter helices, and N-terminal helix that projects away from the remainder of the \textalpha subunit. A large region of the C-
terminus as well as the N-terminus have been implicated in receptor contact (Onrust et al., 1997; Hamm, 1998). The guanine nucleotide binding site is buried deep between the GTPase and helical domains (Lambright et al., 1996). The structural nature of the GTP-mediated switch on the α subunit involves a conformation change in three flexible regions of the subunit termed switches I, II, and III (Lambright et al., 1994). The interaction between the α and βγ subunits occurs at two interfaces. The first involves residues in the switch I and II regions of the α subunit that interact with residues at the top of the β-propeller domain of the β subunit. The second interface is between the N-terminal helix of the α subunit and the β-propeller domain of the β subunit.

1.10.2 Gβγ subunit structure and function

It is thought that the β subunit consists of two structures: an N-terminal helix (1992) and a structure consisting of seven repeating units (β-sheets) of approximately 43 amino acids (WD repeats) (Simon et al., 1991; Lambright et al., 1996), that is commonly found in protein classes that participate in signal transduction, cell division, cytoskeleton fusion, and vesicular fusion (Neer et al., 1994). Each β-sheet has four antiparallel strands that form the “blades” of the β-propeller structure (Lambright et al., 1996). The five known β subunits share approximately 50-83% sequence homology (Watson et al., 1994). The γ subunit contains two helices and has no tertiary structure. The N-terminal helix interacts with the N-terminal helix of the β subunit and the remainder of Gγ interacts and makes extensive contacts with the β-propeller domain of Gβ (Lambright et al., 1996). The twelve identified γ subunits are far more divergent than their α and β counterparts, sharing only 27-75%
homology (Lupas et al., 1992). Thus, it has been suggested that the γ subunit determines the functional specificity of Gβγ complexes (reviewed in Neer, 1995). The γ subunit is also subject to post-translational modification and prenylation of the C-terminus is essential for membrane attachment (Casey, 1994; Wedegaertner et al., 1995).

The β and γ subunits bind very tightly to each other and can only be undissociated by denaturants. Although originally thought to be a passive member of the G-protein heterotrimer, the βγ dimer in fact plays a significant role in cellular signaling (Clapham & Neer, 1993). For example, it is known that βγ can act directly with and modulate the activities of AC (Katada et al., 1987), PLA₂ (Jelsema & Axelrod, 1987), K⁺ channels (Logothetis et al., 1987), Ca²⁺ channels (Dunlap 1997), members of the MAP kinase cascade (Faure et al., 1994), in addition to that of the α subunit.

1.11 EVIDENCE FOR THE INVOLVEMENT OF G-PROTEIN IN DRUG DEPENDENCE

1.11.1 Animal Models

There is strong evidence from animal studies that chronic cocaine and morphine treatment leads to altered G-protein levels in specific brain regions. Decreases of Ga₁ in NACS and increases of Ga₁ and Ga₀ in amygdala have been found in rats following chronic morphine exposure (Terwilliger et al., 1991). Furthermore, increased levels of Ga₁ and Ga₀ have been reported in locus coeruleus, a region implicated in mediating the effects of opiate withdrawal (Nestler et al., 1989). Chronic cocaine treatment to rats has also been found to decrease levels of Ga₁ and/or Ga₀ in brain areas implicated in addiction, specifically the locus
coeruleus, ventral tegmental area and NACS (Nestler et al., 1990; Striplin & Kalivas, 1993).

However, the most compelling evidence supporting a role for G-proteins in addiction comes from studies using CTX and PTX to directly alter specific G-protein activity. As previously discussed, PTX irreversibly inactivates Gαi and Gαo by catalyzing the ADP-ribosylation of the α subunit preventing modulation of respective effectors, such as inhibition of AC by Gαi (Murayama & Ui, 1983). PTX injections directly into the NACS reduce both cocaine and morphine reinforcement in rats (Self et al., 1994) and PTX injections into the ventral tegmental area enhance stimulation of locomotor activity induced by cocaine and amphetamine NACS (Narayanan et al., 1996; Narayanan et al., 1997) and increase dopaminergic transmission to the NACS (Steketee et al., 1992). CTX, which irreversibly activates Gαs leading to increased stimulation of AC (Gill & Meren, 1978), sensitizes locomotor responses to both cocaine and amphetamine (Cunningham & Kelley, 1993), and decreases cocaine reinforcement in rats when injected into the NACS (Self & Nestler, 1995). Activating Gαs increases stimulatory input to AC and inhibition of Gαi results in disinhibition of AC and increased basal cAMP levels, also promoting cAMP signaling.

At this point, it is important to note that the relationship between sensitization, self-administration and reinforcement is unclear. Studies have found that both PTX and CTX lead to enhanced locomotor activity and self-administration in rats treated with psychostimulants. The increased rate of self-administration was interpreted as decreased reinforcement (Self & Nestler, 1995). However, it has been shown that pre-exposure to cocaine (Horger et al., 1990) and amphetamine (Pierre & Vezina, 1997) sensitizes rats to the rewarding effects of these drugs, leading to an increase
in self-administration of these drugs. These authors interpret the increased rate of self-administration as increased reinforcement.

1.11.2 Human Studies

Despite evidence from rodent models of signal transduction abnormalities resulting from chronic drug treatment, few studies have tested whether postreceptor changes in G-protein levels/function occur in drug users. Increased Gαs, Gαi, Gαo and Gβ immunolabelling have been reported in frontal cortex (Escriba et al., 1994), and increased Gβ in temporal cortex from heroin addicts who had died of opiate intoxication (Hashimoto et al., 1996). Yet, it is unclear if these G-protein changes resulted from chronic heroin use or from acute drug overdose. A recent study examined platelet G-protein levels from living heroin addicts stabilized on methadone and found increased immunolabelling of the short (45 kDa) isoform of Gαs, Gαs45, and decreases of Gαi1,2 (Manji et al., 1997), also suggesting that chronic drug exposure modifies G-protein expression. To my knowledge, there have been no studies on G-protein status in cocaine and methamphetamine users or of G-protein subunit immunoreactivities in subcortical regions of autopsied human brain, specifically the NACS.

1.12 SIGNIFICANCE OF AN UPREGULATED DOPAMINE D1 RECEPTOR-LINKED cAMP SYSTEM

The normal activity of AC reflects a balance between all sources of input, whether stimulatory or inhibitory. It is hypothesized that the decreases of Gαi levels observed in NACS of rats following chronic cocaine and/or morphine administration lead to supersensitivity of the enzyme AC making it even more responsive to excitatory input from Gαs (Terwilliger et al., 1991). Increased activity of AC then
leads to increased activity of the second messenger cAMP. Most effects of cAMP are mediated by cAMP dependent protein kinases, in particular PKA, that are activated upon binding cAMP, whereas, the effects of dopaminergic transmission are mediated by at least two sets of receptors. D1-like receptors are linked positively via Goα to AC, whereas D2-like receptors are linked negatively via Goi to AC (reviewed in Missale et al., 1998). Therefore, the increased dopaminergic transmission induced by cocaine, methamphetamine, and morphine is hypothesized to be regulated via upregulation of D1 linked pathways (Terwilliger et al., 1991).

Support for the notion that opiates can upregulate the cAMP system comes from investigations showing that rats treated with chronic morphine have altered levels of AC and protein kinase A immunolabelling in the amygdala, thalamus and NACS (Terwilliger et al., 1991). Cocaine treatment also leads to similar increases in AC and protein kinase A immunoreactivities and activity in the NACS (Terwilliger et al., 1991), whereas inhibition of protein kinase A at the level of the NACS decreases behavioral sensitization to the locomotor activating effects of cocaine (Miserendino & Nestler, 1995). Collectively, the above observations suggest sensitization of the cAMP system may underlie behavioral correlates of psychostimulant and opiate addiction.

The long-term supersensitivity reported for post-synaptic D1 dopamine receptor-mediated responses in NACS following chronic cocaine and morphine administration would be expected to have marked consequences on neuronal physiology. Yet, the mechanisms by which chronic drug exposure elicits the upregulation of the cAMP system remain poorly understood. Regulation of receptor-effector response can be accomplished by alterations in AC activity and several
investigations have shown that prolonged agonist exposure can lead to decreases in AC inhibition, suggesting a mechanism by which cells adapt to long-term inhibition of CAMP synthesis. For example, exposure of NG108-15 cells to agonists acting at opiate (Sharma et al., 1975), muscarinic (Nathanson et al., 1978), and α2-adrenergic (Thomas et al., 1986) receptors results in reduced receptor-stimulated inhibition of AC and similar effects on AC have been observed upon treatment of mouse anterior pituitary cells with somatostatin (Reisine & Takahashi, 1984), and upon treatment of CHO cells with opiates (Avidor-Reiss et al., 1995). Long-term activation of both D2S and D2L dopamine receptors has been shown to enhance both basal and forskolin-stimulated AC activity in cell cultures, effects that are attenuated by pretreatment with pertussis toxin (Bates et al., 1991; Filtz et al., 1994; Zhang et al., 1994), implicating the involvement of inhibitory G-proteins.

Signal regulation via G-proteins may also occur further downstream. Activation of PKA leads to phosphorylation of numerous target proteins and protein phosphorylation is a common cellular regulatory mechanism. Several neurotransmitter receptors, including the β-adrenergic receptor, are desensitized by phosphorylation following ligand binding (Benovic et al., 1996; reviewed in Premont et al., 1995). Ca²⁺ channels that are regulated by Gαs are phosphorylated by PKA, leading to further increases in Ca²⁺ conductance (reviewed in Lefkowitz, 1993). Neurotransmitter synthesis is also modulated by phosphorylation and decreased levels of tyrosine hydroxylase have been observed in NACS from rats treated with long term cocaine or morphine treatment (Beitner-Johnson & Nestler, 1991). A recent study has also shown a new cross-regulatory role for PKA in signaling cascades. Daaka et al., (1997) observed that not only does PKA receptor-mediated
phosphorylation mediate uncoupling of the $\beta_2$-adrenergic receptor from $G_{\alpha}s$ (heterologous desensitization), but also serves to switch coupling of this receptor from $G_{\alpha}s$ to $G_{\alpha}i$, and the $\beta_{\gamma}$ subunit of $G_{\alpha}i$ is then able to activate the MAP kinase pathway.

