

**FUNCTIONAL NEUROGENOMICS OF COCAINE**

BY

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## **DEDICATION**

This dissertation is dedicated to my grandfather, Willard L. Morgan, whose genes for science and good humor have provided me with innate assistance and my parents, Joseph F. Freeman IV and Marjorie M. Freeman, who have supported me in all of my efforts and whose desire for learning lead me to science.

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## **ABSTRACT**

Willard Morgan Freeman

### **THE FUNCTIONAL NEUROGENOMICS OF COCAINE**

Dissertation prepared under the direction of  
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Chronic cocaine abuse causes altered neuronal gene expression, morphology, and physiology. These changes are thought to contribute to an allostatic state of behavior characterized by compulsive drug seeking, sensitization, tolerance, withdrawal and psychological dependence. Through the use of cDNA hybridization arrays and immunoreactive protein quantification, gene expression analysis can be carried out on a functional neurogenomic scale. This dissertation research identifies changes in gene expression across three animal models of chronic cocaine administration (non-contingent non-human primate, non-contingent rat, and self-administering rat), and three brain regions, (nucleus accumbens, frontal cortex, and hippocampus).

In the non-human primate, chronic cocaine induced the expression of protein tyrosine kinase 2, mitogen activated protein kinase kinase,  $\beta$ -catenin, and protein kinase A  $\alpha$  catalytic subunit. While each of these genes has important cellular effects, the prime finding of the study was that they all serve to activate cyclic AMP response element binding protein or activator protein 1, known mediators of cocaine-responsive gene expression

and behavior. In the non-contingent rodent model, hippocampal expression of protein kinase C $\alpha$ , protein kinase C $\epsilon$ , metabotropic glutamate receptor 5, potassium channel Kv1.1, protein tyrosine kinase 2, and  $\beta$ -catenin were induced by cocaine. Each of these genes could potentially have a number of effects, but, interestingly, some of the changes observed could act in an antagonistic manner. In the rat frontal cortex, induction of protein tyrosine kinase 2, activity-regulated cytoskeletal protein, and a nuclear receptor 77 related antigen were seen with chronic cocaine administration. In the rat nucleus accumbens, protein tyrosine kinase 2 protein was shown to be significantly up-regulated. Initial hybridization array analysis of cocaine self-administering rats has produced a number of potentially cocaine-responsive genes, some of which were observed in the non-contingent rat model.

These studies demonstrate that some changes in gene expression are specific to certain regions of the brain and others are more ubiquitous. These changes in gene expression provide hypotheses for future research into the role of functional neurogenomics in physiology and behavior, and may provide potential targets for pharmacotherapeutic intervention.

# Preface to the Introduction



While it is clear that cocaine is a serious societal problem, the underlying mechanisms responsible for its abuse potential are poorly understood. These mechanisms involve psychosocial, behavioral, neuroanatomical, *and* molecular components. This last facet, the molecular component, is the subject of the research presented in this dissertation. While the social and behavioral components of cocaine abuse are substantial, growing evidence suggests that long-term drug administration produces lasting changes in brain gene expression. These responsive changes in gene expression may contribute to an allostatic state characterized by physiological and behavioral phenomena such as physical dependence, tolerance, withdrawal, craving, sensitization, and psychological addiction.

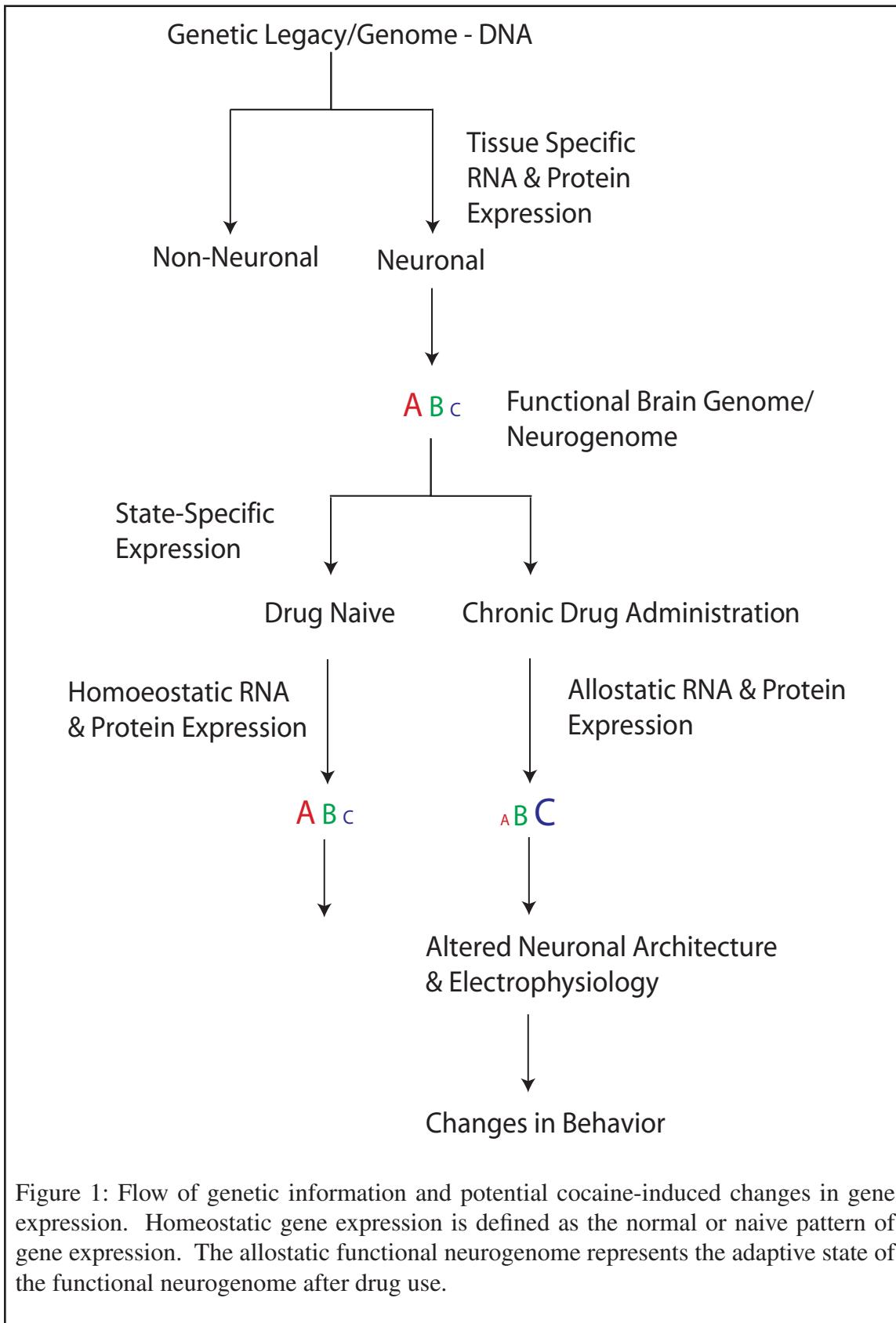
Every cell in an organism contains the same complement of DNA. Tissue identity and function is largely determined by the unique pattern of genes expressed within the cells of the tissue. If a pattern of gene expression (as represented by **A B c**) is taken to represent a normal or homeostatic pattern of expression, a disease state may manifest (or be caused by) an altered, state-specific pattern of gene expression (as represented by **A B C**). This central premise of the present research is set forth in Figure 1. Cocaine-induced, neuro-specific changes in gene expression are the subject of this dissertation, entitled *The Functional Neurogenomics of Cocaine*.

Determining what genes change in

response to cocaine administration in various animal models will provide insights

of how to analyze gene expression on a large scale. Given the tens of thousands of genes encoded within the human genomes, traditional models of examining one gene at a time are inadequate. Therefore, large-scale gene expression analysis (simultaneous testing of hundreds to thousands of genes) is required. DNA hybridization arrays provide the experimental technology for this type of analysis and are the predominant experimental technique in this work.

This dissertation is presented in the following manner: (1) An Introduction follows this Preface that (a) provides information on what is currently known about cocaine and altered gene expression; and that (b) describes in detail the new technology of hybridization arrays. (2) Five research chapters (each a first-author work in press, submitted, or in preparation) detail a series of experiments on cocaine-responsive gene expression. These studies address the following fundamental questions about the functional neurogenomics of cocaine. (1) What are the changes in CNS gene expression in behaviorally relevant brain regions? (2) How do these changes differ with various administration paradigms? (3)



What changes are seen in common? (4) What changes are specific to discrete brain regions, animal models or treatment paradigms? The research chapters are followed by a brief summarizing Discussion Chapter. Finally, an appendix chapter has been included that focuses on issues of experimental design and functional neurogenomics. This work advances the understanding of the functional neurogenomics of cocaine and identifies new avenues of research in pharmacology, behavior, and functional genomics.



# Introduction, Part A: Cocaine and Neuronal Gene Expression



## Introduction- Cocaine:

Cocaine is the purified extract of the coca plant, *Erythroxylum coca*, native to the highlands of South America. Coca leaves have been part of the culture of the Inca, Ayamara and Quechua peoples for centuries, if not millennia. Coca leaves are chewed in a manner similar to tobacco in the United States for its stimulatory effects, appetite suppression, and mild euphoria. South American people also used coca in religious ceremonies as a sacrifice to gods. Coca was originally used only in its leaf form and the active ingredient was never purified. In 1860, Albert Neiman isolated the pure drug of the coca leaf, cocaine. This was followed by use of purified cocaine around the world. Both real people, Sigmund Freud and Pope Leo XIII, and fictional characters like Sherlock Holmes, extolled the benefits of cocaine. Cocaine became available in the U.S. in everything from pills to the now famous CocaCola in the late 19th century. With increased use of cocaine, concern about the deleterious effects of cocaine and heroin/opium in the U.S. led to the Har-

rison Narcotics Act of 1914. This law provided for the regulation of narcotics and appropriated \$150,000 for the implementation of the law. This was the beginning of attempts in the U.S. to control and limit the use of cocaine. Cocaine use diminished until the 1970s when there was a generalized increased interest in and use of recreational drugs. Cocaine use peaked in the 1980s, which also saw the introduction of crack cocaine, a cheaper form of cocaine that could be smoked. During the “War on Drugs” of the 1980’s and 1990’s, cocaine use decreased from a high of almost 10 million users to the level of around 4 million users seen today (SAMHSA, 1999).

The 2000 National Household Survey on Drug Abuse estimates the number of new cocaine users at over 900,000 annually, with the number of regular cocaine users remaining constant. Cocaine is the most common cause of drug-related emergency department admissions (currently over 150,000 annual admissions). Clearly, cocaine abuse remains a major societal problem, and successful treatment of cocaine addiction remains difficult since recidivism rates are high. While the dopamine transporter is known to be a primary site of action for the acute reinforcing effects of cocaine, much remains to be learned about the neurobiological effects of chronic cocaine use, and how these effects contribute to the behavioral aspects of cocaine abuse. There exists growing evidence that different abused substances (e.g. cocaine, nicotine, and morphine) cause common neurobiological changes (Pich et al., 1997; Picciotto, 1998). The identification of the molecular biologi-

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Abbreviations: AP-1, activator protein complex 1; ARC, activity-regulated cytoskeletal protein; Cdk5, cyclin-dependent kinase 5; CREB, cyclic AMP response element binding protein; D1-3, dopamine receptors 1-3; ERK, extracellular signalling related kinase; FRA, fos-related antigen; IEG, immediate early gene; JAK, janus kinase; mGluR5, metabotropic glutamate receptor 5; MAPK, mitogen activated protein kinase; NAcc, nucleus accumbens; NMDAR1, NMDA Receptor 1; PFC, prefrontal cortex; PKA, protein kinase A; PKC, protein kinase C; RGS2, regulator of G-protein signaling; SN, substantia nigra; TH, tyrosine hydroxylase; VTA, ventral tegmental area.

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cal components underlying cocaine addiction is a critical step in identifying potential targets for therapeutic intervention (Koob, 1992).

### **Cocaine- Pharmacodynamics and Pharmacokinetics:**

Cocaine acts through blocking the reuptake of the monoamines, dopamine (Moore et al., 1977; Heikkila et al., 1979; Ritz et al., 1987) norepinephrine (Herrting et al., 1961; Moore et al., 1977) and serotonin (Ross and Renyi, 1967). While cocaine exhibits comparable potency at these three transporters, many of the behavioral effects (DeWit and Wise, 1977; Colpaert et al., 1978; Miczek and Yoshimura, 1982; Ritz et al., 1987), and locomotor activating effects (Kelly and Iverson, 1976; Giros et al., 1996) of cocaine have been attributed to dopamine uptake blockade.

Following administration, cocaine shows a rapid onset of action by blocking the reuptake of dopamine by presynaptic dopamine transporters, and thus increasing synaptic dopamine. Peak levels of synaptic dopamine can be seen within 5 minutes of administration, and a return to baseline dopamine levels within 30 minutes (Bradberry, 2000). Depending on the route of administration peak plasma cocaine concentrations are reached within 30 minutes when cocaine is administered either intranasally or intravenously (Shuster, 1992). Cocaine's half-life is short, estimated at between 15 minutes to 1 hour in rats - depending on route of administration (Nayak et al., 1976). These pharmacokinetic characteristics of rapid induction of synaptic dopamine, a short drug half-life, and therefore repeated administration, have been hypothesized to contribute to the intense reinforcing effects of cocaine and the frequency of its repeated use (Volkow et al., 1999). The hydrolysis

of cocaine's ester linkages is the major route of metabolism (Shuster, 1992). The methylecgonine moiety shares a similar structure to atropine and the synthesis of cocaine analogs has centered on these tropanes for the preparation of drugs with different pharmacokinetic and dynamic characteristics. One tropane analog,  $2\beta$ -propanoyl- $3\beta$ -(4-tolyl)-tropane (PTT or WF11), (Davies et al., 1994; Davies et al., 1993) does not have these ester linkages and exhibits a much longer half-life and potency (Bennett et al., 1998; Bennett et al., 1995). These differences have been shown to manifest themselves in behavioral measures (Nader et al., 1997; Porrino et al., 1994; Porrino et al., 1995). The molecular consequences of this long-acting cocaine analog are the subject of Chapter 6.

### **Animal models of cocaine abuse:**

There are a number of models for cocaine abuse, and each of these animal models has particular benefits and limitations. Consideration of the animal model used for studies of cocaine is critical and is dependent on the goal of the study and the variables measured. Human subjects are naturally the ideal for studying cocaine abuse, but only non-invasive studies can be conducted in living humans. Also, the high comorbidity of cocaine use with other drugs of abuse and psychiatric disorders creates confounds when studying the human population. As well, the need for neuronal samples for molecular biological experiments makes human studies problematic because postmortem tissues are notoriously difficult to work with in a proper experimental manner. Non-human primates, due to their close evolutionary proximity to humans, have the most similar physiology to humans and represent an excellent laboratory animal model of cocaine abuse. Chap-

ter 2 in the present dissertation makes use of this model. Extensive use of primates is difficult due to the high expense of subjects, housing and experimental designs requiring several different groups of subjects. This limits both the number of animals that can be included in a study and the number of paradigms that can be used. Rats therefore represent the next level of animals used in the study of cocaine (as presented in Chapters 3-6). Rats can be implanted with indwelling cannulas, and will perform operant tasks for cocaine. As well, analogous brain structures exist in rat and primate which allow for general comparisons across species. Mice are another commonly-used animal for cocaine studies, but due to their size, they are not optimal for indwelling i.v. lines. Moreover, the size of the brain makes microdissection of specific brain regions difficult. Therefore, mice were not used in any of the studies detailed here.

### **Pharmacological & Behavioral Models of Cocaine Administration:**

The two major types of models to study chronic cocaine administration, which can be achieved in a variety of laboratory animal species are non-contingent drug administration and drug self-administration. Non-contingent administration of cocaine involves the investigator delivering the drug to the animal in a response-independent manner by an appropriate route of administration (*i.v.*, *i.m.*, *i.p.*). In the case of cocaine, self-administration requires the animal to perform a task to receive an *i.v.* injection of drug. This operant task can take a number of different forms, and the nature of the task is used as a tool for quantifying different behavioral phenomenon.

The differences between these two modes of administration are important in light of a growing body of evidence that has

described biochemical and molecular differences between drug self-administration and non-contingent drug administration (Dworkin et al., 1995; Hemby et al., 1997; Dworkin and Smith, 1986; Mark et al., 1999; Wilson et al., 1994). Moreover, response-independent administration can be stressful and aversive. Stress and differences in neuro-transmitter levels probably contribute to differences in gene expression between self-administering and response-independent rats (Kuzmin and Johansson, 1999). Because stress is known to increase the expression of some genes, including those thought to play a role in cocaine-responsive behavior (Fitzgerald et al., 1996; Nankova et al., 2000), it is important to differentiate between gene expression changes induced by stress and those changes which result from drug self-administration. Increased sensitization to cocaine is seen after social stress and parallels fos induction (Miczek et al., 1999). On the other hand, non-contingent studies do not require invasive surgery on the laboratory animal and the animal is not burdened with indwelling i.v. lines or other materials. Furthermore, handling stress in non-contingent studies is controlled by giving control groups injections of saline.

As a pharmacological model, non-contingent cocaine administration can be delivered in a pattern that resembles the style of self-administration of cocaine seen in humans. The model of non-contingent administration in non-human primates used in Chapter 2 is that of a high dose given chronically. This resembles the human condition in that drug abuse takes place over a long period of time and intake of cocaine escalates as abuse continues. In rats (Chapters 3-5), the non-contingent administration paradigm used is also intended to resemble human intake. Rats are given a large dose of cocaine over 3 hours a day for 14 days. This

dosing regimen is intended to be analogous to cocaine ‘binges’ seen in the human population (Spangler et al., 1993; Yuferov et al., 1999; Unterwald et al., 1996). In total, non-contingent paradigms are useful models bearing in mind that they do not provide direct insight into behavior. As pharmacological models, both non-contingent models used in the studies described here (Chapters 2-5) represent chronic intake of high doses of cocaine.

Because of the biochemical and molecular differences previously described, a self-administration model of cocaine is needed to examine both pharmacological and behavioral aspects of cocaine abuse. A widely used behavioral paradigm for cocaine administration is the limited access procedure (Woolverton et al., 1982; Hurd et al., 1992; Daunais and McGinty, 1995). This procedure uses a fixed ratio of responses in order for the animal to receive an injection of cocaine over a limited session (typically 4-6 hours) with a maximum number of injections. This paradigm creates relatively constant intakes and rates so that changes seen are more likely to represent group means and not outlying behaviors or dose differences. While an excellent self-administration model, the limited access paradigm does not resemble the human condition in some aspects. Human cocaine intake is often quite high with administration lasting for hours and days at a time. Human intake patterns will often progress from recreational to compulsive drug-seeking behavior (Gawin, 1991). A compulsive drug-seeking state is often characterized by long periods of drug intake, a binge, followed by a crash and abstinence (Risner and Jones, 1976). To more adequately resemble the human condition, the binge-abstinence model has been developed (Roberts et al., 2001). This procedure involves continuous,

24hr/day, access to cocaine. The number of trials, or times which the rat can press on the lever on a fixed ratio schedule to receive the cocaine injection per hour is controlled to prevent toxicity. The result of this paradigm is self-administration of high dosages (80-100mg/kg/day) of cocaine for several weeks in a manner more analogous to humans (Roberts et al., 2001). This model of self-administration was used for self-administration studies described here (Chapter 5). Therefore, the two paradigms used in the present studies can be described as a behavioral/pharmacological model (self-administration; Chapter 5) and a pharmacological model (non-contingent administration; Chapters 2-5).

### **Neuroanatomy:**

It is well understood that the brain is a heterogenous organ in which different regions contain specialized sets of neuronal and non-neuronal cells that subserve specific functions. The neuroanatomical substrates for the behavioral effects of cocaine have received a great deal of attention. Because of the mechanism of action of cocaine, much of this attention has focused on dopaminergic pathways (Figure 2). The mesolimbic, ventral tegmental area (VTA) to nucleus accumbens (NAcc), mesocortical (VTA to prefrontal cortex (PFC), and nigrostriatal (substantia nigra (SN) to striatum) dopaminergic pathways have been examined in detail for their role in cocaine’s behavioral effects. For instance, lesion studies of the NAcc produced an inhibition of the rewarding aspects of cocaine (Roberts et al., 1977). Rats will self-administer microinjections of cocaine into the prefrontal cortex (Goeders and Smith, 1983). The nigrostriatal pathway subserves many motor functions and has the largest concentration of dopaminergic cells. While these brain regions play a major

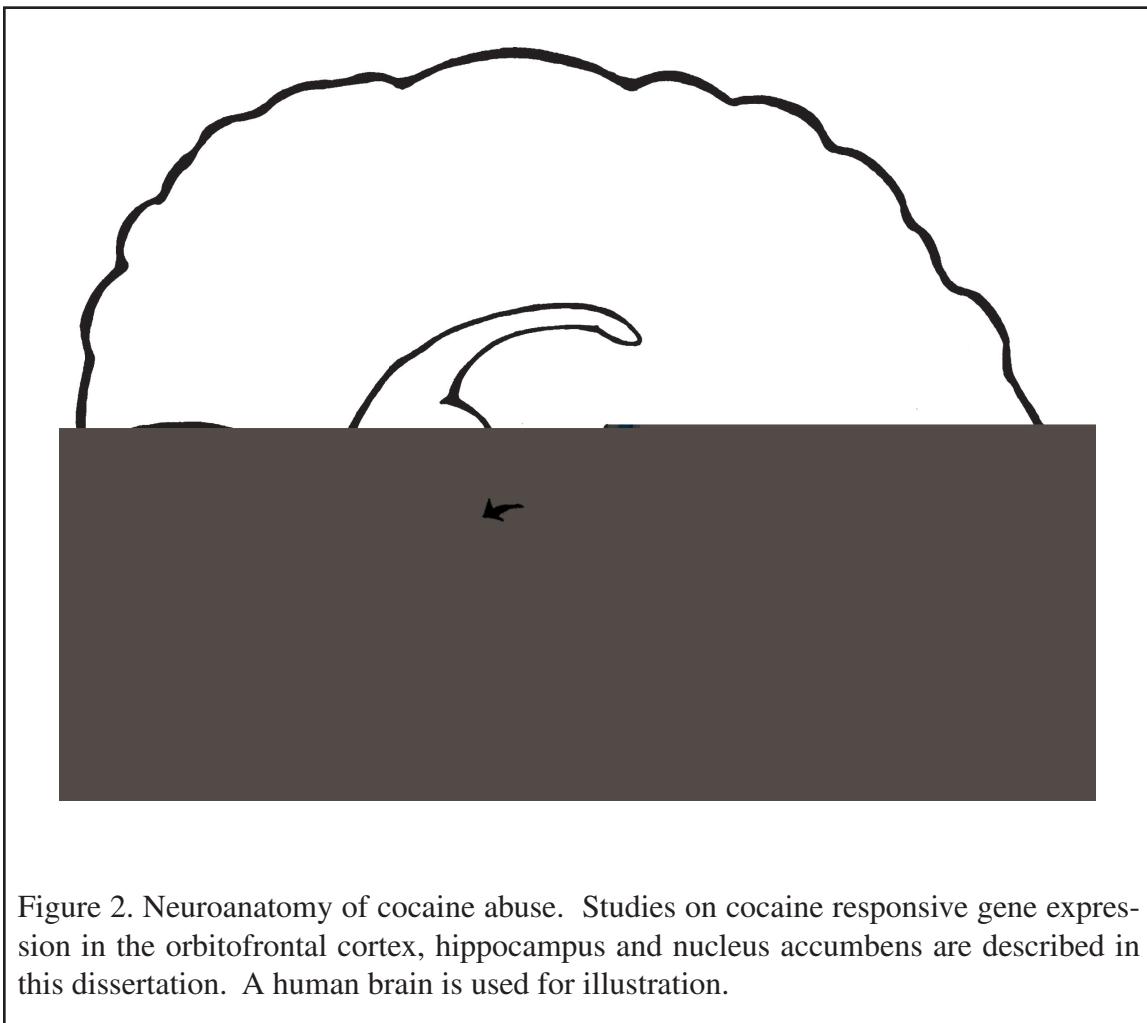


Figure 2. Neuroanatomy of cocaine abuse. Studies on cocaine responsive gene expression in the orbitofrontal cortex, hippocampus and nucleus accumbens are described in this dissertation. A human brain is used for illustration.

role in cocaine's behavioral effects, they are not the only relevant regions (Bardo, 1998). The amygdala and hippocampus play critical roles in learning and memory and the hippocampus has connections with both the NAcc and PFC (O'Donnell and Grace, 1995; Volkow and Fowler, 2000). On balance, these regions will undoubtably work in concert to determine the reinforcing characteristics and learned behaviors relevant to cocaine abuse. In this dissertation, the effects of cocaine within the NAcc, frontal cortex and hippocampus are examined in Chapters 2-5.

#### Gene expression and cocaine:

Cocaine is capable of alter-

ing the expression of a variety of genes in a number of different brain regions (for review see (Nestler and Aghajanian, 1997; White and Kalivas, 1998; Torres and Horowitz, 1999; Nestler, 2001)). The extent to which these numerous changes take place is variable and dependent on dose, animal model, length of administration, and other environmental factors. Acute and chronic administration of cocaine produce differential changes in gene expression. For example, many immediate early genes (IEGs) show a rapid and large induction with acute cocaine, but demonstrate a diminished induction with chronic treatment (Hope, 1998; Hope, 1996). Changes in the expression of some genes are only observed after chronic administration

(for example see (Fitzgerald et al., 1996). Changes in gene expression are also dependent on the time since the last administration of cocaine. Some genes are induced only during withdrawal from chronic cocaine (Bennett et al., 1999) and other changes gradually disappear during withdrawal (Denovan-Wright et al., 1998; Churchill et al., 1999). In the studies described here, the time from last cocaine administration at which changes are measured is 30 minutes for rats and 24 hours for monkeys and is intended to study the effects of maintained cocaine administration and not withdrawal from cocaine administration. Genes whose expression is responsive to cocaine can be grouped into families of genes, moving from the cell membrane to the nucleus, represented by: (1) neurotransmitter receptors and transporters; (2) ion channels; (3) architectural proteins; (4) signal transduction (both secondary and tertiary); (5) biosynthetic enzymes; and finally, (6) transcription factors (e.g. immediate early genes).

### **Dopamine Receptors and Transporters:**

The responsiveness of dopamine receptor mRNA levels to cocaine has been extensively examined, but no clear picture has emerged. A human postmortem study indicated no changes in dopamine receptor 1 (D1) or dopamine receptor 2 (D2) mRNA or binding sites in cocaine addicts vs matched controls (Medor-Woodruff et al., 1993). This study concentrated on the NAcc, corpus striatum (CS), and SN using *in situ* hybridization and receptor autoradiography. D1 mRNA was reported to increase in forebrain tissue, while D1 and D2 mRNA were increased in limbic areas after four weeks of cocaine self-administration in rat (Laurier et al., 1994). A reduction followed by induction of D2 mRNA that is dependent on time from acute cocaine administration has

also been described (Przewlocka and Lason, 1995). Dopamine receptor 3 (D3) mRNA has been reported to increase in the NAcc of human post mortem (Segal et al., 1997). The factors responsible for these differences remain unclear, but may relate to differing assessment methodologies (homogenate receptor binding vs quantitative receptor autoradiography; northern/dot blots vs *in situ* hybridization vs RT-PCR), different brain regions studied, or differences in drug dose, administration schedule, and route of administration.

Dopamine transporter (DAT) mRNA levels have been reported to decrease (Letchworth et al., 1997) or not to change (Maggos et al., 1997) with cocaine. Recent experiments simultaneously examining DAT mRNA, protein, and binding indicate that these parameters are not coordinately regulated (Letchworth et al., 1999). A quantitative *in vitro* receptor autoradiography study in non-human primates showed time- and dose-dependent changes in dopamine transporter binding (Letchworth et al., 2001). In the chronic, high cocaine dose group, increases in DAT binding were seen in the striatal regions and decreases in the NAcc (Letchworth et al., 2001). In total, the work on the dopamine receptors and transporter has shown that alterations exist but are variable and the definitive role of these changes in behavior is still unknown.

### **Other Neurotransmitter Receptors :**

Despite its central role in drugs of abuse, dopamine is a minority neurotransmitter in the brain. Glutamate is the major excitatory neurotransmitter in the brain. GluR1 has been shown to increase in the VTA with chronic treatment (Fitzgerald et al., 1996; Churchill et al., 1999). The AMPA receptor subunit, GluR2, has been shown to be increased with cocaine (Kelz

et al., 1999), while GluR4 has been seen by RT-PCR to be down-regulated in the NAcc after acute and chronic cocaine (Ghasemzadeh et al., 1999). With chronic cocaine administration, metabotropic glutamate receptor 5 (mGluR5) has been shown to be induced in the NAcc shell and striatum (Ghasemzadeh et al., 1999). NMDA receptor 1 subunit (NMDAR1) is also increased after chronic cocaine in the VTA (Fitzgerald et al., 1996). Increases in  $\mu$ -opioid receptor mRNA have also been observed in frontal cortex, amygdala and accumbens of rat (Yuferov et al., 1999). These changes could have potentially important effects on neuronal firing and communication. The fact that a multitude of changes are occurring in neurotransmitter receptor changes points towards a need to simultaneously analyze as many of these genes as possible for changes in expression. Indeed, changes in glutamate receptors are reported in Chapters 3 and 5.

### **Ion channels:**

There have been extensive studies of cocaine using electrophysiology. While the details of these studies are beyond the scope of this introduction, the changes in electrophysiological properties of neurons after cocaine administration may be caused by differential expression of ion channels after cocaine treatment (White and Kalivas, 1998). Alterations in neuronal firing could also be the result of other changes described in this introduction. For example, changes in  $\text{Na}^+$  channel currents seen with cocaine appear to be protein kinase A (PKA)-mediated. Therefore, the induction of the cAMP/PKA system by cocaine could alter the electrophysiological properties of neurons without changes in the expression of the ion channels (Zhang et al., 1998; White et al., 1998). With changes in the electrophysiological properties seen following cocaine

administration, it is important to find the basis for these changes. They could be the result of alterations in gene expression of ion channels themselves (Chapter 3) or in kinases which regulate ion channels (Chapters 2-5).

### **Architectural proteins:**

One hypothesized mechanism for the long-lasting changes in behavior seen with chronic drug use is that there are plastic changes in the organization of the brain. Recently, changes in dendritic structure have been described after chronic cocaine (Robinson et al., 2001; Robinson and Kolb, 1999). These changes could be caused by altered expression of neurofilament proteins, cytoskeletal proteins, and/or gap junctions.

Neurofilament proteins are involved in neuronal morphology and axonal transport. In rats treated non-contingently for 14 days, reduced levels of three different neurofilament proteins in the VTA have been described (Beitner-Johnson et al., 1992). The immediate early effector gene, activity-regulated cytoskeletal protein (Arc), is induced by a number of stimuli. Because de novo Arc mRNA and protein localize to activated post-synaptic zones, Arc is a putative maker of activated synapses (Steward et al., 1998; Wallace et al., 1998). Cocaine administration has been shown to induce Arc (Fosnaugh et al., 1995; Tan et al., 2000).

Recent findings also show that the cyclin dependent kinase 5 (Cdk5) is induced by chronic cocaine in a FosB-dependent manner (Bibb et al., 2001). Cdk5 could play dual roles in regulating dopaminergic signaling and regulating  $\beta$ -Catenin and adherens junctions (Bibb et al., 1999; Kwon et al., 2000).

Non-synaptic connections between cells could also be altered as seen by the differential expression of the Gap-junction pro-

tein, connexin 32, during withdrawal after cocaine self-administration (Bennett et al., 1999). While there has been limited investigation of these architectural proteins and cocaine, the studies described in this dissertation make them a valuable avenue of interest, and are described in Chapters 2-4.

### **Signal transduction:**

Another area of gene expression which is receiving increased interest is signal transduction. These functional responses can be further differentiated into two groups of genes: (1) those secondary signal transduction mechanisms which subserve receptors to transmit their signal into the cell; and (2) tertiary signal transduction, the kinases and phosphatases that regulate a wide range of protein activities within the cell and activate transcription factors. Many of the genes described as mediating neuronal plasticity and function were examined in these studies in order to gain a better understanding of the neurogenomic adaptations that occur with chronic cocaine.

### **Secondary signal transduction:**

For metabotropic receptors, G-proteins represent the first step in transmitting a signal into the cell. The first changes in adenylyl cylase and G-proteins were described in 1990-1991 (Terwilliger et al., 1991; Nestler et al., 1990). Changes in G $\beta$ 1 subunit (Wang et al., 1997) and G $i\alpha$ 1 subunits (Striplin and Kalivas, 1993) have been described in the rat NAcc. Levels of hippocampal G $s$  and G $o$  have also been demonstrated to change with cocaine treatment (Przewlocka et al., 1994). Regulators of G-proteins, like rG $\beta$ 1 (Wang et al., 1997), and regulators of G-protein signalling 2 (RGS2) (Burchett et al., 1999), are also altered in their expression with cocaine administration. These changes could play important roles in signal

transduction pathways that alter behavior (Yang et al., 2000). As well, changes in G-proteins could be important in light of elevated cyclic AMP levels increasing rewarding properties of cocaine (Kelley and Holahan, 1997). Changes in G-protein expression were analyzed in Chapters 3 and 5.

### **Tertiary signal transduction:**

Further along in signal transduction, cellular kinases and phosphatases are critical players both in signal transduction and in affecting the activity of different proteins. The cocaine-responsive nature of the cAMP/PKA pathway in rodents is well known (Terwilliger et al., 1991; Miserendino and Nestler, 1995; Unterwald et al., 1996; Tolliver et al., 1996; Self et al., 1998). Cocaine-related behavior has also been shown to be modified by interference with PKA activators and inhibitors (Self et al., 1998). In addition to the PKA pathway modifications, the protein kinase C (PKC) family has been shown to be responsive to cocaine administration (Steketee et al., 1998).

In the mitogen activated protein kinase (MAPK) pathway, changes in signaling kinases have also been shown in response to chronic cocaine. For example, increased extracellular signal related kinase (ERK) expression has been observed following chronic cocaine administration (Berhow et al., 1996b). The Janus kinase (JAK) pathway has also been shown to be altered with cocaine treatment in the VTA (Berhow et al., 1996a). Inhibition of the MAPK cascade by infusions of the inhibitor PD98059 into the VTA reduced behavioral sensitization to cocaine (Pierce et al., 1999). These changes in signal transduction cascades could play multiple roles in modifying the signal of neuronal activity or receptor/ligand binding, changing the activity of cytoplasmic proteins

and regulating transcription. For example, MAPK has been shown to induce zif268 (Kumahara et al., 1999) and other cocaine-responsive genes (Pierce et al., 1999; Yan et al., 1999). Because of the involvement of these cascades in regulating almost all aspects of cellular function, understanding changes in expression of this group of genes will increase the knowledge of intracellular adaptations to chronic cocaine. A number of changes in kinase expression were seen in the studies described in this dissertation (Chapters 2-5).