Perhaps the most critical action of PKA for cells undergoing long-term adaptations is the ability of its catalytic subunit to translocate to the nucleus and directly influence gene expression (Hyman & Nestler, 1996). Such long-lived adaptations involving stable changes is gene expression may underlie aspects of drug dependence. Alterations in signal transduction cascades can exert effects on gene transcription factors, which are proteins that regulate the expression of a given gene by binding to specific recognition sites in the promoter of the gene. There are two general types of mechanisms that appear to be involved in gene regulation via upregulation of the cAMP pathway. The first involves the phosphorylation of the transcription factor CREB by PKA. CREB binds to genes with a specific response element termed CRE to regulate transcription (Montminy et al., 1990). In neurons within the striatum and NACS, stimulation of dopamine $D_1$ receptors leads to CREB phosphorylation (Konradi et al., 1994), which is then able to activate a number of genes. In the second mechanism, protein kinases, via phosphorylation, stimulate the expression of a family of genes, known as immediate early genes, which encode transcription factors, for example c-fos, c-jun and zif-68. These newly synthesized transcription factors then translocate back into the nucleus where they can regulate the expression of other genes by forming homodimeric or heterodimeric complexes that bind specific DNA sequences called AP-1 binding sites (Sheng & Greenburg, 1990; reviewed in Nestler, 1992).
Both acute and chronic treatment of rats with drugs of abuse have been shown to alter gene expression in the NACS. Given that animal studies have shown that upregulation of the cAMP pathway via \( \alpha_2 \) receptor systems (Konradi et al., 1994) can lead to CREB activation and the \( \alpha_2 \) gene has a CRE element (Brann et al., 1987), it is reasonable to speculate that alterations at the gene level may in part mediate G-protein changes seen following drug administration. Acute administration of cocaine, amphetamine and morphine leads through the phosphorylation of CREB to the induction of c-Fos, c-jun, and other IEG's and to increase AP-1 binding activity in the NACS (reviewed in Self & Nestler, 1995). The ability to induce c-fos and other IEG's in the NACS is attenuated upon chronic cocaine treatment, yet the AP-1 binding activity persists for up to one week, suggesting the induction of other IEG's (Hope et al., 1992). Furthermore, in CREB\( \Delta \) mutant mice, the main symptoms of opiate withdrawal are attenuated, implicating CREB dependent gene transcription in the onset of behavioral manifestations of opiate dependence (Maldonado et al., 1996). Chronic exposure to cocaine (Hope et al., 1994) and to morphine (Nye & Nestler, 1996) lead to the accumulation of different Fos-like proteins, called chronic FRAs, which have long half-lives in the brain. Although the functional role of the FRAs remains to be identified, the accumulation of these novel proteins make them attractive candidates for participating in the molecular adaptations seen after chronic drug use (Hyman & Nestler, 1996).

### 1.13 Behavioral Consequences of an Upregulated Dopamine \( D_1 \) Receptor-Linked System

One of the greatest challenges in the field of drug addiction is linking the well-documented behavioral plasticity of chronic drug use in the human with specific
molecular changes. Although there is animal evidence that the up-regulated cAMP system in the nucleus accumbens both opposes drug reinforcement (Self et al., 1994) as well as contributes to more long term adaptations such as sensitization to the locomotor activating effects of psychostimulants (Cunningham & Kelly, 1993; Miserendino & Nestler, 1995), there is little data on the effects of upregulated cAMP systems in humans.

In the striatum and NACS, levels of dynorphin mRNA and peptides increase following chronic cocaine and amphetamine administration in rats and in post mortem human brain (Hanson, et al., 1988; Hurd & Herkenham, 1993). Dynorphin is selective for the κ opioid receptor, which mediates cAMP inhibition through pertussis sensitive G-proteins and inhibition of Ca\(^{2+}\) entry (reviewed in Reisine & Bell, 1993). Overactivation of the D\(_1\) dopamine system following psychostimulant or opiate administration or sensitization of the system as a compensatory mechanism consequent to reduced inhibitory G-protein levels, leads to activation of CREB. CREB in turn activates the transcription of the prodynorphin gene (Cole et al., 1995) and the resulting peptides can then act through κ opioid receptors in the NACS to decrease dopamine release. It has been suggested that this compensatory mechanism may contribute to the anhedonia, dysphoria and other withdrawal symptoms seen upon cessation of drug taking (Hyman & Nestler, 1996).

There is also speculation that the enhanced D\(_1\) receptor linked cAMP upregulation observed after chronic psychostimulant and opiate administration that has been well characterized in rodent models may underlie certain aspects of sensitization in the human, particularly sensitization to drug craving as proposed by Robinson & Berridge, (1993). The pathologically strong craving seen during
withdrawal may be in part due to an overactive mesolimbic dopamine system and therefore, the up-regulated cAMP system seen following repeated drug exposure in rats may represent a parallel mechanism which contributes to excessive drug craving in humans (Koob & Le Moal, 1997).

1.14 MECHANISMS OF G-PROTEIN REGULATION

The mechanism(s) responsible for the observed decreases in G\(\alpha_{i1}\) and G\(\alpha_{i2}\) immunoreactivity levels is of particular interest to understanding the molecular basis of addiction to the psychostimulants cocaine and methamphetamine and to heroin. Decreasing inhibitory G-protein immunoreactivity levels in the NACS may lead to enhanced dopamine-receptor-linked stimulation, thereby representing a compensatory mechanism by which cells adapt to chronic heroin and methamphetamine exposure. Desensitization of G-protein coupled receptor-effector interaction following prolonged agonist exposure is an ubiquitous regulatory mechanism that modulates transmembrane signaling in cells (Milligan, 1993; Milligan et al., 1995).

Downregulation of signal transduction cascades involves a series of processes including loss/modulation of receptors, alterations in receptor-G-protein or G-protein AC coupling efficiency, and modulation in downstream target proteins that regulate receptor, G-protein and/or AC sensitivity. A variety of data indicate that changes in G-protein subunit levels are particularly important in homologous and heterologous desensitization of the receptor-G-protein–effector complex. For example, prolonged exposure of rat adipocytes to the A\(_1\) adenosine receptor agonist PIA (Green et al., 1990) or EP prostanoid receptor agonists (Green et al., 1992) leads to inhibition of AC and a time- and concentration-dependent downregulation of
Gαi1-3, with no effects on Gαs immunolabelling. Down regulation of Gαi by PIA does not involve alterations in the levels of the corresponding mRNA's (Longabaugh et al., 1988), suggesting that regulation occurs at the level of protein turnover. Furthermore, reduction of Gαi by PIA also reduced the cell’s ability to respond to PGE1, resulting from the fact that both receptor types share the same complement of inhibitory G-proteins, and thus provide an example of heterologous desensitization. Hadcock et al., (1991) also found that PIA increased the rate of Gαi degradation in hamster smooth muscle cells. Other studies in CHO cells have shown that agonist activation of α2-adrenoceptors leads to selective downregulation of inhibitory G-proteins (Eason et al., 1992).

Desensitization of cellular pathways linked to Gαs in certain cell lines also involves decreases in Gαs levels. McKenzie & Milligan (1990) and Adie et al., (1992) found that treating NG108-15 cells with prostaglandin E1 produced a marked reduction in Gαs immunoreactivity levels, whereas similar treatment had no effect on Gαi, Gαo, or Gβ levels (Mckenzie & Milligan, 1990). Prostaglandin E1-mediated downregulation of Gαs did not result in concomitant decreases in Gαs mRNA, thereby demonstrating the effect was not produced at the transcriptional level and suggesting such agonist treatment leads to enhanced Gαs degradation (McKenzie & Milligan, 1990). Furthermore, isoprenaline treatment of NG108-15 cells expressing the β2 adrenergic receptor leads to large, selective decreases of Gαs (Adie & Milligan, 1994) and chronic ethanol treatment of NG108-15 cell results in Gαs downregulation, an effect accompanied by a reduction in Gαs mRNA levels (Mochly-Rosen et al., 1988). Several studies have shown that treating a variety of cells with
GTP analogues or agonists can lead to time and dose-dependent removal of Gαs from the membrane fraction, thereby demonstrating that G-protein translocation may be a potential mechanism for controlling cellular sensitivity (reviewed in Milligan 1993).

Signal transduction pathways are also subject to regulation via indirect input from neighboring pathways, an event termed cross-talk. Persistent activation of AC and PKA leads both to desensitization of the stimulatory pathway and to an increase in the responsiveness of the inhibitory pathway. For example, prolonged activation of AC by the β-adrenergic agonist isoproterenol or direct activation of AC by forskolin leads to decreases in Gαs levels and increases in Gαi protein levels in several cell lines (Hadcock et al., 1990; reviewed in Hadcock & Malbon, 1993). This effect appears to be a compensatory mechanism that increases the function of the inhibitory pathway of AC (Hadcock & Malbon, 1993). Levels of Gαi2 mRNA were also increased when the Gαs-mediated pathway was stimulated, suggesting a role for PKA (Hadcock et al., 1990). It is further suggested that the CRE in the Gαi2 gene (Brann et al., 1987) is responsible for the increased protein levels of Gαi in cells stimulated with forskolin or β-adrenergic agonists (Hadcock & Malbon, 1993).

There have also been reports of cross regulation from the inhibitory to the stimulatory G-protein-coupled AC pathway AC. For example, in rat adipocytes, persistent activation of the inhibitory pathway of AC using PIA leads to an increase in cAMP accumulation in response to isoproterenol, ACTH and forskolin (Hoffman et al., 1986). Moreover, in DDT1 MF-2 cells persistent activation of inhibitory AC by PIA leads to upregulation of the β2-adrenergic receptor and down-regulation of both
A1 adenosine receptors and the inhibitory G-protein Gαi2 (Haddock et al., 1991). Integration of stimulatory and inhibitory input to AC is a dynamic process that depends on the enzyme type and the phosphorylation status of the cell (Marjamaki et al., 1997).