### **Biosynthetic enzymes:**

Tyrosine hydroxylase (TH) is the rate-limiting enzyme of catecholamine biosynthesis. In the NAcc core, decreases in TH protein have been observed, while in the NAcc shell, TH protein has been shown to increase after chronic cocaine (Todtenkopf et al., 2000). Increases in TH have also been seen in the VTA and SN (Vrana et al., 1993; Beitner-Johnson and Nestler, 1991). Changes in catecholamine biosynthesis could result in lasting alterations in neurotransmitter levels and therefore be important brokers of behavior. Changes in TH are addressed in Chapter 6.

### **Transcription Factors/Immediate Early Genes (IEG):**

The transcription factor group of genes has received a great deal of attention in cocaine-responsive gene expression studies. These genes are often induced rapidly with cocaine administration and are thought to underlie some of the changes described in the previous groups of genes by altering their transcription. This role of transcription factors is important in understanding cocaine-responsive gene expression. Transcription factors do not directly cause any behavior or change in neuronal phenotype

themselves; rather, these genes are part of the cascades whose endpoint may cause changes in behavior and cellular function.

Of particular interest has been the Fos- and Jun-like families that comprise the activator protein 1 (AP-1) complex (Morgan and Curran, 1991). Members of this family (c-fos, FosB, and c-jun) show a differential time course of expression with acute vs. chronic cocaine (Hope, 1998; Hope, 1996). c-fos is induced after acute and chronic cocaine in the cerebellum (Couceyro et al., 1994; Klitenick et al., 1995), caudate/putamen (Ennulat et al., 1994), parietal cortex (Daunais and McGinty, 1994), and striatum (Young et al., 1991; Couceyro et al., 1994; Daunais and McGinty, 1995; Rosen et al., 1994). FosB induction has also been observed following acute cocaine in the striatum (Rosen et al., 1994; Chen et al., 1995). Immediate early c-fos and FosB then show a reduced induction or desensitization with chronic treatment, whereby the increase in these genes is less with continuing cocaine administration (Couceyro et al., 1994; Rosen et al., 1994; Nestler et al., 1999). In contrast, chronic Fos-related antigens (FRAs) - stable  $\Delta$ FosB isoforms - are only induced by chronic cocaine and persist after withdrawal of the stimulus (Chen et al., 1995). Stimuli which induce chronic FRAs include cocaine (Hope et al., 1994; Nye et al., 1995), nicotine (Pich et al., 1997), and opiates (Nye and Nestler, 1996). These chronic FRAs, along with JunD, form heterodimers to make the chronic AP-1 complex (Chen et al., 1995; Hiroi et al., 1998). The transcriptional targets of chronic AP-1 complexes that underlie plasticity are yet to be determined; however, changes have been observed with NMDA glutamate receptor subunits (Hiroi et al., 1998; Kelz et al., 1999) and with cyclin dependent kinase 5 (Bibb et al., 2001). Behaviorally, FosB knockout mice (Hiroi

et al., 1997) show initial sensitivity to cocaine, while inducible ΔFosB mice exhibit increased responsiveness to the locomotor and rewarding aspects of cocaine, indicating a pivotal role in cocaine's actions (Kelz et al., 1999).

Phosphorylation of the cyclic-AMP response element binding protein (CREB) is the endpoint of the PKA pathway for transcriptional regulation. Increased CREB phosphorylation and therefore activity is thought to play a role in cocaine reinforcement. Overexpression of CREB decreases cocaine conditioned place preference and overexpression of negative mutant of CREB in the NAcc increases cocaine conditioned place preference (Carlezon et al., 1998). A number of other immediate early genes have been described to be induced by cocaine: Zif/268 (Daunais and McGinty, 1994; Daunais and McGinty, 1995), nur77 (Werme et al., 2000), and various anai isoforms (Berke et al., 1998). The robust and widespread nature of changes in transcription factor expression with chronic cocaine administration warrant further examination because of their potential role in the cascade of events resulting in cocaine-responsive behavior. Induction of transcription factors could be responsible for the changes in gene expression of other groups of genes previously described. As well, because transcription factors show a well-described change in their pattern of induction, they could play a role in the switch from drug abuse to compulsive drug-taking behavior. Changes in transcription factor expression are examined in Chapters 4 and 5.

### Cocaine-Responsive Gene Expression and Behavior:

While it is obvious that cocaine causes a number of changes in gene expression across a wide range of genes, the util-

ity of this knowledge is limited without

strated following chronic cocaine.

### **Summary:**

While many of the changes described so far could explain the behaviors associated with drug use, a recurrent question is how these changes contribute to the long lasting changes in behavior that can be seen in humans years after cessation of drug use. Gene expression itself is unlikely to be the direct cause of these long-lasting behaviors because the expression of mRNAs and proteins have relatively short half-lives. A viable explanation is that there are changes which alter the circuitry of the brain. Chronic cocaine has now been shown to alter dendritic morphology, a possible end result of the changes in gene expression (Robinson et al., 2001; Robinson and Kolb, 1999). Investigation of what these changes in morphology mean for behavior, and what role changes in gene expression have in altering morphology will need to be examined in detail.

The panoply of changes seen with cocaine administration points towards a more complex cocaine-responsive epigenetic imprint than may have been first imagined. While the traditional approach of examining one gene or gene family at a time for changes in expression has advanced the field greatly, it is clear that larger scale analysis of cocaine-responsive gene expression is needed. Genomic-scale gene expression analysis has become possible due to the advances of genome projects and laboratory robotics. Functional genomics, the study of all the genes expressed by a cell, is now becoming possible through hybridiza-

so e gene expression will allow a a ger

## References:

- Bardo MT (1998) Neuropharmacological mechanisms of drug reward: beyond dopamine in the nucleus accumbens. *Crit Rev Neurobiol* 12: 37-67.
- Beitner-Johnson D, Guitart X, Nestler EJ (1992) Neurofilament proteins and the meso-limbic dopamine system: common regulation by chronic morphine and chronic cocaine in the rat ventral tegmental area. *J Neurosci* 12: 2165-2176.
- Beitner-Johnson D, Nestler EJ (1991) Morphine and cocaine exert common chronic actions on tyrosine hydroxylase in dopaminergic brain reward regions. *J Neurochem* 57: 344-347.
- Bennett BA, Hollingsworth CK, Martin RS, Childers SR, Ehrenkaufer RE, Porrino LJ, Davies HML (1998) Prolonged dopamine and serotonin transporter inhibition after exposure to tropanes. *Neuropharmacology* 37: 123-130.
- Bennett BA, Wichems CH, Hollingsworth CK, Davies HM, Thornley C, Sexton T, Childers SR (1995) Novel 2-substituted cocaine analogs: uptake and ligand binding studies at dopamine, serotonin and norepinephrine transport sites in the rat brain. *J Pharmacol Exper Ther* 272: 1176-1186.
- Bennett SA, Arnold JM, Chen J, Stenger J, Paul DL, Roberts DC (1999) Long-term changes in connexin32 gap junction protein and mRNA expression following cocaine self-administration in rats. *Eur J Neurosci* 11: 3329-3338.
- Berhow MT, Hiroi N, Kobierski LA, Hyman SE, Nestler EJ (1996a) Influence of cocaine on the JAK-STAT pathway in the meso-limbic dopamine system. *J Neurosci* 16: 8019-8026.
- Berhow MT, Hiroi N, Nestler EJ (1996b) Regulation of ERK (extracellular signal regulated kinase), part of the neurotrophin signal transduction cascade, in the rat meso-limbic dopamine system by chronic exposure to morphine or cocaine. *J Neurosci* 16: 4707-4715.
- Berke JD, Paletzki RF, Aronson GJ, Hyman SE, Gerfen CR (1998) A complex program of striatal gene expression induced by dopaminergic stimulation. *J Neurosci* 18: 5301-5310.
- Bibb JA, Chen J, Taylor JR, Svenningsson P, Nishi A, Synder GL, Nestler EJ, Greengard P (2001) Effects of chronic exposure to cocaine are regulated by the neuronal protein Cdk5. *Nature* 410: 376-380.
- Bibb JA, Snyder GL, Nishi A, Yan Z, Meijer L, Fienberg AA, Tsai LH, Kwon YT, Girault JA, Czernik AJ, Huganir RL, Hemmings HC, Nairn AC, Greengard P (1999) Phosphorylation of DARPP-32 by Cdk5 modulates dopamine signalling in neurons. *Nature* 402: 669-671.
- Bradberry CW (2000) Acute and chronic dopamine dynamics in a nonhuman primate model of recreational cocaine use. *J Neurosci* 20: 7109-7115.
- Burchett SA, Bannon MJ, Granneman JG (1999) RGS mRNA expression in rat striatum: modulation by dopamine receptors and effects of repeated amphetamine administration. *J Neurochem* 72: 1529-1533.
- Berhow MT, Hiroi N, Kobierski LA, Hyman SE, Nestler EJ (1996a) Influence of cocaine on the JAK-STAT pathway in the meso-limbic dopamine system. *J Neurosci* 16: 8019-8026.

- Carlezon WA, Thome J, Olson VG, Lane-Ladd SB, Brodkin ES, Hiroi N, Duman RS, Neve RL, Nestler EJ (1998) Regulation of cocaine reward by CREB. *Science* 282: 2272-2275.
- Cervo L, Mukherjee S, Bertaglia A, Samanin R (1997) Protein kinases A and C are involved in the mechanisms underlying consolidation of cocaine place conditioning. *Brain Res* 775: 30-36.
- Chen J, Nye HE, Kelz MB, Hiroi N, Nakabeppu Y, Hope BT, Nestler EJ (1995) Regulation of delta FosB and FosB-like proteins by electroconvulsive seizure and cocaine treatments. *Mol Pharmacol* 48: 880-889.
- Churchill L, Swanson CJ, Urbina M, Kalivas PW (1999) Repeated cocaine alters glutamate receptor subunit levels in the nucleus accumbens and ventral tegmental area of rats that develop behavioral sensitization. *J Neurochem* 72: 2397-2403.
- Colpaert FC, Niemegeers CJ, Janssen PA (1978) Neuroleptic interference with the cocaine cue: internal stimulus control of behavior and psychosis. *Psychopharmacology* 58: 247-255.
- Couceyro P, Pollock KM, Drews K, Douglass J (1994) Cocaine differentially regulates activator protein-1 mRNA levels and DNA-binding complexes in the rat striatum and cerebellum. *Mol Pharmacol* 46: 667-676.
- Daunais JB, McGinty JF (1994) Acute and chronic cocaine administration differentially alters striatal opioid and nuclear transcription factor mRNAs. *Synapse* 18: 35-45.
- Daunais JB, McGinty JF (1995) Cocaine binges differentially alter striatal preprodynorphin and zif/268 mRNAs. *Mol Brain Res* 29: 201-210.
- Davies HM, Saikali E, Huby NJ, Gilliatt VJ, Matasi JJ, Sexton T, Childers SR (1994) Synthesis of 2 beta-acyl-3beta-aryl-8-azabi-cyclo[3.2.1] octanes and their binding affinities at dopamine and serotonin transport sites in rat striatum and frontal cortex. *J Med Chem* 37: 1262-1268.
- Davies HM, Saikali E, Sexton T, Childers SR (1993) Novel 2-substituted cocaine analogs: binding properties at dopamine transport sites in rat striatum. *Eur J Pharmacol* 244: 93-97.
- Denovan-Wright EM, Newton RA, Armstrong JN, Babity JM, Robertson HA (1998) Acute administration of cocaine, but not amphetamine, increases the level of synaptotagmin IV mRNA in the dorsal striatum of rat. *Mol Brain Res* 55: 350-354.
- DeWit H, Wise R (1977) Blockade of cocaine reinforcement in rats with the dopamine blocker, pimozide, but not with the noradrenergic blockers phentolamine or phenoxybenzamine. *Canadian J Psychol* 31: 195-303.
- Dworkin SI, Co C, Smith JE (1995) Rat brain neurotransmitter turnover rates altered during withdrawal from chronic cocaine administration. *Brain Res* 682: 116-126.
- Dworkin SI, Smith JE (1986) Behavioral contingencies involved in drug-induced neurotransmitter turnover changes. *NIDA Res Monogr* 74: 90-106.
- Ennulat DJ, Babb S, Cohen BM (1994) Persistent reduction of immediate early gene

- mRNA in rat forebrain following single or multiple doses of cocaine. *Mol Brain Res* 26: 106-112.
- Fitzgerald LW, Ortiz J, Hamedani AG, Nestler EJ (1996) Drugs of abuse and stress increase the expression of GluR1 and NMDAR1 glutamate receptor subunits in the rat ventral tegmental area: common adaptations among cross-sensitizing agents. *J Neurosci* 16: 274-282.
- Fosnaugh JS, Bhat RV, Yamagata K, Worley PF, Baraban JM (1995) Activation of arc, a putative "effector" immediate early gene, by cocaine in rat brain. *J Neurochem* 64: 2377-2380.
- Gawin FH (1991) Cocaine addiction: psychology and neurophysiology. *Science* 251: 1580-1586.
- Ghasemzadeh MB, Nelson LC, Lu XY, Kalivas PW (1999) Neuroadaptations in ionotropic and metabotropic glutamate receptor mRNA produced by cocaine treatment. *J Neurochem* 72: 157-165.
- Goeders NE, Smith JE (1983) Cortical dopaminergic involvement in cocaine reinforcement. *Science* 221: 773-775.
- Heikkila RE, Cabbat FS, Manzino L, Duvoisin RC (1979) Rotational behavior induced by cocaine analogs in rats with unilateral 6-hydroxy-dopamine lesions of the substantia nigra: dependence upon dopamine uptake inhibition. *J Pharmacol Exper Ther* 211: 189-194.
- Heilig M, Engel JA, Soderpalm B (1993) C-fos antisense in the nucleus accumbens blocks the locomotor stimulant action of cocaine. *Eur J Pharmacol* 236: 339-340.
- Hemby SE, Co C, Koves TR, Smith JE, Dworkin SI (1997) Differences in extracellular dopamine concentrations in the nucleus accumbens during response-dependent and response-independent cocaine administration in the rat. *Psychopharmacology* 133: 7-16.
- Herrting G, Axelrod J, Whitby L (1961) Effect of drugs on the uptake and metabolism of <sup>3</sup>H-norepinephrine. *J Pharmacol Exper Ther* 134: 146-153.
- Hiroi N, Brown JR, Haile CN, Ye H, Greenberg ME, Nestler EJ (1997) FosB mutant mice: loss of chronic cocaine induction of Fos-related proteins and heightened sensitivity to cocaine's psychomotor and rewarding effects. *Proc Natl Acad Sci U S A* 94:10397-10402.
- Hiroi N, Marek GJ, Brown JR, Ye H, Saudou F, Vaidya VA, Duman RS, Greenberg ME, Nestler EJ (1998) Essential role of the fosB gene in molecular, cellular, and behavioral actions of chronic electroconvulsive seizures. *J Neurosci* 18: 6952-6962.
- Hope BT (1996) Novel transcription factors are induced by chronic cocaine treatment. *Ann N Y Acad Sci* 801: 1-12.
- Hope BT (1998) Cocaine and the AP-1 transcription factor complex. *Ann N Y Acad Sci* 844: 1-6.
- Hope BT, Kelz MB, Duman RS, Nestler EJ (1994) Chronic electroconvulsive seizure (ECS) treatment results in expression of a long-lasting AP-1 complex in brain with altered composition and characteristics. *J Neurosci* 14: 4318-4328.

- Hurd YL, Brown EE, Finlay JM, Fibiger HC, Gerfen CR (1992) Cocaine self-administration differentially alters mRNA expression of striatal peptides. *Mol Brain Res* 13: 165-170.
- Kalivas PW, Duffy P, Mackler SA (1999) Interrupted expression of NAC-1 augments the behavioral responses to cocaine. *Synapse* 33: 153-159.
- Kelley AE, Holahan MR (1997) Enhanced reward-related responding following cholera toxin infusion into the nucleus accumbens. *Synapse* 26: 46-54.
- Kelz MB, Chen J, Carlezon WA, Whisler K, Gilden L, Beckmann AM, Steffen C, Zhang YJ, Marotti L, Self DW, Tkatch T, Baranauskas G, Surmeier DJ, Neve RL, Duman RS, Picciotto MR, Nestler EJ (1999) Expression of the transcription factor deltaFosB in the brain controls sensitivity to cocaine. *Nature* 401: 272-276.
- Klitenick MA, Tham CS, Fibiger HC (1995) Cocaine and d-amphetamine increase c-fos expression in the rat cerebellum. *Synapse* 19: 29-36.
- Kumahara E, Ebihara T, Saffen D (1999) Nerve growth factor induces zif268 gene expression via MAPK-dependent and -independent pathways in PC12D cells. *J Biochem* 12: 541-553.
- Kwon YT, Gupta A, Zhou Y, Nikolic M, Tsai LH (2000) Regulation of N-cadherin-mediated adhesion by the p35-Cdk5 kinase. *Curr Biol* 10: 363-372.
- Laurier LG, Corrigall WA, George SR (1994) Dopamine receptor density, sensitivity and mRNA levels are altered following self-administration of cocaine in the rat. *Brain Res* 634: 31-40.
- Letchworth SR, Daunais JB, Hedgecock AA, Porrino LJ (1997) Effects of chronic cocaine administration on dopamine transporter mRNA and protein in the rat. *Brain Res* 750: 214-222.
- Letchworth SR, Nader MA, Smith HR, Friedman DP, Porrino LJ (2001) Progression of Changes in Dopamine Transporter Binding Site Density as a Result of Cocaine Self-Administration in Rhesus Monkeys. In press: *J Neurosci*.
- Letchworth SR, Sexton T, Childers SR, Vrana KE, Vaughan RA, Davies HML, Porrino LJ (1999) Regulation of rat dopamine transporter mRNA and protein by chronic cocaine administration. *J Neurochem* 73: 1982-1989.
- Lockhart DJ, Winzeler EA (2000) Genomics, gene expression and DNA arrays. *Nature* 405: 827-836.
- Maggos CE, Spangler R, Zhou Y, Schlussman SD, Ho A, Kreek MJ (1997) Quantitation of dopamine transporter mRNA in the rat brain: mapping, effects of "binge" cocaine administration and withdrawal. *Synapse* 26: 55-61.
- Mark GP, Hajnal A, Kinney AE, Keys AS (1999) Self-administration of cocaine increases the release of acetylcholine to a greater extent than response-independent cocaine in the nucleus accumbens of rats. *Psychopharmacology (Berl)* 143: 47-53.
- Meador-Woodruff JH, Little KY, Damask SP, Mansour A, Watson SJ (1993) Effects of cocaine on dopamine receptor gene expres-

- sion: a study in the postmortem human brain. *Biol Psychiatry* 34: 348-355.
- Miczek KA, Yoshimura H (1982) Disruption of primate social behavior by d-amphetamine and cocaine: differential antagonism by antipsychotics. *Psychopharmacol* 76: 163-171.
- Miserendino MJ, Nestler EJ (1995) Behavioral sensitization to cocaine: modulation by the cyclic AMP system in the nucleus accumbens. *Brain Res* 674: 299-306.
- Moore K, Chiueh C, Zeldes G (1977) Release of neurotransmitters in the brain in vivo by amphetamine, methylphenidate and cocaine. In: *Cocaine and other stimulants* (Ellinwood E, Kilbey M, eds), pp 143-160. New York: Plenum.
- Morgan JI, Curran T (1991) Stimulus-transcription coupling in the nervous system: involvement of the inducible proto-oncogenes fos and jun. *Annu Rev Neurosci* 14: 421-451.
- Nader MA, Grant KA, Davies HM, Mach RH, Childers SR (1997) The reinforcing and discriminative stimulus effects of the novel cocaine analog 2beta-propanoyl-3beta-(4-tolyl)-tropane in rhesus monkeys. *J Pharmacol Exper Ther* 280: 541-550.
- Nayak PK, Misra AL, Mule SJ (1976) Physiological disposition and biotransformation of (3H) cocaine in acutely and chronically treated rats. *J Pharmacol Exper Ther* 196: 556-569.
- Nestler EJ (2001) Molecular basis of long-term plasticity underlying addiction. *Nat Neurosci Rev* 2: 119-128.
- Nestler EJ, Aghajanian GK (1997) Molecular and cellular basis of addiction. *Science* 278: 58-63.
- Nestler EJ, Kelz MB, Chen J (1999) DeltaFosB: a molecular mediator of long-term neural and behavioral plasticity. *Brain Res* 835: 10-17.
- Nestler EJ, Terwilliger RZ, Walker JR, Sevarino KA, Duman RS (1990) Chronic cocaine treatment decreases levels of the G protein subunits Gi alpha and Go alpha in discrete regions of rat brain. *J Neurochem* 55: 1079-1082.
- Nye HE, Hope BT, Kelz MB, Iadarola M, Nestler EJ (1995) Pharmacological studies of the regulation of chronic FOS-related antigen induction by cocaine in the striatum and nucleus accumbens. *J Pharmacol Exper Ther* 275: 1671-1680.
- Nye HE, Nestler EJ (1996) Induction of chronic Fos-related antigens in rat brain by chronic morphine administration. *Mol Pharm* 49: 636-645.
- O'Donnell P, Grace AA (1995) Synaptic interactions among excitatory afferents to nucleus accumbens neurons: hippocampal gating of prefrontal cortical input. *J Neurosci* 15: 3622-3639.
- Peris J, Jung BJ, Resnick A, Walker P, Malakhova O, Bokrand Y, Wielbo D (1998) Antisense inhibition of striatal GABA<sub>A</sub> receptor proteins decreases GABA- stimulated chloride uptake and increases cocaine sensitivity in rats. *Mol Brain Res* 57: 310-320.
- Picciotto MR (1998) Common aspects of the action of nicotine and other drugs of abuse. *Drug Alcohol Depend* 51: 165-172.

- Pich EM, Pagliusi SR, Tessari M, Talabot-Ayer D, Hooft v, Huijsdijnen R, Chiampulera C (1997) Common neural substrates for the addictive properties of nicotine and cocaine. *Science* 275: 83-86.
- Pierce RC, Pierce-Bancroft AF, Prasad BM (1999) Neurotrophin-3 contributes to the initiation of behavioral sensitization to cocaine by activating the Ras/Mitogen-activated protein kinase signal transduction cascade. *J Neurosci* 19: 8685- 8695.
- Pierce RC, Quick EA, Reeder DC, Morgan ZR, Kalivas PW (1998) Calcium-mediated second messengers modulate the expression of behavioral sensitization to cocaine. *J Pharmacol Exper Ther* 286: 1171- 1176.
- Porrino LJ, Davies HM, Childers SR (1995) Behavioral and local cerebral metabolic effects of the novel tropane analog, 2 beta-propanoyl-3 beta-(4-tolyl)-tropane. *J Pharmacol Exper Ther* 272: 901-910.
- Porrino LJ, Migliarese K, Davies HM, Saikali E, Childers SR (1994) Behavioral effects of the novel tropane analog, 2beta-propanoyl-3beta-(4-tolyl)-tropane (PTT). *Life Sci* 54: PL511-7.
- Przewlocka B, Lason W (1995) Adaptive changes in the proenkephalin and D2 dopamine receptor mRNA expression after chronic cocaine in the nucleus accumbens and striatum of the rat. *Eur Neuropsychopharmacol* 5: 465-469.
- Przewlocka B, Lason W, Przewlocki R (1994) The effect of chronic morphine and cocaine administration on the Gs and Go protein messenger RNA levels in the rat hippocampus. *Neurosci* 63: 1111-1116.
- Risner ME, Jones BE (1976) Characteristics of unlimited access to self-administered stimulant infusions in dogs. *Biol Psychiatry* 11: 625-634.
- Ritz MC, Lamb RJ, Goldberg SR, Kuhar MJ (1987) Cocaine receptors on dopamine transporters are related to self-administration of cocaine. *Science* 237: 1219-1223.
- Roberts DCS, Corcoran ME, Fibiger HC (1977) On the role of ascending catecholaminergic systems in intravenous self-administration of cocaine. *Pharmacol Biochem & Behav* 6: 615-620.
- Roberts DCS, Brebner K, Vincler M, Lynch WJ (2001) Binge cocaine self-administration in rats responding under a discrete trials procedure. Submitted: *J Pharmcol Exp Ther*.
- Robinson TE, Gorny G, Mitton E, Kolb B (2001) Cocaine self-administration alters the morphology of dendrites and dendritic spines in the nucleus accumbens and neocortex. *Synapse* 39: 257-266.
- Robinson TE, Kolb B (1999) Alterations in the morphology of dendrites and dendritic spines in the nucleus accumbens and prefrontal cortex following repeated treatment with amphetamine or cocaine. *Eur J Neurosci* 11: 1598-1604.
- Rocha BA, Fumagalli F, Gainetdinov RR, Jones SR, Ator R, Giros B, Miller GW, Caron MG (1998) Cocaine self-administration in dopamine transporter knockout mice. *Nat Neurosci* 1: 132-137.
- Rosen JB, Chuang E, Iadarola MJ (1994) Differential induction of Fos protein and a Fos-related antigen following acute and

- repeated cocaine administration. *Mol Brain Res* 25:168-172.
- Ross SB, Renyi AL (1967) Inhibition of the uptake of tritiated catecholamines by anti-depressant and related agents. *Eur J Pharmacol* 2: 181-186.
- SAMHSA. National Household Survey on Drug Abuse. 1999.
- Segal DM, Moraes CT, Mash DC (1997) Up-regulation of D3 dopamine receptor mRNA in the nucleus accumbens of human cocaine fatalities. *Mol Brain Res* 45: 335-339.
- Self DW, Genova LM, Hope BT, Barnhart WJ, Spencer JJ, Nestler EJ (1998) Involvement of cAMP-dependent protein kinase in the nucleus accumbens in cocaine self-administration and relapse of cocaine-seeking behavior. *J Neurosci* 18: 1848-1859.
- Shuster L (1992) Pharmacokinetics, metabolism, and disposition of cocaine. In: Cocaine: Pharmacology, Physiology and Clinical Strategies (Lakowski JM, Galloway MP, White FJ, eds), pp 1-14. Boca Raton, FL: CRC Press.
- Sora I, Wichems C, Takahashi N, Li XF, Zeng Z, Revay R, Lesch KP, Murphy DL, Uhl GR (1998) Cocaine reward models: conditioned place preference can be established in dopamine and in serotonin-transporter knockout mice. *Proc Natl Acad Sci U S A* 95: 7699-7704.
- Spangler R, Unterwald EM, Kreek MJ (1993) 'Binge' cocaine administration induces a sustained increase of prodynorphin mRNA in rat caudateputamen. *Mol Brain Res* 19: 323-327.
- Steketee JD, Rowe LA, Chandler LJ (1998) The effects of acute and repeated cocaine injections on protein kinase C activity and isoform levels in dopaminergic brain regions. *Neuropharmacology* 37: 339-347.
- Steward O, Wallace CS, Lyford GL, Worley PF (1998) Synaptic activation causes the mRNA for the IEG Arc to localize selectively near activated post-synaptic sites on dendrites. *Neuron* 21: 741-751.
- Striplin CD, Kalivas PW (1993) Robustness of G protein changes in cocaine sensitization shown with immunoblotting. *Synapse* 14: 10-15.
- Tan A, Moratalla R, Lyford GL, Worley P, Graybiel AM (2000) The activity-regulated cytoskeletal-associated protein arc is expressed in different striosome-matrix patterns following exposure to amphetamine and cocaine. *J Neurochem* 74: 2074-2078.
- Terwilliger RZ, Beitner-Johnson D, Sevareno KA, Crain SM, Nestler EJ (1991) A general role for adaptations in G-proteins and the cyclic AMP system in mediating the chronic actions of morphine and cocaine on neuronal function. *Brain Res* 548: 100-110.
- Todtenkopf MS, De Leon KR, Stellar JR, (2000) Repeated cocaine treatment alters tyrosine hydroxylase in the rat nucleus accumbens. *Brain Res Bull* 52: 407-411.
- Tolliver BK, Ho LB, Reid MS, Berger SP (1996) Evidence for involvement of ventral tegmental area cyclic AMP systems in behavioral sensitization to psychostimulants. *J Pharmacol Exper Ther* 278: 411-420.
- Torres G, Horowitz JM (1999) Drugs of abuse and brain gene expression. *Psycho-*

- som Med 61: 630-650.
- Unterwald EM, Fillmore J, Kreek MJ (1996) Chronic repeated cocaine administration increases dopamine D1 receptor-mediated signal transduction. Eur J Pharmacol 318: 31-35.
- Volkow ND, Fowler JS (2000) Addiction, a disease of compulsion and drive: Involvement of the orbitofrontal cortex. Cerebral Cortex 10: 318-325.
- Volkow ND, Fowler JS, Wang GJ (1999) Imaging studies on the role of dopamine in cocaine reinforcement and addiction in humans. J Psychopharmacol 13: 337-345.
- Vrana SL, Vrana KE, Koves TR, Smith JE, Dworkin SI (1993) Chronic cocaine administration increases CNS tyrosine hydroxylase enzyme activity and mRNA levels and tryptophan hydroxylase enzyme activity levels. J Neurochem 61: 2262-2268.
- Wallace CS, Lyford GL, Worley PF, Steward O (1998) Differential intracellular sorting of immediate early gene mRNAs depends on signals in the mRNA sequence. J Neurosci 18: 26-35.
- Wang XB, Funada M, Imai Y, Revay RS, Ujike H, Vandenberghe DJ, Uhl GR (1997) rGbeta1: a psychostimulant-regulated gene essential for establishing cocaine sensitization. J Neurosci 17: 5993-6000.
- Werme M, Olson L, Brene S (2000) NGFI-B and Nor1 mRNAs are upregulated in brain reward pathways by drugs of abuse: different effects in Fischer and Lewis rats. Mol Brain Res 76: 18-24.
- White FJ, Hu XT, Zhang XF (1998) Neuroadaptations in nucleus accumbens neurons resulting from repeated cocaine administration. Advan Pharmacol (New York) 42: 1006-1009.
- White FJ, Kalivas PW (1998) Neuroadaptations involved in amphetamine and cocaine addiction. Drug Alcohol Depend 51: 141-153.
- Wilson JM, Nobrega JN, Corrigall WA, Coen KM, Shannak K, Kish SJ (1994) Amygdala dopamine levels are markedly elevated after self- but not passive-administration of cocaine. Brain Res 668: 39-45.
- Woolverton WL, Wessinger WD, Balster RL (1982) Reinforcing properties of clonidine in rhesus monkeys. Psychopharmacol (Berl) 77: 17-23.
- Yan Z, Feng J, Fienberg AA, Greengard P (1999) D(2) dopamine receptors induce mitogen-activated protein kinase and cAMP response element binding protein phosphorylation in neurons. Proc Natl Acad Sci USA 96: 11607-11612.
- Yang J, Wu J, Kowalska MA, Dalvi A, Prevost N, O'Brien PJ, Manning D, Poncz M, Lucki I, Blendy JA, Brass LF (2000) Loss of signaling through the G protein, Gz, results in abnormal platelet activation and altered responses to psychoactive drugs. Proc Natl Acad Sci USA 97: 9984-9989.
- Young ST, Porrino LJ, Iadarola MJ (1991) Cocaine induces striatal c-fos-immunoreactive proteins via dopaminergic D1 receptors. Proc Natl Acad Sci USA 88: 1291-1295.
- Yuferov V, Zhou Y, Spangler R, Maggos CE, Ho A, Kreek MJ (1999) Acute "binge" cocaine increases mu-opioid receptor mRNA levels in areas of the rat mesolimbic mesocortical dopamine system. Brain Res Bull

24

48: 109-112.

Zhang XF, Hu XT, White FJ (1998) Whole-cell plasticity in cocaine withdrawal: reduced sodium currents in nucleus accumbens neurons. *J Neurosci* 18: 488-498.

# Introduction, Part B: Fundamentals of DNA Hybridization Arrays for Gene Expression Analysis



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## Abstract:

DNA hybridization arrays (also known as macroarrays, microarrays, and/or high-density oligonucleotide arrays (gene chips)) bring gene expression analysis to a genomic scale by permitting investigators to simultaneously examine changes in the expression of literally thousands of genes. For hybridization arrays, the general approach is to immobilize gene-specific sequences (probes) on a solid state matrix (nylon membranes, glass microscope slides, silicon/ceramic chips). These sequences are then queried with labeled copies of nucleic acids from biological samples (targets). The underlying theory is that the greater the expression of a gene, the greater the amount of labeled target, and hence, the greater output signal. In spite of the simplicity of the experimental design, there are at least four different platforms and several different approaches to processing and labeling the biological samples. Moreover, investigators must also determine whether they will utilize commercially-available arrays or generate their own. This review will cover the status of the hybridization array field with an eye toward underlying principles and available technologies. Future developments are evaluated.

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The diagram consists of three horizontal boxes stacked vertically. The top box contains the word "DNA". The middle box contains the word "mRNA". The bottom box contains the word "Protein".

**DNA**

**mRNA**

**Protein**

**Figure 1:** Genetic information flows from DNA into mRNA through transcription and then from mRNA to protein through translation. The different technologies for each level of analysis are indicated. It should be noted that there is some controversy over whether polymorphism analysis should be included in functional genomics. For the present discussion, we chose to include this under genomics as it represents structural variations in DNA sequence - albeit with the potential to represent functional changes.

technology as expensive, non-hypothesis-driven, descriptive research - the ultimate 'fishing experiment' (47; 10). Both views have valid points and this controversy can be typical of any new field of study. Although gene expression studies using multiplex hybridization arrays have been performed on a wide range of research topics including cell biology, aging, cancer, environmental toxicity, and drug abuse (20; 29; 37; 42; 54; 63; 76), performing these experiments in a manner that yields accurate results pres-

ents a unique technical challenge.

This review will elaborate the technical underpinnings of hybridization arrays and describe potential problems that must be addressed for reliable determination of gene expression changes. The basics of multiplex hybridization arrays will be presented first, followed by the descriptions of the four different types of array platforms: macroarrays, microarrays, high density oligonucleotide arrays (Gene Chip), and microelectronic arrays. Next, probe selec-



**Figure 2:** All hybridization arrays are based on the same four steps. 1) RNA is isolated from samples. 2) An array with many gene specific probes for the organism being studied is purchased or constructed. 3) Labeled targets are created from the sample RNA. 4) The targets are hybridized to the probes and the relative signals are measured. In the case of microarrays fluorescent dyes are used to label the RNA targets (one color for each condition). These are then mixed and used to query the array in a competitive manner. The detection system discriminates the relative signal outputs. High density oligonucleotide arrays and microelectronic arrays use the same fluorescent dye with a separate array for each sample. In the case of macroarrays, the RNA is converted to radioactively-labeled cDNAs and the control and experimental targets are used in separate hybridization and washing experiments.

tion and design will be discussed. Subsequently, because all arrays employ the same four basic components: target labeling, target-probe hybridization, detection, and data analysis (Figure 2), these steps will be individually discussed. Finally, central aspects of experimental design will be reviewed. This presentation is not intended to advocate a specific experimental approach or any single version of the technology. Neither will this commentary address uses of arrays for multiplex sequencing or polymorphism detection (32; 21) (important topics worthy of independent discussion in their own right; ‘genomics’ in Figure 1). Rather, this discussion focuses on the ‘good practice’ use of arrays for monitoring differential gene expression with the realization that the best choice of experimental options depends on the specific application.

## II. Array Basics

In the past, analysis of gene expression (through measurement of steady-state levels of mRNA) was conducted one gene at a time. Northern blotting, dot blots, and quantitative reverse transcription-polymerase chain reaction (QRT-PCR) were the common methods for investigating such changes in gene expression. Northern blot analysis (3) works with sample RNA that has been resolved by agarose electrophoresis and bound to a membrane. A gene-specific probe is then labeled and hybridized to the immobilized RNA, but large amounts of RNA are frequently required, only one gene is analyzed at a time, and the approach requires the production of an individual and specific probe for each gene of interest. Dot blots (35; 59) are an attempt to increase the throughput of northern blots by eliminating the need for electrophoretic resolution, but they suffer from the same problems of single-gene analysis. Quantitative RT-PCR

was subsequently developed in the hope that it would increase the throughput and reduce the sample size needed to measure gene expression. Quantitative RT-PCR certainly is more sensitive and so requires less RNA than blotting methods, but this procedure presents unique problems in the form of designing appropriate amplification standards and characterizing reaction kinetics for each gene of interest (30).

Differential display (43; 44), Serial Analysis of Gene Expression (SAGE) (56), and Total Gene Expression Analysis (TOGA) (64) offer great promise because they are multiplex technologies, but they have limited development and acceptance. The very advantage of these approaches (unbiased screening) is the source of their greatest limitation in that identified genes must then be sequenced, identified, and analyzed in a serial fashion. These procedures can be time- and labor-intensive and prone to false positives. However, these techniques are still the best for gene discovery - that is, finding unknown genes. Hybridization array technology, on the other hand, offers to bypass many of the limitations of these techniques by simultaneously creating labeled copies of multiple sample RNAs and then hybridizing them to many different, gene-specific, fixed DNA molecules (Figure 2). The nomenclature has developed whereby the labeled sample RNA is termed the target and the individual gene sequences placed on the array are termed probes (Figure 2) (50).

It is noteworthy that, while arrays are increasingly used for gene expression analysis, they only measure relative and not absolute levels of gene transcription. That is, the relative levels of RNAs can be described (Sample A has 50% more of the specific RNA than Sample B), but absolute amounts (Sample A has 1000 copies of the RNA and Sample B has 500 copies of the tran-

script) can not be determined. As well, most hybridization arrays are not designed to differentiate between alternatively spliced transcripts of the same gene and, in some cases, between highly homologous members of a gene family. Finally, a change in messenger RNA does not necessarily correlate with a change in protein (4), and the translated protein often requires further modification to realize its full activity. These latter two points are a common, and legitimate criticism of the technology. However, until proteomic technologies (49) (Figure 1) become universally accessible to the research community, hybridization arrays are the best opportunity for studying gene expression on a genomic scale.

### **III. Array Types**

The hybridization array, an ingenious inversion of the northen blot (frequently considered the ‘reverse northern’), has spawned a number of different formats. Current array formats can be categorized into four groups: macroarrays, microarrays, high-density oligonucleotide arrays (Gene Chips), and microelectronic arrays. While the nomenclature in the field is vague and variable, we use these terms to describe specific formats. In many places in the literature, all of these formats are referred to as microarrays; however, we will use the term hybridization array to describe the technology as a whole, while the term microarray will describe a specific subset of hybridization arrays. These varying platforms differ according to matrix, probe number/density, array size, and type of label. An understanding of the strengths and weakness of each platform is necessary to decide which is appropriate for an individual investigator’s research aims.

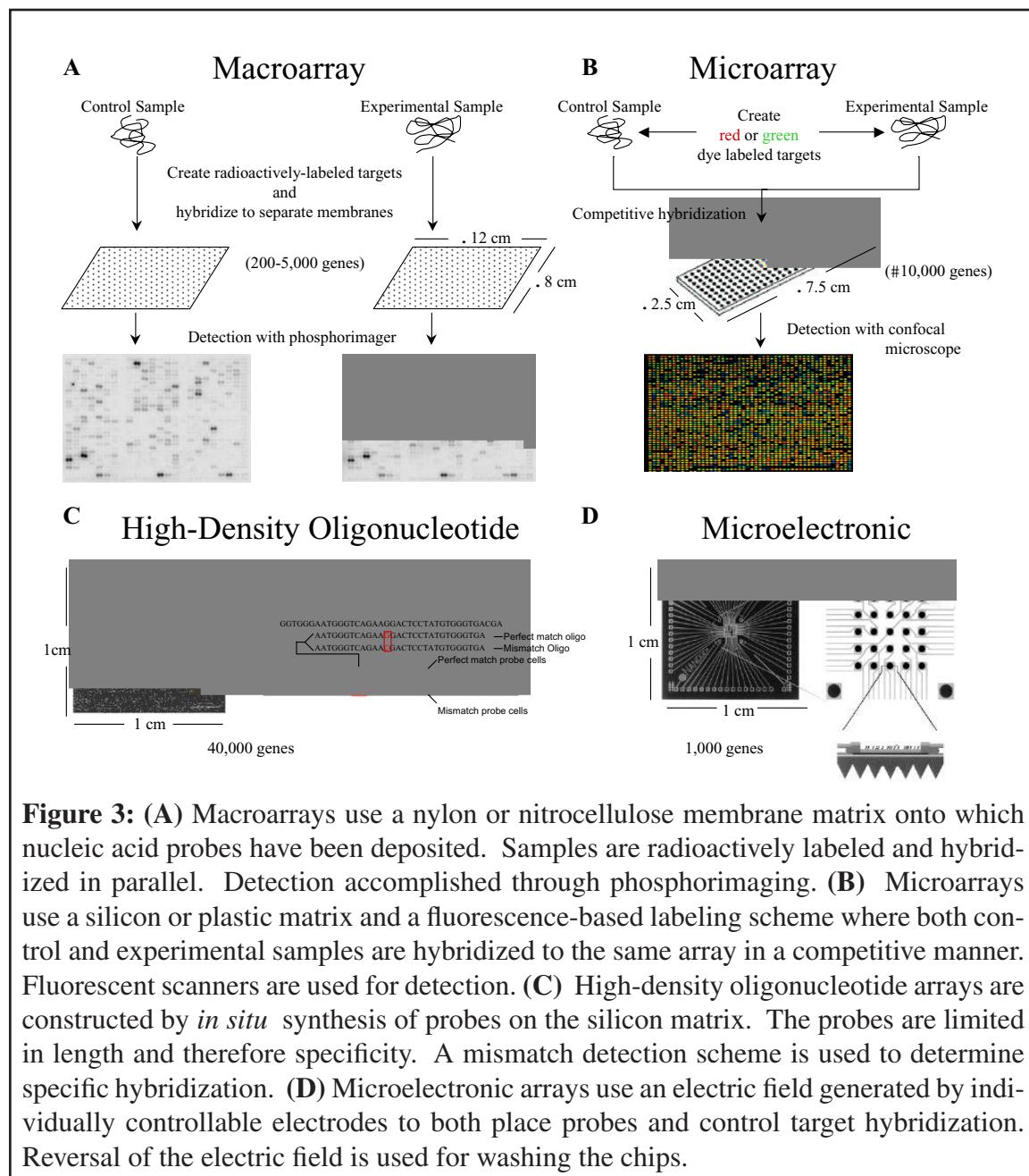
#### **A. Macroarrays**

Macroarrays (Figure 3A) are generally defined as those arrays that rely on robotically spotted probes that have been immobilized on a membrane-based matrix. The term macroarray as opposed to microarray (discussed next) refers to the generally lower probe density on these arrays. While density varies among arrays, the term macroarray is useful because of other inherent differences of membrane-based arrays. The idea of macroarrays grew from early hybridization experiments against cDNA libraries (31; 38). These experiments were combined with improvements in molecular biology and the use of robotic workstations to spot individual cDNA clones onto membranes (48; 67; 81). Currently, DNA clones, PCR amplicons or oligonucleotides are all spotted onto membranes as probes. Macroarrays are unique among hybridization arrays in that they use radioactive target labeling, although chemiluminescent labeling has also been described (52). After radioactively labeling the target, different samples are hybridized to individual separate arrays. Phosphorimagers (or less frequently X-ray film) are then used to detect the bound target. These arrays, typically containing between 200 and 5,000 genes, are commercially available for a wide variety of organisms and genes and can be obtained from a number of companies. The number of commercially-available macroarrays is rapidly expanding and these companies’ offerings are listed on the internet (82). ‘Custom’ macroarrays can also be constructed in-house by hand or by robot and can contain as few as a dozen or as many as thousands of genes. The complexity of generating these arrays is a topic unto itself (15; 16; 24).

## B. Microarrays

Microarrays (Figure 3B) can be differentiated from macroarrays in three ways. First, microarrays use a glass or plastic slide as a matrix and, second, they use fluorescent dye-labeling detection. Microarrays also tend to have a larger number and higher density of probes than macroarrays. As with macroarrays, probes are made from either

clones, PCR amplicons or oligonucleotides spotted robotically. A leader in this area has been P.O. Brown at Stanford University who introduced this form of microarrays in 1995 (20; 55; 58). This approach has advantages in that hybridization takes place in a flow cell or small hybridization chamber, which uses a much smaller hybridization solution volume as compared to macroarrays, thereby



increasing the relative target concentration. A competitive fluorescent scheme allows both sample groups (control and treated) to be hybridized to the same array and is discussed further in the section on detection. Like macroarrays, an ever-expanding number of microarrays are commercially-available and listed on the internet (82). Many research institutions are currently investing heavily in the equipment to produce custom microarrays in-house. This process has been reviewed (15, 16; 24) and allows researchers to focus on specific genes of particular interest to their research. One caveat with custom production however, is that it is an involved effort (requiring intensive bioinformatics and molecular biology) and many groups find that scale-up times are about a year.

### C. High Density Oligonucleotide/Gene Chip Arrays

High-density oligonucleotide arrays (Figure 3C) differ from other formats in that the probe is generated *in situ* on the surface of the matrix. The leader in these arrays is Affymetrix (Santa Clara, CA) and their combinatorial synthesis method (40). This method makes use of a process, called photolithography, to construct probes on the array surface by making oligonucleotides one base at a time. This synthetic scheme has been described in detail elsewhere (28; 46). Because the combinatorial synthesis scheme has a 95% efficiency at each step, synthesis of oligonucleotides longer than 25 bases is problematic. As a result of using these 25-mer oligonucleotides for gene expression analysis, mismatches and spurious target-probe binding can take place because of the limited specificity and binding affinity for a 25 residue oligonucleotide. To overcome this problem, a series of oligonucleotides that differ by a one base mis-

match from the gene-specific probe are also included on the array and can be used to determine the amount of mismatch hybridization, which can then be subtracted from the signal (41; 79). These arrays, which are available only from Affymetrix, contain between 40,000 and 60,000 probes (including multiple mismatch controls for each gene) and provide the highest density of probes of any array. The greatest hindrance to widespread use of this technology has been the high cost and limited organisms available.

### D. Microelectronic Arrays

Microelectronic chips (Figure 3D) are one of the newer formats for hybridization arrays. They are the result of a combination of advances in molecular biology and semiconductor microfabrication techniques. Instead of a membrane or a glass slide platform, these arrays consist of sets of electrodes covered by a thin layer of agarose coupled with an affinity moiety (permitting biotin-avidin immobilization of probes). Each microelectrode is 80  $\mu$ m in diameter and is capable of generating a current (22; 60). The incorporation of controllable electric fields gives a new degree of control over probe deposition and target hybridization (see hybridization section) (14; 33). This technology is still under development by Nanogen (San Diego, CA), but has great potential because of its ability to move nucleic acids (and proteins) around on the surface of the chip.

### E. Other Formats

A number of alternative formats to hybridization arrays are under development. Xanthon (Research Triangle Park, NC) uses a microtiter plate and electrochemical detection. Lynx Therapeutics (Hayward, CA) has developed a cell-sorter based scheme (9).

Interactiva (Ulm, Germany) employs a gold matrix for arrays and finally, Gene Logic (Berkeley CA) has produced fiber optic bundle, bead-based arrays. All of these technologies have interesting capabilities, but few published reports are available on their use in gene expression experiments. Their future is dependent on being easier, cheaper and more reliable than current hybridization arrays.

#### IV. Probes

Probe design and production are the key developments that have made hybridization arrays possible. Without the wealth of sequence data available from various genome projects and sequence databases, the generation of probes would not be possible. Using these genetic databases, specific probes can be constructed for each gene to be placed on the array. Probes should be both organism- and gene-specific, otherwise non-specific hybridization can compromise the experiment. Some probes can be used across species, amongst humans and higher primates for example, where there is a high degree of sequence identity (29). Probes for homologous gene families should also be constructed to hybridize to regions with the lowest sequence conservation. Physically, probes take three different forms: complex cDNA clones, PCR amplicons, and oligonucleotides. Clones, amplicons, and oligonucleotides are used with macroarrays, microarrays, and microelectronic arrays, whereas high-density oligonucleotide arrays use only short oligonucleotide probes. Clone-based probes are spotted onto arrays as whole genes or fragments of genes and are usually obtained from clone libraries. Frequently, the sequences of these clone-based probes are not specifically designed for regions of low-homology to other genes. This can result in non-specific hybridiza-

tion. Clone probes can be anywhere from a few hundred base pairs to several kilobases. Amplicon probes, when properly designed, represent non-homologous regions of a gene generated by PCR from a cDNA clone, library, or RNA and tend to be around 200 to 500 base pairs in length. Oligonucleotide probes differ from the other probes in that they can be deposited by printing or synthesized *in situ* on the array matrix by a process called photolithography (28; 45). *In situ* synthesized oligonucleotide probes generally do not exceed 25 bases in length because of reaction efficiency limits. Because 25 bases are not often enough to be gene-specific, a number of different oligonucleotide probes are used per gene on high-density oligonucleotide arrays with deliberate mismatches incorporated to dis-

for which there are probes on the hybridization array, their use also requires the production of hundreds or thousands of primers. Other labeling techniques include amplified antisense (aRNA) (71, 74), Tyramide Signal Amplification (TSA) (36), Strand Displacement Amplification (SDA) (77) and polymerase chain reaction (PCR) (62; 70). These methods have the advantage of signal amplification during labeling by creating multiple copies or labels. This helps overcome problems associated with low expression of some genes or small sample sizes. The kinetics of the amplification step, however, must be exactly reproducible in these approaches, otherwise changes observed on the array could be the result of differential amplification artifacts.

## VI. Hybridization

Hybridization and washing are critical to the generation of high-quality hybridization array gene expression data. Regardless of the format, the even distribution of both hybridization and wash solutions is important for optimization of target-probe interactions and minimization of background, respectively. Recall that the purpose of hybridization is to enable complementary target and probe sequences to specifically bind. To accomplish this, the hybridization solution must be evenly mixed to permit targets (in the hybridization solution) to be evenly distributed over the probes (on the solid matrix). Similarly, wash solutions must be evenly distributed over the array to remove non-hybridized probes, reduce non-specific hybridization, and minimize background. In that there are several physical types of arrays, there are also different ways in which they can be hybridized and washed. In this section, three different hybridization techniques will be reviewed: 1) traditional nylon membrane methods, 2)

glass microscope slides in flow cells, and 3) electric field-enhanced methods. Generally, macroarrays use traditional membrane methods, microarrays and high-density oligonucleotide arrays use flow cells, and microelectronic chips use a directed electric field in solution.

### A. Traditional Methods

Since Edwin Southern's introduction of nucleic acid blotting (61), most hybridization schemes have relied on hybridization taking place in sealed bags or solution-filled bottles. Hybridization bottles are designed to roll or agitate in a temperature-controlled environment. These bottles are also used for the sequentially more stringent washes (lower salt concentration and higher temperatures) that serve to minimize background. Conveniently, most nylon membrane-based arrays are hybridized in this way. Therefore, molecular biology researchers familiar with membrane hybridization techniques can immediately use existing hybridization equipment for macroarray experiments.

A technical challenge exists, however, in that arrays are hybridized and washed in different bottles, and yet are compared to each other. Differences in hybridization or washing between the two membranes can therefore result in inconsistent and poor quality data. Therefore, when using this technique, great care must be taken to ensure identical conditions during hybridization and washing of both macroarrays in an experiment.

### B. Flow Cells

Microarrays and high-density oligonucleotide arrays use a glass slide or silicon chip matrix that is anchored to the bottom of a sealed chamber through which solutions can be directed. Temperature is controlled by a metal plate beneath the slide. Alter-

natively, small hybridization cassettes, into which the solutions can be added, can be used in water baths. In either format, temperature and volume control are optimized, and consistent hybridization and washing steps are attainable. Though most laboratories are not equipped with this hardware, many researchers believe that the increased throughput and hybridization efficiency make the technology worth the investment.

### C. Electric Field

The previous two hybridization techniques rely on the random, ‘passive’, interaction between matched targets and probes. The passive approach has inherent difficulties. Different target-probe pairs have different melting temperatures and hybridization kinetics. The temperature and salt concentrations of hybridization solutions represent the best conditions for the probe population mean and therefore will not be optimal for some pairs. In addition, the kinetics of hybridization are dependent on the target concentration in solution and low concentration targets will require a longer time to hybridize if they hybridize at all. Using an electric field to control hybridization can potentially eliminate many of the problems of passive hybridization.

During electric field-driven hybridization (or ‘active’ hybridization), a current is generated at a single test site drawing all targets in the hybridization solution toward the site due to DNA’s inherent negative charge. Active attraction increases the effective concentration of target at a probe site and thus speeds the process to such an extent that only minutes are required for hybridization, compared to hours for passive hybridization. Next, by reversing the current, the site can be ‘washed’ of any mismatches and spurious binding because any incom-

pletely hybridized targets will not possess the hydrogen bonding strength required to overcome the repulsive forces of the current. A further advantage of this technique is that multiple samples can be assayed by the sequential hybridization of individual samples to different sets of probes on the same array. This technology is currently under development (Nanogen, San Diego, CA) and if it can be made inexpensive enough could represent the future of hybridization arrays (14; 33).

## VII. Detection

Whether elucidating developmental gene expression changes or characterizing a tumor cell line, array experiments all share a common thread in their design - creating a clear picture. Thus, sensitive and accurate signal output detection is imperative. In this section, two methods of detection will be discussed: 1) radioisotopic and 2) fluorescence.

### A. Radioisotopic Detection

Detection of nucleic acids on macroarrays is most commonly accomplished through the use of a radioactive isotope in conjunction with an imaging plate/phosphorimaging system. After hybridization and washing, the array is apposed to an image plate (IP) and the signal intensity detected when the plate emulsion is excited by the high-energy radioactive particles (recall that the labeling step yielded a radiolabeled cDNA) (53). Following an appropriate exposure time, the IP is developed by scanning with a laser of an appropriate wavelength thus releasing the energy of the excited electrons. This release is detected by the phosphorimager yielding a digital ‘image’ of the radioactivity (both location and intensity). After image capture, analysis is performed using computer

software that ascribes signal intensities to all of the pixel within each spot. In this way, the intensity of a gene-specific spot on one array, the control array, can be compared to the same spot on the other, experimental array. Classically, x-ray film has been used to image radioactivity; however, phosphorimaging's superior dynamic range, shorter exposure times, and direct digital output have made it the method of choice.

### B. Fluorescence Detection

Fluorescent detection is used with microarrays, high-density oligonucleotide arrays and microelectronic chips. The main reason for using fluorescence is that gene expression experiments can use a competitive hybridization scheme, in which both experimental and control samples are hybridized to the same array. It is possible to differentiate the signals from the control and experimental samples because of a fluorescent phenomenon known as the Stokes shift. The Stokes shift of a fluorescent tag is the difference between its excitation and emission wavelengths. Fluorescent tags are compounds that absorb light of a certain wavelength, exciting electrons within the compound to a higher energy state. From this higher energy state, photons are emitted at a specific wavelength for each compound. Therefore, if two samples (control and experimental), are labeled with different fluorescent tags, having different emission wavelengths, their respective signals can be detected separately within the same experiment. Because the emission of energy is generally linear, the amount of signal detected is relative to the amount of fluorescently-labeled cDNA hybridized to the probe. For example, one sample is labeled with a red dye and the other is labeled with a green dye. When the samples are mixed and used to query an array, if there is more

mRNA for a given gene in one sample, there will be more dye of that particular color, and following hybridization, a predominance of that dye signal over the other. The advantage of a competitive hybridization scheme such as this is that comparison of inter-array hybridization, as seen with the macroarray format, is eliminated.

Fluorescence is generally detected using a confocal scanning microscope which uses lasers for generating light of the correct excitation wavelength, and photomultiplier tubes for detection (57). The confocal nature of these devices is such that having a pair of focal points restricts the detection of light to one direction and reduces light from spurious sources above, below, or to the side of the part of the array being scanned. A drawback of confocal scanners, however, is that each excitation wavelength must have its own expensive laser and they therefore tend to be costly. Charge coupled device (CCD) detectors have been used in an attempt to provide more flexibility, since they can use a single light source with different filters for different excitation wavelengths. The CCD detectors are hampered by hardware issues however, and time will tell if they can become a competitor of the confocal microscope.

### VIII. Data Analysis

The creation, hybridization, and detection of hybridization arrays may seem to be a daunting task. It would appear that once an image of the array, with relative intensities for each sample, has been generated, the experiment would nearly be finished. Unfortunately, this is not the case as scientists are now learning that the massive amounts of data generated by arrays pose a new challenge (6; 11; 26; 66; 73). In this section, basics in array normalization and data management will be presented, along

with a look at the mathematical models being developed to help create a relevant biological story from expression data.

### A. Basics

The first steps in data analysis are background subtraction and normalization. The principals of both are similar to the techniques used with conventional nucleic acid or protein blotting. Background subtraction pulls the non-specific background noise out of the signal detected for each spot and allows comparison of specific signals. For illustration, if the signal intensities for the control and experimental spots are 4 and 6, respectively, it would appear that the experimental is 50% higher. However, if a background of 2 is subtracted from both signal intensities, the experimental value is actually 100% higher than control. Background is often taken from the blank areas on the array. A complication to background subtraction is that variations in background across the array can affect some spots more than others. An alternative is to use either a local background for the area around each spot or spots with the lowest signal intensities for background determination. The latter, may be a more accurate determination of non-specific background because it represents the non-specific binding of targets to probe. Background intensities from blank areas (no nucleic acids) do not contain probe, and are arguably a different form of background.

Normalization is the process by which differences between separate arrays are accounted for. All macroarray and high-density oligonucleotide experiments and any other multiple array experiments may require the use of normalization for consistent comparisons. For example, when a pair of macroarrays representing control and treated samples show a difference in overall

or total signal intensity, such differences can arise from unequal starting amounts of RNA or cDNAs, from labeling reactions of different efficiencies, or from subtle differences in hybridization efficiencies. Any of these factors can skew the results. One common method of normalization is to use a housekeeping gene(s), a gene thought to be invariant under experimental conditions, for comparison (75). If the signal for this gene is higher on one array than the other it can be used to normalize the data. Housekeeping genes are problematic for many experiments because housekeeping genes do in fact vary under some experimental conditions (65). To overcome the variability of these genes, some researchers have turned to a ‘basket’ or ‘sum’ approach for normalization. This strategy is based on the precept that the overall or global levels of gene expression is unaffected by the experimental conditions, and changes are equally distributed between inductions and reductions. Therefore, arrays can be normalized by taking the sum of the intensities for all control and experimental spots, and equilibrating them. Even this approach is invalid for some experiments, however, and exogenous synthetic RNA standards have been used to normalize (23).

### B. Clustering analysis

Because the sheer quantity of data generated by arrays exceeds the ability of manual human assessment, new modes of analysis are needed to effectively and exhaustively explore hybridization array results. To this end, there have been advances in computational biology and bioinformatics that have helped to create a biological story out of the databases generated by hybridization array data (6; 17; 80). As this topic warrants an in-depth review of its own, the current discussion will provide

only a basic overview of histograms and clustering algorithms.

For binary experiments, where there is a control and a single experimental condition, a common first step in data analysis is to create a histogram. Histograms serve to rank genes in order of magnitude of change from greatest induction to greatest reduction (29; 51; 63). Moreover, the histogram provides a visual sense of the distribution (between induction and reduction) and any skew to the results. Such a comparison highlights those genes that show the greatest fold (+ or -) changes. These genes can subsequently be made into a simple list and examined further at the level of protein or activity.

For more complex experimental designs, where there are two or more experimental conditions (typically, an experiment looking at multiple timepoints, doses, or groups), the computational requirements are much greater. The question then becomes not one of a simple change under one condition, but how does one gene (out of thousands) change over multiple conditions. The first step in understanding such complex relationships is to plot the expression of each gene over the various conditions. With large experiments analyzing thousands of genes, these data increase dramatically and as a result it can be difficult to find patterns in the data. To this end, clustering algorithms have been employed. Stated simply, these approaches seek to find groups of genes, or clusters, that behave similarly across the experimental conditions. Clusters, and the genes within them, can subsequently be examined for commonalities in function or sequence to better understand how and why they behave similarly (18). A number of different methods: k-means, self-organizing maps, hierarchical clustering, vector machines, and Bayesian statis-

tics are employed for clustering analysis (2; 7; 12; 25; 34; 68; 69; 76; 78). In contrast to an expression histogram that focuses only on the genes whose expression changes, an advantage of clustering analysis is that it uses all of the data generated. In a multi-endpoint experiment, clustering analysis can also identify groups of genes that do not change their expression pattern under some, or all, of the conditions. The fact that a group of genes is unperturbed or unresponsive in a specific experimental condition can lead to valuable biological insights. Clustering analyses will be critical for the mining of public expression databases that are being generated (8; 17; 83).

Clustering techniques are also being used to combine gene expression data generated from hybridization arrays with other experimental data. This is the likely direction of functional genomic research in the future. The goal is being able to make correlations between seemingly disparate measures, such as biochemical, electrophysiological, or behavioral observations for example. This type of clustering which combines gene expression with other data has begun to be demonstrated in the cancer field (54; 56).

## **IX. Experimental Design**

All successful science is based on sound experimental design. From a practical standpoint, this is especially true of hybridization array experiments because the time and resources that can be wasted on poorly designed functional genomic research is staggering. For both the beginning researcher and those already conducting experiments using hybridization arrays, it is worth examining the concerns of sample collection, fold changes, sensitivity, post-hoc confirmation and application-specific questions.

### A. Sample collection

Sample collection is a basic element of experimental design for many molecular biological experiments, but it is worth reiterating. Depending on the cells or tissue being examined, it is often unavoidable that a sample will contain multiple cell types. In complex samples, such as brain tissue, there is routinely a heterogeneous cell population. Therefore, observed changes may represent a change in one cell type or all cell types. Similarly, smaller changes occurring in only one type of cell may be hidden. Thus, researchers must be mindful of this heterogeneous population when drawing conclusions. Similarly, in comparing normal and cancer samples, there will be obvious differences in the proportion of the cell types (i.e., cancer cells will be over-represented). Therefore, interpretations of differences in gene expression may be complicated by the sheer mass of one cell over another. A promising technological solution is laser capture microdissection which allows very small and identified cellular populations to be dissected (42).

The timing of tissue collection goes hand-in-hand with the nature of the collected tissue and therefore sample collection times will be important. For example, in an experiment in which cells undergo programmed cell death, the collection time point will determine if causative changes or end-point changes are observed. If a late timepoint is chosen, it becomes increasingly difficult to distinguish changes due to the general breakdown of cellular processes from those which have triggered the cell death.

### B. Detection Sensitivity

Detection sensitivity in array research takes two very distinct forms. The first, termed threshold sensitivity, is the ability to detect one RNA species out of a population and is a concern for rarely expressed messages, for small sample sizes, and is the traditional issue of sensitivity common to other techniques. The second sensitivity parameter is ‘fold-change sensitivity’, or the ability of hybridization arrays to reliably determine a certain magnitude difference in expression. The claimed fold-change sensitivity of different platforms varies from 2- to 0.75- fold for fluorescence-based protocols and 1.5- to 0.66- fold for radioactivity-based methods. Determination of this parameter is crucial to characterizing the technology and ensuring that researchers choose the technology most appropriate to their goals (5). For research involving systems that undergo large gene expression changes (e.g. yeast cell-cycle regulation, or organ developmental processes where 10-fold changes are expected), one can detect such changes with fluorescent protocols. Other research efforts, for example in neuroscience, where gene changes are less dramatic, may find radioactivity-based methods more applicable.

### C. Post-hoc Confirmation

One of the most common criticisms of hybridization arrays is that when hundreds or thousands of genes are examined at once, some will appear changed by random chance. This is a statistical reality and highlights the requirement for post hoc confirmation of changes seen with arrays. This is because a single array experiment, representing an *n* of one, lacks the sample size needed for statistical analysis. Therefore, tests on individual samples themselves are necessary to produce statistical significance. Such corroborating experiments can examine the gene changes at the level of mRNA (northern blot, QRT-PCR), protein (immunoblot), or activity (enzymatic activity, DNA

binding, or other measures). The protein and activity tests are recommended because they assess the gene of interest at a level closer to function or actually address the function itself. Protein analysis is important because increased levels of transcription do not always translate into increased levels of protein (4). In addition, protein assessment is achieved with fundamentally different experimental techniques and may not be subject to the same sources of error as the array. Unfortunately, immunoblotting and activity assays would appear to return researchers to the single gene assay that hybridization arrays were intended to avoid. This is not true in practice because large amounts of genes have already been screened by the array. The optimal solution to ascribing relevance to the data is to develop techniques by which confidence intervals for individual genes can be generated from arrays and these results can be combined with proteomic techniques under development (49). Alternatively, as costs are decreased, experiments will ultimately permit hybridization arrays for each sample. As well, many researchers are exploring the use of small (in the number of genes) arrays that focus on a specific gene family or pathway. Regardless of the confirmation strategy used, the ultimate goal of hybridization array-based experiments is not technical showmanship, but the creation of biological narratives to illuminate the issue at hand.

## X. Conclusion

Potential applications for hybridization arrays exist anywhere the levels of gene expression are of interest. As delineated in the discussion paragraphs, the technology will have to be optimized and tested for each area of application. Foremost is the need for probes specific to many different animal and plant models. This will be made

possible by the growing number of genome projects for model organisms (e.g., *Arabidopsis thaliana* (39) and *Drosophila melanogaster* (1)). Similarly, targeted (custom) arrays will need to be generated for areas such as cancer biology, neuroscience, and developmental biology.

As detailed in this review, DNA hybridization arrays will revolutionize gene expression research. Like any new method, researchers will have to be simultaneously aware of its power and its limitations. The scientific community and marketplace have embraced this technology as witnessed by the research funding opportunities to academics and capitalization of array-based companies. Hybridization arrays will therefore clearly be a major tool of the post-genome era.

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## References

1. Adams M.D., S.E. Celniker, R.A. Holt, C.A. Evans, J.D. Gocayne, P.G. Amanatides, S.E. Scherer, P.W. Li, R.A. Hoskins, R.F. Galle, R.A. George, S.E. Lewis, S. Richards, M. Ashburner, S.N. Henderson, G.G. Sutton, J.R. Wortman, Q. Zhang, L.X. Chen, R.C. Brandon, Y.H.C. Rogers, R.G. Blazej, M. Champe and B.D. Pfeiffer. 2000. The genome sequence of *Drosophila melanogaster*. *Science* 287:2185-2195.
2. Alon U., N. Barkai, D.A. Notterman, K. Gish, S. Ybarra, D. Mack and A.J. Levine. 1999. Broad patterns of gene expression revealed by clustering analysis of tumor and normal colon tissues probed by oligonucleotide arrays. *Proc. Natl. Acad. Sci. USA* 96:3745-3750.
3. Alwine J.C., D.J. Kemp and G.R. Stark. 1977. Method for detection of specific RNAs in agarose gels by transfer to diazobenzyloxymethane to obMEiet

- Cahill, K. Bussow and H. Lehrach. 1999. Construction and analysis of arrayed cDNA libraries. *Methods in Enzymology.* 303:205-33.
17. Claverie J.M. 2000. Do we need a huge new centre to annotate the human genome? *Nature* 403:12.
18. Claverie J.M. 1999. Computational methods for the identification of differential and coordinated gene expression. *Human Mol. Gen.* 8:1821-1832.
19. DeRisi J.L., V.R. Iyer and P.O. Brown. 1997. Exploring the metabolic and genetic control of gene expression on a genomic scale. *Science* 278:680-686.
20. DeRisi J., L. Penland, P.O. Brown, M.L. Bittner, P.S. Meltzer, M. Ray, Chen, Y, Y.A. Su and J.M. Trent. 1996. Use of a cDNA microarray to analyse gene expression patterns in human cancer. *Nat. Genetics* 14:457-460.
21. Drmanac R. and S. Drmanac. 1999. cDNA screening by array hybridization. *Methods in Enzymology.* 303:165-78.
22. Edman C.F., D.E. Raymond, D.J. Wu, E. Tu, R.G. Sosnowski, W.F. Butler, M. Nerenberg and M.J. Heller. 1997. Electric field directed nucleic acid hybridization on microchips. *Nucleic Acids Res.* 25:4907-4914.
23. Eickhoff B., B. Korn, M. Schick, A. Poustka and J. van der Bosch. 1999. Normalization of array hybridization experiments in differential gene expression analysis. *Nucleic Acids Res.* 27:e33.
24. Eisen M.B. and P.O. Brown. 1999. DNA arrays for analysis of gene expression. *Methods in Enzymology.* 303:179-205.
25. Eisen M.B., P.T. Spellman, P.O. Brown and D. Botstein. 1998. Cluster analysis and display of genome-wide expression patterns. *Proc. Natl. Acad. Sci. USA* 95:14863-14868.
26. Ermolaeva O., M. Rastogi, K.D. Pruitt, G.D. Schuler, M.L. Bittner, Y. Chen, R. Simon, P. Meltzer, J.M. Trent and M.S. Boguski. 1998. Data management and analysis for gene expression arrays. *Nat. Genetics* 20:19-23.
27. Fields S., Y. Kohara and D.J. Lockhart. 1999. Functional genomics. *Proc. Natl. Acad. Sci. USA* 96:8825-8826.
28. Fodor S.P., J.L. Read, M.C. Pirrung, L. Stryer, A.T. Lu and D. Solas. 1991. Light-directed, spatially addressable parallel chemical synthesis. *Science* 251:767-773.
29. Freeman W.M., D.J. Robertson, S.H. Nader, M.A. Nader, L. Gioia, J.B. Daunais, L.J. Porrino, D.P. Friedman, G.M. Hellmann and K.E. Vrana. 2000. Chronic Cocaine-Mediated Changes in Non-Human Primate Nucleus Accumbens Gene Expression. (In Press).
30. Freeman W.M., S.J. Walker and K.E. Vrana. 1999. Quantitative RT-PCR: pitfalls and potential. *Biotechniques* 26:112-122.
31. Gress T.M., J.D. Hoheisel, G.G. Lennon, G. Zehetner and H. Lehrach. 1992. Hybridization fingerprinting of high-density cDNA-library arrays with cDNA pools derived from whole tissues. *Mammalian Genome* 3:609-619.