It is now becoming clear, however, that novel protein families, such as regulators of G-protein signaling (RGS proteins) also modulate G-protein activity and may thereby play a role in signal control. RGS proteins modulate the functioning of G-proteins by activating the intrinsic GTPase activity of G-protein α subunits. Thus, the duration of the activated GTP-bound state of the α subunit is reduced, inhibiting G-protein function (Dohlman & Thorner, 1997). Recently, at least 18 RGS proteins have been shown to serve as GTPase activating proteins for both Gαi, Gαo (Berman et al., 1996; Dohlman & Thorner, 1997; Hunt et al., 1996; Watson et al., 1996) and Gαq (Heximer et al., 1997; Shuey et al., 1998). Several RGS protein subtypes have been characterized and these proteins show highly specific brain distribution; RGS9 mRNA is nearly exclusively located in striatal regions, including the NACS (Gold et al., 1997). Furthermore, RGS7, RGS8 and RGS10 have all been shown to be altered by acute seizure in rats demonstrating the ability of RGS proteins to be dynamically regulated in the nervous system (Gold et al., 1997). The fact that RGS proteins specific for Gαi and Gαo G-protein subunits have been characterized combined with the ability of these novel proteins to be modulated by seizure activity make them attractive targets for drug adaptations (Nestler & Aghajanian, 1997).

1.15 OBJECTIVES AND HYPOTHESES OF THIS STUDY

From the above discussion, it can be seen that chronic agonist exposure can lead to adaptations in G-protein levels in a variety of cell systems. Animal
investigations have shown reduced Gαi and Gαo immunoreactivity levels in the NACS following chronic cocaine and morphine exposure. Repeated psychostimulant and heroin use leads to increased extracellular dopamine levels selectively in the NACS and VTA (Di Chiara, 1995). The increased level of the agonist dopamine is in turn hypothesized to promote reduced inhibitory G-protein levels. It is further speculated that such decreased Gαi and Gαo immunoreactivity levels are part of a molecular chain of compensatory events leading to supersensitization of the dopamine D1 receptor-mediated cAMP system. As reviewed, studies have found that chronic drug treatment leads to a compensatory upregulation of the D1 receptor-linked cAMP system.

Thus, my objective was to undertake a systematic and comprehensive examination of G-protein immunoreactivity in autopsied brain from cocaine, methamphetamine and heroin users compared with non-drug user controls in order to determine if brain G-protein levels are altered following chronic use of these addictive drugs in humans, as implied by findings of animal studies. Based on animal studies that have shown reduced inhibitory G-protein levels following chronic cocaine and morphine treatment, it was hypothesized that these inhibitory G-proteins would be decreased in NACS from human cocaine, methamphetamine and heroin users.
CHAPTER 2: MATERIALS AND METHODS
2.1 MATERIALS FOR ASSAY

Electrophoresis reagents (SDS, acrylamide, bisacrylamide, ammonium persulphate, TEMED), and bovine serum albumin (BSA) were obtained from BioRad (Richmond, CA). PVDF membrane (0.2 μm) and Enhanced Chemiluminescent (ECL) detection reagents were purchased from Mandel Scientific (Boston, MA). Horseradish peroxidase-conjugated protein A was obtained from Sigma (St. Louis, MI) and hyperfilm-MP was obtained from Amersham (Oakville, ON). All reagents were of analytical grade.

2.2 ANTISERA

Antisera RM/1, AS/7, GC/2, and SW/1 were obtained from Mandel Scientific (Boston, MA) and were used to detect and quantify \( \text{G} \alpha_{s(52-45)} \), \( \text{G} \alpha_{i(1-2)} \), \( \text{G} \alpha_{o} \), and \( \text{G} \beta \), respectively. Specific G-protein synthetic peptide sequences and the corresponding terminus used to raise the antisera are summarized in Table 2.1. The specificity of these antisera has been previously established (Goldsmith et al., 1987; Spiegel et al., 1990).

Table 2.1: Synthetic peptide sequences used to generate specific G-protein subunit antisera

<table>
<thead>
<tr>
<th>Antisera</th>
<th>G-protein subunit</th>
<th>Peptide sequence</th>
<th>Terminal</th>
</tr>
</thead>
<tbody>
<tr>
<td>RM/1</td>
<td>( \text{G} \alpha_{s(52-45)} )</td>
<td>RMHLRQYELL</td>
<td>carboxy</td>
</tr>
<tr>
<td>AS/7</td>
<td>( \text{G} \alpha_{i(1-2)} )</td>
<td>KENLKDCGFL</td>
<td>carboxy</td>
</tr>
<tr>
<td>GC/2</td>
<td>( \text{G} \alpha_{o} )</td>
<td>GCTLSAEERAALCRS</td>
<td>amino</td>
</tr>
<tr>
<td>SW/1</td>
<td>( \text{G} \beta )</td>
<td>GSWDSFLKIWN</td>
<td>carboxy</td>
</tr>
</tbody>
</table>
2.3 HUMAN POSTMORTEM BRAIN

Postmortem brain material from a total of 32 chronic drug users (12 cocaine; 12 methamphetamine; 8 heroin) and 12 controls was obtained from Medical Examiner offices in Little Rock, Arkansas; Sacramento, California; Tampa and Orlando, Florida; Baltimore, Maryland; Toronto, Ontario, and New Westminster, British Columbia. The control subjects were not significantly different from the cocaine, methamphetamine and heroin groups with respect to mean age, interval between death and freezing of the brain, and sex (see Table 2.2).

Table 2.2: Subject characteristics

<table>
<thead>
<tr>
<th>Group</th>
<th>Number</th>
<th>Age (mean± SEM)*</th>
<th>Sex (M:F)*</th>
<th>PMI (h) (mean± SEM)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12</td>
<td>35.4 ± 2.8</td>
<td>11:1</td>
<td>15.3 ± 2</td>
</tr>
<tr>
<td>Cocaine</td>
<td>12</td>
<td>33 ± 2</td>
<td>10:2</td>
<td>18 ± 2</td>
</tr>
<tr>
<td>Methamphetamine</td>
<td>12</td>
<td>33 ± 2</td>
<td>9:3</td>
<td>15 ± 2</td>
</tr>
<tr>
<td>Heroin</td>
<td>8</td>
<td>35 ± 2</td>
<td>11:1</td>
<td>14.3 ± 2</td>
</tr>
</tbody>
</table>

* (p > 0.05, Student's two-tailed t-test).

At autopsy, each brain was halved mid-sagitally. One half-brain was fixed in formalin and the other half was frozen at -80°C for subsequent dissection. The interval between death and freezing of the brain was less than 24 hours. Cerebral cortical divisions were excised using the Brodmann classification. Approximately 15 coronal sections of subcortical brain were taken from which distinct brain areas were dissected according to the Atlas of Riley (1960). All brain dissections were
performed by Dr. S. Kish at the Human Brain Lab at the Clarke Institute of Psychiatry, Toronto, ON. Dissected brain tissue was stored at -80°C until analyzed. Neuropathological examination of the formalin-fixed half of the brain was performed after a minimum of 14 days of fixation. Samples of cardiac blood and brain tissue were obtained from all subjects for drug screening, and where possible, scalp hair samples were also analyzed. Clinical, toxicological, and pathological data for all subjects, including controls, are included in Tables 2.3-2.7.

2.4 CONTROL SUBJECTS

Autopsied brain was obtained from 12 normal subjects who tested negative for drugs of abuse in blood and urine at the time of death. Furthermore, drugs of abuse and their metabolites could not be detected either in autopsied brain or scalp hair and these subjects had no neuropathology related to drug use.

2.5 DRUG USERS

Post-mortem brain samples were obtained from 32 chronic drug users who met the following criteria: 1) presence of drug (cocaine or methamphetamine or heroin) and/or respective metabolites in blood or urine; 2) absence of other drugs in bodily fluids with the exception of ethanol; 3) evidence from case records of cocaine or methamphetamine or heroin as the primary drug of abuse for at least one year prior to death; and 4) absence of brain pathology related to chronic drug use. Most potential subjects were rejected because of a history of polydrug abuse and the detection of multiple drugs of abuse in bodily fluids. Clinical information was obtained by the medical examiners using a questionnaire format inquiring about specific patterns of drug use and medical/social problems, including how much and how often drugs were used, and if drug use had any impact on daily functioning.
Furthermore, where possible, information was also obtained from structured telephone interviews with the next of kin. All family members who participated provided consent under the guidelines approved by the institutional review board of the University of Toronto.