32. Hacia J.G. 1999. Resequencing and mutational analysis using oligonucleotide microarrays. *Nat. Genetics* 21:42-47.
33. Heller M.J., A.H. Forster and E. Tu. 2000. Active microelectronic chip devices which utilize controlled electrophoretic fields for multiplex DNA hybridization and other genomic applications. *Electrophoresis* 21:157-164.
34. Hilsenbeck S.G., W.E. Friedrichs, R. Schiff, P. O'Connell, R.K. Hansen, C.K. Osborne and S.A.W. Fuqua. 1999. Statistical analysis of array expression data as applied to the problem of tamoxifen resistance. *J. Natl. Cancer Inst.* 91:453-459.
35. Kafatos F.C., C.W. Jones and A. Efstratiadis. 1979. Determination of nucleic acid sequence homologies and relative concentrations by a dot hybridization procedure. *Nucleic Acids Res.* 7:1541-1552.
36. Kerstens H.M., P.J. Poddighe and A.G. Hanselaar. 1995. A novel *in situ* hybridization signal amplification method based on the deposition of biotinylated tyramine. *J. Histochem. & Cytochem.* 43:347-352.
37. Lee C.K., R.G. Klopp, R. Weindruch and T.A. Prolla. 1999. Gene expression profile of aging and its retardation by caloric restriction. *Science* 285:1390-1393.
38. Lennon G.G. and H. Lehrach. 1991. Hybridization analyses of arrayed cDNA libraries. *Trends Genetics* 7:314-317.
39. Lin X.Y., S.S. Kaul, S. Rounsley, T.P. Shea, M.I. Benito, C.D. Town, C.Y. Fujii, T. Mason, C.L. Bowman, M. Barnstead, T.V. Feldblyum, C.R. Buell, K.A. Ketchum, J. Lee, C.M. Ronning, H.L. Koo, K.S. Moffat, L.A. Cronin, M. Shen, G. Pai, S. Aken, L. Umayam, L.J. Tallon, J.E. Gill, M.D. Adams and J.C. Venter. 1999. Sequence and analysis of chromosome 2 of the plant *Arabidopsis thaliana*. *Nature* 402:761-768.
40. Lipshutz R.J., S.P. Fodor, T.R. Gingers and D.J. Lockhart. 1999. High density synthetic oligonucleotide arrays. *Nat. Genetics* 21:20-24.
41. Lockhart D.J., H. Dong, M. Bryne, M. Follettie, M. Gallo, M. Chee, M. Mittmann, C. Wang, M. Kobayashi, H. Horton and E. Brown. 1996. Expression monitoring by hybridization to high-density oligonucleotide arrays. *Nat. Biotech.* 14:1675-1680.
42. Luo L., R.C. Salunga, H.Q. Guo, A. Bittner, K.C. Joy, J.E. Galindo, H.N. Xiao, K.E. Rogers, J.S. Wan, M.R. Jackson and M.G. Erlander. 1999. Gene expression profiles of laser-captured adjacent neuronal subtypes. *Nat. Med.* 5:117-122.
43. Matz M.V. and S.A. Lukyanov. 1998. Different strategies of differential display: areas of application. *Nucleic Acids Res.* 26:5537-5543.
44. Martin K.J. and A.B. Pardee. 1999. Principles of differential display. *Methods in Enzymology*. 303:234-58.
45. McGall G., A. Barone, M. Diggemann, S.P. Fodor, E. Gentalen and N. Ngo. 1997. The Efficiency of Light-Directed Synthesis of DNA Arrays on Glass Substrates. *J. Amer. Chem. Soc.* 119:5081-5090.
46. McGall G., J. Labadie, P. Brock, G. Wallraff, T. Nguyen and W. Hinsberg. 1996. Light-directed synthesis of high-den-

- sity oligonucleotide arrays using semiconductor photoresists. *Proc. Natl. Acad. Sci. USA* 93:13555-13560.
47. Mir K.U. 2000. The hypothesis is there is no hypothesis. *Trends in Genetics* 16:63-64.
48. Nguyen C., D. Rocha, S. Granjeaud, M. Baldit, K. Bernard, P. Naquet and B.R. Jordan. 1995. Differential gene expression in the murine thymus assayed by quantitative hybridization of arrayed cDNA clones. *Genomics* 29:207-216.
49. Pandey A. and M. Mann. 2000. Proteomics to study genes and genomes. *Nature* 405:837-846.
50. Phimister B. 2000. Going global. *Nat. Genetics* 21:1.
51. Pietu G., O. Alibert, V. Guichard, B. Lamy, F. Bois, E. Leroy, R. Mariage-Sampson, R. Houlgate, P. Soularue and C. Auffray. 1996. Novel gene transcripts preferentially expressed in human muscles revealed by quantitative hybridization of a high density cDNA array. *Genome Res.* 6:492-503.
52. Rajeevan M.S., I.M. Dimulescu, E.R. Unger and S.D. Vernon. 1999. Chemiluminescent analysis of gene expression on high-density filter arrays. *J. Histochem. & Cytochem.* 47:337-342.
53. Robertson D.J., W.M. Freeman and K.E. Vrana. 2000. Phosphorimaging. In: *Encyclopedia of Life Sciences*. (Submitted)
54. Ross D.T., U. Scherf, M.B. Eisen, C.M. Perou, C. Rees, P. Spellman, V. Iyer, S.S. Jeffrey, M. Van de Rijn, M. Waltham, A. Pergamenschikov, J.C.E. Lee, D. Lash-  
kari, D. Shalon, T.G. Myers, J.N. Weinstein, D. Botstein and P.O. Brown. 2000. Systematic variation in gene expression patterns in human cancer cell lines. *Nat. Genetics* 24:227-235.
55. Schena M., D. Shalon, R.W. Davis and P.O. Brown. 1995. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* 270:467-470.
56. Scherf U., D.T. Ross, M. Waltham, L.H. Smith, J.K Lee, L. Tanabe, K.W. Kohn, W.C. Reinhold, T.G. Myers, D.T. Andrews, D.A. Scudiero, M.B. Eisen, E.A. Sausville, Y. Pommier, D. Botstein, P.O. Brown, J.N. Weinstein. 2000. A gene expression database for the molecular pharmacology of cancer. *Nature Genetics* 24:236-244.
57. Schermer M.J. 1999. Confocal scanning microscopy in array detection. In: Schena M., ed. *DNA Microarrays: a Practical Approach*. Oxford University Press.
58. Shalon D., S.J. Smith and P.O. Brown. 1996. A DNA microarray system for analyzing complex DNA samples using two-color fluorescent probe hybridization. *Genome Res.* 6:639-645.
59. Sim G.K., F.C. Kafatos, C.W. Jones, M.D. Koehler, A. Efstratiadis and T. Maniatis. 1979. Use of a cDNA library for studies on evolution and developmental expression of the chorion multigene families. *Cell* 18:1303-1316.
60. Sosnowski R.G., E. Tu, W.F. Butler, J.P. O'Connell and M.J. Heller. 1997. Rapid determination of single base mismatch mutations in DNA hybrids by direct electric field control. *Proc. Natl. Acad. Sci. USA*

- 94:1119-1123.
61. Southern E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98:503-517.
62. Spirin K.S., A.V. Ljubimov, R. Castellon, O. Wiedoeft, M. Marano, Sheppard, M.C. Kenney and D.J. Brown. 1999. Analysis of gene expression in human bullous keratopathy corneas containing limiting amounts of RNA. *Invest. Ophthal. & Vis. Sci.* 40:3108-3115.
63. Stokes A.H., W.M. Freeman, S.G. Mitchell, T.A. Burnette, G.M. Hellmann and K.E. Vrana. 2000. Induction of GADD45 and GADD153 in neuroblastoma cells by dopamine-induced toxicity. (Submitted)
64. Sutcliffe J.G., P.E. Foye, M.G. Erlander, B.S. Hilbush, L.J. Bodzin, J.T. Durham, and K.W. Hasel. 2000. TOGA: An automated parsing technology for analyzing expression of nearly all genes. *Proc Natl. Acad. Sci., USA* 97:1976-1981.
65. Suzuki T., P.J. Higgins and D.R. Crawford. 2000. Control Selection for RNA Quantification. *BioTechniques.* 29:332-337.
66. Szallasi Z. 1999. Genetic network analysis in light of massively parallel biological data acquisition. *Pacific Symp. Bio-comp.* 5-16.
67. Takahashi N., H. Hashida, N. Zhao, Y. Misumi and Y. Sakaki. 1995. High-density cDNA filter analysis of the expression profiles of the genes preferentially expressed in human brain. *Gene* 164:219-227.
68. Tamayo P., D. Slonim, J. Mesirov, Q. Zhu, S. Kitareewan, E. Dmitrovsky, E.S. Lander and T.R. Golub. 1999. Interpreting patterns of gene expression with self-organizing maps: Methods and application to hematopoietic differentiation. *Proc. Natl. Acad. Sci. USA* 96:2907-2912.
69. Toronen P., M. Kolehmainen, C. Wong and E. Castren. 1999. Analysis of gene expression data using self-organizing maps. *FEBS Letters* 451:142-146.
70. Trenkle T., J. Welsh and M. McClelland. 1999. Differential display probes for cDNA arrays. *Biotechniques* 27:554-560.
71. Van Gelder R.N., M.E. von Zastrow, A. Yool, W.C. Dement, J.D. Barchas and J.H. Eberwine. 1990. Amplified RNA synthesized from limited quantities of heterogeneous cDNA. *Proc. Natl. Acad. Sci. USA* 87:1663-1667.
72. Velculescu V.E., L. Zhang, B. Vogelstein and K.W. Kinzler. 1995. Serial analysis of gene expression. *Science* 270:484-487.
73. VingronM. and J. Hoheisel. 1999. Computational aspects of expression data. *J. Mol. Med.* 77:3-7.
74. Wang E., L.D. Miller, G.A. Ohnmacht, E.T. Liu and F.M. Marincola. 2000. High-fidelity mRNA amplification for gene profiling. *Nature Biotech.* 18:457-459.
75. Warrington J.A., A. Nair, M. Mahadevappa and M. Tsyganskaya. 2000. Comparison of human adult and fetal expression and identification of 535 housekeeping maintenance genes. *Physio. Genomics.* 2:143-147.

76. Weinstein J.N., T.G. Myers, P.M. O'Connor, S.H. Friend, A.J. Fornace, K.W. Kohn, T. Fojo, S.E. Bates, L.V. Rubinstein, N.L. Anderson, J.K. Buolamwini, van, W.W. Osdol, A.P. Monks, D.A. Scudiero, E.A. Sausville, D.W. Zaharevitz, Bunow, B, V.N. Viswanadhan, G.S. Johnson, R.E. Witten and K.D. Paull. 1997. An information-intensive approach to the molecular pharmacology of cancer. *Science* 275:343-349.
77. Westin L., X. Xu, C. Miller, L. Wang, C.F. Edman and M. Nerenberg. 2000. Anchored multiplex amplification on a microelectronic chip array. *Nature Biotech.* 18:199-204.
78. Witten J. and H.P. Friedman. 1999. Searching for evidence of altered gene expression: a comment on statistical analysis of microarray data. *J. Natl. Cancer Inst.* 91:400-401.
79. Wodicka L., H. Dong, M. Mittmann, M. Ho and D.J. Lockhart. 1997. Genome-wide expression monitoring in *Saccharomyces cerevisiae*. *Nat. Biotech.* 15:1359-1366.
80. Zhang M.Q. 1999. Large-scale gene expression data analysis: a new challenge to computational biologists. *Genome Res.* 9:681-688.
81. Zhao N., H. Hashida, N. Takahashi, Y. Misumi and Y. Sakaki. 1995. High-density cDNA filter analysis: a novel approach for large- scale, quantitative analysis of gene expression. *Gene* 156:207-213.
82. [www.gene-chips.com/](http://www.gene-chips.com/),  
[www.wfubmc.edu/physpharm/genetech/](http://www.wfubmc.edu/physpharm/genetech/),  
[www.people.cornell.edu/pages/alm13/chips.html](http://www.people.cornell.edu/pages/alm13/chips.html)
83. [cmgm.stanford.edu/pbrown/](http://cmgm.stanford.edu/pbrown/),  
[www.nhgri.nih.gov/DIR/LCG/15K/HTML/index.html](http://www.nhgri.nih.gov/DIR/LCG/15K/HTML/index.html), [www.arraydata.org](http://www.arraydata.org)

## STATEMENT OF PURPOSE

The overall goal of this dissertation research is to identify changes in neuronal gene expression after chronic cocaine administration. Specifically, the new technology of hybridization arrays was used to screen large numbers of genes for cocaine-responsiveness using *in vivo* models. Apparent changes in gene expression illuminated by hybridization analysis were then confirmed by immunoblotting to both provide statistical confidence and confirmation that the apparent change in mRNA was recapitulated at the level of protein. These changes in gene expression could represent key mediators of behavioral and physiological phenomenon seen with drug abuse. A greater knowledge of these gene expression changes creates greater insight into the neurobiology of cocaine abuse and opens avenues for development of pharmacotherapeutics.

The first aspect of this study was to apply, for the first time, hybridization array analysis to a non-human primate model of cocaine abuse. Using a primate model of chronic cocaine administration would provide changes in gene expression that may represent the human condition. Hybridization array and protein confirmation of changes seen with the array analysis, identified novel changes in gene expression that integrate with existing knowledge of cocaine-responsive gene expression and are presented in Chapter 2.

The second intent of this dissertation research was to use a rat model of chronic non-contingent cocaine administration to identify gene expression changes in the hippocampus, frontal cortex and nucleus accumbens and are presented in Chapters 3-5, respectively. These regions represent important parts of reinforcement-related dopaminergic pathways. Gene expression changes within this pathway may subserve

changes in behavior seen with chronic cocaine administration. Hybridization array analysis and protein confirmation illuminated a number of novel cocaine-responsive changes in gene expression. Common changes were also found among these brain regions and changes seen in the non-human primate model.

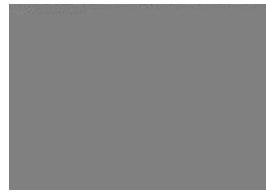
A third undertaking of this study was to begin applying functional genomics and protein analysis to rats that self-administer cocaine in a discrete trial/binge-abstinence paradigm. Due to known differences in neurochemistry and behavior between non-contingent cocaine administration and cocaine self-administration, a self-administration paradigm is necessary to analyze direct behaviorally-relevant changes in gene expression. Hybridization array analysis of the nucleus accumbens of these animals showed both similarities and differences as compared to this brain region in the other animal model and administration paradigm used.

A fourth goal of this study was to examine tyrosine hydroxylase, the rate limiting enzyme in dopamine biosynthesis, in the mesolimbic system of rats treated with a long-acting cocaine analog. Cocaine is known to alter tyrosine hydroxylase expression and activity within this system and a long-lasting and potent cocaine analog was hypothesized to act on tyrosine hydroxylase in a manner similar to cocaine. In fact, such a cocaine analog produced the inverse effect of cocaine on tyrosine hydroxylase activity and expression. This study is described in Chapter 6.

On balance, these studies were undertaken with the intent of identifying novel aspects of the functional neurogenomics of cocaine. It is hoped that the results will contribute to the compendium of knowledge

concerning the epigenetic imprinting by this drug of abuse. Moreover, such knowledge of specific molecular targets may ultimately permit development of pharmacotherapeutics for cocaine abuse.

# Chapter 2: Chronic Cocaine-Mediated Changes in Non-Human Primate Nucleus Accumbens Gene Expression



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## Abstract:

Chronic cocaine use elicits changes in the pattern of gene expression within reinforcement-related, dopaminergic regions. cDNA hybridization arrays were used to illuminate cocaine-regulated genes in the nucleus accumbens (NAcc) of non-human primates (*macaca fascicularis*; *cynomolgus macaque*), treated daily with escalating doses of cocaine over one year. Changes seen in mRNA levels by hybridization array analysis were confirmed at the level of protein (via specific immunoblots). Significantly upregulated genes included: protein kinase A  $\alpha$  catalytic subunit (PKAc $\alpha$ ); cell adhesion tyrosine kinase beta (PYK2); mitogen activated protein kinase kinase 1 (MEK1); and  $\beta$ -catenin. While some of these changes exist in previously described cocaine-responsive models, others are novel to any model of cocaine use. All of these adaptive responses coexist within a signaling scheme that could account for known inductions of genes (e.g., fos and jun proteins, and cyclic AMP response element binding protein) previously shown to be relevant to cocaine's behavioral actions. The complete data set from this experiment has been posted to the newly created Drug & Alcohol Abuse Array Data Consortium ([www.arraydata.org](http://www.arraydata.org)) for mining by the general research community.

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The following chapter is published in the *Journal of Neurochemistry* 77:542-849 (2001). The materials have been reprinted with permission of the publisher. Stylistic variations are due to the requirements of the journal and publisher. Michael A. Nader and Susan H. Nader were responsible for the design and execution of the animal treatment. Linda J. Porrino, James B. Daunais, and Davis P. Friedman performed the necropsy, brain dissection, and established the non-human primate tissue bank. Daniel J. Robertson, Lynda Gioia, and Samara M. Mitchell assisted in the development of the hybridization array and immunoblot protocols. Kent E. Vrana served in an advisory position on experimental design and manuscript preparation. Willard M. Freeman performed all other experimental work.

## Introduction:

Cocaine-induced molecular alterations are believed to be responsible for many of the drug's behavioral consequences (Nestler and Aghajanian, 1997; Carlezon et al., 1998; White and Kalivas; 1998, Kelz et al., 1999). The behavioral effects of cocaine are mediated, in large measure, by the mesolimbic dopamine system which has been shown to be critical for the reinforcing aspects of many abused substances (Koob and Bloom, 1988; Koob, 1992a, 1992b). Specifically, the NAcc is a key anatomical substrate underlying drug-seeking behavior (Roberts et al., 1977; Koob and Le Moal, 1997; Wise, 1998).

Attention has therefore been focused on determining the changes in gene expression that take place in reinforcement-related areas of the brain. Changes in expression of a number of genes have been observed in response to cocaine (e.g., tyrosine hydroxylase, neurofilament proteins, neurotrophins, connexins, etc). Two areas that have been the most extensively investigated are the cyclic AMP (cAMP) pathway, that can activate transcription through CREB, and the activator protein 1 (AP1) family of transcription factors (fos and jun), also called immediate early genes (Hope, 1996,1998). The AP-1 complex is usually a dimer of fos and jun proteins (or a homodimer of jun proteins), each of which has multiple iso-

forms.

Immediate early genes are known to increase with acute cocaine administration. One member of the AP-1 family,  $\Delta$ FosB, has been shown to be induced following chronic, but not acute cocaine administration (Pich et al., 1997; Nestler et al., 1999). Additionally, mouse mutants of  $\Delta$ FosB show increased sensitivity to the psychomotor and rewarding aspects of cocaine (Hiroi et al., 1997, Kelz et al., 1999). The mechanisms by which cocaine induces AP-1 related genes have not been elucidated; however, the CREB complex and upstream activators of CREB have been shown to modulate the reinforcing effects of cocaine (Carlezon et al., 1998). To illuminate potential mechanisms which accompany and possibly underlie these changes, we used DNA hybridization arrays to examine mRNA expression in the NAcc of cynomolgus macaques that were chronically treated with cocaine.

## Experimental Procedures:

### *Animals:*

Nine adult male cynomolgus macaques (age 8-15, mean saline group weight = 5.7 kg, mean cocaine group weight = 6.7 kg) were group housed for one year. Five animals received increasing doses of cocaine (1.0 to 10.0 mg/kg/day, BID, i.m.) to a maximally tolerated dose as determined by behavioral assessment of stereotypes, such as huddling in a corner. Doses were similar to those observed in self-administration studies (Letchworth, et al. In press) and total dose ranged from 10.7 to 26.6 g. The other four animals received 0.5 ml/day of saline, equivalent to the volume of the cocaine injections. Approximately 24 hours after the last administration of cocaine or

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**Abbreviations:** AP-1, activator protein 1; CREB, cyclic AMP response element binding protein; GADD 153, Growth Arrest and DNA Damage Protein 153; JAK1, Janus Kinase 1; MAPK, mitogen activated protein kinase; MEK1, mitogen activated protein kinase kinase 1; NAcc, nucleus accumbens; NF1-X, Nuclear Factor 1-X; PAGE, polyacrylamide gel electrophoresis; PKA $\alpha$ , protein kinase A  $\alpha$  catalytic subunit; PYK2, protein tyrosine kinase 2; SDS, sodium dodecyl sulfate; SSC, saline sodium citrate

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saline, animals were sedated with 10-15 mg/kg (i.m.) ketamine and euthanized with 100 mg/kg (i.v.) pentobarbital. The animals were perfused with ice cold saline, the cranium removed and the brains immediately extracted from the skull (8-10 min). Each brain was blocked into three pieces and bisected. One half of each brain was placed on ice and the NAcc was removed by manual dissection. The NAcc was identified by direct observation with reference to surrounding structures. The dissected sample was then immediately frozen in liquid nitrogen. All experiments were conducted with approval of the institutional animal care and use committee and performed in AAALAC-approved facilities.

#### *RNA Isolation and DNA hybridization arrays*

Tissue from the NAcc of each animal was ground with liquid nitrogen in a chilled mortar. The resulting dry homogenate was divided into two pools. Total RNA was isolated from one ground tissue aliquot of each animal according to the method of Chomczynski and Sacchi (1987), and then quantified by spectroscopy and denaturing agarose gel electrophoresis. The array analysis was performed using human Atlas cDNA arrays according to the manufacturer's protocol (Clontech, Palo Alto, CA). These arrays contain gene fragments corresponding to 588 known genes (kinases, transcription factors, cell-cycle proteins, receptors and others). Equal amounts of total RNA from each animal in a group (cocaine or saline) was used to create a total RNA pool for each group. Each pool was treated with 5 $\mu$ L DNase I for 30 min at 37°C (1mg/ml Boehringer Mannheim GmbH, Mannhem Germany).  $^{32}$ P-labeled cDNA probes were synthesized by reverse transcription of 5  $\mu$ g total RNA from each pool, a mixture

of gene-specific primers, dNTPs,  $^{32}$ P-dATP (DuPont NEN Research Products, Boston, MA), and Moloney-murine-leukemia virus (MMLV) reverse transcriptase. These radio-labeled cDNAs (from treated and control samples) were purified by column chromatography and equivalent amounts of radioactivity ( $5 \times 10^6$  cpm) from control or treated cDNA were hybridized to the arrays overnight at 68°C. Arrays were prehybridized with salmon sperm DNA in UltraHyb buffer (Clontech). Following hybridization, the arrays were washed three times at 68°C in 2X SSC (0.3 M NaCl/ 0.03 M sodium citrate), and 1% sodium dodecyl sulfate (SDS), followed by three additional washes at 68°C in 0.1X SSC, 0.5% SDS. The radioactive signals were detected and quantified using GLEAMS (NuTec Services, Stafford TX) and Atlas Image (Clontech) array anal-

to a nitrocellulose membrane (Duralose, Stratagene, La Jolla, CA). PKAc immunoreactive protein was detected using a monoclonal antibody generated against residues 18 through 347 of the human PKAc $\alpha$  subunit (P7340, Transduction Laboratories, Lexington, KY). MEK1 immunoreactive protein was detected using an monoclonal antibody generated against residues 2 through 124 of human MEK1 (M17020, Transduction Laboratories, Lexington, KY). PYK2 immunoreactive protein was visualized using a polyclonal antibody generated against the carboxy terminus of human PYK2 (p47120, Transduction Laboratories, Lexington, KY).  $\beta$ -Catenin was detected using a monoclonal antibody generated against residues 571 to 781 of mouse  $\beta$ -Catenin (C19220, Transduction Laboratories, Lexington, KY). All immunoblot signal values were quantified (TINA, Fuji Medical, Stamford, CA) and are expressed as a mean  $\pm$  S.E.M. and analyzed with a two-tailed student's t-test at a 0.05 level of significance.

## Results:

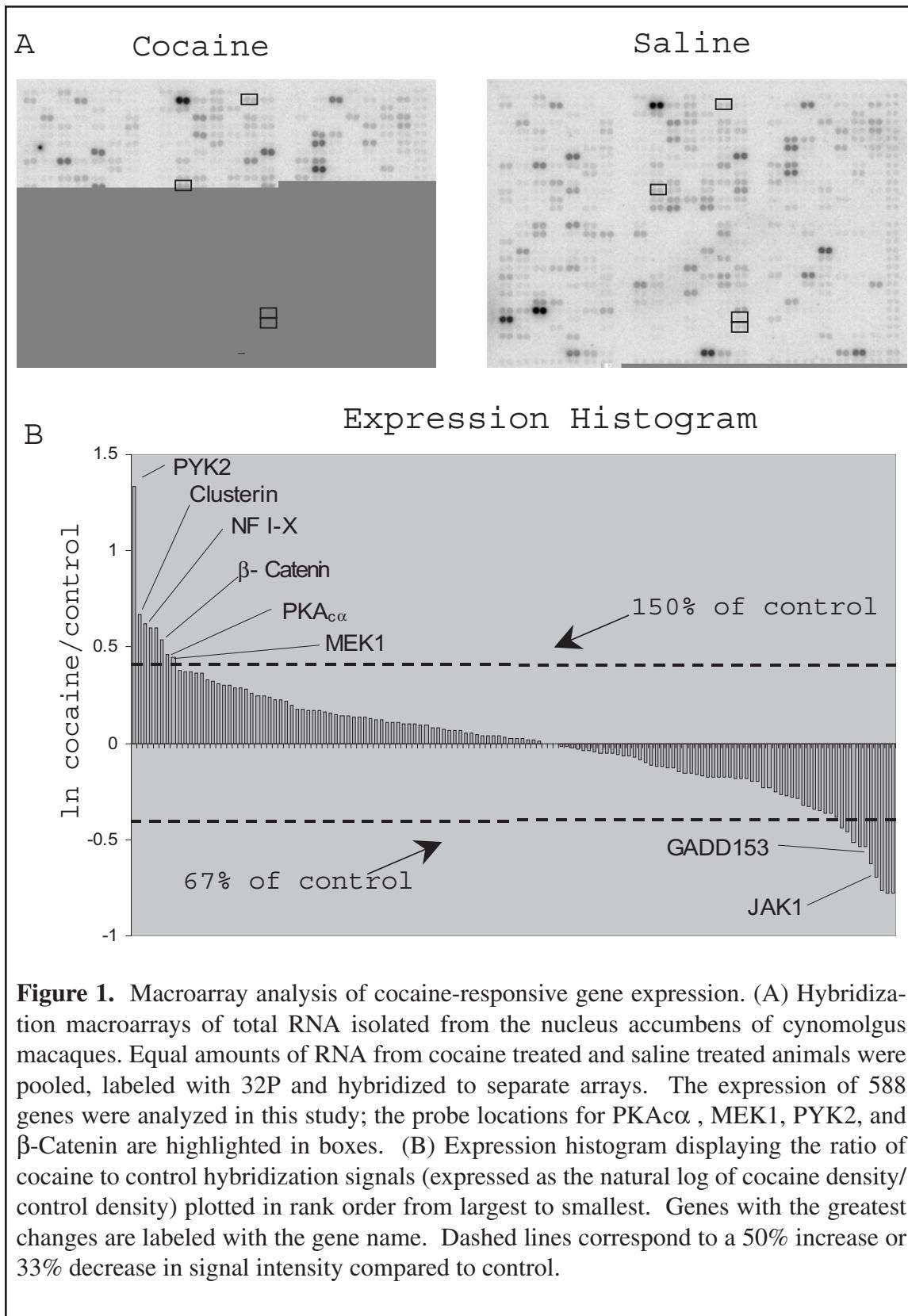
### *Cocaine-responsive changes in mRNA levels*

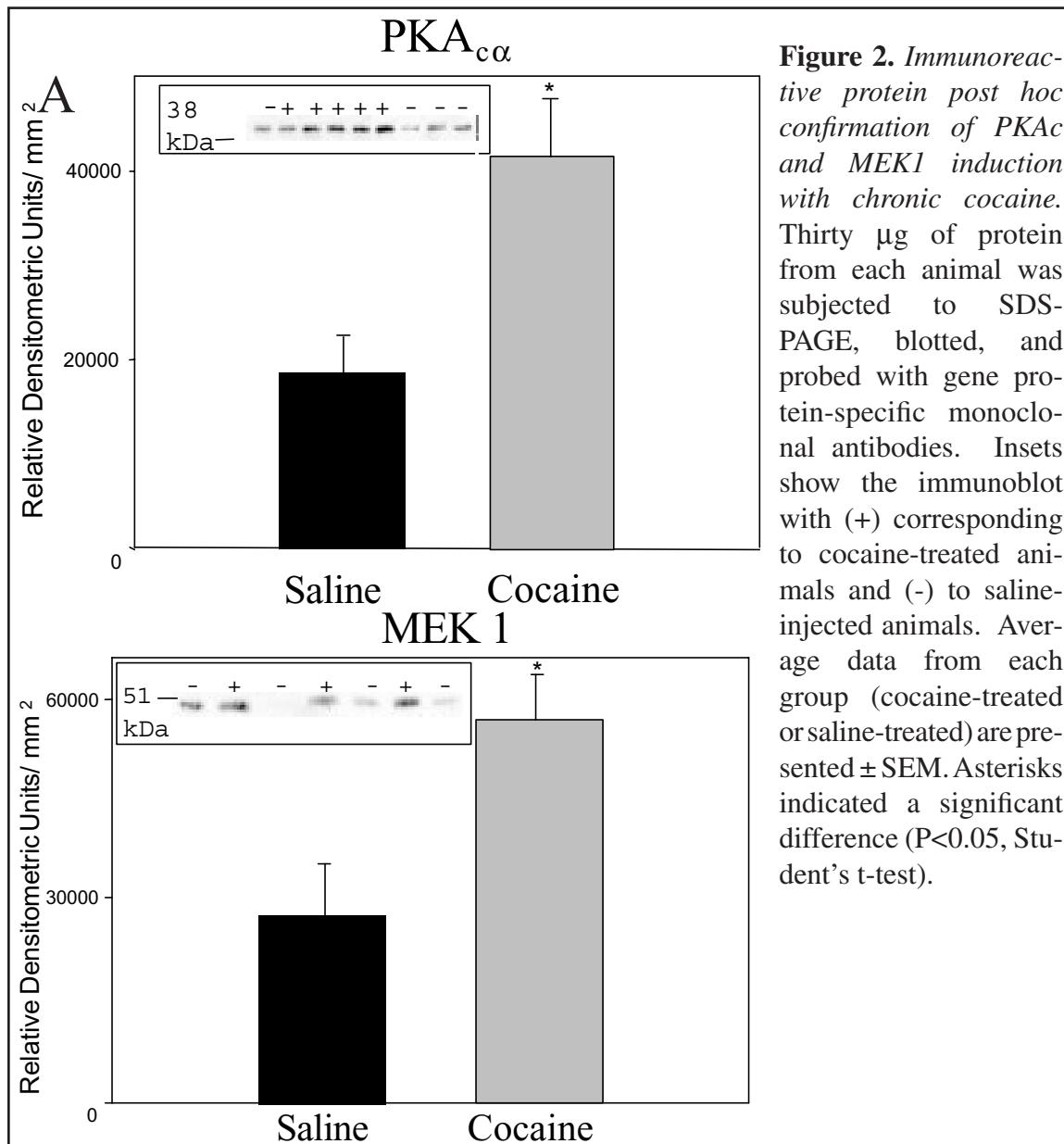
Atlas human arrays (Clontech Laboratories), containing 588 gene-specific probes spotted in duplicate, were used to screen for changes in mRNA expression. This array, while limited in scope, reflects a variety of receptors, second messengers, cytokines, transcription factors, and other genes. Of these 588 probes, 138 produced a signal when hybridized with macaque NAcc  $^{32}\text{P}$ -labeled cDNA. The remaining genes were either not expressed within the NAcc, or were expressed at levels below detection (Figure 1A). Two fos-related antigen genes

(FRA-1 and FRA-2) were present on the array, but did not display a signal above background. This therefore illustrates one often-overlooked problem with DNA array technology; namely, a reduced signal sensitivity (compared with RT-PCR and northern blots). A histogram output generated by rank ordering of the expression ratios highlights the sigmoidal distribution of expression ratios. Previous macroarray work and experience with cocaine-responsive gene expression in the CNS (Beitner-Johnson et al.; 1991, Vrana et al., 1993, 1995) has established that reliably detectable and biologically-appropriate changes in gene expression may be found at 50% induction or 33% reduction in expression. This is especially true when concerning the central nervous system which exhibits a high level of control with disease states producing relatively small changes in gene expression. Because no statistical measures can be drawn from array analysis, this screen was used to prioritize potentially cocaine-regulated genes for subsequent immunoblot analysis.

### *Cocaine-responsive changes in protein expression*

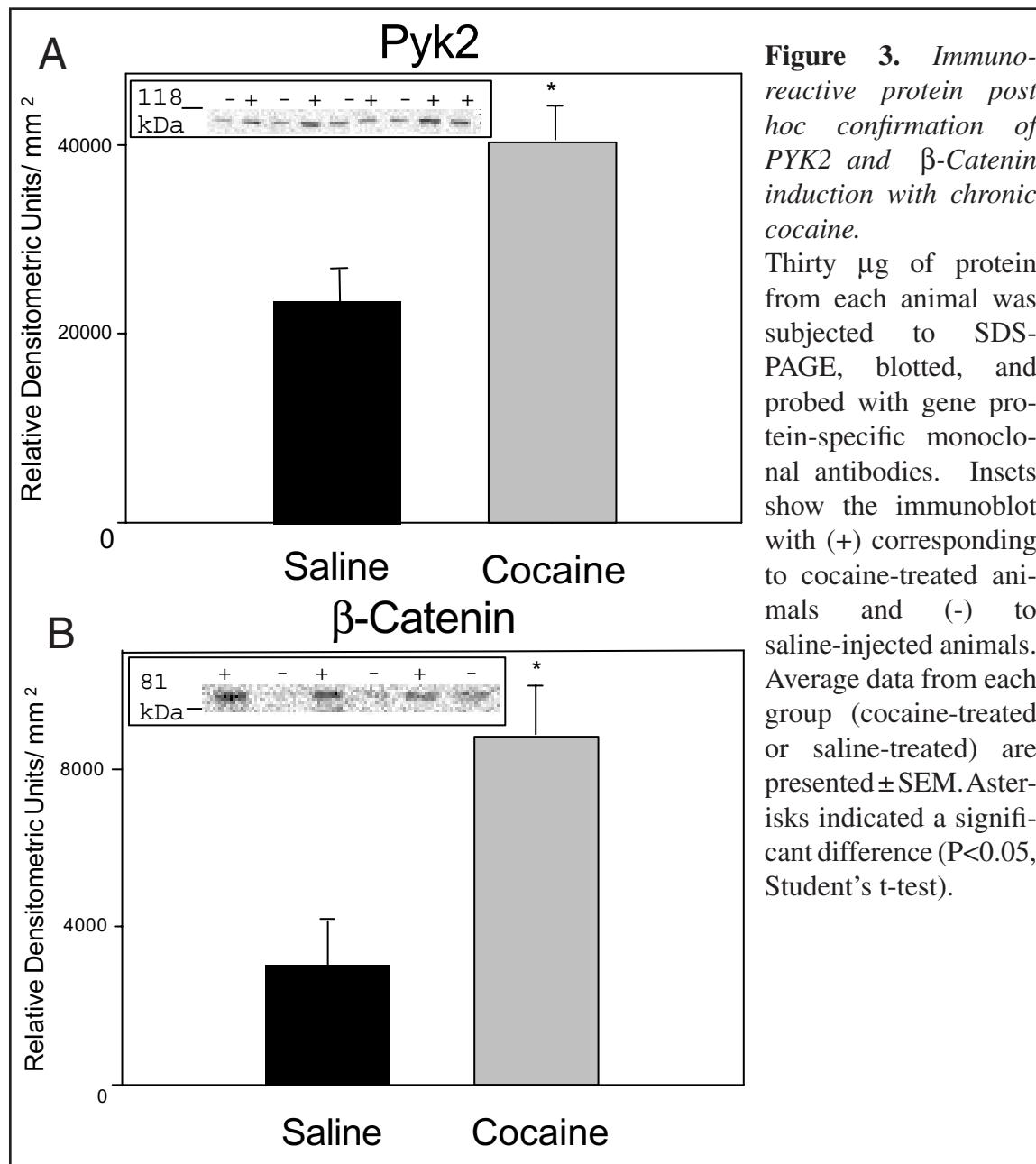
Eighteen genes exceeded a >50% induction or 33% reduction from the macroarray analysis, and of these, eight genes (PKAc $\alpha$ , PYK2,  $\beta$ -catenin, MEK1, NF1-X, Clusterin, GADD153, and JAK1) were chosen for post-hoc immunoblot confirmation based on their biological relevance and the availability of protein antibodies. Post-hoc confirmation is required to provide statistical values on changes seen and to control for false positive, type II, statistical errors. Confirmation at the level of protein assures that increased (or decreased) mRNA levels are reflected by protein levels (Anderson and Seilhamer, 1997). Our findings showed





120% increase ( $t=3.005$ , d.f.=7,  $p<0.025$ ) in  $\text{PKA}_{\text{c}\alpha}$  immunoreactive protein in chronic cocaine animals as compared to controls (Figure 2A). Following chronic cocaine, MEK1 immunoreactive protein was increased by 103% ( $t=2.671$ , d.f.=5,  $p<0.05$ ) compared to controls (Figure 2B). PYK2 immunoreactive protein was up-regulated 70% in cocaine treated animals ( $t=3.076$ , d.f.=7,  $p<0.025$ ) (Figure 3A) and  $\beta$ -Catenin immunoreactive protein increased 180% ( $t=2.863$ , d.f.=5,

$p<0.05$ ) (Figure 3B) in the monkey NAcc. The level of protein induction seen with immunoblots corresponded to the level of induction on the array (Figure 4) but with some variance, possibly due to differing rates of protein translation per mRNA copy. Additional changes illuminated by the array were also tested by immunoblot analysis. Nuclear Factor 1-X (NF1-X), a transcription complex factor, and Clusterin (also known as apolipoprotein J) showed increases in



protein that did not reach statistical significance (data not shown). Janus Kinase 1 (JAK1) and Growth Arrest and DNA Damage Protein 153 (GADD 153) displayed non-significant decreases in protein by immunoblot analysis (data not shown). Although, these changes may not have reached statistical significance due to the limited number of samples available in this primate experimental model, they do

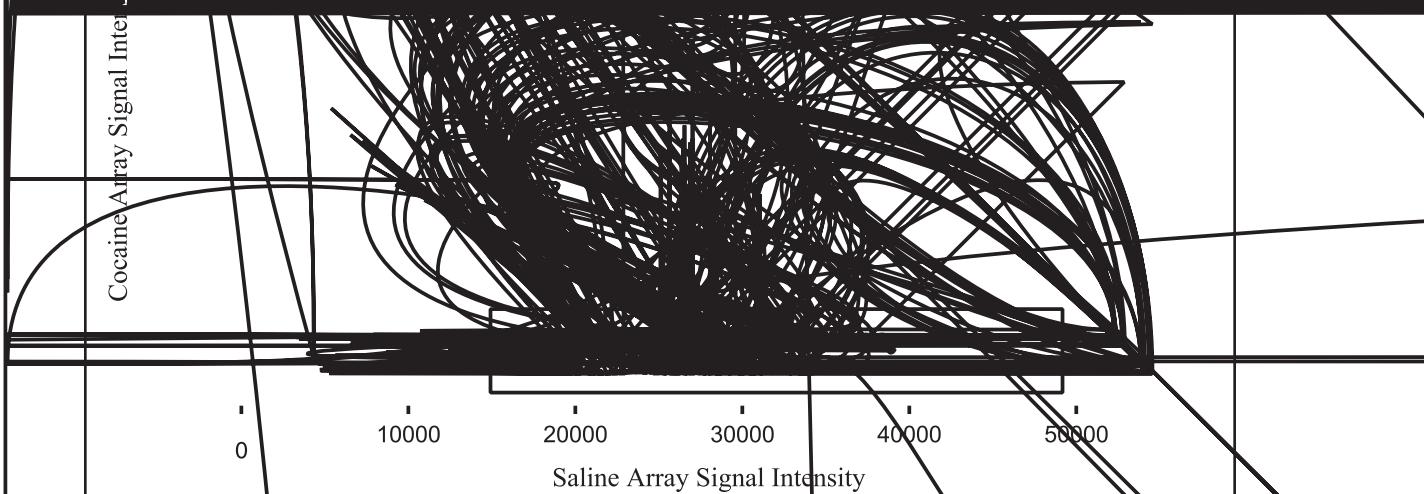
fit into the overall scheme of synergistic cocaine-responsive gene expression changes.

#### Discussion:

The primary purpose of this study was to screen a large number of genes for cocaine-responsive changes in gene expression. Separate groups of macaque monkeys were exposed to escalating doses

of cocaine (1.0 to 10.0 mg/kg/day, BID, i.m.) or saline (0.5mL, BID, i.m.) for one year and then RNA from the NACC was examined for novel cocaine-responsive genes. A membrane-based/radioactive detection format DNA hybridization array, or macroarray (Freeman et al., 2000), with gene-specific human probes was used for the analysis. Xenohybridization, the hybridization of targets from one species to probes from another species, is possible, in this case of the high sequence

between humans and non-human primates (Hacia et al., 1998) and the fact that the array employ large (200-500bp) gene specific probes. Moreover, the non-human primate serves as a close biological model of human cocaine abuse. Macroarray analysis has an advantage of being able to screen hundreds of genes simultaneously using only microgram amounts.



**Figure 4. Hybridization array and immunoblot scatter plot.** Array data is plotted as a function of the cocaine array signal (y-axis) versus saline array signal (x-axis) in solid circles. The black line is draw through unity (equal cocaine to saline signal ratio) and 50% induction and 33% reduction cut off lines are drawn in gray. Immunoblot signal is plotted as a function of cocaine signal (y-axis) to saline signal (x-axis) with hollow circles. The four genes confirmed by post-hoc analysis are color-coded.

Results in the present study suggest that many of the changes observed on macroarrays, and confirmed at the protein level, would fall below the reliable two-fold-change detection limit of many fluorescence-based microarrays.

After hybridization array and specific immunoblot analysis, four genes were found to be significantly induced: PKAc $\alpha$ , MEK1, PYK2 and  $\beta$ -Catenin (Figure 4). These changes are of significant potential importance individually and as members of a common regulatory pathway. One of the key components of this analysis is that changes identified by array experiments are confirmed by quantitative immunoblot analysis (with attendant statistical power). Therefore, we not only have statistical confidence in the findings, but have also established that the changes in mRNA are recapitulated at the level of protein.

The cocaine-responsive nature of the cAMP pathway in rodents is well known (Terwilliger et al., 1991; Miserendo and Nestler, 1995; Tolliver et al., 1996; Unterwald et al., 1996; Self et al., 1998). The PKA holoenzyme consists of two regulatory and two catalytic subunits which dissociate when activated by cAMP. The free catalytic subunits can then act directly through phosphorylation of proteins (cytoplasmic or nuclear). The heightened levels of synaptic dopamine caused by cocaine-mediated blockade of neurotransmitter re-uptake work through G<sub>S</sub>-linked D<sub>1</sub> receptors to increase cAMP levels and therefore PKA activity (Sibley et al., 1993). Functional D<sub>1</sub> receptor supersensitivity has been observed following chronic cocaine and may be explained through an upregulation of the cAMP pathway as well as increases in voltage-gated Na<sup>+</sup> channel phosphorylation (Henry and White, 1995; White et al., 1998). Dopamine and cocaine have been shown

to increase Ser845 phosphorylation of the GluR1 AMPA receptor (Snyder et al., 2000) through a PKA mechanism in the neostriatum.

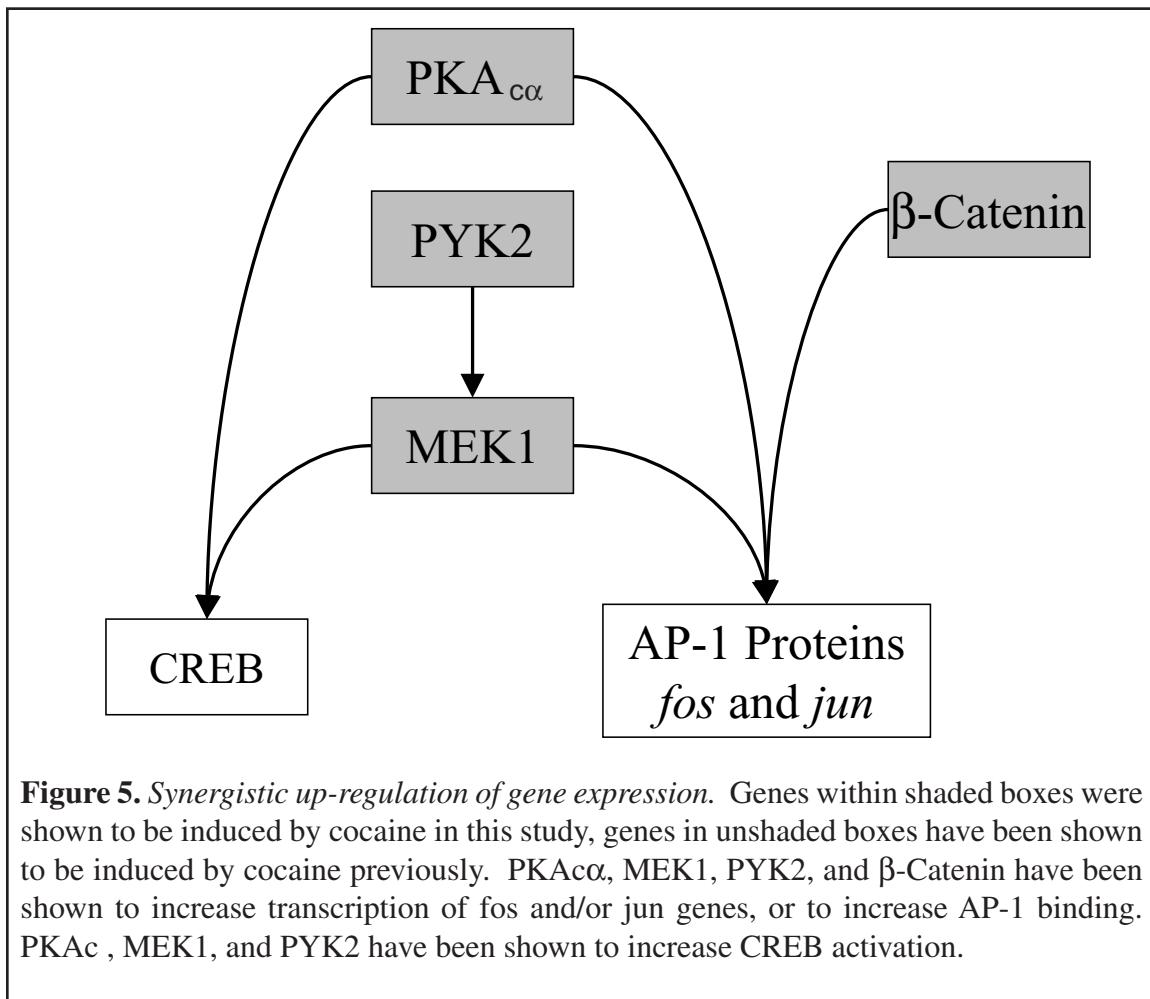
For PKA-mediated transcriptional regulation, one crucial phosphorylation target for the PKA catalytic subunit is CREB, a transcription factor that has been shown to alter behavioral responsiveness to cocaine (Carlezon et al., 1998). Transcriptional activation can also work through both AP-1 mechanisms (Comb et al., 1992) and modulation of the mitogen-activated protein kinase (MAPK) pathway (Yao et al., 1998; Roberson et al., 1999). A link between cocaine and the PKA axis in the NAcc has been reported (Terwilliger et al., 1991; Nestler et al., 1996), including interference by PKA activators and inhibitors (Self et al., 1998).

MEK1, also known as MAPKK1 and MAPK/ERK Kinase 1, is a member of the MAPK family. MEK1 is downstream from Raf and upstream of ERK in the MAPK cascade (Seger and Krebs, 1995). As with PKA, MEK1 can directly alter the activity of cytoplasmic proteins or indirectly activate transcription. Working indirectly through phosphorylation of ERK, MEK1 can activate AP-1 mediated transcription of tyrosine hydroxylase (TH) (Guo et al., 1998) or the phosphorylation of TH - the rate-limiting enzyme in catecholamine biosynthesis (Haycock et al., 1992; Kumer and Vrana, 1996). Increased ERK and TH activity have been demonstrated in other brain regions with chronic cocaine (Beitner-Johnson et al., 1991; Sorg et al., 1993; Vrana et al., 1993; 1995; Berhow et al., 1996). In relation to AP-1 mediated transcription, inhibition of MEK, by PD98059, inhibits c-fos expression (Dudley et al., 1995; Cook et al., 1999; Berhow et al., 1996; Cook et al., 1997). MAPK has also been shown

to induce zif268 (Kumahara et al., 1999) and other cocaine-responsive genes (Pierce et al., 1999; Yan et al., 1999). Inhibition of the MAPK cascade by infusions of the inhibitor PD98059 into the ventral tegmental area reduced behavioral sensitization to cocaine (Pierce et al., 1999). Systemic administration of the MEK inhibitor, SL327, blocked cocaine-induced ERK activation, hyperlocomotion and place-conditioning (Valjent, et al. 2000).

Protein tyrosine kinase 2, PYK2, also known as cell adhesion kinase beta (CAK $\beta$ ), RAFTK, CADTK, or FAK2, is a non-receptor tyrosine kinase (Derkinderen et al., 1999; Girault et al., 1999). PYK2 is activated by increases in intracellular cal-

cium, integrins, protein kinase C (PKC) and by depolarization in hippocampal slices (Lev et al., 1995; Siciliano et al., 1996; Derkinderen et al., 1998; Blaukat et al., 1999). MAPK/ERK kinases are activated by PYK2 (Blaukat et al., 1999; Girault et al., 1999; Pandey et al., 1999). However, a negative mutant of MEK1 does not inhibit PYK2-induced p38MAPK induction (Lev et al., 1995). It has been shown that although PYK2 is activated by D<sub>2</sub> binding, it is not required for MAPK activation by D<sub>2</sub> (Yan et al., 1999). An increased level of PYK2 could play a role in increasing and amplifying the signals of depolarization/calcium influx and g-protein coupled receptors binding through MAPK mediated fos/fra (fos-



related antigen) induction. Additionally, because of the hypothesized roles of PYK2 in long-term potentiation, synaptic plasticity and neuronal survival, it may have relevant actions outside of signal transduction (Girault et al., 1999; Avraham et al., 2000).

$\beta$ -Catenin, also known as cadherin-associated protein beta, plays roles both in adherin junctions and in signal transduction to the nucleus where it interacts with the LEF, TCF, and ELK transcription factors to stimulate the Wnt target genes (Ben-Ze'ev and Geiger, 1998; Willert and Nusse, 1998; Eastman and Grosschedl, 1999). Overexpression of  $\beta$ -Catenin has been shown to stimulate c-jun and fra-1 gene expression (Mann et al., 1999; Kikuchi 2000). Valproic acid has been shown to increase  $\beta$ -Catenin protein levels which antagonize glycogen synthase kinase (GSK-3 $\beta$ ) inhibition of AP-1 DNA binding activity (Chen et al., 1997). Cocaine has also been shown to increase ELK-1 phosphorylation acutely in the Nacc (Valjent, et al. 2000).  $\beta$ -Catenin could serve as a vital intermediary of cocaine-responsive gene expression.

Each of the genes found to be upregulated in this study has the dual capability to both modulate cellular activities in the cytoplasm or to indirectly induce gene transcription. Some genes were members of pathways previously known to be cocaine-responsive (PKA $\alpha$  and MEK1), while other changes ( $\beta$ -catenin and PYK2) were novel findings for cocaine-induced gene expression. The phosphorylation targets of these cocaine-responsive genes, which include ion channels and biosynthetic enzymes, could have behaviorally relevant actions on their own. More study will be needed to assess these actions. While individual changes in gene expression are of importance, the most significant finding is that the genes confirmed by immunoblot analysis shared the

characteristics of being related to the activation of the CREB and/or AP-1 family of transcription factors (Figure 5). These genes were not initially selected for their synergistic properties, but were among the largest changes seen in a 'blind' screen of cocaine-responsive gene expression. All confirmed changes are related to increased expression or activation of AP-1 proteins (fos and jun). The importance of fosB, a longer-lasting, stable transcription factor created by alternative splicing of fosB, in stable neuronal modification of a number of brain functions, including cocaine-abuse, is becoming clear (Nestler et al., 1999). Additionally, PKA $\alpha$ , MEK1 and PYK2 increases could all play a role in increasing CREB activation and its attendant behavioral qualities (Carlezon et al., 1998). The changes in additional genes studied by immunoblot analysis (NF1-X, Clusterin, JAK1, and GADD153), while non-statistically significant, were observed in two independent experimental modalities and correspond to the confirmed changes in their relationship to CREB and/or AP-1. CREB and AP-1 binding sites exist on a number of potential behaviorally-relevant genes, including the serotonin transporter (Bradley and Blakely 1997) and the metabotropic glutamate receptor 1, mGluR1 (Nestler et al., 1999). Depending on the cells within the brain region of interest, these binding sites could have differential effects as typified by the TH gene (Ghee et al., 1998).

Taken as a whole, these results suggest a complex cocaine epigenetic imprint. Genes with functions as varied as signal transduction, transcription activation, and cell adhesion are induced by cocaine. The exact behavioral influence of these changes will have to be determined. These changes,

in the case of PKAcc $\alpha$  and MEK1, extend findings in rodents to non-human primates treated chronically for one year with high doses of cocaine. The findings with PYK2 and  $\beta$ -Catenin are ma

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**References:**

- Anderson L. and Seilhamer J. (1997) A comparison of selected mRNA and protein abundances in human liver. *Electrophoresis* 18, 533-537.
- Avraham H., Park S.Y., Schinkmann K., and Avraham S. (2000) RAFTK/Pyk2-mediated cellular signalling. *Cellular Signalling* 12, 123-133.
- Beitner-Johnson D., Guitart X., and Nestler E.J. (1991) Dopaminergic brain reward regions of Lewis and Fischer rats display different levels of tyrosine hydroxylase and other morphine- and cocaine-regulated phosphoproteins. *Brain Res.* 561, 147-150.
- Ben-Ze'ev A. and Geiger B. (1998) Differential molecular interactions of beta-catenin and plakoglobin in adhesion, signaling and cancer. *Curr. Opin. Cell Bio.* 10, 629-639.
- Berhow M.T., Hiroi N., Kobierski L.A., Hyman S.E., and Nestler E.J. (1996) Influence of cocaine on the JAK-STAT pathway in the mesolimbic dopamine system. *J. Neurosci.* 16, 8019-8026.
- Blaukat A., Ivankovic-Dikic I., Gronroos E., Dolfi F., Tokiwa G., Vuori K., and Dikic I. (1999) Adaptor proteins Grb2 and Crk couple Pyk2 with activation of specific mitogen-activated protein kinase cascades. *J. Biol. Chem.* 274, 14893-14901.
- Bradley C.C., and Blakely R.D. (1997) Alternative splicing of the human serotonin transporter gene. *J. Neurochem.* 69, 1356-1367.
- Carlezon W.A.J., Thome J., Olson L.G., Lane-Ladd S.B., Brodkin E.S., Hiroi N., Duman R.S., Neve R.L., and Nestler E.J. (1998) Regulation of cocaine reward by CREB. *Science* 282, 2272-2275.
- Chen G., Yuan P., Hawver D.B., Potter W.Z., Manji H.K. (1997) Increase in AP-1 transcription factor DNA binding activity by valproic acid. *Neuropsychopharmacol.* 16, 238-245.
- Chomczynski P., and Sacchi N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162, 156-159.
- Comb M.J., Kobierski L., Chu H.M., Tan Y., Borsook D., Herrup K., and Hyman S.E. (1992) Regulation of opioid gene expression: a model to understand neural plasticity. *NIDA Res. Monograph* 126, 98-112.
- Cook S.J., Beltman J., Cadwallader K.A., McMahon M., and McCormick F. (1997) Regulation of mitogen-activated protein kinase phosphatase-1 expression by extracellular signal-related kinase-dependent and Ca<sup>2+</sup>-dependent signal pathways in Rat-1 cells. *J. Biol. Chem.* 272, 13309-13319.
- Cook S.J., Aziz N., and McMahon M. (1999) The repertoire of fos and jun proteins expressed during the G1 phase of the cell cycle is determined by the duration of mitogen-activated protein kinase activation. *Mol. Cell. Bio.* 19, 330-341.
- Derkinderen P., Siciliano J., Toutant M., and Girault J.A. (1998) Differential regulation of FAK+ and PYK2/Cakbeta, two related tyrosine kinases, in rat hippocampal slices: effects of LPA, carbachol, depolarization and hyperosmolarity. *Eur. J. Neurosci.* 10, 1667-1675.
- Derkinderen P., Enslen H., and Girault

- J.A. (1999) The ERK/MAP-kinases cascade in the nervous system. *Neuroreport* 10, R24-R34.
- Dudley D.T., Pang L., Decker S.J., Bridges A.J., and Saltiel A.R. (1995) A synthetic inhibitor of the mitogen-activated protein kinase cascade. *Proc. Natl. Acad. Sci. USA.* 92, 7686-7689.
- Eastman Q., and Grosschedl R. (1999) Regulation of LEF-1/TCF transcription factors by Wnt and other signals. *Curr. Opin. Cell. Bio.* 11, 233-240.
- Freeman W.M., Robertson D.J., and Vrana K.E. (2000) Fundamentals of DNA hybridization arrays for gene expression analysis. *BioTechniques* 29, 1042-1055.
- Ghee M., Baker H., Miller J.C., and Ziff E.B. (1998) AP-1, CREB and CBP transcription factors differentially regulate the tyrosine hydroxylase gene. *Brain Res.* 55, 101-114.
- Girault J.A., Costa A., Derkinderen P., Studler J.M., and Toutant M. (1999) FAK and PYK2/CAKbeta in the nervous system: a link between neuronal activity, plasticity and survival? *Trend. Neurosci.* 22, 257-263.
- Guo Z., Du X., and Iacovitti L. (1998) Regulation of tyrosine hydroxylase gene expression during transdifferentiation of striatal neurons: changes in transcription factors binding the AP-1 site. *J. Neurosci.* 18, 8163-8174.
- Hacia J.G., Makalowski W., Edgemon K., Erdos M.R., Robbins C.M., Fodor S.P., Brody L.C., and Collins F.S. (1998) Evolutionary sequence comparisons using high-density oligonucleotide arrays. *Nature Gen.* 18, 155-158.
- Haycock J.W., Ahn N.G., Cobb M.H., and Krebs E.G. (1992) ERK1 and ERK2, two microtubule-associated protein 2 kinases, mediate the phosphorylation of tyrosine hydroxylase at serine-31 in situ. *Proc. Natl. Acad. Sci. USA.* 89, 2365-2369.
- Henry D.J., and White F.J. (1995) The persistence of behavioral sensitization to cocaine parallels enhanced inhibition of nucleus accumbens neurons. *J. Neurosci.* 15, 6287-6299.
- Hiroi N., Brown J.R., Haile C.N., Ye H., Greenberg M.E., and Nestler E.J. (1997) FosB mutant mice: loss of chronic cocaine induction of Fos-related proteins and heightened sensitivity to cocaine's psychomotor and rewarding effects. *Proc. Natl. Acad. Sci. USA.* 94, 10397-10402.
- Hope B.T. (1996) Novel transcription factors are induced by chronic cocaine treatment. *Annal. New York Acad. Sci.* 80, 1-12.
- Hope B.T. (1998) Cocaine and the AP-1 transcription factor complex. *Annal. New York Acad. Sci.* 844, 1-6.
- Kelz M.B., Chen J., Carlezon W.A.J., Whisler K., Gilden L., Beckmann A.M., Steffen C., Zhang Y.J., Marotti L., Self D.W., Tkatch T., Baranauskas G., Surmeier D.J., Neve R.L., Duman R.S., Picciotto M.R., and Nestler E.J. (1999) Expression of the transcription factor deltaFosB in the brain controls sensitivity to cocaine. *Nature* 401, 272-276.
- Kikuchi A. (2000) Regulation of beta-catenin signaling in the Wnt pathway. *Biochem. Biophys. Res. Comm.* 268, 243-248.

- Koob G.F., and Bloom F.E. (1988) Cellular and molecular mechanisms of drug dependence. *Science* 242, 715-723.
- Koob G.F. (1992a) Drugs of abuse: anatomy, pharmacology and function of reward pathway. *Trend. Pharmacol. Sci.* 13, 177-184.
- Koob G.F. (1992b) Neural mechanisms of drug reinforcement. *Annal. New York Acad. Sci.* 654, 171-191.
- Koob G.F., and Le Moal M. (1997) Drug abuse: hedonic homeostatic dysregulation. *Science* 278, 52-58.
- Kumahara E., Ebihara T., and Saffen D. (1999) Nerve growth factor induces zif268 gene expression via MAPK-dependent and -independent pathways in PC12D cells. *J. Biochem.* 125, 541-553.
- Kumer S.C. and Vrana K.E. (1996) Intricate regulation of tyrosine hydroxylase activity and gene expression. *J. Neurochem.* 67:443-62.
- Laemmli U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685.
- Letchworth S.R., Nader M.A., Smith H.R., Vinsant S.L., Moore R.J., Friedman D.P. and Porrino L.J. Cocaine self-administration in rhesus monkeys: progression of changes in dopamine transporter binding site density. *J. Neurosci.* Accepted pending revision.
- Lev S., Moreno H., Martinez R., Canoll P., Peles E., Musacchio J.M., Plowman G.D., Rudy B., and Schlessinger J. (1995) Protein tyrosine kinase PYK2 involved in Ca(2+)-induced regulation of ion channel and MAP kinase functions. *Nature* 376, 737-745.
- Mann B., Gelos M., Siedow A., Hanski M.L., Gratchev A., Ilyas M., Bodmer, W.F., Moyer M.P., Riecken E.O., Buhr H.J., and Hanski C. (1999) Target genes of beta-catenin-T cell-factor/lymphoid-enhancer-factor signaling in human colorectal carcinomas. *Proc. Natl. Acad. Sci. USA.* 96, 1603-1608.
- Miserendino M.J., and Nestler E.J. (1995) Behavioral sensitization to cocaine: modulation by the cyclic AMP system in the nucleus accumbens. *Brain Res.* 674, 299-306.
- Nestler E.J., Berhow M.T., and Brodkin E.S. (1996) Molecular mechanisms of drug addiction: adaptations in signal transduction pathways. *Mol. Psychiatry* 1, 190-199.
- Nestler E.J., and Aghajanian G.K. (1997) Molecular and cellular basis of addiction. *Science* 278:58-63.
- Nestler E.J., Kelz M.B., and Chen J. (1999) DeltaFosB: a molecular mediator of long-term neural and behavioral plasticity. *Brain Res.* 835, 10-17.
- Pandey P., Avraham S., Kumar S., Nakazawa A., Place A., Ghanem L., Rana A., Kumar V., Majumder P.K., Avraham H., Davis R.J., and Kharbanda S. (1999) Activation of p38 mitogen-activated protein kinase by PYK2/related adhesion focal tyrosine kinase-dependent mechanism. *J. Biol. Chem.* 274, 10140-10144.
- Pich E.M., Pagliusi S.R., Tessari M., Talabot-Ayer D., Hooft v., Huijsdijnen R., and Chiamulera C. (1997) Common neural substrates for the addictive properties of nicotine and cocaine. *Science* 275, 83-86.
- Pierce R.C., Pierce-Bancroft A.F., and Prasad B.M. (1999) Neurotrophin-3 contributes to

- the initiation of behavioral sensitization to cocaine by activating the Ras/Mitogen-activated protein kinase signal transduction cascade. *J. Neurosci.* 19, 8685-8695.
- Roberson E.D., English J.D., Adams J.P., Selcher J.C., Kondratick C., and Sweatt, J.D. (1999) The mitogen-activated protein kinase cascade couples PKA and PKC to cAMP response element binding protein phosphorylation in area CA1 of hippocampus. *J. Neurosci.* 19, 4337-4348.
- Roberts D.C., Corcoran M.E., and Fibiger H.C. (1977) On the role of ascending catecholaminergic systems in intravenous self-administration of cocaine. *Pharmacol. Biochem. Behav.* 6, 615-620.
- Seger R., and Krebs E.G. (1995) The MAPK signaling cascade. *FASEB Journal* 9, 726-735.
- Self D.W., Genova L.M., Hope B.T., Barnhart W.J., Spencer J.J., and Nestler E.J. (1998) Involvement of cAMP-dependent protein kinase in the nucleus accumbens in cocaine self-administration and relapse of cocaine-seeking behavior. *J. Neurosci.* 18, 1848-1859.
- Sibley D.R., Monsma F.J.J., and Shen Y. (1993) Molecular neurobiology of dopaminergic receptors. *Internatl. Rev. Neurobio.* 35, 391-415.
- Siciliano J.C., Toutant M., Derkinderen P., Sasaki T., and Girault J.A. (1996) Differential regulation of proline-rich tyrosine kinase 2/cell adhesion kinase beta (PYK2/CAKbeta) and pp125(FAK) by glutamate and depolarization in rat hippocampus. *J. Biol. Chem.* 271, 28942-28946.
- Snyder G. L., Allen P. B., Fienberg A.A., Valle C. G., Huganir R. L., Nairn A. C., and Greengard P. (2000) Regulation of phosphorylation of the GluR1 AMPA receptor in the neostriatum by dopamine and psychostimulants in vivo. *J. Neurosci.* 20, 4480-4488.
- Sorg B.A., Chen S.Y., and Kalivas P.W. (1993) Time course of tyrosine hydroxylase expression after behavioral sensitization to cocaine. *J. Pharmacol. Exp. Ther.* 266, 424-430.
- Terwilliger R.Z., Beitner-Johnson D., Sevarino K.A., Crain S.M., and Nestler E.J. (1991) A general role for adaptations in G-proteins and the cyclic AMP system in mediating the chronic actions of morphine and cocaine on neuronal function. *Brain. Res.* 548, 100-110.
- Tolliver B.K., Ho L.B., Reid M.S., and Berger S.P. (1996) Evidence for involvement of ventral tegmental area cyclic AMP systems in behavioral sensitization to psychostimulants. *J. Pharmacol. Exper. Ther.* 278, 411-420.
- Unterwald E.M., Fillmore J., and Kreek M.J. (1996) Chronic repeated cocaine administration increases dopamine D1 receptor-mediated signal transduction. *Euro. J. Pharmacol.* 318, 31-35.
- Valjent E., Corvol J.C., Pages C., Besson M.J., Maldonado R. and Caboche J. (2000) Involvement of the extracellular signal-regulated kinase cascade for Cocaine-Rewarding properties. *J. Neurosci.* 20:8701-8709.
- Vrana S.L., Vrana K.E., Kovacs T.R., Smith J.E., and Dworkin S.I. (1993) Chronic cocaine administration increases CNS

tyrosine hydroxylase enzyme activity and mRNA levels and tryptophan hydroxylase enzyme activity levels. *J. Neurochem.* 61, 2262-2268.

Vrana S.L., Klutts B.W., and Vrana K.E. (1995) Application of quantitative RT-PCR to the analysis of dopamine receptor mRNA levels in rat striatum. *Brain. Res.* 34, 127-134.

White F.J., Hu X.T., and Zhang X.F. (1998) Neuroadaptations in nucleus accumbens neurons resulting from repeated cocaine administration. *Advances. Pharmacol. (New York)* 42, 1006-1009.

White F.J., and Kalivas P.W. (1998) Neuroadaptations involved in amphetamine and cocaine addiction. *Drug & Alcohol Depend.* 51, 141-153.

Willert K., and Nusse R. (1998) Beta-catenin: a key mediator of Wnt signaling. *Current Opin. Gene. & Develop.* 8, 95-102.

Wise R.A. (1998) Drug-activation of brain reward pathways. *Drug & Alcohol Depend.* 51, 13-22.

Yan Z., Feng J., Fienberg A.A., and Greengard P. (1999) D(2) dopamine receptors induce mitogen-activated protein kinase and cAMP response element-binding protein phosphorylation in neurons. *Proc. Natl. Acad. Sci. USA.* 96, 11607-11612.

Yao H., York R.D., Misra-Press A., Carr D.W., and Stork P.J. (1998) The cyclic adenosine monophosphate-dependent protein kinase (PKA) is required for the sustained activation of mitogen-activated kinases and gene expression by nerve growth factor. *J. Biol. Chem.* 273, 8240-8247.