Table 2.3: Patient information and cause of death for control subjects

<table>
<thead>
<tr>
<th>Case</th>
<th>Sex</th>
<th>Age</th>
<th>PMI (h)</th>
<th>Cause of Death</th>
<th>Toxicology</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>30</td>
<td>15.5</td>
<td>leukemia</td>
<td>negative</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>28</td>
<td>14</td>
<td>drowning (accidental)</td>
<td>negative</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>40</td>
<td>18</td>
<td>atherosclerotic cardiovascular disease</td>
<td>negative</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>19</td>
<td>16.5</td>
<td>gunshot wound to chest and abdomen</td>
<td>negative</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>47</td>
<td>23</td>
<td>atherosclerotic cardiovascular disease</td>
<td>negative</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>34</td>
<td>5</td>
<td>valvular disease</td>
<td>negative</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>40</td>
<td>5.5</td>
<td>atherosclerotic cardiovascular disease</td>
<td>negative</td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>31</td>
<td>13</td>
<td>massive cardiomegaly</td>
<td>negative</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>18</td>
<td>16.5</td>
<td>multiple injuries (accident)</td>
<td>negative</td>
</tr>
<tr>
<td>10</td>
<td>M</td>
<td>48</td>
<td>5.25</td>
<td>cardiomyopathy</td>
<td>negative</td>
</tr>
<tr>
<td>11</td>
<td>F</td>
<td>48</td>
<td>22.25</td>
<td>atherosclerotic cardiovascular disease</td>
<td>negative</td>
</tr>
<tr>
<td>12</td>
<td>M</td>
<td>36</td>
<td>23</td>
<td>asphyxia</td>
<td>negative</td>
</tr>
<tr>
<td>Case</td>
<td>Sex</td>
<td>Age</td>
<td>PMI (H)</td>
<td>Duration of use (y)</td>
<td>Pattern of recent drug use</td>
</tr>
<tr>
<td>------</td>
<td>-----</td>
<td>-----</td>
<td>---------</td>
<td>---------------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>1&lt;sup&gt;e&lt;/sup&gt;</td>
<td>M</td>
<td>26</td>
<td>18</td>
<td>2</td>
<td>unknown&lt;sup&gt;3,4&lt;/sup&gt;</td>
</tr>
<tr>
<td>2&lt;sup&gt;e,g&lt;/sup&gt;</td>
<td>M</td>
<td>31</td>
<td>16</td>
<td>6</td>
<td>&gt;10 hits/week; daily with weekend binges&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>3&lt;sup&gt;none&lt;/sup&gt;</td>
<td>M</td>
<td>21</td>
<td>6</td>
<td>3</td>
<td>$150/month, some times daily, weekends</td>
</tr>
<tr>
<td>4&lt;sup&gt;a,c,d&lt;/sup&gt;</td>
<td>F</td>
<td>26</td>
<td>18</td>
<td>8</td>
<td>when she could get it; binge until all gone; limited by funds&lt;sup&gt;3,4&lt;/sup&gt;</td>
</tr>
<tr>
<td>5&lt;sup&gt;c,e&lt;/sup&gt;</td>
<td>M</td>
<td>39</td>
<td>24 years</td>
<td>unknown; appeared high on weekends&lt;sup&gt;1&lt;/sup&gt;</td>
<td>&gt;24</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>36</td>
<td>24</td>
<td>3</td>
<td>unknown&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>7&lt;sup&gt;e&lt;/sup&gt;</td>
<td>M</td>
<td>36</td>
<td>26</td>
<td>&gt;1</td>
<td>unknown&lt;sup&gt;1,2,4&lt;/sup&gt;</td>
</tr>
<tr>
<td>8&lt;sup&gt;g&lt;/sup&gt;</td>
<td>M</td>
<td>31</td>
<td>22</td>
<td>&gt;2</td>
<td>limited by funds&lt;sup&gt;1,3&lt;/sup&gt;</td>
</tr>
<tr>
<td>9&lt;sup&gt;h&lt;/sup&gt;</td>
<td>M</td>
<td>40</td>
<td>16</td>
<td>&gt;2</td>
<td>unknown&lt;sup&gt;1,3&lt;/sup&gt;</td>
</tr>
<tr>
<td>10&lt;sup&gt;a,e&lt;/sup&gt;</td>
<td>M</td>
<td>40</td>
<td>9</td>
<td>&gt;10</td>
<td>binge 2-3 weeks until no funds; limited by funds&lt;sup&gt;1,3&lt;/sup&gt;</td>
</tr>
<tr>
<td>11&lt;sup&gt;g&lt;/sup&gt;</td>
<td>M</td>
<td>36</td>
<td>23</td>
<td>&gt;2</td>
<td>unknown&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>12&lt;sup&gt;i&lt;/sup&gt;</td>
<td>F</td>
<td>30</td>
<td>20</td>
<td>&gt;1</td>
<td>unknown&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
Table 2.5: Patient information for methamphetamine users

<table>
<thead>
<tr>
<th>Case</th>
<th>Sex</th>
<th>Age</th>
<th>PMI (h)</th>
<th>Duration of use (y)</th>
<th>Pattern of recent drug use</th>
<th>Estimated interval since last admin. (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1(^{none})</td>
<td>F</td>
<td>34</td>
<td>14</td>
<td>10</td>
<td>$10/day; daily(^1)</td>
<td>16</td>
</tr>
<tr>
<td>2(^{d,k})</td>
<td>M</td>
<td>36</td>
<td>5</td>
<td>&gt;10</td>
<td>once/month(^{1,2})</td>
<td>12</td>
</tr>
<tr>
<td>3(^{a,b,d})</td>
<td>M</td>
<td>22</td>
<td>16</td>
<td>8</td>
<td>75cc hit daily if funds available(^2)</td>
<td>3</td>
</tr>
<tr>
<td>4(^a)</td>
<td>M</td>
<td>42</td>
<td>10</td>
<td>&gt;20</td>
<td>3-4 times/week(^{1,4})</td>
<td>6</td>
</tr>
<tr>
<td>5(^a)</td>
<td>M</td>
<td>20</td>
<td>21</td>
<td>1</td>
<td>unknown(^4)</td>
<td>1</td>
</tr>
<tr>
<td>6(^{a,b,c,e,f})</td>
<td>M</td>
<td>28</td>
<td>14</td>
<td>16</td>
<td>daily; binges 2-3 days, 7-10 days apart(^2,3)</td>
<td>3</td>
</tr>
<tr>
<td>7(^{c,d,f})</td>
<td>M</td>
<td>39</td>
<td>19</td>
<td>23</td>
<td>a line every two weeks(^1)</td>
<td>2 weeks</td>
</tr>
<tr>
<td>8(^{a,d,j})</td>
<td>M</td>
<td>28</td>
<td>4</td>
<td>10</td>
<td>4-5 hits/day(^2)</td>
<td>recent</td>
</tr>
<tr>
<td>9(^d)</td>
<td>F</td>
<td>44</td>
<td>23</td>
<td>10</td>
<td>every two weeks(^2)</td>
<td>hours</td>
</tr>
<tr>
<td>10(^{unknown})</td>
<td>M</td>
<td>33</td>
<td>7</td>
<td>18</td>
<td>1-2 lines crank/day; marijuana daily(^1), daily(^1,4)</td>
<td>8</td>
</tr>
<tr>
<td>11(^{a,d,e})</td>
<td>M</td>
<td>20</td>
<td>21</td>
<td>4</td>
<td>recent</td>
<td></td>
</tr>
<tr>
<td>12(^k)</td>
<td>F</td>
<td>44</td>
<td>24</td>
<td>15</td>
<td>daily(^1,3,4)</td>
<td>6</td>
</tr>
</tbody>
</table>
Table 2.6: Patient information for heroin users

<table>
<thead>
<tr>
<th>Case</th>
<th>Sex</th>
<th>Age</th>
<th>PMI (h)</th>
<th>Duration of Use (y)</th>
<th>Pattern of recent drug use</th>
<th>Estimated interval since last admin. (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1&lt;sup&gt;d,e,g&lt;/sup&gt;</td>
<td>M</td>
<td>43</td>
<td>13</td>
<td>27</td>
<td>unknown&lt;sup&gt;2&lt;/sup&gt;</td>
<td>12-48</td>
</tr>
<tr>
<td>2&lt;sup&gt;a,d&lt;/sup&gt;</td>
<td>M</td>
<td>34</td>
<td>23</td>
<td>years</td>
<td>unknown&lt;sup&gt;2&lt;/sup&gt;</td>
<td>seconds to minutes</td>
</tr>
<tr>
<td>3&lt;sup&gt;b,c,e,m&lt;/sup&gt;</td>
<td>M</td>
<td>34</td>
<td>10.5</td>
<td>&gt;1</td>
<td>unknown&lt;sup&gt;2&lt;/sup&gt;</td>
<td>minutes</td>
</tr>
<tr>
<td>4&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>M</td>
<td>41</td>
<td>5</td>
<td>20</td>
<td>daily&lt;sup&gt;2&lt;/sup&gt;</td>
<td>minutes</td>
</tr>
<tr>
<td>5&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>F</td>
<td>40</td>
<td>18.5</td>
<td>23</td>
<td>daily&lt;sup&gt;2&lt;/sup&gt;</td>
<td>unknown</td>
</tr>
<tr>
<td>6&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>M</td>
<td>43</td>
<td>21</td>
<td>years</td>
<td>daily&lt;sup&gt;2&lt;/sup&gt;</td>
<td>unknown</td>
</tr>
<tr>
<td>7&lt;sup&gt;a,i&lt;/sup&gt;</td>
<td>M</td>
<td>28</td>
<td>8</td>
<td>4</td>
<td>daily&lt;sup&gt;2&lt;/sup&gt;</td>
<td>unknown</td>
</tr>
<tr>
<td>8&lt;sup&gt;a,m&lt;/sup&gt;</td>
<td>M</td>
<td>42</td>
<td>11</td>
<td>10</td>
<td>unknown&lt;sup&gt;2&lt;/sup&gt;</td>
<td>unknown</td>
</tr>
</tbody>
</table>

Legend to Tables 2.4-2.6: Abbreviations: <sup>a</sup> prior arrest for drug related offenses; <sup>b</sup> prior accidental overdose; <sup>c</sup> prior admission to rehabilitation for substance abuse treatment; <sup>d</sup> unemployed; <sup>e</sup> depression; <sup>f</sup> receiving lithium therapy; <sup>g</sup> seizures; <sup>h</sup> homeless; <sup>i</sup> prostitute; <sup>j</sup> paranoid; <sup>k</sup> history of chest pain; <sup>l</sup> asthma; <sup>m</sup> marijuana; M, male; F, female; PMI, postmortem interval. Routes of administration: <sup>1</sup> nasal; <sup>2</sup> intra-venous; <sup>3</sup> smoking; <sup>4</sup> oral. Cause of death: Cocaine users: cocaine intoxication (case nos. 1,2,4,6-8,11); hypertensive cardiac disease with chronic cocaine use as a contributing factor (case no. 3); hypertensive cardiac disease with metoprolol/diltiazem intoxication (case no. 5); multiple stab wounds to chest (case no. 9); gunshot wound to chest (case no. 10); rupture aneurysm of right carotid artery with chronic cocaine use as a contributing factor (case no. 12). Methamphetamine users: methamphetamine intoxication (case nos. 1-5,8,11,12); gunshot wounds to chest (case nos. 6,7); severe coronary atherosclerosis with methamphetamine toxicity as a contributing factor (case nos. 9,10). Heroin users: opiate intoxication (case nos. 1-8).
Table 2.7: Toxicology results for drug users