# Chapter 3: Cocaine-Responsive Gene Expression Changes in Rat Hippocampus.



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## Abstract:

Chronic cocaine use is known to elicit changes in the pattern of gene expression within the brain. The hippocampus plays a critical role in learning and memory and may also play a role in mediating behaviors associated with cocaine abuse. To profile the gene expression response of the hippocampus to chronic cocaine, cDNA hybridization arrays were used to illuminate cocaine-regulated genes in rats treated non-contingently with a binge model of cocaine (45mg/kg/day, IP) for fourteen days. Validation of mRNA changes seen by hybridization array analysis was accomplished by measuring immunoreactive protein (via specific immunoblots). A number of genes: protein tyrosine kinase 2, beta-catenin, protein kinase C $\alpha$ , protein kinase C $\epsilon$ , potassium channel 1.1, and metabotropic glutamate receptor 5, were shown to be significantly induced by cocaine administration.

While some of these changes exist in previously described cocaine-responsive models, others are novel to any model of cocaine use. The induction of protein tyrosine kinase 2 and beta-catenin have been observed previously in the primate nucleus accumbens after chronic cocaine. Protein kinase C isoforms  $\alpha$  and  $\epsilon$ , and metabotropic glutamate receptor 5 are novel findings to hippocampal cocaine-responsive gene expression, and are known to subserve learning and memory functions within the hippocampus. Potassium channel 1.1, a shaker family  $\alpha$  subunit, alterations could alter the firing pattern of the cell. Additionally, these genes are known to interact with one another, forming a more complex pattern of gene expression changes that may alter neuronal plasticity. These findings suggest a wide range of protein changes in the rat hippocampus after a ‘binge’ style of non-contingent cocaine administration that may play roles in neuronal plasticity and the behavioral phenomenon associated with cocaine abuse.

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The following chapter is in press in *Neuroscience*. Stylistic variations are due to the requirements of the journal and publisher. David C. S. Roberts, Karen Brebner, Wendy J. Lynch were responsible for execution of the animal treatments. Daniel J. Robertson assisted in the execution of the immunoblot protocols. Willard M. Freeman performed all other experimental work. Kent E. Vrana served in an advisory position on experimental design and manuscript preparation.

## Introduction:

Chronic psychomotor stimulant use is well known to alter neuronal gene expression (for review see: Nestler<sup>37</sup>, White and Kalivas<sup>52</sup>). Changes in gene expression have also been shown to subserve the behavioral effects of cocaine.<sup>7,23</sup> Cocaine administration has been shown to affect a number of genes in different brain regions. These genes encode a wide variety of molecular functions, ranging from transcription factors, to neurotransmitter receptors and transporters, and signal transduction mechanisms. While the identification of this gene set represents a significant step forward in understanding cocaine's neurobiological actions, it is likely that there are many other cocaine-induced changes in gene expression that have yet to be discovered.

Since the dopamine (DA) transporter has been shown to be the site of action involved in mediating cocaine's reinforcing effects, much of the gene expression literature has focused on areas of the brain that receive a dense DA innervation, such as the dorsal and ventral striatum. However, other areas of the brain associated with the meso-

limbic DA system are also involved in processing information related to cocaine use. Hence, increased attention is now being directed towards the involvement of a number of limbic system structures, such as the hippocampus, and amygdala as well as cognition-related regions such as prefrontal cortex. Electrophysiological data have indicated that the hippocampus and prefrontal cortex send converging afferents to the nucleus accumbens (NAcc), and the prefrontal cortex and hippocampus have reciprocal connections.<sup>38,49</sup> We and others have shown cocaine-induced changes in gene expression in the hippocampus.<sup>4,39,42</sup> Additionally, because of the critical role of the hippocampus in learning and memory, it has been suggested that the hippocampus plays an important role in the acquisition of cocaine-related acquisition behaviors.

A new method for identifying novel changes in gene expression is the use of cDNA hybridization arrays.<sup>16,17</sup> This method allows the parallel analysis of hundreds to thousands of genes. Genes that show an apparent change on hybridization arrays must then be confirmed with other methods to show statistically significant differences. Confirming changes at the level of protein allows both confirmation of the change, and proof that differences in mRNA are recapitulated at the protein level. Using this approach of mRNA screening and protein confirmation, the hippocampus was examined for changes in gene expression after a well characterized, 'binge', model of chronic (14 day) non-contingent intraperitoneal (IP) administration of cocaine.<sup>46,47</sup>

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### Abbreviations:

AP-1, activator protein complex 1; Cdk5, cyclin-dependent kinase 5; CREB, cyclic AMP response element binding protein; Dopamine (DA); ELK, ets oncogene proteins; Gαi, G-protein inhibiting alpha 1; Kv1.1 shaker-related voltage-gated potassium channel member; MEK1, mitogen activated protein kinase kinase 1; mGluR5, metabotropic glutamate receptor 5; LTD, long-term depression; LTP, long-term potentiation; NAcc, nucleus accumbens; OATP2, organic anion transporting polypeptide 2; PAGE, polyacrylamide gel electrophoresis; PKA $\alpha$ , protein kinase A  $\alpha$  catalytic subunit; PKC $\alpha$ , protein kinase C alpha; PKC $\epsilon$ , protein kinase C epsilon; PP2A, protein phosphatase 2A, PYK2, protein tyrosine kinase 2; Ras-GAP, ras-GTPase-activating protein, SDS, sodium dodecyl sulfate; SMN, survival of motor neuron; SSC, saline sodium citrate

## Experimental Procedures:

### *Animals*

Subjects were male Sprague-Dawley rats (Harlan, Indianapolis, IN) weigh-

ing 300g at the initiation of the experiment. Upon arrival at the facility, rats were individually housed in polypropylene cages and placed on a 12 h light:dark cycle (lights on at 900 h). Rats were allowed to acclimate to the facility for 7 days before the start of the experiment. Food and water were available ad libitum throughout the experiment. Care and treatment of all animals conformed to the standards and guidelines promulgated by the Wake Forest University Animal Care and Use Committee and the National Institutes of Health. All possible efforts were made to minimize the number of animals used and their suffering.

#### *Procedure*

Rats were randomly assigned to two treatment groups ( $n=6$ ) that received IP injections of cocaine or saline for 14 days. Rats were weighed at 10:00 every morning, immediately prior to the first injection. Rats in each group received 3 hourly injections of cocaine HCl (15 mg/kg), or saline (1ml/kg) beginning at 10:00. Thirty minutes after the final cocaine or saline injection, rats were deeply anaesthetized with sodium pentobarbital (IP) and decapitated. Brains were rapidly removed and placed on ice for dissection. To dissect the hippocampus, the corpus callosum was transected, and the hippocampus was rolled away from the cortex by freehand dissection and immediately frozen on dry ice. A second set of animals ( $n=6$  per saline and cocaine group) was treated identically as above for the protein immunoblot confirmations.

#### *Drugs*

Cocaine HCl was supplied by the National Institute on Drug Abuse (Research Triangle, NC) and dissolved in 0.9% sterile saline. Dosages are expressed as the salt.

#### *RNA Isolation and DNA hybridization array*

Tissue from the hippocampus of each animal was ground with liquid nitrogen in a dry ice chilled mortar. Total RNA was isolated from the ground tissue of each animal according to the method of Chomczynski.<sup>10</sup> Hybridization array analysis was performed twice using Rat 1.2 (I) Atlas cDNA arrays according to the manufacturer's protocol (Clontech, Palo Alto, CA) and similarly to as previously described.<sup>16</sup> These arrays contain gene fragments corresponding to 1176 known regulatory genes (kinases, transcription factors, cell-cycle proteins, receptors and others). Equal amounts of total RNA from each animal in a group (cocaine or saline) were used to create a total RNA pool for each group.  $^{32}\text{P}$ -labeled cDNA probes were synthesized by reverse transcription of 5 $\mu\text{g}$  total RNA from each pool, a mixture of gene-specific primers, dNTPs,  $^{32}\text{P}$ -dATP (DuPont NEN Research Products, Boston, MA), and Moloney-murine-leukemia virus (MMLV) reverse transcriptase. These radiolabeled cDNAs (from treated and control samples) were purified by column chromatography and equal amounts of radioactivity (3-5 x 10<sup>6</sup> cpm) from control or treated cDNA were hybridized to the arrays overnight at 68°C. Arrays were prehybridized overnight with salmon sperm DNA in UltraHyb buffer (Clontech). Following hybridization, the arrays were washed three times at 68°C in 2X SSC (0.3 M NaCl/ 0.03 M sodium citrate), and 1% sodium dodecyl sulfate (SDS), followed by three additional washes at 68°C in 0.1X SSC, 0.5% SDS and one wash in 2X SSC at room temperature. The radioactive signals were detected with a Storm Phosphorimager (Molecular Dynamics, Sunnyvale, CA) and quantified using GLEAMS (NuTec Services, Stafford TX)

and Atlas Image2.0 (Clontech) array analysis software. Background was determined from the blank areas of the array and subtracted from density measurements for each gene on that array. A normalization factor for each pair of arrays was determined from the ratio of the sums of the specific signals for each array. The normalization factor was then applied to the signal for each spot of one array as described by the AtlasImage2.0 array analysis software. Genes which demonstrated a consistent up-regulation or down-regulation across the duplicate experiments were chosen for post-hoc immunoblot analysis. The complete data set is available at the Drug and Alcohol Abuse Array Data Consortium website ([www.arraydata.org](http://www.arraydata.org)).

#### *Immunoblotting*

Protein was isolated from a second set of identically-treated animals as the cDNA hybridization array analysis. Tissue was ground with liquid nitrogen in a dry ice chilled mortar. A portion of the dry homogenate was then homogenized by sonication in protein buffer (25mM N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)(pH=7.5), 250mM Sucrose, 100µM Ethylenediaminetetraacetic acid, 1µg/mL Leupeptin, 0.5µg/mL Pepstatin A, 500µM PMSF, 1mM 1,4-Dithio-DL-threitol, 10µM Fe(NH4)2(SO4)2, 0.2% Triton X-100). Total protein concentration was determined with the bicinchoninic acid assay 45 (BCA protein assay, Pierce Chemical, Rockford IL) of brain homogenates from each animal. Equal amounts of protein from each animal (30-60µg) were resolved by denaturing polyacrylamide gel electrophoresis (SDS-PAGE) 27, and transferred to a polyvinylidene fluoride membrane (Immobilon P, Millipore, Bedford, MA) by semidry transfer (TE-70, Amersham Pharmacia Biotech, Piscataway, NJ). Immunoreactive

protein corresponding to the protein molecular weight was detected with antibodies at the following concentrations: β-Catenin (92kDa) 0.33µg/mL (C19220, Transduction Laboratories); G-protein inhibiting alpha 1, Gi $\alpha$ 1, 1.0µg/mL (SA-281, Biomol, Plymouth Meeting, PA); Kv1.1 (90kDa) 1.0µg/ml (05407, Upstate Biotechnology); mitogen-activated protein kinase kinase 1, MEK1, (45kDa) 0.165 µg/mL (M17020, Transduction Laboratories); metabotropic glutamate receptor 5, mGluR5, (120kDa) 0.8µg/mL (06451, Upstate Biotechnology, Lake Placid, NY); organic anion transporter polypeptide 2, OATP2, 3.33 µg/mL, (OATP21-A, Alpha Diagnostic International, San Antonio, TX); protein kinase A alpha catalytic subunit, PKAc $\alpha$ , (40kDa) 0.165µg/mL (P73420, Transduction Laboratories, Lexington, KY); protein kinase C alpha, PKC $\alpha$ , (82kDa) 0.165µg/mL (P16520, Transduction Laboratories); protein kinase C epsilon, PKC $\epsilon$ , (90kDa) 0.165µg/mL (P14820, Transduction Laboratories); protein phosphatase 2A, PP2A, (36kDa) 0.033 µg/mL (P47720, Transduction Laboratories); protein tyrosine kinase 2, PYK2, (116kDa) 0.165µg/mL (P47120, Transduction Laboratories); ras-GTPase-activating protein, Ras-GAP (120kDa) 0.165µg/mL (G12920, Transduction Laboratories), and survival of motor neuron, SMN, (40kDa) 0.165µg/mL (S55920, Transduction Laboratories). Visualization was accomplished using a horseradish peroxidase-coupled secondary antibody, either mouse or rabbit IgG (AP Biotech) and a chemiluminescent substrate (Pico Signal, Pierce Chemical). All immunoblot signal values were quantified by transmissive densitometry (TINA, Fuji Medical Systems, Stamford, CA). All values are expressed as a mean ± S.E.M. Immunoblots for targets seen from the hybridization array, SMN, mGluR5, OATP2, PKC $\alpha$ , Gi $\alpha$ 1, Ras-GAP,

PP2A  $\alpha$  catalytic, and Kv1.1, were analyzed with a one-tailed student's t-test (with a 0.05 level of significance) to test the hypothesis that the changes in RNA levels seen on the array were re-capitulated at the protein level. Immunoblots for genes, (MEK1, PYK2,  $\beta$ -Catenin, PKAc $\alpha$ , PKC $\epsilon$ ), that were present on the hybridization array or did not show a consistent change on the hybridization array but were identified in other models or systems as cocaine-responsive were analyzed with a two-tailed student's t-test at a 0.05 level of significance.

## Results:

### *Cocaine-responsive changes in mRNA levels*

Array analysis, performed in duplicate, demonstrated a number of potential cocaine-responsive genes (Figure 1). Of the 1176 genes on the array, 628 showed a discernable signal on at least one of the arrays. The remaining genes were either not expressed in the hippocampus or at levels below the sensitivity of the array. Previous macroarray studies<sup>16,17</sup> and experiments with cocaine-responsive CNS gene expression<sup>50</sup> have shown that reliable and biologically significant changes can be seen at the level of 50% induction and 33% reduction (equivalent change on a natural log scale). Five genes, SMN, mGluR5, OATP2, PKC $\alpha$ , and Gia1 exhibited a 50% or greater induction with cocaine treatment across the replicate arrays. No genes showed a 33% or greater reduction with cocaine treatment across replicate arrays.

An alternative method of hybridization array data analysis is to use the mean ratio and the standard deviation from it for determining which genes are responsive to treatment. This analysis involves taking the natural log of the ratio for each gene which

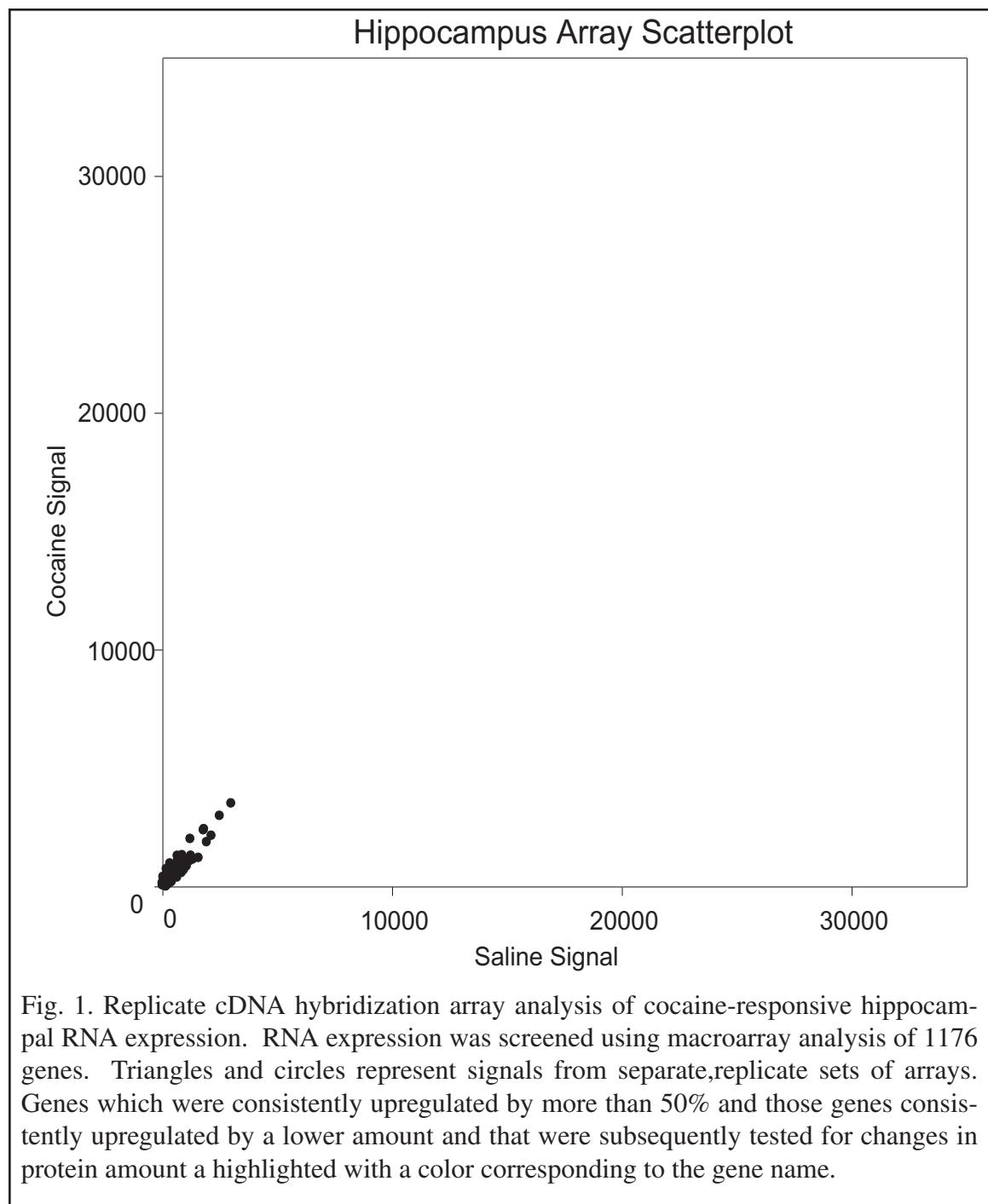
was 50% over background on both of the arrays in a pair. From the natural log value of the ratio for all of these genes, a mean and standard deviation is derived. Genes whose ratio is greater than two standard deviations from the mean represent a significant deviation from the mean. This analysis is then repeated on the duplicate array experiment. Genes which deviate significantly from the mean in both sets of arrays represent high quality targets for post-hoc confirmation. In the present study, this analysis was not superior to the standard 50% induction - 33% reduction cutoff values from our previous experiments.<sup>16</sup> The standard deviation analysis produced cutoff values on a linear scale of 51.2% and 54.78% increase for the two sets of arrays. In fact, this approach identified the same set of genes for further study as the discretionary 50% threshold. As well, because the duplicate experiments did not show the exact same sets of genes with a signal 50% over background, many of the genes whose ratios deviated from the mean in one experiment, were not above background in the other experiment. While statistical means are needed to assess the reliability of changes seen with many hybridization array experiments, in this study they were of limited utility.

Hybridization arrays lack power to assign statistical significance because of the lack of multiple samples combined with the large excess of dependent variables. Therefore, post-hoc confirmation is needed to determine which of the changes seen on the array represent a true up-regulation. Post-hoc tests were performed using specific immunoblots. This serves the dual purposes of providing a statistical measure of changes and, secondly, to confirm that changes in mRNA levels correlate to changes in protein levels which is not necessarily the case.<sup>1,28</sup>

### Cocaine-responsive changes in protein expression

Specific immunoblots were performed on protein homogenates from individual animals. From array analysis, eight genes were chosen to be tested at the protein level. SMN, mGluR5, OATP2, PKC $\alpha$ ,

and Gi $\alpha$ 1 showed a greater than 50% induction on both sets of arrays. To test the ability of hybridization arrays to detect smaller magnitude changes (<50%), Ras-GAP, PP2A  $\alpha$  catalytic subunit, and Kv1.1, which showed consistent changes below 40%, were chosen for immunoblot anal-



ysis. These three genes were of interest because they have been seen to be induced by cocaine in hybridization array analysis of other brain regions (Freeman, W.M., Roberts, D.C.S, and Vrana, K.E., unpublished observations). MEK1, PYK2,  $\beta$ -Catenin and PKAc $\alpha$  have been shown to be increased with cocaine administration in non-human primates<sup>16</sup> and were assayed by immunoblot to see if these changes replicated in a different brain region and animal model. PKC was also tested because it has been seen to change on arrays of other brain regions with chronic cocaine (Freeman, W.M. and Vrana, K.E., unpublished observations). MEK1 was present on the arrays and showed a greater than 50% induction in one replicate but not the other. PKAc $\alpha$  was present on the array, but did not have a signal 50% over background on either of the replicate experiments. PYK2 was present on the arrays used and showed a change in expression in only one of the replicates.  $\beta$ -Catenin was not present on the arrays and PKC $\epsilon$  showed a slight (10%) average induction on the two arrays.

PKC $\alpha$  immunoreactive protein was increased by 32% ( $p<0.025$ ) following cocaine administration (figure 2A), while immunoreactive protein levels of mGluR5 increased by 38% ( $p<0.025$ ) (figure 2B). SMN (figure 2C) was unchanged and antibodies to OATP2 or Gia1 were not found to reliably or specifically detect their antigens.

Kv1.1 immunoblot analysis showed a statistically significant 25% increase ( $p<0.05$ ) in protein (Figure 3A). PP2A  $\alpha$  catalytic exhibited a non-significant 13% induction in protein levels (Figure 3B). Ras-GAP showed a non-significant 45% induction at the protein level (Figure 3C).

Our findings also demonstrated that two of the four proteins previously documented<sup>16</sup> to be increased with chronic

cocaine in the non-human primate nucleus accumbens were induced. PYK2 increased by 45% ( $p<0.025$ ) and  $\beta$ -Catenin increased by 340% ( $p<0.05$ ) (Figure 4A&B). MEK1 and PKAc $\alpha$  were unchanged with cocaine treatment (Figure 4C&D). PKC $\epsilon$  immunoreactive protein increased 48% ( $p<0.05$ ) (Figure 4E).

## Discussion:

The results of this study have several implications for the functional genomics of neuronal cocaine-responsive gene expression. Out of the three genes (SMN, mGluR5, PKC $\alpha$ ) that showed a greater than 50% induction on both arrays and to which a reliable antibody was available, two (mGluR5, PKC $\alpha$ ) showed a statistically significant change at the level of protein. Of the three genes (Ras-GAP, PP2A  $\alpha$  catalytic, and Kv1.1) that showed a smaller increase on the arrays and were chosen for protein post-hoc confirmation, one, Kv1.1, was observed to have significantly induced immunoreactive protein levels. Five other proteins, (MEK1, PYK2,  $\beta$ -Catenin, PKAc $\alpha$ , PKC $\epsilon$ ) were also assayed because of their cocaine-responsiveness in other brain regions. PYK2,  $\beta$ -Catenin, and PKC $\epsilon$  showed significant increases in immunoreactive protein. The fact that some apparent changes in mRNA were not recapitulated at the level of protein could be the result of several factors. First, some changes in mRNA may be subject to additional regulation at the levels of translation and degradation. Second, the sensitivity of hybridization arrays may be such that smaller magnitude changes are subject to higher variability. Finally, mRNA changes present in the brain region assayed by hybridization array may only be manifested in projections from that region to other regions. Therefore, a

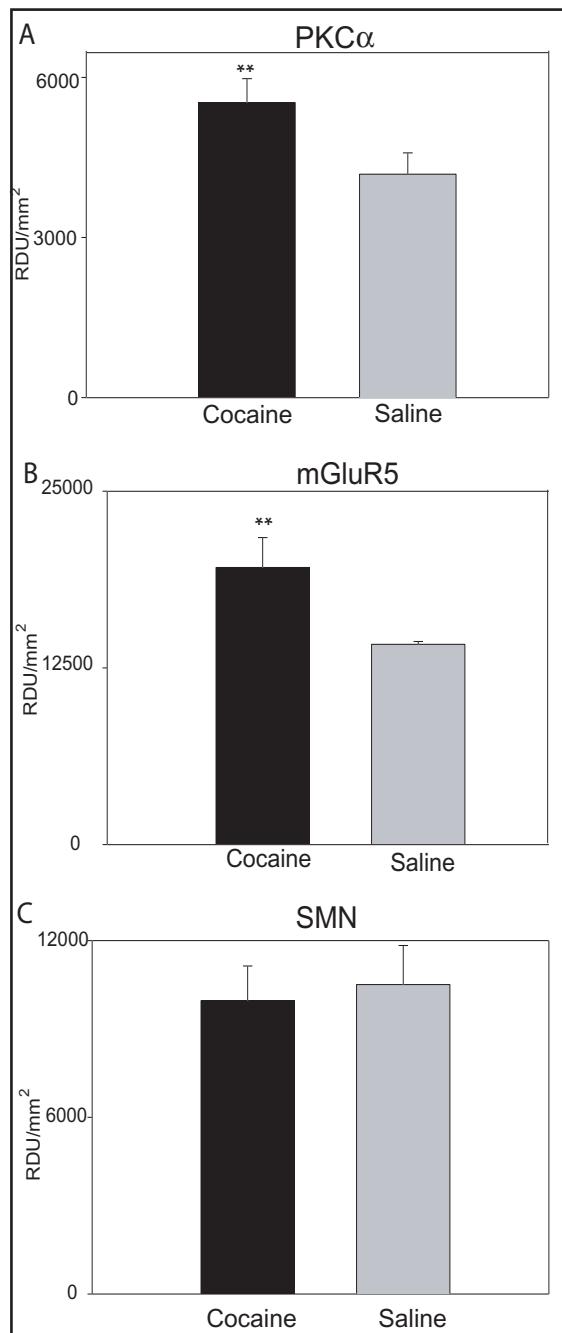


Fig. 2. Immunblot post-hoc analysis of genes consistently upregulated by 50% or more in cDNA array analysis. Results are expressed as the mean optical density of the gene-specific band for all animals in the each treatment group (n=6/group) ( $\pm$ S.E.). \* p<0.05, \*\*p<0.025 (Student's T-Test, one-tailed).

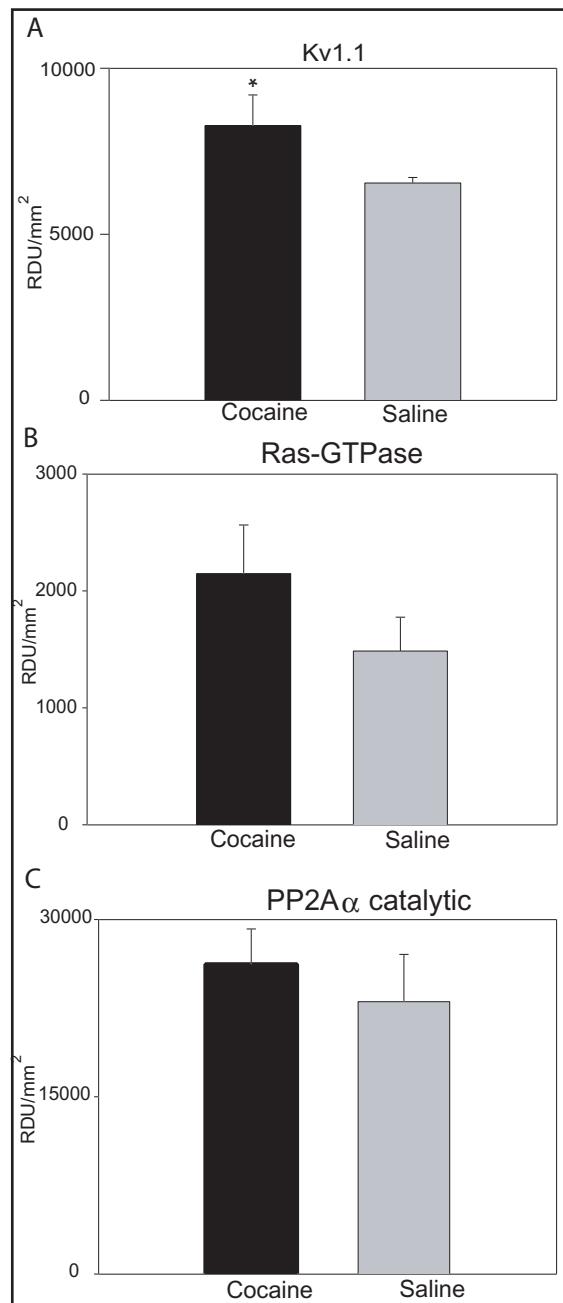


Fig. 3. Immunblot post-hoc analysis of gene consistently upregulated by less than 50% in cDNA array analysis. Results are expressed as the mean optical density of the gene-specific band for all animals in the treatment group (n=6/group) ( $\pm$ S.E.). \* p<0.05, (Student's T-Test, one-tailed).

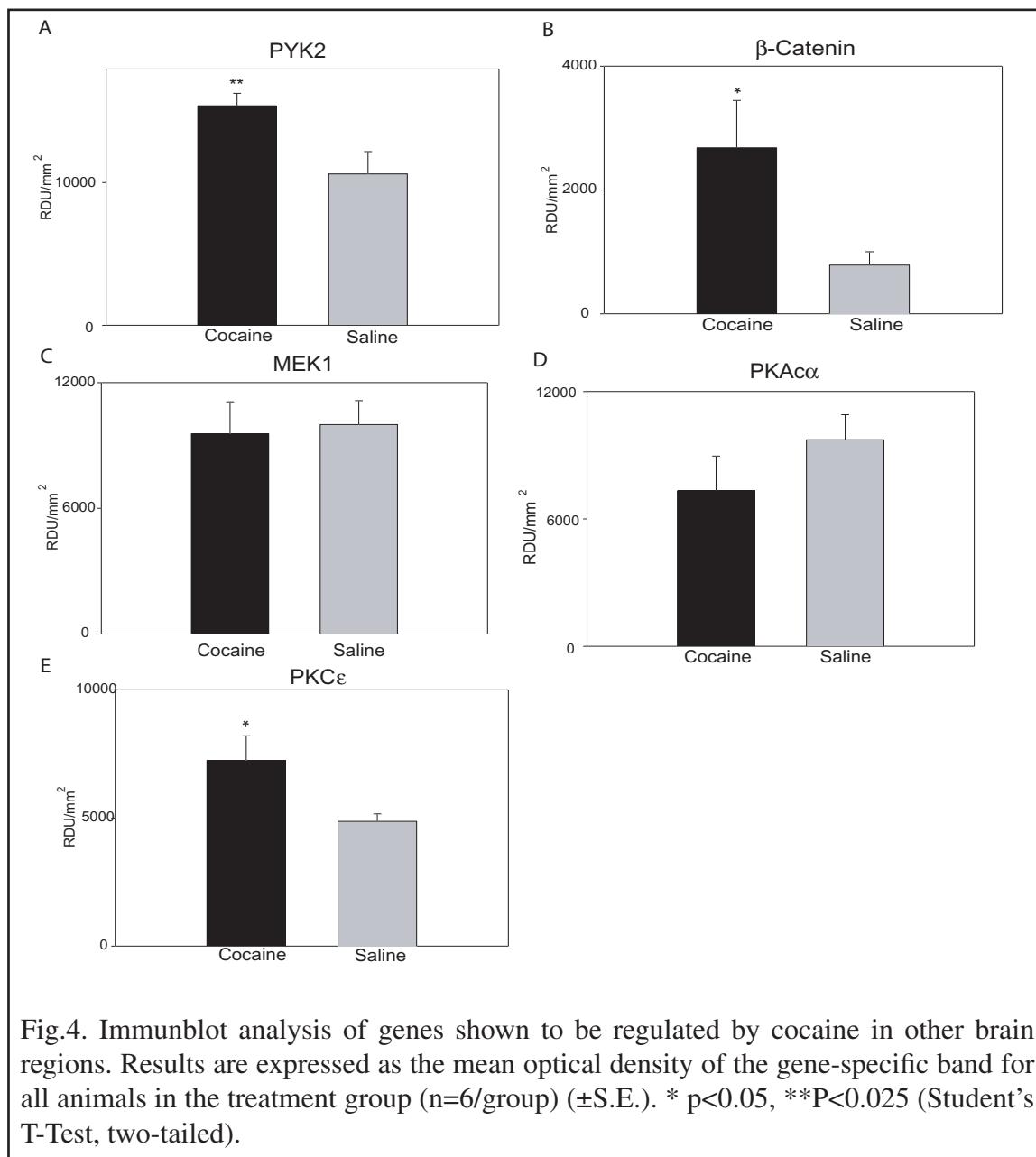


Fig.4. Immunoblot analysis of genes shown to be regulated by cocaine in other brain regions. Results are expressed as the mean optical density of the gene-specific band for all animals in the treatment group ( $n=6$ /group) ( $\pm$ S.E.). \*  $p<0.05$ , \*\* $P<0.025$  (Student's T-Test, two-tailed).

change in somatic mRNA of a protein found primarily on the axon or in the presynaptic terminal would not be detectable by our methods. Conversely, a change in protein could also be seen when there is no apparent change in mRNA. These factors could explain why some protein species seemed to be upregulated when their mRNA is unchanged (PKC $\epsilon$ ), or why some proteins were not changed while their mRNA was

apparently increased (SMN). These considerations emphasize the importance of protein post-hoc confirmation of changes in mRNA seen on hybridization arrays. In total, this study demonstrates the utility of hybridization array analysis combined with protein post-hoc confirmation.

### *Functions of proteins induced by cocaine*

PKC $\alpha$  is a member of the classical or typical family of PKC isoforms, including  $\alpha, \beta I, \beta II, \gamma$ . These PKC isoforms are characterized by Ca<sup>2+</sup>-dependence and sensitivity to diacylglycerol and phorbol esters. PKC $\epsilon$  is a member of the novel or new family of PKC isoforms ( $\delta, \epsilon, \mu, \theta, \eta$ ). Novel PKC isoforms are not Ca<sup>2+</sup>-dependent or sensitive to diacylglycerol and phorbol esters. Twenty-one-day IP administration of haloperidol, a selective D2-like dopamine receptor antagonist, decreases PKC $\alpha$  and PKC $\epsilon$  cytosolic and membrane immunoreactive protein in the rat hippocampus.<sup>14</sup> Haloperidol treatment also decreased PKC $\alpha$  and PKC $\epsilon$  mRNA and general PKC activity in the hippocampus in response to cocaine.<sup>14</sup> If taken generally, haloperidol treatment could be seen as the inverse of cocaine (an indirect dopamine agonist) and it is therefore not surprising to see an induction of these PKC isozymes in the present studies. Moreover, chronic administration of both valproate and lithium have been shown to decrease PKC $\alpha$  and PKC $\epsilon$  in the rat hippocampus,<sup>32,33</sup> demonstrating the responsiveness of these kinases to pharmacological treatment. PKC may also play a role in the behavior of cocaine as demonstrated by intracerebroventricular injections of chelerythrine, a specific PKC inhibitor, reducing cocaine-mediated conditioned place preference.<sup>8</sup>

The glutamate receptor, mGluR5, belongs to Group I (mGluR1 and mGluR5) metabotropic receptors which are positively coupled to phospholipase C through G<sub>q</sub>.<sup>41</sup> In the hippocampus mGluR5 is localized to postsynaptic elements.<sup>43</sup> The mGluR5 receptor plays an important role in learning, memory, and long-term potentiation (LTP) in the rat hippocampus. Mice lacking mGluR5 show impaired learning and

reduced long-term potentiation in hippocampal CA1 fields.<sup>30</sup> In addition, a mGluR5-selective antagonist inhibits LTP in hippocampal CA1 fields.<sup>22</sup> With chronic cocaine administration, mGluR5 has been shown to be induced in the NAcc shell and striatum.<sup>19</sup> Therefore, with the known functions of mGluR5 in the hippocampus, increased levels of this receptor could contribute to a mechanism for neuroadaptive changes.