<table>
<thead>
<tr>
<th>Case</th>
<th>Source</th>
<th>Cocaine</th>
<th>Methamphetamine</th>
<th>Heroin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>COC</td>
<td>BZE</td>
<td>EME</td>
</tr>
<tr>
<td>1</td>
<td>blood</td>
<td>50</td>
<td>75</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>brain</td>
<td>44.272</td>
<td>1.848</td>
<td>2.185</td>
</tr>
<tr>
<td></td>
<td>hair</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>2</td>
<td>blood</td>
<td>0.25</td>
<td>0.7</td>
<td>NE</td>
</tr>
<tr>
<td></td>
<td>brain</td>
<td>ND</td>
<td>0.322</td>
<td>0.486</td>
</tr>
<tr>
<td></td>
<td>hair</td>
<td>44.7</td>
<td>12.2</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>blood</td>
<td>ND</td>
<td>ND</td>
<td>NE</td>
</tr>
<tr>
<td></td>
<td>brain</td>
<td>0.122</td>
<td>3.925</td>
<td>1.339</td>
</tr>
<tr>
<td></td>
<td>hair</td>
<td>117-159</td>
<td>6.0-8.1</td>
<td>ND</td>
</tr>
<tr>
<td>4</td>
<td>blood</td>
<td>0.08</td>
<td>NE</td>
<td>NE</td>
</tr>
<tr>
<td></td>
<td>brain</td>
<td>1.359</td>
<td>1.681</td>
<td>0.416</td>
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<tr>
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<td>hair</td>
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<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>5</td>
<td>blood</td>
<td>ND</td>
<td>ND</td>
<td>NE</td>
</tr>
<tr>
<td></td>
<td>brain</td>
<td>0.001</td>
<td>0.516</td>
<td>0.322</td>
</tr>
<tr>
<td></td>
<td>hair</td>
<td>ND</td>
<td>8.25</td>
<td>ND</td>
</tr>
<tr>
<td>6</td>
<td>blood</td>
<td>5.415</td>
<td>2.649</td>
<td>2.329</td>
</tr>
<tr>
<td></td>
<td>brain</td>
<td>34.207</td>
<td>4.472</td>
<td>4.762</td>
</tr>
<tr>
<td></td>
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<td>NE</td>
</tr>
<tr>
<td></td>
<td>brain</td>
<td>1.12</td>
<td>1.12</td>
<td>0.067</td>
</tr>
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<td></td>
<td>hair</td>
<td>6.23</td>
<td>1.62</td>
<td>ND</td>
</tr>
<tr>
<td>8</td>
<td>blood</td>
<td>5.4</td>
<td>6.2</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>brain</td>
<td>38.2</td>
<td>1.643</td>
<td>4.848</td>
</tr>
<tr>
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<td>hair</td>
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<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>9</td>
<td>blood</td>
<td>0.013</td>
<td>0.057</td>
<td>0.021</td>
</tr>
<tr>
<td></td>
<td>brain</td>
<td>0.104</td>
<td>0.146</td>
<td>0.476</td>
</tr>
<tr>
<td></td>
<td>hair</td>
<td>5.5-24.2</td>
<td>0.7-1.8</td>
<td>ND</td>
</tr>
<tr>
<td>10</td>
<td>blood</td>
<td>0.034</td>
<td>3.537</td>
<td>0.736</td>
</tr>
<tr>
<td></td>
<td>brain</td>
<td>1.92</td>
<td>4.05</td>
<td>3.941</td>
</tr>
<tr>
<td></td>
<td>hair</td>
<td>36.8-70</td>
<td>0.5-3.2</td>
<td>ND</td>
</tr>
<tr>
<td>11</td>
<td>blood</td>
<td>0.046</td>
<td>NE</td>
<td>NE</td>
</tr>
<tr>
<td></td>
<td>brain</td>
<td>0.518</td>
<td>0.089</td>
<td>0.435</td>
</tr>
<tr>
<td></td>
<td>hair</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>12</td>
<td>blood</td>
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<td>4.787</td>
<td>0.909</td>
</tr>
<tr>
<td></td>
<td>brain</td>
<td>0.649</td>
<td>3.82</td>
<td>0.701</td>
</tr>
<tr>
<td></td>
<td>hair</td>
<td>102-183</td>
<td>17-50</td>
<td>ND</td>
</tr>
</tbody>
</table>

Legend to Table 2.7: Abbreviations: COC, cocaine; BZE, benzoylcegonine; EME, ecegonine methylster; AMP, amphetamine; METH, methamphetamine, MOPH, morphine; 6-MAM, 6-N-acetylmorphine; NA, not available; ND, none detected; NE, not examined; blood levels, mg/L; brain levels, ng/mg; hair levels, ng/mg.
2.6 DETERMINATION OF DRUG AND METABOLITE LEVELS IN HAIR AND BRAIN

Brain and hair drug metabolites were measured by Dr. Kathryn Kalasinsky at the Armed Forces Institute in Washington, DC, using gas chromatography/mass spectroscopy as described in Wilson et al., (1996). Briefly, samples were quantitated using a Hewlett Packard (HP) 5958A mass spectrometer operated in the electron ionization selected monitoring mode. The spectrometer was interfaced to a HP 5890 gas chromatograph operated with a J&W Scientific DB-5MS capillary column using helium as the carrier gas.

2.7 QUANTIFICATION OF BRAIN G-PROTEINS

Brain samples for all regions (48-60 mg) were sonicated (Vibra-Cell Sonicator; 30% output, 2 x 10 sec with 2 sec pulse) in microcentrifuge tubes containing 350 µL of TME buffer (50 mM Tris, 2mM MgCl₂, 1 mM EDTA, pH 7.4) on ice. The homogenates were centrifuged at 12,000 g for 15 min at 4°C and the pellets containing the crude membrane fraction were resonicated (1 x 10 sec with 2 sec pulse) in 350 µL of TME buffer. The homogenates were further diluted with 350 µL of TME. Due to the limited amount of striatal tissue, smaller samples of NACS and putamen were used (26-39 mg) and were homogenized in 250 mL of TME buffer as above. Protein concentration was determined using BSA as the standard as described by Bradford (1976). Samples were aliquoted (100 µg homogenate protein/tube) and stored at -20°C for Western immunoblot assay.

Aliquots of membrane preparation (100 µg protein) were centrifuged at 12,000 g for 10 min at 4°C and the pellets were resuspended in 30 µL of Tris-SDS (40 mM Tris HCL, 1 mM DDT, 2 % SDS, pH 6.8), and heated for 5 min at 75°C.
Solubilized proteins were then alkylated using 20 μL of 0.1M N-ethylmaleamide and left to stand at room temperature for 15 min. Fifty μL of sample buffer (62.5 mM tris HCL, 3% SDS, 20% glycerol, 5% 2-mercaptoethanol, 0.15% bromophenol blue, pH 6.8) were then added and the samples boiled at 100 °C for 3 min. Equivalent amounts of membrane proteins were loaded onto acrylamide gels (10% acrylamide/0.26% bisacrylamide for Gαs52, Gαs45), Gαo and Gβ subunits or 12.5% acrylamide/0.0625% bisacrylamide for Gαsolf and Gαi1 and Gαi2, and separated by SDS-PAGE. The resolved proteins were electroblotted onto PVDF membrane (0.45mm) using a wet transfer cell (Pharmacia; 32V, 18 hours).

Following transfer, membranes were blocked with 5% BSA in PBS-T buffer (80mM Na2HPO4, 20 mM NaH2PO4, 100 mM NaCl, 1% Tween 20, pH 7.5) for 1 hour at 21°C. The blots were then washed with PBS-T (3 x 5 min) and immunolabeled with antiserum (Gαs52,olf-RM/1; Gαi1-2)-AS/7; Gαo-GC/2; Gβ-SW/1; NEN Dupont) in PBS-T (1:5000 dilution) for 2 hours. Blots were again washed thoroughly in PBS-T and incubated with HRP-conjugated protein A at 1:3000 dilution in PBS-T for 30 min. Following extensive washing in PBS-T, the blots were soaked in ECL western blot detection solution (Renaissance, NEN Dupont) for 1 min and luminographs were taken using Hyperfilm-MP (Amersham). Exposure time was adjusted from 2 to 120 sec depending on signal strength. Immunoreactive bands were quantified using a computer-based image analysis system (MCID, St. Catherine’s, ON). To ensure that the immunoreactive signal was in range of luminographic detection, a pooled human membrane sample containing a range of protein was included on each gel. Subject samples from each group (control, cocaine, methamphetamine, heroin) were run in duplicate side by side on each gel. Additional samples in duplicate of the same
pooled human cortical membranes were also included on each gel, thus allowing normalization of subject samples. Inter- and intra-blot variation was estimated to be 10-15% and 4-10%, respectively.

2.8 STATISTICS

G-protein subunit immunolabelling in brain regions from cocaine, methamphetamine, heroin, and control subjects was compared using an ANOVA followed by post-hoc comparisons of cell means by the least significant difference test (Winer et al., 1971). Correlations between inhibitory G-protein immunoreactivity levels in the NACS and drug metabolite levels and between NACS inhibitory G-protein immunoreactivity levels and dopamine levels in the NACS were assessed using the Spearman rank correlation test for the methamphetamine and heroin users. Drug levels were obtained from the parietal cortex, putamen and caudate from the methamphetamine users and from the cerebellar cortex from the heroin group. Total drug levels for the groups were calculated for the correlations (methamphetamine, amphetamine + methamphetamine; heroin, morphine + 6-N-acetylmorphine). P-values less than 0.05 were accepted as statistically significant.
CHAPTER 3: RESULTS
3.1 CLINICAL CHARACTERISTICS AND TOXICOLOGY

Summaries of clinical and toxicological characteristics of the drug users are presented in Tables 2.3-2.7. All subjects included had drug and/or metabolite in brain, blood, urine, and where possible hair. Ethanol was detected in the blood of one cocaine user (#6) and in four heroin users (#'s 2, 4, 9, 12). Furthermore, evidence of marijuana use was detected in three of the methamphetamine users (#'s 4, 10, 11) and in two heroin users (#'s 4, 12). Drugs of abuse and their metabolites could not be detected either in autopsied brain or scalp hair of the control subjects and these subjects had no neuropathology related to drug use. The control subjects were matched to the cocaine, methamphetamine and heroin groups with respect to mean age, interval between death and freezing of the brain (PMI), and sex (See Table 2.2).

3.2 G-PROTEIN SUBUNIT IMMUNOREACTIVITY LEVELS

In all instances, distinct immunoreactive bands were resolved, migrating at the expected molecular weights of 52 and 45 kDa for Gαs, 46 kDa for Gαoff, 41 kDa for Gαi1, 40 kDa for Gαi2, 39 kDa for Gαo, and 35-36 kDa for Gβ, respectively, as previously reported by our group for postmortem brain (Young et al., 1993). The long and short forms of Gαs (52 and 45 kDa, respectively) are the predominant stimulatory G-protein subunits expressed in the cerebral cortical regions, hippocampus, amygdala, and cerebellum, whereas Gαoff is predominant as homolog expressed in the NACS and putamen (see Figure 3.1).

The immunolabelling results for each of the G-protein subunits measured in the individual comparison groups for the brain regions examined are presented in Table 3.1. Significant drug by group effects were found in the NACS for Gαi1
(F=3.33, df=3.40, p=.029), Gαi2 (F=2.85, df=3.40, p=0.049 and Gαo (F=2.97, 3.40, p=0.049). Post-hoc least significant difference tests revealed significant reductions in mean immunolabelling of Gαi1 (47%, p<0.05) and Gαi2 (49%, p<0.05) in NACS from the heroin group and of Gαi1 (32%, p<0.05) and Gαo (18%, p<0.05) from methamphetamine users compared to controls. Gαi2 immunoreactivity was also decreased (36%, p>0.05), but non-significantly, in NACS from the amphetamine group compared with controls (see Figure 3.3) but this difference did not reach statistical significance (0.1>p>0.05). In the methamphetamine users, there also were notable, but non-significant (p=0.06) increases of temporal cortical Gαs45 (47%) immunolabelling. In contrast, no alterations were found in of G-protein subunit immunolabelling in brain regions from the cocaine users compared with the control group. Furthermore, there were no significant differences of any G-protein subunit immunoreactivity levels in cerebellum, frontal and occipital cortices, putamen, amygdala, or hippocampus among comparison groups.

3.3 CORRELATIONS BETWEEN G-PROTEIN IMMUNOREACTIVITY LEVELS AND DOPAMINE AND DRUG METABOLITE LEVELS

There were no significant correlations between Gαi1,2 or Gαo levels in NACS and brain drug levels for either the methamphetamine (parietal cortex, caudate, and putamen) or heroin (cerebellar cortex) groups. There were no significant correlations between NACS dopamine levels and NACS Gαi1,2 or Gαo levels from the methamphetamine or heroin users.
Figure 3.1: Brain distribution of stimulatory G-protein subunits $G\alpha_{s45,52}$ and $G\alpha_{olf}$.

Figure 1: Stimulatory G-protein Distribution

Legend to Figure 3.1: $G\alpha_{s45,52}$ are the predominant stimulatory subunits in the frontal, temporal, occipital and cerebellar cortices, and amygdala and hippocampus, whereas $G\alpha_{olf}$ is predominant in the NACS and putamen.
**Table 3.1:** G-protein subunit immunolabelling in postmortem brain regions from non-drug, cocaine, methamphetamine and heroin users

<table>
<thead>
<tr>
<th>NACS</th>
<th>Gal₁</th>
<th>Gal₂</th>
<th>Gal₃/off</th>
<th>Gal₄/6</th>
<th>Ga₀</th>
<th>Gβ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>91±12</td>
<td>120±15</td>
<td>78±7</td>
<td>-</td>
<td>90±2</td>
<td>93±15</td>
</tr>
<tr>
<td>Cocaine</td>
<td>85±11</td>
<td>115±20</td>
<td>90±9</td>
<td>-</td>
<td>86±4</td>
<td>88±4</td>
</tr>
<tr>
<td>Methamphetamine</td>
<td>61±8*</td>
<td>76±12</td>
<td>65±11</td>
<td>-</td>
<td>76±4*</td>
<td>80±5</td>
</tr>
<tr>
<td>Heroin</td>
<td>48±11*</td>
<td>61±18*</td>
<td>76±8</td>
<td>-</td>
<td>84±4</td>
<td>85±3</td>
</tr>
<tr>
<td><strong>Putamen</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>88±10</td>
<td>88±10</td>
<td>79±8</td>
<td>-</td>
<td>89±8</td>
<td>89±8</td>
</tr>
<tr>
<td>Cocaine</td>
<td>78±10</td>
<td>88±12</td>
<td>93±9</td>
<td>-</td>
<td>87±7</td>
<td>96±6</td>
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<td>114±13</td>
<td>107±10</td>
<td>-</td>
<td>89±11</td>
<td>95±12</td>
</tr>
<tr>
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<td>100±18</td>
<td>98±15</td>
<td>-</td>
<td>99±8</td>
<td>109±12</td>
</tr>
<tr>
<td><strong>Hippocampus</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
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<td>141±14</td>
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<td>93±7</td>
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<td>76±6</td>
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<td>66±6</td>
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<tr>
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<td>146±17</td>
<td>86±6</td>
<td>126±8</td>
<td>105±3</td>
<td>106±4</td>
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<td>99±5</td>
<td>117±12</td>
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<td>102±4</td>
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<td>139±15</td>
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(normalized ROD)
Figure 3.2: Representative immunoblot of $\alpha_i_1$ and $\alpha_i_2$ in NACS from controls and drug users
Figure 3.3: G-protein subunit immunoreactivity levels in NACS of drug users expressed as percent of control
CHAPTER 4: DISCUSSION
4.1 OVERVIEW

The present study demonstrates for the first time that inhibitory G-protein immunolabelling is selectively reduced in the NACS of chronic heroin or methamphetamine users. There were no differences in immunolabelling of other G-protein subunits in this or other brain regions examined in these drug abuse groups compared with controls. Furthermore, no changes were observed in immunolabelling of any G-protein subunits in brain regions from cocaine users examined. The marked reduction in Ga immunolabelling in NACS from heroin users is consistent with observations in animal studies; chronic morphine administration to rats has been shown to decrease Ga immunoreactivity levels in this brain region (Terwilliger et al., 1991).

4.2 WHAT ABOUT COCAINE?

Interestingly, several animal studies have found that chronic cocaine administration also reduces Ga immunolabelling in the NACS (Nestler et al., 1990; Striplin & Kalivas, 1993; Terwilliger et al., 1991), whereas we observed no such alterations in cocaine users. The reason for this difference in findings between rats and humans is not clear. One possibility, however, may be related to differences in drug dosage. The amount of drug used by subjects in this study was not subject to control as in animal studies, and it is possible that the amount of cocaine was not sufficient to induce changes in G-protein immunolabelling. Another possibility stems from animal studies that have found that methamphetamine administration leads to larger increases in NACS (DiChiara & Imperato, 1988) and in striatal (Kuczenski et al., 1991) synaptic dopamine levels than does cocaine or morphine treatment.
Since changes in G-protein levels can be dependent on agonist concentration (Green et al., 1991), it is possible that dopamine levels in the cocaine group were not high enough to cause such subsequent postreceptor adaptations. A third possibility is related to the longer half-life of methamphetamine compared with cocaine: 12 hours versus 40 minutes. Because methamphetamine is in the body longer, it may induce greater effects on neuronal physiology. Several studies have shown that agonist regulation of G-protein levels is time dependent (Green et al., 1990; Green et al., 1992).

It should also be noted that the brain cocaine levels in the cocaine group were extremely variable, ranging from 0.001 ng/mg to 44.272 ng/mg. The very low levels seen in some subjects may in part explain why no alterations in inhibitory G-proteins were seen in the cocaine group.

I did, however, find significantly lower Gαi immunolabelling in the NACS of methamphetamine users, which in part supports findings from animal studies, since cocaine and methamphetamine are closely related psychostimulants (Gropetti et al., 1973).

4.3 WHY THE NACS?

Why G-protein alterations are found solely in the NACS and not in the putamen which has a similar complement of G-proteins (McLeman 1997, unpublished observations) and even higher levels of dopamine D₁ and D₂ receptors (Hall et al., 1994) is interesting. However, animal investigations have also found selective decreases of inhibitory G-proteins in the NACS following chronic drug administration. Nestler et al., (1990) and Terwilliger et al., (1991) both examined the effects of abused drugs in rats on G-protein levels across several brain regions
including the neostriatum and changes were only seen in the NACS. Moreover, other studies (DiChiara & Imperato, 1988; Kuczenski et al., 1991) have shown that dopamine levels are preferentially increased in the NACS compared with the putamen and caudate in rats treated with psychostimulants or morphine. As changes in G-protein levels may be agonist concentration dependent (Green et al., 1991), the levels of dopamine needed to induce such changes may only be attained in the NACS following drug exposure.

4.4 COMORBIDITY

Of concern is the fact that several subjects in this study from each of the three drug groups were polydrug users with ethanol and/or marijuana detected in their bodily fluids. Furthermore, several of the subjects had a history of mental illness and two methamphetamine users were receiving lithium therapy (#s 6 & 7). Although it is possible that these drugs and/or comorbid mental illnesses may have influenced G-protein status, the results of several studies argue against such possibilities.

While no G-protein alterations have been found in the cerebral cortex, hippocampus, or cerebellum, chronic ethanol increases Gαi and Gαo levels in the striatum of ethanol fed mice (Tabakoff et al., 1995), alters Gαs levels in cell lines (Mochly-Rosen et al., 1988; Rabin 1993; Williams et al., 1993), and decreases of Gαs, but not of Gαi, were observed in post-mortem temporal cortex from alcoholics (Ozawa et al., 1993). G-protein subunit immunolabelling differences have been reported in postmortem brain studies of bipolar disorder and depressed suicide patients. In the former mood disorder, higher Gαs52, but not Gαi, Gαo or Gβ immunolabelling was observed in prefrontal, temporal, and occipital cortex, but not subcortical regions or cerebellum compared with nonpsychiatric non-neurological
subjects (Young et al. 1993), whereas higher Gα45 and reductions in Gαi2, but not Gαi1, in prefrontal (Brodman area 8/9) cortex have been observed in some (Pacheco et al., 1996) but not all (Cowburn et al., 1994; Ozawa et al., 1993) studies of depressed suicide subjects. Unfortunately, none of these latter depressed suicide studies have examined G-proteins in other brain regions including NACS as in this study. Thus, it is not possible to rule out completely an effect of comorbid psychiatric disorder in producing the changes in NACS G-protein subunit immunolabelling observed here. Finally, studies of a variety of antidepressant drugs including imipramine, desipramine, fluoxetine, amitriptyline, and tranylcypromine and chronic lithium treatment have reported no consistent effect of these agents on G-protein immunoreactivity levels in rat brain (Terwilliger et al., 1991; Li et al., 1993; Emamghoreishi et al., 1996). Taken together, these findings suggest that comorbid psychiatric disorders including alcohol abuse, depression or bipolar disorder, or the medications used in treating these disorders do not account for the discreet NACS changes in Gαi1, Gαi2 and Gαo immunolabelling observed in this study.