PYK2, proline-rich tyrosine kinase 2, also known as cell adhesion kinase beta (CAK $\beta$ ), related adhesion focal tyrosine kinase (RAFTK) or focal adhesion kinase 2 (FAK2), is a non-receptor tyrosine kinase<sup>12,20</sup> which we have previously demonstrated to be induced in the non-human primate nucleus accumbens after chronic cocaine.<sup>16</sup> Expression of PYK2 is highest during adulthood in the rat; and is seen at high levels in the hippocampus, concentrated primarily in the soma and dendrites of hippocampal neurons.<sup>36</sup> PYK2 is activated by increases in intracellular calcium, integrins, PKC and by depolarization in hippocampal slices.<sup>5,13,20,29,44</sup> PYK2 also plays an important role in growth factor induced neurite outgrowth and differentiation.<sup>21,40</sup> Additionally, because of the hypothesized roles of PYK2 in long-term potentiation, synaptic plasticity, and neuronal survival, it may have relevant actions outside of signal transduction.<sup>2,20</sup>

$\beta$ -Catenin, also known as cadherin-associated protein beta, plays a role in both adherin junctions and interacts in the nucleus with the lymphoid enhancer-binding factor, ternary complex factor, and ets oncogene proteins (ELK) to stimulate the Wnt target genes.<sup>3,15,53</sup> In rat hippocampal cultures, overexpression of  $\beta$ -Catenin has been shown to stimulate c-jun and fra-1 gene expression.<sup>24,34</sup>  $\beta$ -Catenin is induced

by cocaine<sup>16</sup> and cocaine has also been shown to increase ELK-1 phosphorylation acutely in the NAcc.<sup>48</sup> Furthermore, the increase in  $\beta$ -Catenin protein is interesting in light of its interaction with cyclin-dependent kinase 5 (Cdk5). Cdk5 has been shown to increase in the NAcc and prefrontal cortex after chronic cocaine (Bibb, J.A., et al. In press, Nature), after fosB transgenic induction, and in an activator protein complex 1 (AP-1) dependent manner.<sup>9</sup> Because Cdk5 can destabilize cadherin -  $\beta$ -Catenin association it could lead to decreases in cell adhesion.<sup>26</sup>  $\beta$ -Catenin could also play a role in increasing Cdk5 expression because of its ability to increase transcription factors which bind to the AP-1 complex. The

fact that  $\beta$ -Catenin and Cdk5 regulate one another and are both induced by cocaine reveals a potentially important pathway.

Kv1.1 is a member of the shaker family of potassium channel  $\alpha$  subunits. Fully functional potassium channels are composed of four subunits which may be homomeric or heteromeric with other subunits. Kv1.1 channels give rise to delayed-rectifying currents through K<sup>+</sup> outflow in a fast-activating, slow-inactivating manner.<sup>35</sup> Kv1.1 is widely expressed in the mouse brain and in the hippocampus Kv1.1 is most often found on axons or at synaptic terminals.<sup>51</sup> Heterotetramers containing Kv1.1 and other subunits are more likely to be expressed at the cell surface as opposed to

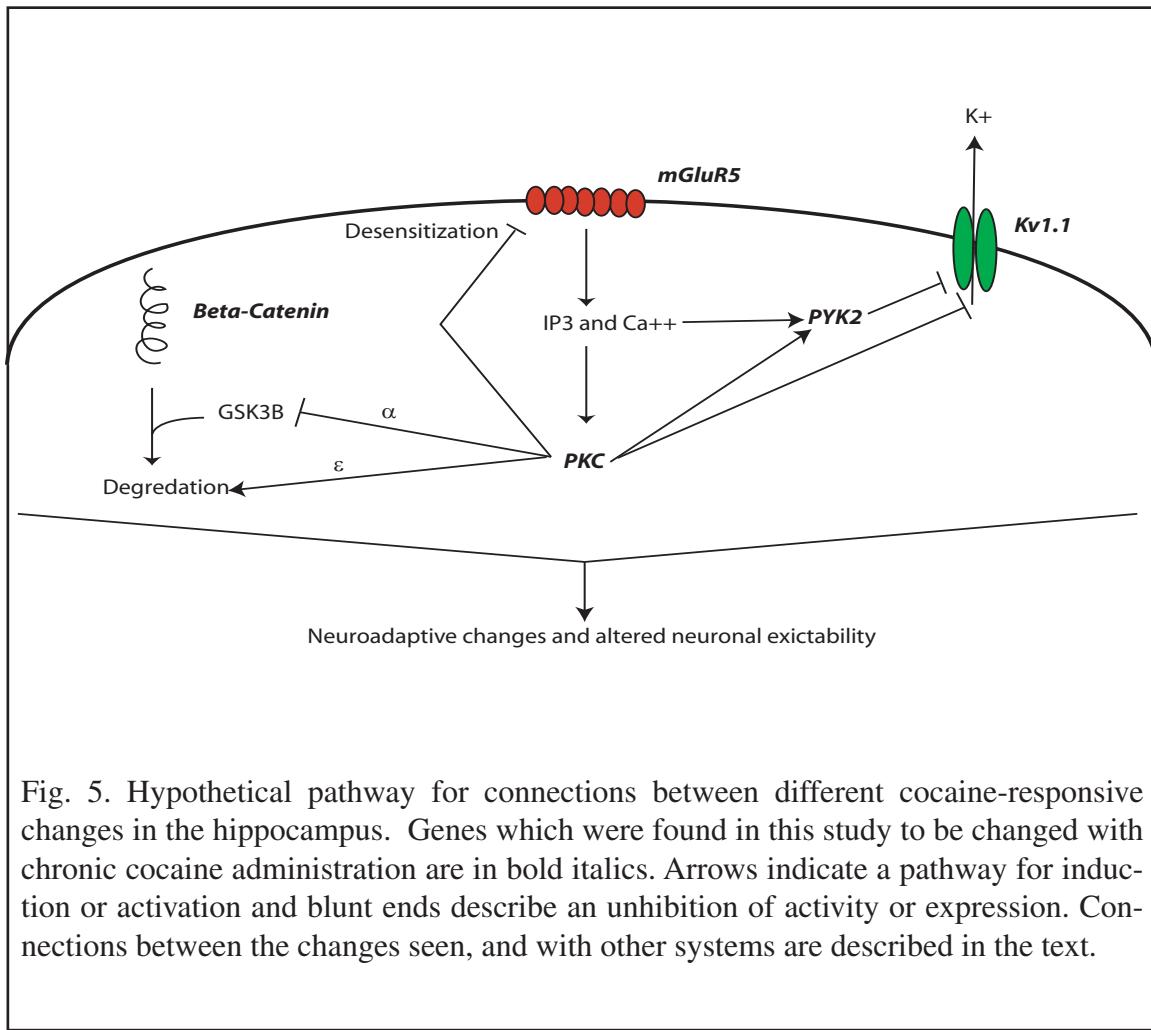


Fig. 5. Hypothetical pathway for connections between different cocaine-responsive changes in the hippocampus. Genes which were found in this study to be changed with chronic cocaine administration are in bold italicics. Arrows indicate a pathway for induction or activation and blunt ends describe an inhibition of activity or expression. Connections between the changes seen, and with other systems are described in the text.

the endoplasmic reticulum.<sup>31</sup> Blockade of Kv1.1 and Kv1.3 in an olfactory learning paradigm improved associative learning in rats.<sup>25</sup>

#### *Interactions between proteins induced by cocaine*

In addition to the individual actions of each of these genes, there are a number of connections between the observed changes (Figure 5). mGluR5 works through G<sub>q</sub> to increase phospholipase C activity, which generates diacylglycerol and releases Ca<sup>2+</sup> from internal stores, and in turn increases PKC activity. At the same time, mGluR5 has been shown to be desensitized by glutamate through a PKC mechanism in a Xenopus model.<sup>18</sup> Phorbol ester-sensitive PKC isoforms, like PKC $\alpha$ , may be involved in the accumulation of  $\beta$ -Catenin through inactivation of the glycogen synthase kinase-3 mediated  $\beta$ -Catenin degradation.<sup>11</sup> Diacylglycerol independent PKC isoforms (novel and atypical classes), like PKC $\epsilon$ , may decrease  $\beta$ -Catenin by targeting it for ubiquination and degradation. In PC12 cells, overexpression of PYK2 inhibits K<sup>+</sup> channels via increased phosphorylation of tyrosine residues.<sup>29</sup> Activation of PYK2 by Ca<sup>2+</sup>, is thought to occur through an indirect, PKC-mediated mechanism.<sup>44</sup> Kv1.1 channel currents have been shown to be inhibited by PKC through an indirect g-protein dependent mechanism.<sup>6</sup> Figure 5 demonstrates a post-synaptic model in which some changes show an antagonistic relationship. For example, there is both an increase in Kv1.1 protein and an increase in PKC( $\alpha$  and  $\epsilon$ ) and PYK2 which are respectively, indirect and direct, negative regulators of Kv1.1 channel currents.  $\beta$ -Catenin degradation is induced by PKC isoforms like PKC $\epsilon$  but indirectly inhibited by typical PKC isoforms like PKC $\alpha$ . These inductions may

therefore represent both initial responses to chronic drug administration and secondary compensatory mechanisms.

Taken as a whole, the changes in hippocampal gene expression seen after chronic cocaine administration point towards changes in neuronal plasticity. Some of these changes (PYK2,  $\beta$ -Catenin, and mGluR5) have been seen with cocaine administration in other animal models and brain regions. Because these common changes occur in different regions with distinct neuronal cell types and functions, they may reflect specific molecular substrates of neuronal plasticity. Other changes in protein seen in this study are novel to the cocaine literature (PKC $\alpha$ , PKC $\epsilon$ , Kv1.1). It is not possible from this study to determine the time course of changes but their connections warrant further investigation. More examination of these changes will be needed to assess the functions of these proteins in the behavioral aspects of cocaine abuse, but because of the role of the hippocampus in learning and memory, these changes may represent molecular aspects of learning associated with cocaine administration.

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**References:**

1. Anderson L. and Seilhamer J. (1997) A comparison of selected mRNA and protein abundances in human liver. *Electrophoresis* 18, 533-537.
2. Avraham H., Park S.Y., Schinkmann K., Avraham S., Tyrosine k., Raftk, Fak, and Signalling (2000) RAFTK/Pyk2-mediated cellular signalling. *Cellular Signalling* 12, 123-133.
3. Ben-Ze'ev A. and Geiger B. (1998) Differential molecular interactions of beta-catenin and plakoglobin in adhesion, signalling and cancer. *Curr. Opin. Cell Bio.* 10, 629-639.
4. Bennett S.A., Arnold J.M., Chen J., Stenger J., Paul D.L., and Roberts D.C. (1999) Long-term changes in connexin32 gap junction protein and mRNA expression following cocaine self-administration in rats. *Eur. J. Neurosci.* 11, 3329-3338.
5. Blaukat A., Ivankovic-Dikic I., Gronroos E., Dolfi F., Tokiwa G., Vuori K., and Dikic I. (1999) Adaptor proteins Grb2 and Crk couple Pyk2 with activation of specific mitogen-activated protein kinase cascades. *J. Biol. Chem.* 274, 14893-14901.
6. Boland L.M. and Jackson K.A. (1999) Protein kinase C inhibits Kv1.1 potassium channel function. *Am. J. Physiol.* 2770, C100-C110.
7. Carlezon W.A., Thome J., Olson V.G., Lane-Ladd S.B., Brodkin E.S., Hiroi N., Duman R.S., Neve R.L., and Nestler E.J. (1998) Regulation of cocaine reward by CREB. *Science* 282, 2272-2275.
8. Cervo L., Mukherjee S., Bertaglia A., and Samanin R. (1997) Protein kinases A and C are involved in the mechanisms underlying consolidation of cocaine place conditioning. *Brain Res.* 775, 30-36.
9. Chen J., Zhang Y., Kelz M.B., Steffen C., Ang E.S., Zeng L., and Nestler E.J. (2000) Induction of cyclin-dependent kinase 5 in the hippocampus by chronic electro-convulsive seizures: role of DeltaFosB. *J. Neurosci.* 20, 8965-8971.
10. Chomczynski P. and Sacchi N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate- phenol-chloroform extraction. *Anal. Biochem.* 162, 156-159.
11. Cook D., Fry M.J., Hughes K., Sumathipala R., Woodgett J.R., and Dale T.C. (1996) Wingless inactivates glycogen synthase kinase-3 via an intracellular signalling pathway which involves a protein kinase C. *EMBO J.* 15, 4526-4536.
12. Derkinderen P., Enslen H., and Girault J.A. (1999) The ERK/MAP-kinases cascade in the nervous system. *Neuroreport* 10, R24-R34.
13. Derkinderen P., Siciliano J., Toutant M., and Girault J.A. (1998) Differential regulation of FAK+ and PYK2/Cakbeta, two related tyrosine kinases, in rat hippocampal slices: effects of LPA, carbachol, depolarization and hyperosmolarity. *Eur. J. Neurosci.* 10, 1667-1675.
14. Dwivedi Y. and Pandey G.N. (1999) Effects of treatment with haloperidol, chlorpromazine, and clozapine on protein kinase C (PKC) and phosphoinositide-specific phos-

- pholipase C (PI-PLC) activity and on mRNA and protein expression of PKC and PLC isozymes in rat brain. *J. Pharmacol. Exp. Ther.* 291, 688-704.
15. Eastman Q. and Grosschedl R. (1999) Regulation of LEF-1/TCF transcription factors by Wnt and other signals. *Curr. Opin. Cell Bio.* 11, 233-240.
16. Freeman W.M., Nader M.A., Nader S.H., Robertson D.J., Gioia L., Mitchell S.M., Porrino L.J., Friedman D.P., and Vrana K.E. (2001) Chronic Cocaine-Mediated Changes in Non-Human Primate Nucleus Accumbens Gene Expression. *J. Neurochem.* In Press
17. Freeman W.M., Robertson D.J., and Vrana K.E. (2000) Fundamentals of DNA hybridization arrays for gene expression analysis. *Biotechniques* 29, 1042-1055.
18. Gereau R.W. and Heinemann S.F. (1998) Role of protein kinase C phosphorylation in rapid desensitization of metabotropic glutamate receptor 5. *Neuron* 20, 143-151.
19. Ghasemzadeh M.B., Nelson L.C., Lu X.Y., and Kalivas P.W. (1999) Neuroadaptations in ionotropic and metabotropic glutamate receptor mRNA produced by cocaine treatment. *J. Neurochem.* 72, 157-165.
20. Girault J.A., Costa A., Derkinderen P., Studler J.M., and Toutant M. (1999) FAK and PYK2/CAKbeta in the nervous system: a link between neuronal activity, plasticity and survival? *Trends in Neurosciences* 22, 257-263.
21. Ivankovic-Dikic I., Gronroos E., Blaukat A., Barth B.U., and Dikic I. (2000) Pyk2 and FAK regulate neurite outgrowth induced by growth factors and integrins. *Nat. Cell Biol.* 2, 574-581.
22. Izumi Y., Zarrin A.R., and Zorumski C.F. (2000) Arachidonic acid rescues hippocampal long-term potentiation blocked by group I metabotropic glutamate receptor antagonists. *Neuroscience* 100, 485-491.
23. Kelz M.B., Chen J., Carlezon W.A., Whisler K., Gilden L., Beckmann A.M., Steffen C., Zhang Y.J., Marotti L., Self D.W., Tkatch T., Baranauskas G., Surmeier D.J., Neve R.L., Duman R.S., Picciotto M.R., and Nestler E.J. (1999) Expression of the transcription factor deltaFosB in the brain controls sensitivity to cocaine. *Nature* 401, 272-276.
24. Kikuchi A. (2000) Regulation of beta-catenin signaling in the Wnt pathway. *Biochem. Biophys. Res. Comm.* 268, 243-248.
25. Kourrich S., Mourre C., and Soumireu-Mourat B. (2001) Kaliotoxin, a Kv1.1 and Kv1.3 channel blocker, improves associative learning in rats. *Behav. Brain Res.* 120, 35-46.
26. Kwon Y.T., Gupta A., Zhou Y., Nikolic M., and Tsai L.H. (2000) Regulation of N-cadherin-mediated adhesion by the p35-Cdk5 kinase. *Curr. Biol.* 10, 363-372.
27. Laemmli U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685.
28. Letchworth S.R., Sexton T., Childers S.R., Vrana K.E., Vaughan R.A., Davies H.M., and Porrino L.J. (1999) Regulation of

- rat dopamine transporter mRNA and protein by chronic cocaine administration. *J. Neurochem.* 73, 1982-1989.
29. Lev S., Moreno H., Martinez R., Canoll P., Peles E., Musacchio J.M., Plowman G.D., Rudy B., and Schlessinger J. (1995) Protein tyrosine kinase PYK2 involved in Ca(2+)-induced regulation of ion channel and MAP kinase functions. *Nature* 376, 737-745.
30. Lu Y.M., Jia Z., Janus C., Henderson J.T., Gerlai R., Wojtowicz J.M., and Roder J.C. (1997) Mice lacking metabotropic glutamate receptor 5 show impaired learning and reduced CA1 long-term potentiation (LTP) but normal CA3 LTP. *J. Neurosci.* 17, 5196-5205.
31. Manganas L.N. and Trimmer J.S. (2000) Subunit composition determines Kv1 potassium channel surface expression. *J. Biol. Chem.* 275, 29685-29693.
32. Manji H.K., Bebchuk J.M., Moore G.J., Glitz D., Hasanat K.A., and Chen G. (1999) Modulation of CNS signal transduction pathways and gene expression by mood-stabilizing agents: therapeutic implications. *J. Clin. Psychiatry* 60 Suppl 2, 27-39.
33. Manji H.K., Bersudsky Y., Chen G., Belmaker R.H., and Potter W.Z. (1996) Modulation of protein kinase C isozymes and substrates by lithium: the role of myoinositol. *Neuropsychopharmacol.* 15, 370-381.
34. Mann B., Gelos M., Siedow A., Hanski M.L., Gratchev A., Ilyas M., Bodmer, WF, Moyer M.P., Riecken E.O., Buhr H.J., and Hanski C. (1999) Target genes of beta-catenin-T cell-factor/lymphoid-enhancer-factor signaling in human colorectal carcinomas. *Proc. Natl. Acad. Sci. U.S.A.* 96, 1603-1608.
35. Mathie A., Wooltorton J.R., and Watkins C.S. (1998) Voltage-activated potassium channels in mammalian neurons and their block by novel pharmacological agents. *Gen. Pharmacol.* 30, 13-24.
36. Menegon A., Burgaya F., Baudot P., Dunlap D.D., Girault J.A., and Valtorta F. (1999) FAK+ and PYK2/CAKbeta, two related tyrosine kinases highly expressed in the central nervous system: similarities and differences in the expression pattern. *Eur. J. Neurosci.* 11, 3777-3788.
37. Nestler E.J. (2001) Molecular basis of long-term plasticity underlying addiction. *Nature Neurosci. Reviews* 2, 119-128.
38. O'Donnell P. and Grace A.A. (1995) Synaptic interactions among excitatory afferents to nucleus accumbens neurons: hippocampal gating of prefrontal cortical input. *J. Neurosci.* 15, 3622-3639.
39. Onaivi E.S., Bishop-Robinson C., Motley E.D., Chakrabarti A., and Chirwa S.S. (1996) Neurobiological actions of cocaine in the hippocampus. *Ann. N. Y. Acad. Sci.* 801, 76-94.
40. Park S.Y., Avraham H., and Avraham S. (2000) Characterization of the tyrosine kinases RAFTK/Pyk2 and FAK in nerve growth factor-induced neuronal differentiation. *J. Biol. Chem.* 275, 19768-19777.
41. Pin J.P. and Duvoisin R. (1995) The metabotropic glutamate receptors: structure and functions. *Neuropharmacol.* 34, 1-26.

42. Sevarino K.A. and Primus R.J. (1993) Cocaine regulation of brain prepro-thyrotropin-releasing hormone mRNA. *J. Neurochem.* 60, 1151-1154.
43. Shigemoto R., Kinoshita A., Wada E., Nomura S., Ohishi H., Takada M., Flor P.J., Neki A., Abe T., Nakanishi S., and Mizuno N. (1997) Differential presynaptic localization of metabotropic glutamate receptor subtypes in the rat hippocampus. *J. Neurosci.* 17, 7503-7522.
44. Siciliano J.C., Toutant M., Derkinderen P., Sasaki T., and Girault J.A. (1996) Differential regulation of proline-rich tyrosine kinase 2/cell adhesion kinase beta (PYK2/CAKbeta) and pp125(FAK) by glutamate and depolarization in rat hippocampus. *J. Biol. Chem.* 271, 28942-28946.
45. Smith P.K., Krohn R.I., Hermanson G.T., Mallia A.K., Gartner F.H., Provenzano M.D., Fujimoto E.K., Goeke N.M., Olson B.J., and Klenk D.C. (1985) Measurement of protein using bicinchoninic acid. *Anal. Biochem.* 150, 76-85.
46. Spangler R., Unterwald E.M., and Kreek M.J. (1993) 'Binge' cocaine administration induces a sustained increase of prodynorphin mRNA in rat caudate-putamen. *Brain Res. Mol. Brain Res.* 19, 323-327.
47. Unterwald E.M., Ho A., Rubenfeld J.M., and Kreek M.J. (1994) Time course of the development of behavioral sensitization and dopamine receptor up-regulation during binge cocaine administration. *J. Pharmacol. Exp. Ther.* 270, 1387-1396.
48. Valjent E., Corvol J.C., Pages C., Besson M.J., Maldonado R., and Caboche J. (2000) Involvement of the extracellular signal-regulated kinase cascade for cocaine-rewarding properties. *J. Neurosci.* 20, 8701-8709.
49. Volkow N.D. and Fowler J.S. (2000) Addiction, a disease of compulsion and drive: Involvement of the orbitofrontal cortex. *Cerebral Cortex* 10, 318-325.
50. Vrana S.L., Vrana K.E., Kovacs T.R., Smith J.E., and Dworkin S.I. (1993) Chronic cocaine administration increases CNS tyrosine hydroxylase enzyme activity and mRNA levels and tryptophan hydroxylase enzyme activity levels. *J. Neurochem.* 61, 2262-2268.
51. Wang H., Kunkel D.D., Schwartzkroin P.A., and Tempel B.L. (1994) Localization of Kv1.1 and Kv1.2, two K channel proteins, to synaptic terminals, somata, and dendrites in the mouse brain. *J. Neurosci.* 14, 4588-4599.
52. White F.J. and Kalivas P.W. (1998) Neuroadaptations involved in amphetamine and cocaine addiction. *Drug Alcohol Depend.* 51, 141-153.
53. Willert K. and Nusse R. (1998) Beta-catenin: a key mediator of Wnt signaling. *Curr. Opin. in Gene. & Develop.* 8, 95-102.

# **Chapter 4: Changes in Rat Frontal Cortex Gene Expression Following Chronic Cocaine**

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## **Abstract:**

Alterations in gene expression caused by chronic cocaine administration have been implicated in the behavioral aspects of cocaine abuse. The frontal cortex plays an important role in the mesocortical dopamine system which has reinforcement, sensory, associative, and executive functions. Rats treated non-contingently with a binge model of cocaine (45mg/kg/day, i.p.) for fourteen days were screened for changes in relative mRNA abundance by cDNA hybridization arrays. To confirm changes, immunoreactive protein was measured (via specific immunoblots). Protein tyrosine kinase 2 (PYK2), activity regulated cytoskeletal protein (ARC), and an antigen related to nerve growth factor 1-B (NGF1B) were shown to be significantly induced after cocaine administration. These findings have potential roles in synaptic plasticity, cell signaling and the regulation of other genes.

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The following chapter is in preparation for *Neuroreport*. Stylistic variations are due to the requirements of the journal and publisher. Karen Brebner, Wendy J. Lynch and David C.S. Roberts were responsible for execution of the animal treatment. Daniel J. Robertson assisted in the performance of the hybridization array and immunoblot protocols. Kent E. Vrana served in an advisory position on experimental design and manuscript preparation. Willard M. Freeman performed all other experimental work.

## Introduction:

Changes in gene expression are hypothesized to be one of the mediators of the behavioral effects of chronic drug abuse (Nestler 2001). For example, using inducible transgenic  $\Delta$ fosB mice, differences in behavioral sensitization to cocaine have been seen (Kelz et al. 1999; Bibb et al. 2001). Alterations in gene expression could also change the functionality of neurons, (e.g. electrophysiology) or cascades involved in synaptic remodeling/plasticity. The finding that both chronic non-contingent and chronic self-administered cocaine alter neuronal morphology establishes the potential that changes in gene expression could be a mechanism by which these remodeling events take place (Robinson and Kolb 1999; Robinson et al. 2001).

Many of the previous studies of cocaine-responsive gene expression have focused on the anatomical compartments of the striatum (caudate and putamen), ventral tegmental area (VTA) and nucleus accumbens (NAcc), because of their dopaminergic cell content and the results of lesion studies that demonstrated altered drug self-administration after anatomical ablation (Roberts

et al. 1977). Another dopaminergic region also studied is the frontal cortex. The frontal cortex has extensive connections with ‘reward’-related regions. The frontal cortex receives indirect afferents from the mesolimbic dopamine system (VTA-NAcc) through the thalamus and sends efferents to the NAcc (Volkow and Fowler 2000). In addition, the frontal cortex has reciprocal links with the amygdala and hippocampus. Beyond these direct connections with regions involved in reinforcement, the associative, sensory, and executive functions of the frontal cortex could play a role in behaviors associated with drug abuse (Volkow and Fowler 2000). Previously, changes in the expression of genes, such as GluR2 (Ghasemzadeh et al. 1999), have been observed in the frontal cortex after chronic cocaine administration.

To assess changes in gene expression on a larger scale, cDNA hybridization arrays (Freeman et al. 2000) were used to analyze changes in gene expression after a ‘binge’ model (Spangler et al. 1993) of non-contingent cocaine administration. Subsequent immunoblot post-hoc analysis was used to statistically confirm changes at the level of protein.

## Material and Methods:

### *Animals*

Male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing an average of 300g were individually housed in polypropylene cages; placed on a 12hr light:dark cycle (lights on at 900 h) and were allowed to acclimate to the facility for 7 days before the start of the experiment. Food and water were available ad libitum throughout the experiment. Care and treatment of all animals conformed to the standards and guidelines promulgated by the Wake Forest

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**Abbreviations:** ARC, activity regulated cytoskeletal protein; CKII $\alpha$ , casein kinase II $\alpha$ ; CREBP, cyclic AMP response element binding protein; FRA-1, fos-related antigen 1; GluR1, ionotropic glutamate receptor 1; Kv1.1 shaker-related voltage-gated potassium channel member; MAPK, mitogen activated protein kinase; MEK1, mitogen activated protein kinase kinase 1; mGluR5, metabotropic glutamate receptor 5; NAcc, nucleus accumbens; NGF1-B, nerve growth factor 1-B; NGF1B-RA, nerve growth factor 1-B related antigen; PKA $\alpha$ , protein kinase A  $\alpha$  catalytic subunit; PKC $\alpha$ , protein kinase C alpha; PKCe, protein kinase C epsilon; PYK2, protein tyrosine kinase 2; ventral tegmental area, VTA

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University Animal Care and Use Committee and the National Institutes of Health.

#### *Procedure*

Two treatment groups (n=6) of randomly-assigned rats received i.p. injections of cocaine or saline for 14 days. Rats in each group received 3 *i.p.* injections of cocaine HCl (15 mg/kg), or saline (1ml/kg) on an hourly schedule beginning at 10:00. Rats were deeply anaesthetized with sodium pentobarbital (*i.p.*) and decapitated thirty minutes after the final cocaine or saline injection. Brains were rapidly removed and placed in an ice-chilled ASI brain slicer (ASI Instruments, Warren MI). The dissected frontal cortex consisted of the cortex from +2.2mm rostral of the anterior commissure with the olfactory bulbs removed. A second and independent set of identically-treated animals (n=6 per treatment group) were used for protein immunoblot confirmations.

#### *Drugs*

Cocaine HCl was supplied by the National Institute on Drug Abuse (Research Triangle, NC) and dissolved in 0.9% sterile saline. Dosages are expressed as the salt.

#### *RNA Isolation and DNA hybridization arrays*

Frontal cortex from each animal was ground with liquid nitrogen in a dry ice chilled mortar. Total RNA was isolated from the ground tissue of each animal and hybridization array analysis was performed in triplicate as previously described (Freeman et al. 2001b; Freeman et al. 2001a). Atlas Rat 1.2 (I) Atlas cDNA arrays (Clontech, Palo Alto, CA) were used which contain gene fragments corresponding to 1176 known genes. The arrays' radioactive signals were detected with a Storm Phosphorimager (Molecular Dynamics, Sunnyvale,

CA) and quantified using GLEAMS (NuTec Services, Stafford TX) and Atlas Image2.0 (Clontech) array analysis software. Background and normalization factors were generated as previously described (Freeman et al. 2001b; Freeman et al. 2001a). The complete data set is available at the Drug and Alcohol Abuse Array Data Consortium website ([www.arraydata.org](http://www.arraydata.org)).

#### *Immunoblotting*

Frontal cortex from the second set of identically-treated animals was ground with liquid nitrogen in a dry ice-chilled mortar. A portion of the dry homogenate was then homogenized by sonication in protein buffer. Total protein concentration was determined with the bicinchoninic acid assay (BCA protein assay, Pierce Chemical, Rockford IL) and equal amounts of protein from each animal (30-75 $\mu$ g) were resolved by denaturing polyacrylamide gel electrophoresis as previously described (Freeman et al. 2001a). Protein was then transferred to a polyvinylidene fluoride membrane (Immobilon P, Millipore, Bedford, MA) by semidry transfer (TE-70, Amersham Pharmacia Biotech, Piscataway, NJ). Immunoreactive protein corresponding to each proteins molecular weight was detected with antibodies at the following concentrations: ARC (55kDa) 0.66  $\mu$ g/mL (A43220, Transduction Laboratories, Lexington, KY);  $\beta$ -Catenin (92kDa) 0.33 $\mu$ g/mL (C19220, Transduction Laboratories); casein kinase II  $\alpha$ , CKII $\alpha$  (45kDa) 0.5 $\mu$ g/mL (C11320, Transduction Laboratories); fos-related antigen 1, FRA-1, (46kDa) 1 $\mu$ g/mL (SC-605, Santa Cruz Biotechnology, Santa Cruz, CA); glycogen synthase kinase 3 alpha/beta, GSK3  $\alpha/\beta$ , (51 and 47kDa) 1 $\mu$ g/mL (MAB3195, Chemicon, Temecula, CA); potassium channel Kv1.1 (90kDa) 1 $\mu$ g/ml (05407, Upstate Biotechnology, Lake Placid,

NY); mitogen-activated protein kinase kinase 1, MEK1, (45kDa) 0.165 $\mu$ g/mL (M17020, Transduction Laboratories); metabotropic glutamate receptor 5, mGluR5, (120kDa) 0.8 $\mu$ g/mL (06451, Upstate Biotechnology); NGF1-B, (77kDa) 2 $\mu$ g/mL (1600045, Geneka Biotechnology, Montréal, Québec, Canada) and 2 $\mu$ g/mL (55408, BD PharMingen, San Diego, CA); protein kinase A alpha catalytic subunit, PKA $\alpha$ , (40kDa) 0.165 $\mu$ g/mL (P73420, Transduction Laboratories, Lexington, KY); protein kinase C alpha, PKC $\alpha$ , (82kDa) 0.165 $\mu$ g/mL (P16520, Transduction Laboratories); protein kinase C epsilon, PKC $\epsilon$ , (90kDa) 0.165 $\mu$ g/mL (P14820, Transduction Laboratories); protein tyrosine kinase 2, PYK2, (116kDa) 0.165 $\mu$ g/mL (P47120, Transduction Laboratories). Immunoreactive bands were visualized using a horseradish peroxidase-coupled secondary antibody, either mouse or rabbit IgG (Amersham Pharmacia Biotech) and a chemiluminescent substrate (Pico Signal, Pierce Chemical). All immunoblot signal values were quantified by transmissive densitometry (TINA, Fuji Medical Systems, Stamford, CA). All values are expressed as a mean  $\pm$  S.E.M. Immunoblots were analyzed with a two-tailed student's t-test (with a 0.05 level of significance).