There are little data on the neurochemical effects of marijuana, particularly on post-receptor signaling processes. However, in light of the recent finding that the active ingredient in marijuana (Δ9-tetrahydrocannabinol) increases mesolimbic accumbens DA transmission via μ opioid receptor activation in a manner similar to heroin (Tanda et al., 1997), the possibility that marijuana may affect G-protein status cannot be ruled out.

4.5 STUDY COMPARISON

A previous study reported significant increases of Gαs, Gαi1-2, Gαo, and Gβ in the frontal cortex of heroin addicts (Escriba et al., 1994), whereas in the present
study, no such alterations were observed in this region. In the former study, the subjects included also showed evidence of polydrug abuse with several drugs in addition to morphine detected in their bodily fluids including codeine, cocaine, benzodiazepines, amphetamines, cannabinoids, and ethanol. In this study, for each subject, the brain, blood, hair, and urine were tested, and with the exception of 5 who had evidence of ethanol use and 5 whose toxicology screens revealed marijuana use, there was no evidence of polydrug abuse. It is unlikely that the G-protein alterations reported here were the result of comorbid alcohol abuse, as the latter appears to have selective effects on G-protein subunits, none of which were detected in this study (discussed above). However, there are no reported studies on the effects of benzodiazepines or codeine on brain G-protein levels and it may be possible that these drugs also influence G-protein levels.

A further explanation stems from the fact that Escriba et al. (1994) suggest that several of the subjects used may have been depressed or suffered from bipolar disorder. As noted, several studies have shown that G-protein levels, specifically of Gαs (Cowburn et al., 1994; Pacheo et al., 1996; and Gαi (Pacheo et al., 1996), are altered in the frontal cortex of autopsied human brain from depressed subjects. Moreover, higher Gαs52, but not Gαi, Gαo or Gβ immunolabelling was also observed in prefrontal, temporal, and occipital cortex, but not subcortical regions or cerebellum of autopsied brain from bipolar patients (Young et al. 1993). Perhaps the altered levels of G-proteins seen in the study of Escriba et al. (1994) may reflect in part the result of a comorbid mood disorder.

It is important to note that the decreased levels of inhibitory G-proteins in NACS from methamphetamine and heroin users observed in my study are in accord
with animal investigations that have found that chronic, but not acute, cocaine and morphine treatment leads to decreased levels of Gαi/o in the NACS. (Terwilliger et al., 1991).

4.6 POST-RECEPTOR REGULATORY SITES

Traditionally, research into the mechanism of how cells regulate the intensity and duration of signaling has focused largely on receptors, yet throughout the past decade, studies have revealed that there are several post-receptor mechanisms that could potentially underlie drug-induced changes in opioid and D₁ and D₂ like dopamine receptor sensitivity.

As previously reviewed, several investigations have found that chronic drug treatment alters Gαi immunoreactivity levels in rats (Terwilliger et al., 1991; Striplin & Kalivas, 1993) and humans (Escriba et al., 1994). Hashimoto et al., 1996 also found altered Gβ levels in postmortem brain of opiate addicts. The present study demonstrates for the first time that chronic methamphetamine and heroin use in humans leads to selective decreases in Gαi and Gαo immunolabelling in the NACS. Studies in rats have also shown that AC and PKA immunoreactivity and activity levels in the NACS are increased after repeated cocaine and morphine treatment (Terwilliger et al., 1991). Together these studies indicate that both the α subunits and βγ dimers of G-proteins, the effector enzyme, AC, and the downstream regulatory protein, PKA, may be involved in drug dependence.

4.7 MECHANISMS OF G-PROTEIN DOWN REGULATION

Protein levels can be regulated by turnover and degradation, by gene transcription and mRNA translation. As previously discussed, prolonged agonist treatment in many cell types leads to receptor desensitization via various
mechanisms which include G-protein removal from the membrane and degradation, as occurs in heterologous desensitization (Longabaugh et al., 1988; Hadcock et al., 1991; reviewed in Milligan, 1993), and decreased levels of G-protein mRNA (transcriptional control) (Mochly-Rosen et al., 1988; McKenzie & Milligan, 1990). However, G-protein function and levels are also regulated by post-translational modifications, including palmitoylation and prenylation, proteolysis, subcellular trafficking, and associations with various cellular organelles (Nestler et al., 1993). They may also be regulated through several processes involving mRNA handling, including mRNA transport from the nucleus to the cytoplasm, targeting to dendrites, assembly into polysomes, and rate of translation (Hentze, 1991; Steward & Banker, 1992).

The relationships between the aforementioned processes/mechanisms and drug use remain to be elucidated. Levels of mRNA have been quantitated in few studies investigating drug abuse. Mochly-Rosen et al., (1988) found that ethanol decreases Gαs and its mRNA in NG108-15 cells. Because Gαi1 and Gαi2 have CRE binding sites in their promotor regions (Brann et al., 1987), it is reasonable to speculate that the reduced inhibitory G-protein immunolabelling seen in the methamphetamine and heroin subjects in this study may be regulated at the gene level.

4.8 FUNCTIONAL CONSEQUENCES OF DECREASED Gαi AND Gαo IMMUNOLABELING

Reducing the inhibitory influence of Gαi through compensatory downregulation is presumed to render AC supersensitive to the stimulatory effects of Gαs, leading to increased enzyme activity and subsequent upregulation of the cAMP system (see
Figure 4.1). Terwilliger et al. (1991) first proposed this hypothesis as perhaps underlying the addictive properties of morphine and cocaine after demonstrating that chronic administration of these drugs to rats leads to decreases in $G_{\alpha i}$ and $G_{\alpha o}$ levels and increases in AC and PKA levels, and activities in the NACS. Support for this notion comes from further investigations which have shown that exposure to alcohol (Ortiz et al., 1995), morphine (Self et al., 1996; Tjon et al., 1995) and cocaine (Unterwald et al., 1996) leads to upregulated cAMP formation and PKA activity.

The precise mechanisms underlying the inhibitory G-protein downregulation and subsequent supersensitization of the D1 receptor cAMP system following chronic methamphetamine and morphine administration remain unknown. However, it may be that repeated drug treatment (prolonged agonist exposure) leads to overstimulation of the D2 dopamine or $\mu$-opioid receptor systems resulting in downregulation of inhibitory G-protein levels (see introduction section 1.14). Based on several studies demonstrating that neighboring $G_{\alpha i}$ and $G_{\alpha s}$ linked signaling cascades can communicate with each other (crosstalk), it is reasonable to speculate that decreased $G_{\alpha i}$ levels may lead to supersensitization of the D1 receptor linked system via crosstalk from inhibitory to stimulatory G-protein cascades. Communication may occur at the level of the G-proteins or at further downstream targets, including AC or PKA (see introduction section 1.14).

It is important to note that in addition to being coupled to D2 receptors, inhibitory G-proteins are also coupled to other heptahelical receptors including $\alpha_2$-adrenergic and serotonergic 5HT-1a receptors (reviewed in Birbaumer et al., 1990). There is strong evidence implicating a role for dopamine and altered dopaminergic transmission in mediating several of the effects of drugs of abuse (see section 1.6).
However, given the fact that \(G\alpha_i/o\) interact with more than one receptor subtype, the potential involvement of other transmitter systems should not be discounted. It will be important to determine the precise receptor subtypes that are linked to the altered inhibitory \(G\)-proteins that I observed in the methamphetamine and heroin users.

**Figure 4.1:** Normal versus drug-treated state in rats

**Legend to Figure 4.1:** Figure 4.1 depicts a VTA neuron projecting to the NACS in both the normal state and drug-treated state in rats. Shown in the VTA are dopamine and \(D_2\) receptors coupled to \(G\alpha_i\). Shown in the NACS are \(D_1\) and \(D_5\) receptors coupled to \(G\alpha_S\) and \(G\alpha_i\), respectively. Also shown in the NACS are components of the cAMP intracellular system, including AC, PKA and the nuclear transcription factors creb, fos and jun. Following chronic cocaine and heroin treatment, levels of \(G\alpha_i\) are decreased leading to increased levels of dopamine release from the VTA due to decreased autoregulation. Decreased \(G\alpha_i\) also leads to compensatory supersensitization of the \(D_1\) receptor mediated cAMP system and increased levels of AC, PKA, creb, fos and jun are observed.

1) Terwilliger et al., 1991; adapted from Nestler, 1993).
D₂ dopamine receptors and μ opioid receptors are coupled via inhibitory G-proteins to other cellular systems in addition to the cAMP pathway, in particular to ion channels. Neuronal signaling is highly dependent on G-protein-receptor coupled modulation of ion channel activity and activation of both Gαi and Gαo has been shown to modulate both K⁺ and Ca²⁺ channels. Important functions of K⁺ channels include setting the resting potential of cells, shaping the repolarization phase of action potentials and decreasing cellular excitability (reviewed in Wickham & Clapham, 1995). Both Gαi₁₋₃ (Yatani et al., 1988) and Gαo (VanDongen et al., 1988) mediate K⁺ channel activation. Moreover, D₂ dopamine receptor (Freedman & Weight., 1988; Roeper et al., 1990) and μ opioid receptor activation (North et al., 1987) have been shown to increase K⁺ channel conductance in various preparations, an effect which is abolished by PTX thereby implicating the involvement of inhibitory G-proteins (Tatsumi et al., 1990; (reviewed in Wickham & Clapham, 1995). The most well documented function of Ca²⁺ channels is presynaptic inhibition (Shapiro et al., 1980) and studies have linked this phenomenon with Gαo (Hescheler et al., 1987). D₂ dopamine (Biu et al., 1994) and μ opioid receptor (Schroeder et al., 1991) activation leads to inhibition of voltage-dependent Ca²⁺ channels.

Thus, drugs of abuse acting through D₂ dopamine and/or μ opioid receptors may also influence neuronal excitability via K⁺ or Ca²⁺ channels. In fact, acute opiates inhibit locus coeruleus neurons through activation of K⁺ channels and inhibition of Na⁺ channels (Alreja & Aghajanian, 1993). Activation of the K⁺ channel occurs via direct coupling of the channel with Gαi/o (Wickham & Clapham, 1995) and chronic morphine treatment in rats leads to compensatory upregulation of locus
coeruleus neurons, including increased levels of inhibitory G-proteins (Nestler et al., 1989). Upregulation of the cAMP pathway in this region has been implicated in mediating components of drug withdrawal (Self & Nestler, 1995). Furthermore, another study has shown that the μ receptor-linked K⁺ channel (G1RK1) desensitizes after prolonged agonist exposure (Kovoor et al., 1995). Therefore, it is possible that the reductions of Gαi and Gαo immunoreactivity in the NACS that I observed in methamphetamine and heroin users may lead to alterations in ion channel activity, which in turn may influence neuronal activity.

The reductions of Gαi and Gαo observed in the present study were relatively large, ranging from 18-49%, and would be expected to have marked functional consequences on Gαi and Gαo mediated neuronal responsiveness based on findings from several animal studies. For example, in rodents, decreases in Gαi immunoreactivity levels (~20%) following chronic morphine and cocaine administration were accompanied by concomitant increases in both the amounts and activity levels of both AC and PKA (Terwilliger et al., 1991). Moreover, it has been shown that even smaller reductions in Gαi and Gαo levels (~10%) markedly reduce the ability of serotonin and GABA to induce hyperpolarization of rat dorsal raphe neurons, whereas inhibition of these inhibitory G-proteins completely abolishes these responses (Innis et al., 1988). Thus, the decrements in Gαi of the order found in this study would be expected to have marked effects on neuronal function. Further studies will be necessary, however, to determine the exact effects of the Gαi and Gαo changes found in heroin and methamphetamine abusers on AC activity and downstream targets modulated by cAMP.
4.9 FUNCTIONAL CONSEQUENCES OF AN UPREGULATED cAMP SYSTEM

Recent studies demonstrating that chronic psychostimulant and opiate treatment can alter gene expression are particularly relevant. Thus, cocaine, methamphetamine, and morphine induced activation of dopamine D1 receptor systems may lead to increased cAMP formation and stimulation of PKA activity (Terwilliger et al., 1991). Activation of PKA holoenzymes results in dissociation and translocation of their catalytic subunits into the nucleus where they are able to phosphorylate target transcription factors, thereby modulating the expression of immediate early response genes (Hyman & Nestler, 1996). PKA translocation therefore can result in changes of expression of numerous other genes which have CREs in their regulatory regions, in addition to regulating IEG’s as discussed above.

Further support for the notion of an up-regulated dopamine D1 receptor-linked cAMP system comes from the recent finding in our lab of increased levels of D1 receptors (45 %) in the NACS from the methamphetamine users (Worsley & Kish, unpublished observations), which may represent a mechanism by which chronic methamphetamine use leads to supersensitivity of dopamine mediated transmission.

4.10 OTHER SIGNALLING PATHWAYS IMPLICATED IN DRUG DEPENDENCE

There is strong evidence that signaling pathways other than the cAMP system are involved in drug dependence, particularly neurotrophic signaling cascades and neurofilament proteins. Neurotrophic factors participate in neural growth and differentiation during development and are involved in regulating signal transduction in the fully differentiated nervous system. One investigation has shown that intra-VTA infusions of brain-derived neurotrophic factor and of neurotrophin-4 prevented the ability of morphine to upregulate the cAMP pathway in the NACS (Berhow et al.,
demonstrating that these proteins may interact with the mesolimbic dopamine system and may play a role in drug-induced neural plasticity (Nestler et al., 1996).

Neurotrophins exert their effects on the central nervous system via a complex signaling cascade resulting in the activation of the mitogen activated protein (MAP)-kinase or ERK pathway. Activation of ERK mediates an array of responses through the phosphorylation of various effector proteins including tyrosine hydroxylase and specific transcription factors (Crews et al., 1993; Davis, 1993). The ERK pathway also has been shown to be regulated by drugs of abuse. Chronic morphine and cocaine treatment in rats leads to upregulation of ERK phosphorylation levels and catalytic activity in the VTA (Berhow et al., 1995; Ortiz et al., 1995). It is interesting to note that the ERK pathways are not only activated through tyrosine kinase receptors, but are also stimulated by agonists acting via G-protein coupled receptors, including Gαi (Faure et al., 1994). Furthermore, studies have implicated the βγ subunit of Gi in mediating the activation of ERK (Koch et al., 1994). Whether or not the altered levels of Gαi protein that I observed in methamphetamine and heroin users will have an effect on ERK pathways remains to be elucidated. Given the highly integrative and complex nature of intracellular signaling pathways and the fact that there is strong evidence for cross talk between ERK and AC pathways (Daaka et al., 1997), it is likely that several mechanisms, and not specific adaptations in only one pathway, underlie molecular, and thus behavioral, changes seen after chronic drug use.

Finally, cytoarchitectural factors may also contribute to the long-term effects of drugs of abuse. A recent study has shown that neurofilament proteins may also be targets for the long-term actions of morphine. These proteins are a major component of the intermediate filaments of the neuronal cytoskeleton and have been
associated with various cellular functions including axonal transport (Tytell et al., 1981; Hoffman et al., 1984) and determination of neuronal morphology (Hall et al., 1991). One subtype of this family, the NF-L protein, is essential for filament assembly (Robinson & Anderson, 1988). Garcia-Sevilla et al., (1997) found reduced levels of the NF-L protein in the frontal cortex of chronic heroin users. The study complemented previous animal investigations which found decreases in levels of three NF proteins in the VTA of rats treated with chronic morphine or cocaine (Beitner-Johnson et al., 1992). It has been speculated that altered levels of NF proteins could lead to changes in neuronal structure which may contribute to mechanisms of drug dependence (Beitner-Johnson et al., 1992).

4.11 RECEPTORS MEDIATING THE EFFECTS OF MORPHINE

Whether the reduction of G-protein levels in NACS of heroin users occurs as the result of direct action of heroin in this region or from the effects of heroin on synaptic inputs into this brain area is of some interest. Heroin acts indirectly to increase DA in the mesolimbic DA system through μ opioid receptors on GABA inhibitory interneurons that synapse on VTA dendritic processes. Released from inhibition, the VTA neurons act to increase DA mediated neurotransmission to the NACS, and the effects of heroin through this pathway are presumed to be mediated through DA receptors (Johnson et al., 1992; Leone et al., 1991).

However, several lines of evidence suggest that heroin may act directly on NACS neurons that are independent of DA neurons. This includes observations that lesions of the projections from the VTA to the NACS with kainic acid (Zito et al., 1985) or 6-OHDA (Pettit et al., 1984) and injection of DA antagonists (Vaccarino et al., 1985) into the NACS do not disrupt heroin administration, as is the case with
cocaine and amphetamine. It has been suggested that both DA and opiates can influence separate receptor populations in the NACS culminating in the activation of common neurological pathways, which mediate drug reward (Self & Nestler, 1995; see also Altman et al. 1996 for review).

Recent gene knock-out studies in mice have shown that both DA D2 receptors and μ opioid receptors are important in mediating several of morphine's effects. Lack of analgesia, place-preference, and physical dependence to chronic morphine was observed in μ opioid receptor deficient mice (Matthes et al., 1996), whereas DA D2 knock-outs still displayed withdrawal symptoms, but the rewarding effects of morphine were abolished (Maldonado et al., 1997). From these studies, it appears that both μ receptors and DA D2 receptors contribute to different aspects of morphine's behavioral effects. Because both receptor subtypes couple to inhibitory G-proteins (Chen et al., 1993; Senogles 1994), it is impossible for us to conclude whether the downregulation of Gαi we observed in heroin subjects is mediated through coupling with μ receptors and/or DA D2 receptors. Indeed, both receptor subtypes may be involved, as agonist stimulation of both classes of receptors has been shown to lead to supersensitization of AC (discussed above).

4.12 COMPLEXITY OF INTRACELLULAR SIGNALING

It also must be noted that the activity of AC reflects a balance between stimulatory and inhibitory inputs and the enzyme's ability to catalyze the synthesis of cyclic AMP is regulated by several cellular species, including G-protein α subunits and βγ subunits, Ca\(^{2+}\), protein kinases, and peptide inhibitors. Furthermore, at least ten distinct mammalian AC have been cloned to date and each type varies in regulation and anatomical distribution. Thus, it is important when interpreting the
downstream effects of decreased inhibitory G-proteins in NACS of methamphetamine and heroin users on AC to consider the fact that the distinct functional properties of ACs allow them to play an integrative and interpretative role and not simply function as a linear receiver that reflects the activity state of the G-protein (Sunahara et al., 1996). Chronic opiate administration selectively upregulated AC types I and VIII in locus coeruleus neurons and the upregulation of AC type VIII appears to be mediated by CREB (Matsuoka et al., 1994; Lane-Ladd et al., 1997). It will be important for future investigations to determine the subtypes of AC that is specifically regulated by Gαi and Gαo in the NACS and whether its levels and/or activity is altered in methamphetamine and heroin users.

4.13 CONCLUSION

In conclusion, the present study demonstrates that chronic heroin and methamphetamine use in the human lead to selective reductions of Gαi/o immunoreactivity in the NACS. These observations together with those in animals suggest that G-protein disturbances in this region may play a role in the development of dependence to these drugs of abuse. Further investigation into the functional consequences of the decreased levels of inhibitory G-proteins, in particular the effects on downstream targets including cAMP and protein kinase A, is necessary to elucidate the impact of such reductions on cellular signaling. The precise mechanisms underlying the reductions of Gαi and Gαo that I observed must also be determined to fully understand the molecular impact of chronic drug use, as do how these adaptations in signal transduction translate into observable behaviors. Recent observations suggest that upregulation of the cAMP pathway may underlie drug-induced behavior such as sensitization to drug craving (Koob & Le Moal, 1997), and
to the anhedonic and dysphoric components of withdrawal (Hyman & Nestler, 1996). Ultimately, findings such as presented here may help lead to the development of novel pharmacological treatments for drug dependence targeted at the molecular mechanisms which induce these signal transduction disturbances.
REFERENCES:


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