## Results:

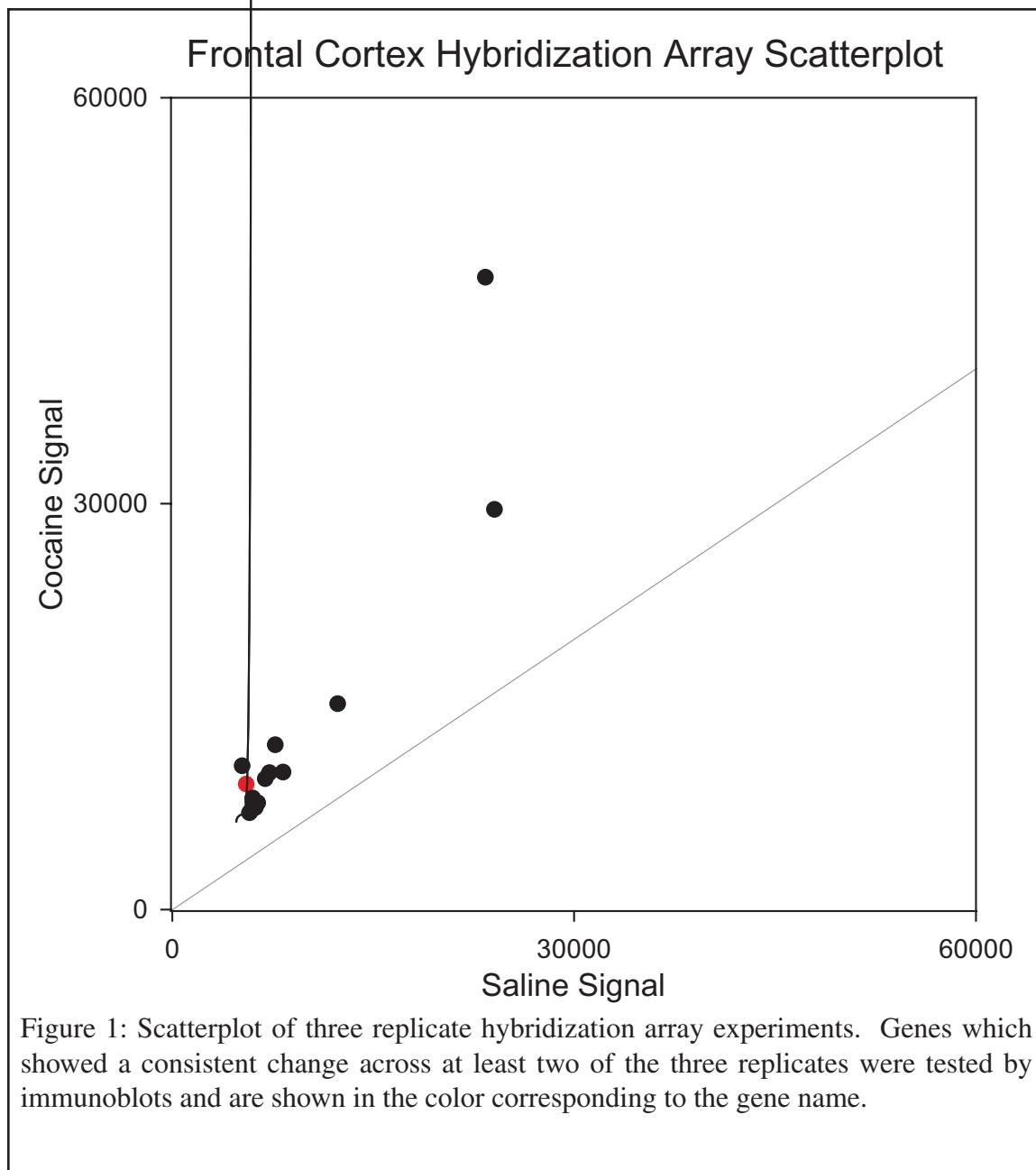
### *Hybridization array analysis*

cDNA hybridization array analysis, performed in triplicate, produced several potential cocaine-regulated transcripts (Figure 1). 461 genes presented a signal 50% above background in at least one of the three replicate experiments. The remaining genes were either not expressed in the frontal cortex or at a level below detectability. Our previous work with cocaine-responsive

gene expression and cDNA hybridization arrays has established that this technology can reliably detect 50% inductions and 33% reductions (equivalent magnitude changes on a natural log scale) (Freeman et al. 2001b). Hybridization array analysis showed ARC and NGF1-B as potentially up-regulated genes. ARC signal was present on all three replicates and showed an average cocaine-to-saline signal ratio of 1.7. NGF1-B signal ratio did not exceed the 50% threshold but consistently showed a cocaine to saline signal ratio of 1.4, and was therefore chosen for post-hoc confirmation. Three potential down-regulated transcripts, CKII $\alpha$ , GSK-3 $\alpha$  and FRA-1, were chosen for post-hoc confirmation. CK II signal was present on all three replicates and showed an average cocaine-to-saline signal ratio of 0.7. GSK-3 $\alpha$  showed a greater than 33% decrease on two replicates, but a 25% increase on the other replicate. FRA1 signal was 50% above background on two replicates with an average cocaine to saline ratio of 0.6. Two potentially cocaine-responsive genes, Von Ebner's gland protein 2, and cytosolic HMG-CoA synthase 1 were not confirmed at the level of protein due to a lack of available antibodies. Primary hybridization array data are available from the Drug and Alcohol Abuse Microarray Data Consortium ([www.arraydata.org](http://www.arraydata.org)) and the Gene Expression Omnibus ([www.ncbi.nlm.nih.gov/geo/](http://www.ncbi.nlm.nih.gov/geo/)).

### *Immunoblot Analysis*

Specific immunoblots were performed on equal amounts of total protein from individual samples of each animal. In addition to testing the targets identified by hybridization analysis (ARC, NGF1-B, CK II $\alpha$ , GSK-3 $\alpha$  and FRA1), eight additional genes (PYK2, PKA $\alpha$ , MEK1,  $\beta$ -Catenin, mGluR5, PKC $\alpha$ , PKC $\epsilon$ , and Kv1.1) which



we have previously documented to be regulated by chronic cocaine (Freeman et al. 2001a; Freeman et al. 2001b) were tested for changes in immunoreactive protein. PYK2 and PKA<sub>cα</sub> did not show a specific signal above background on any of the replicates.

A signal was present in all three replicates for mGluR5, but the cocaine to saline signal ranged widely from 0.88 to 1.75. PKC $\alpha$ , PKC $\epsilon$ , and Kv1.1 presented a signal in

all three replicates and MEK1 showed a signal on two replicates, but none of these genes showed expression ratios outside of the cutoff range (<1.5 and >0.66; cocaine-to-saline).  $\beta$ -Catenin was not present on the hybridization arrays used.

ARC immunoreactive protein was increased by 35% compared to controls ( $p=0.05$ ). NGF1-B was not detected by either of the antibodies used under a

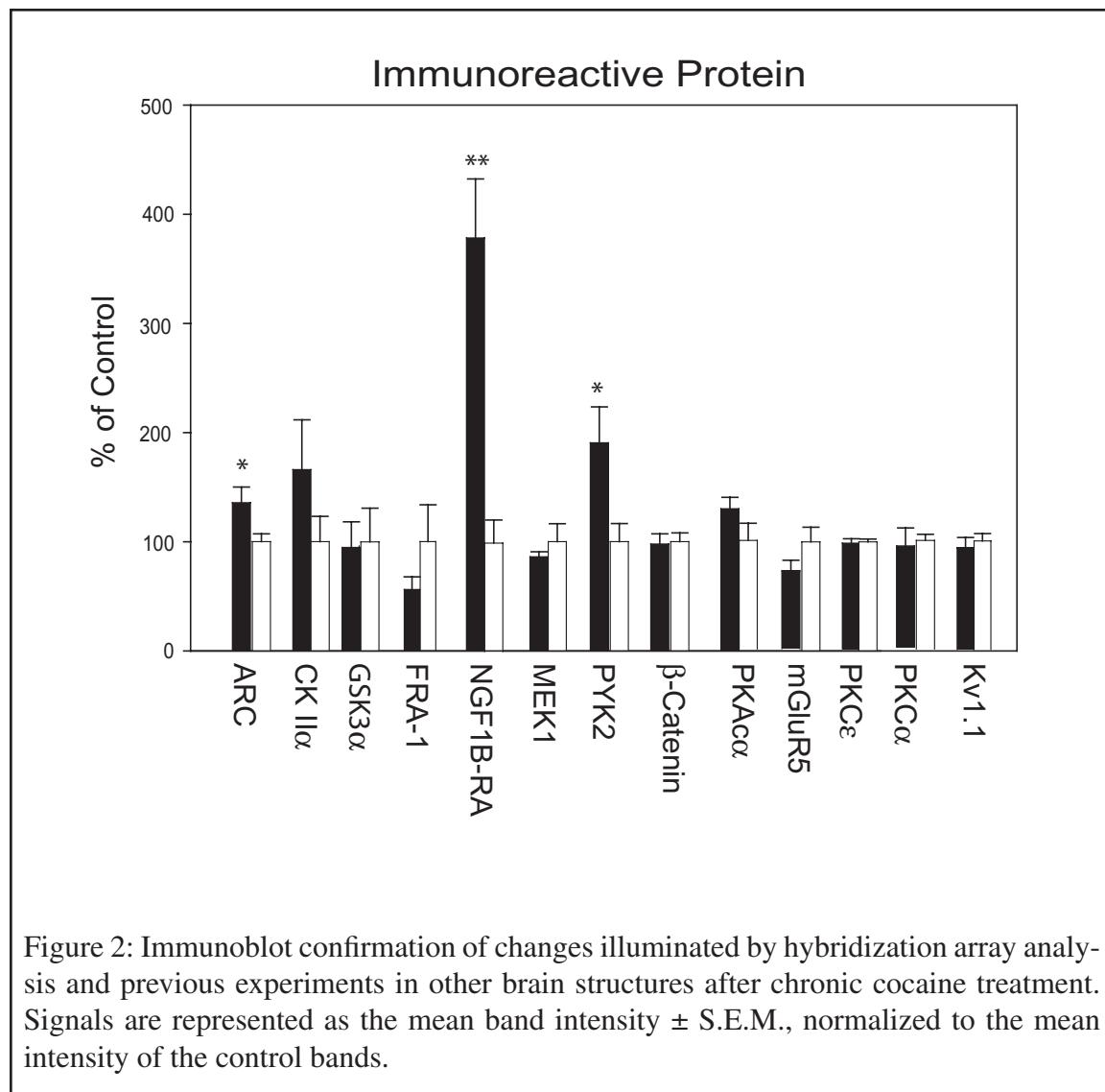


Figure 2: Immunoblot confirmation of changes illuminated by hybridization array analysis and previous experiments in other brain structures after chronic cocaine treatment. Signals are represented as the mean band intensity  $\pm$  S.E.M., normalized to the mean intensity of the control bands.

variety of conditions. A polyclonal antibody (Geneka) for NGF1-B recognized only a protein of 140-150kDa. This band representing an unknown high weight antigen related to NGF1-B (NGF1B-RA) showed a 278% ( $p<0.001$ ) increase. Average CKII $\alpha$  signal was 66% greater than the control, but in a highly variable manner ( $p=0.22$ ). GSK-3 $\alpha$  showed no change in average immunoreactive protein signal. FRA-1 immunoreactive protein was decreased on average by 48% with cocaine administration, but in a highly variable manner and did not reach statistical

significance. Of the genes tested, which we have previously shown to be regulated by cocaine administration, only PYK2 was changed in a significant manner. Chronic cocaine administration increased PYK2 levels 91% ( $p<0.05$ ). MEK1,  $\beta$ -Catenin, PKA $\alpha$ , PKC $\alpha$ , PKC $\epsilon$ , Kv1.1, and mGluR5 did not show significant changes in the amount of protein.

#### Discussion:

ARC, also known as Arg3.1, is an

unusual immediate early gene whose mRNA is found in both the soma and the dendrites (Steward et al. 1998; Wallace et al. 1998). ARC is termed an ‘effector’ immediate early gene because of its rapid and transient induction and the fact that it directly affects cellular processes rather than being a transcription factor. A number of stimuli induce ARC expression including cocaine (Fosnaugh et al. 1995; Tan et al. 2000), and methamphetamine (Kodama et al. 1998), as well as acquisition of a novel behavior (Kelly and Deadwyler 2001). Guzowski et al. (2000) showed that inhibition of ARC expression in the hippocampus impaired maintenance of long-term potentiation and consolidation of long-term spatial memory. This growing body of evidence along with the fact that *de novo* ARC mRNA and protein localize to activated post-synaptic zones, makes Arc a potential player in synaptic plasticity. This study demonstrated an induction in ARC protein, thus validating previous studies that reported mRNA increases following cocaine administration (Fosnaugh et al. 1995).

PYK2, also known as related focal adhesion kinase or cell adhesion kinase beta, is a non-receptor tyrosine kinase implicated in neuronal plasticity (Girault et al. 1999). We have previously shown PYK2 to be induced by chronic cocaine administration in the non-human primate nucleus accumbens (Freeman et al. 2001b), and the rat hippocampus (Freeman et al. 2001a). PYK2 is activated by increases in intracellular calcium, PKC, and by depolarization in hippocampal slices, and can cause MAPK cascade activation (Lev et al. 1995; Derkinderen et al. 1998). PYK2 also plays an important role in growth factor-induced neurite outgrowth and differentiation (Ivankovic-Dikic et al. 2000). The fact that PYK2 is induced in a number of brain regions and in dif-

ferent models points toward a general role of PYK2 in neuronal plasticity rather than a specific response of a particular brain structure to chronic cocaine. This plastic action may belong to any of the hypothesized actions for PYK2 in long-term potentiation, synaptic plasticity, neuronal survival, and signal transduction (Girault et al. 1999).

NGF1-B is the rat homologue of the human NAK1 and mouse Nur77 nuclear receptors. NGF1-B mRNA has been induced by cocaine treatment in the accumbens, caudate, and cerebral cortex (Werme et al. 2000). While we could not detect NGF1-B protein with any of the antibodies used, a high molecular weight related antigen (NGF1B-RA) was detected that was markedly induced in response to cocaine. The exact nature of this protein remains unknown, but due to the large number of orphan nuclear receptors which are related to NGF1-B, and to the peptide sequence (TATKARSGAPGGSE) used to generate the polyclonal antibody, this protein could be a previously uncharacterized nuclear receptor.

There are several possibilities as to why protein levels of GSK3 $\alpha$ , FRA-1, or CKII $\alpha$  were not altered. These apparent changes in mRNA seen from the array analysis could be false positives due to random variance. As well, the mRNA levels of these genes could be reduced, but there could be added modes of regulation at the level of protein synthesis or degradation which prevent changes in protein. In the case of FRA-1, the non-significant reduction in protein matched in magnitude the mRNA change seen with the arrays and may become significant if examined with a larger number of animals, or in a smaller anatomical compartment.

In a larger context, both ARC and PYK2 localize to a quantitative trait loci for

sensitization to cocaine on mouse chromosome 15 (Phillips et al. 1998). A change in prefrontal NGF1-B HWRA could play a role in the changes seen in frontal cortex dopamine transporters because the human dopamine transporter has been shown to be responsive to NGF1-B/NAK1 response elements (Sacchetti et al. 1999).

In total, hybridization array analysis and post-hoc immunoblot confirmation of changes is an efficacious methodology for finding cocaine-responsive changes in gene expression. The limits of the present method are that these changes are not isolated to specific nuclei within the brain region examined or to particular cells. These changes will have to be examined in future studies for their localization and behavioral effects.

**Acknowledgments:**

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## References:

- Bibb J. A., Chen J., Taylor J. R., Svenningsson P., Nishi A., Synder G. L., Nestler E. J., and Greengard P. (2001) Effects of chronic exposure to cocaine are regulated by the neuronal protein Cdk5. *Nature* 410, 376-380.
- Derkinderen P., Siciliano J., Toutant M., and Girault J. A. (1998) Differential regulation of FAK+ and PYK2/Cakbeta, two related tyrosine kinases, in rat hippocampal slices: effects of LPA, carbachol, depolarization and hyperosmolarity. *European Journal of Neuroscience* 10, 1667-1675.
- Fosnaugh J. S., Bhat R. V., Yamagata K., Worley P. F., and Baraban J. M. (1995) Activation of arc, a putative "effector" immediate early gene, by cocaine in rat brain. *J Neurochem* 64, 2377-2380.
- Freeman, W. M., Brebner, K., Lynch, W., Robertson, D. J., Roberts, D.C.S., and Vrana, K. E. (2001a) Cocaine-responsive gene expression changes in rat hippocampus. *Neuroscience*, In press.
- Freeman, W. M., Nader, M. A., Nader, S. H., Robertson, D. J., Gioia, L., Mitchell, S. M., Porrino, L. J., Friedman, D. P., and Vrana, K. E. (2001b) Chronic Cocaine-Mediated Changes in Non-Human Primate Nucleus Accumbens Gene Expression. *J.Neurochem.* 77:542-849.
- Freeman W. M., Robertson D. J., and Vrana K. E. (2000) Fundamentals of DNA hybridization arrays for gene expression analysis. *Biotechniques* 29, 1042-1055.
- Ghasemzadeh M. B., Nelson L. C., Lu X. Y., and Kalivas P. W. (1999) Neuroadaptations in ionotropic and metabotropic glutamate receptor mRNA produced by cocaine treatment. *J Neurochem* 72, 157-165.
- Girault J. A., Costa A., Derkinderen P., Studler J. M., and Toutant M. (1999) FAK and PYK2/CAKbeta in the nervous system: a link between neuronal activity, plasticity and survival? *Trends in Neurosciences* 22, 257-263.
- Guzowski J. F., Lyford G. L., Stevenson G. D., Houston F. P., McGaugh J. L., Worley P. F., and Barnes C. A. (2000) Inhibition of activity-dependent arc protein expression in the rat hippocampus impairs the maintenance of long-term potentiation and the consolidation of long-term memory. *J Neurosci* 20, 3993-4001.
- Ivankovic-Dikic I., Gronroos E., Blaukat A., Barth B. U., and Dikic I. (2000) Pyk2 and FAK regulate neurite outgrowth induced by growth factors and integrins. *Nat Cell Biol* 2, 574-581.
- Kelly, M. P. and Deadwyler, S. A. (2000) Increased mRNA expression of the IEG Arc in limbic structures of newly trained but not overtrained animals: evidence for Arc's role in the acquisition of a new behavior. Society for Neuroscience Abstract.
- Kelz M. B., Chen J., Carlezon W. A., Whisler K., Gilden L., Beckmann A. M., Steffen C., Zhang Y. J., Marotti L., Self D. W., Tkatch T., Baranauskas G., Surmeier D. J., Neve R. L., Duman R. S., Picciotto M. R., and Nestler E. J. (1999) Expression of the transcription factor deltaFosB in the brain controls sensitivity to cocaine. *Nature* 401, 272-276.
- Kodama M., Akiyama K., Ujike H., Shimizu Y., Tanaka Y., and Kuroda S. (1998)

- A robust increase in expression of arc gene, an effector immediate early gene, in the rat brain after acute and chronic methamphetamine administration. *Brain Res* 796, 273-283.
- Lev S., Moreno H., Martinez R., Canoll P., Peles E., Musacchio J. M., Plowman G. D., Rudy B., and Schlessinger J. (1995) Protein tyrosine kinase PYK2 involved in Ca(2+)-induced regulation of ion channel and MAP kinase functions. *Nature* 376, 737-745.
- Phillips T. J., Huson M. G., and McKinnon C. S. (1998) Localization of genes mediating acute and sensitized locomotor responses to cocaine in BXD/Ty recombinant inbred mice. *J Neurosci* 18, 3023-3034.
- Roberts D. C., Corcoran M. E., and Fibiger H. C. (1977) On the role of ascending catecholaminergic systems in intravenous self-administration of cocaine. *Pharmacol Biochem Behav* 6, 615-620.
- Robinson T. E., Gorny G., Mitton E., and Kolb B. (2001) Cocaine self-administration alters the morphology of dendrites and dendritic spines in the nucleus accumbens and neocortex. *Synapse* 39, 257-266.
- Robinson T. E. and Kolb B. (1999) Alterations in the morphology of dendrites and dendritic spines in the nucleus accumbens and prefrontal cortex following repeated treatment with amphetamine or cocaine. *Eur J Neurosci* 11, 1598-1604.
- Sacchetti P., Brownschidle L. A., Granneman J. G., and Bannon M. J. (1999) Characterization of the 5'-flanking region of the human dopamine transporter gene. *Brain Res Mol Brain Res* 74, 167-174.
- Spangler R., Unterwald E. M., and Kreek M. J. (1993) 'Binge' cocaine administration induces a sustained increase of prodynorphin mRNA in rat caudate-putamen. *Brain Res Mol Brain Res* 19, 323-327.
- Steward O., Wallace C. S., Lyford G. L., and Worley P. F. (1998) Synaptic activation causes the mRNA for the IEG Arc to localize selectively near activated postsynaptic sites on dendrites. *Neuron* 21, 741-751.
- Tan A., Moratalla R., Lyford G. L., Worley P., and Graybiel A. M. (2000) The activity-regulated cytoskeletal-associated protein arc is expressed in different striosome-matrix patterns following exposure to amphetamine and cocaine. *J Neurochem* 74, 2074-2078.
- Volkow N. D. and Fowler J. S. (2000) Addiction, a disease of compulsion and drive: Involvement of the orbitofrontal cortex. *Cerebral Cortex* 10, 318-325.
- Wallace C. S., Lyford G. L., Worley P. F., and Steward O. (1998) Differential intracellular sorting of immediate early gene mRNAs depends on signals in the mRNA sequence. *J Neurosci* 18, 26-35.
- Werme M., Olson L., and Brene S. (2000) NGFI-B and Nor1 mRNAs are upregulated in brain reward pathways by drugs of abuse: different effects in Fischer and Lewis rats. *Molecular Brain Research* 76, 18-24.

# Chapter 5: Functional Genomic Analysis of Chronic Contingent and Non-Contingent Cocaine Administration

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**Abstract:**

Functional genomic research is illuminating a variety of gene expression changes that occur following cocaine administration. To analyze gene expression changes in the nucleus accumbens (NAcc) of the rat after chronic non-contingent (NC) cocaine administration and cocaine self-administration (SA), cDNA hybridization arrays were used. Non-contingent cocaine was administered for 14 days (45mg/kg/day) while animals self-administering cocaine were allowed 24 hour access, with a maximum of 5 trials/hour (1.5mg/kg/inj) for 10 days. Potential cocaine regulated genes identified by hybridization array analysis were tested by immunoblotting for changes in protein expression. In addition, proteins previously identified to be cocaine-responsive in the non-human primate NAcc, or in other rat brain regions, were also tested for changes in expression levels.

Hybridization array analysis of mRNA from the NAcc of both animal models highlighted a number of potentially cocaine-regulated transcripts. In the NC-treated animals, NMDA Receptor 1 (NMDAR1), guanine nucleotide-binding protein gamma-7 sub-unit (GNG7), secretogranin V (SgV), c-Jun N-terminal kinase 2 (JNK2), and ras-related GTPase 15 (Rab15), showed a greater than 50% increase in both replicate arrays as compared to saline-injected controls. Sodium hydrogen exchange protein 1 (NHE1), and mitochondrial ATP synthase 5 beta (ATP5B) showed a greater than 33% reduction on both replicates. In SA animals, synaptosomal associated protein of 25kDa (SNAP25), and cocaine and amphetamine induced transcript (CART) showed increased mRNA as compared to control and the sodium and chloride dependent GABA transporter 3 (GABT3) demonstrated a decrease. Specific immunoblot testing of these changes will be needed to determine if these changes are statistically-significant and to establish that changes recapitulated at the level of protein.

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The following chapter is in preparation for *Journal of Neurochemistry*. Additional immunoblot data will be incorporated before submission for publication. Stylistic variations are due to the requirements of the journal and publisher. Karen Brebner, Wendy J. Lynch and David C.S. Roberts were responsible for design and execution of the animal treatments. Daniel J. Robertson assisted in the performance of the experimental protocols. Kent E. Vrana served in an advisory position on experimental design and manuscript preparation. Willard M. Freeman performed all other experimental work.

## Introduction:

A growing body of evidence implicates changes in gene expression as contributing to the altered behavior seen in animals administered cocaine (for review see Torres and Horowitz 1999 and Nestler 2001). For example, changes in the expression of tyrosine hydroxylase (Vrana et al., 1993; Todtenkopf et al. 2000), G1 isoform 1 (Stripling and Kalivas 1993), and the metabotropic glutamate receptor 5 (mGluR5) (Ghasemzadeh et al. 1999) in the NAcc have been described after chronic cocaine administration. These changes are dependent on the duration of cocaine treatment and the duration of withdrawal from cocaine (Ghasemzadeh et al. 1999; Loftis and Janowsky 2000). Immediate early genes such as c-fos and isoforms of c-fos (termed fos-related antigens-FRAs) and FosB have been extensively studied in the NAcc (Nestler et al. 1999) and have been shown to mediate some of the behavioral effects of cocaine (Kelz et

al. 1999). Overexpression of cyclin dependent kinase 5 (Cdk5) in a FosB-dependent manner has been demonstrated to reduce locomotor sensitization to cocaine (Bibb et al. 2001). Inhibition of Cdk5 in the nucleus accumbens (NAcc) increases locomotor sensitization to cocaine (Bibb et al. 2001).

While a number of changes in gene expression have been shown after cocaine administration there are undoubtedly many other changes that have yet to be described. One approach to finding novel changes in gene expression is to use the functional genomic technology of hybridization arrays (Freeman et al. 2000; 2001a; 2001b). In particular, macroarrays offer the advantage of high fold-change sensitivity which allows small changes (50%) to be seen. In order to find novel changes in gene expression hybridization arrays were used to screen for changes in mRNA. Post-hoc confirmation by specific immunoblots of potential changes seen with hybridization arrays is needed to both statistically confirm changes and validate that the change also exists at the level of protein. Immunoblot analysis of previously described changes in other models and brain regions allows for a more complete picture of changes in gene expression.

This study used two models of cocaine administration, NC and SA to study cocaine-responsive gene expression. The contingency of cocaine administration has been found to have effects on neurotransmitter levels (Hemby et al. 1997, Bradberry). These differences could underly some of the dissimilar changes in cocaine-responsive gene expression seen with NC and SA (Kuzmin and Johansson 1999). A 'binge' style of cocaine administration was used for NC animals (Spangler et al. 1993). A new 24 hour access paradigm was used for SA animals (Roberts et al. 2001). While these

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**Abbreviations:** AP-1, activator protein complex 1; ATP5B, mitochondrial ATP synthase beta; CART, cocaine and amphetamine induced transcript; Cdk5, cyclin-dependent kinase 5; CREB, cyclic AMP response element binding protein; GABT3, sodium and chloride dependent GABA transporter 3; GNG7, guanine nucleotide-binding protein, gamma-7 subunit; JNK2, c-Jun N-terminal kinase 2; Kv1.1 shaker-related voltage-gated potassium channel member; MEK1, mitogen activated protein kinase kinase 1; mGluR5, metabotropic glutamate receptor 5; NAcc, nucleus accumbens; NHE1, sodium hydrogen exchange protein 1; NMDAR, NMDA Receptor 1; PAGE, polyacrylamide gel electrophoresis; PKAc, protein kinase A catalytic subunit; PKC, protein kinase C alpha; PKC, protein kinase C epsilon; PYK2, protein tyrosine kinase 2; Rab15, ras-related GTPase; SAPK, stress-activated protein kinase alpha; SDS, sodium dodecyl sulfate; SgV, Secretogranin V; SNAP25, synaptosomal associated protein of 25kDa; SSC, saline sodium citrate

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models are not directly comparable because of differences in dose and time of administration, they both serve as chronic models of cocaine administration and were sacrificed within 30 minutes of their last injection to limit the effects of withdrawal. Changes in gene expression found in these two models will serve to further the knowledge of the molecular events associated with cocaine administration.

### **Experimental Procedures:**

#### *Animals*

Sprague-Dawley rats (Harlan, IN) weighing 275-300g at the start of the experiments served as subjects. All animals were allowed to acclimatize for 3 days following arrival at the facility and were maintained on a 12-h reversed light/dark cycle (lights off at 3:00 am) with food (Purina® Rat Chow) and water available ad libitum for the duration of the experiments. All research was approved by the Animal Care and Use Committee of Wake Forest University and was conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Academy Press, Washington, D.C., 1996) as adopted and promulgated by the U.S. National Institutes of Health.

#### *Surgery*

#### *Cocaine Self-Administering rats: apparatus and measurements*

After a 3-day acclimation period, each rat (5 per treatment group) was anesthetized with sodium pentobarbital (60 mg/kg, supplemented as needed) and implanted with a chronically indwelling Silastic® jugular cannula that exited through the skin on the dorsal surface in the region of the scapulae (Roberts and Goeders 1989).

Following surgery, rats were individually housed in a 25 x 25 x 25 cm test-

ing chamber. The cannula was connected, through a stainless steel protective spring, to a counter-balanced swivel apparatus that allowed freedom of movement within the chamber. Beginning the day after surgery, animals were given access to a response lever that controlled the delivery of cocaine injections on an FR1 schedule. Concurrent with the start of each cocaine injection (1.5 mg/kg/inj), a stimulus light located above the lever was activated to signal a 20-s post-infusion time-out period, during which responses produced no programmed consequence. A computer controlled the dose by adjusting the time each pump was activated according to the body weight of each subject. During training, cocaine was made available on an FR 1 schedule until a total of 40 injections was self-administered. After the animals had established a stable daily pattern of cocaine intake for 7 days (defined as 40 injections self-administered within 6 hours and regular post-infusion pauses), subjects were given 24 hr access to cocaine (1.5 mg/kg/inj) in five 10 minute discrete trials/hr. Rats remained in the discrete trials protocol for 10 days, and were sacrificed immediately (within 30 minutes) following the final discrete trial on day 10. Subjects were deeply anaesthetized with intravenous ketamine to confirm cannula patency, and decapitated. Brains were rapidly removed and dissected on ice. A second set of identically-treated rats was generated for samples used in immunoblot confirmation of mRNA changes observed by hybridization array analysis.

#### *Non-contingently treated rats*

Rats were randomly assigned to two treatment groups (n=6) that received IP injections of cocaine or saline for 14 days. Rats were weighed at 10:00 every morning, immediately prior to the first injection. Rats

in each group received 3 hourly injections of cocaine HCl (15 mg/kg), or saline (1ml/kg) beginning at 10:00. Thirty minutes after the final cocaine or saline injection, rats were deeply anaesthetized with sodium pentobarbital (IP) and decapitated. Brains were rapidly removed and placed on ice for dissection. The NAcc was dissected by free-hand dissection using a brain matrix and landmarks and immediately frozen on dry ice. A second set of animals (n=6 per saline and cocaine group) was treated identically as above for the protein immunoblot confirmations.

#### *Drugs*

Cocaine hydrochloride was obtained from Research Technology Branch of the National Institute on Drug Abuse (Rockville, MD, USA). Cocaine was dissolved in sterile 0.9% saline and passed through a micro-filter (pore size 22 mm). Dosages are expressed as the salt.

#### *RNA Isolation and DNA hybridization arrays*

Tissue from the NAcc of each animal was ground with liquid nitrogen in a dry ice-chilled mortar. Total RNA was isolated from the ground tissue of each animal using a guanadinium thiocyanate method (Chomczynski and Sacchi 1987). Hybridization array analysis was performed twice using Rat 1.2 (I) Atlas cDNA arrays according to the manufacturer's protocol (Clontech, Palo Alto, CA) as previously detailed (Freeman et al. 2001a). These arrays contain gene fragments corresponding to 1176 known regulatory genes (kinases, transcription factors, cell-cycle proteins, receptors and others). Equal amounts of total RNA from each animal in a group (cocaine or saline) were used to create a total RNA pool for each group.  $^{32}$ P-labeled cDNA probes were synthesized by reverse transcription of 5  $\mu$ g

total RNA from each pool, a mixture of gene-specific primers, dNTPs,  $^{32}$ P-dATP (DuPont NEN Research Products, Boston, MA), and Moloney-murine-leukemia virus (MMLV) reverse transcriptase. The resulting radiolabeled cDNAs (from treated and control samples) were purified by column chromatography, and equal amounts of radioactivity (3-5 x 10<sup>6</sup> cpm) from control or treated cDNA were hybridized to the arrays overnight at 68°C. Arrays had been prehybridized overnight with salmon sperm DNA in UltraHyb buffer (Clontech). Following hybridization, the arrays were washed three times at 68°C in 2X SSC (0.3 M NaCl/ 0.03 M sodium citrate), and 1% sodium dodecyl sulfate (SDS), followed by three additional washes at 68°C in 0.1X SSC, 0.5% SDS and one wash in 2X SSC at room temperature. The radioactive signals were detected with a Storm Phosphorimager (Molecular Dynamics, Sunnyvale, CA) or Fuji BAS1500 (Fujimed, Stamford, CT) and quantified using GLEAMS (NuTec Services, Stafford TX) and Atlas Image2.0 (Clontech) array analysis software. Background was determined from the blank areas of the array and subtracted from density measurements for each gene on that array. A normalization factor for each pair of arrays was determined from the ratio of the sums of the specific signals for each array. The normalization factor was then applied to the signal for each spot of one array as described by the AtlasImage2.0 array analysis software. A least square normalization scheme was also employed but did not change the genes selected for further testing. Genes that demonstrated a consistent up-regulation or down-regulation of 50% or 33%, respectively, (equal on a natural log scale) across the duplicate experiments were chosen for post-hoc immunoblot analysis. The complete data set is available at the Drug and

Alcohol Abuse Array Data Consortium website ([www.arraydata.org](http://www.arraydata.org)).

### *Immunoblotting*

Protein was isolated from independent sets of NC and SA animals treated the same as those used in the cDNA hybridization array analysis. Tissue was ground with liquid nitrogen in a dry ice-chilled mortar. A portion of the tissue powder was then homogenized by sonication in protein buffer (25mM N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid); pH=7.5, 250mM Sucrose, 100 M Ethylenediaminetetraacetic acid, 1 g/mL Leupeptin, 0.5 g/mL Pepstatin A, 500 M PMSF, 1mM 1,4-Dithio-DL-threitol, 10 M Fe(NH<sub>4</sub>)<sub>2</sub>(SO<sub>4</sub>)<sub>2</sub>, 0.2% Triton X-100) as described previously (Kumer et al. 1997). Total protein concentration was determined with the bicinchoninic acid assay (Smith et al. 1985) (BCA protein assay, Pierce Chemical, Rockford IL) of brain homogenates from each animal. Equal amounts of protein from each animal (10-30 g) were resolved by denaturing polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970), and transferred to a polyvinylidene fluoride membrane (Immobilon P, Millipore, Bedford, MA) by semidry transfer (TE-70, Amersham Pharmacia Biotech, Piscataway, NJ). Immunoreactive protein corresponding to the appropriate protein molecular weight was detected with antibodies at the following concentrations: -Catenin (92kDa) 0.33 g/mL (C19220, Transduction Laboratories, Lexington, KY); Kv1.1 (90kDa) 1.0 g/ml (05407, Upstate Biotechnology, Lake Placid, NY); mitogen-activated protein kinase kinase 1, MEK1, (45kDa) 0.165 g/mL (M17020, Transduction Laboratories); metabotropic glutamate receptor 5, mGluR5, (120kDa) 0.8 g/mL (06451,Upstate Biotechnology); sodium hydrogen exchange protein 1,

NHE-1, (92kDa) 0.45 g/mL (N12520, Transduction Laboratories) protein kinase A catalytic subunit alpha, PKAc , (40kDa) 0.165 g/mL (P73420, Transduction Laboratories, Lexington, KY); protein kinase C alpha, PKC , (82kDa) 0.165 g/mL (P16520, Transduction Laboratories); protein kinase C epsilon, PKC , (90kDa) 0.165 g/mL (P14820, Transduction Laboratories); protein tyrosine kinase 2, PYK2, (116kDa) 0.165 g/mL (P47120, Transduction Laboratories). Visualization was accomplished using a horseradish peroxidase-coupled secondary antibody, either mouse or rabbit IgG (AP Biotech) and a chemiluminescent substrate (Pico Signal, Pierce Chemical). All immunoblot signal values were quantified by transmissive densitometry (TINA, Fuji Medical Systems, Stamford, CA). All values are expressed as a mean ± S.E.M.

## **Results:**

### *Cocaine self-administration in a discrete trials paradigm*

The discrete trial paradigm produced robust cocaine self-administration. Average daily dose was 92.43 mg/kg with a mean cumulative dose of 924.3 mg/kg over the 10 days (Figure 1). This is a much higher dose of cocaine than in many limited access cocaine administration paradigms (Cha et al. 1997). Within the 10 days of self-administration, there was a significant difference in self-administration between the first day and the remaining days of self-administration. This decrease in self-administration has been ascribed to tolerance (Roberts D.C.S., Brebner K., personal communication)

### *Cocaine-responsive changes in mRNA levels*

Analysis of mRNA from non-contingently treated animals showed 360 genes

Mean cocaine intake of animals used for protein confirmation during  
10 days of self-administration

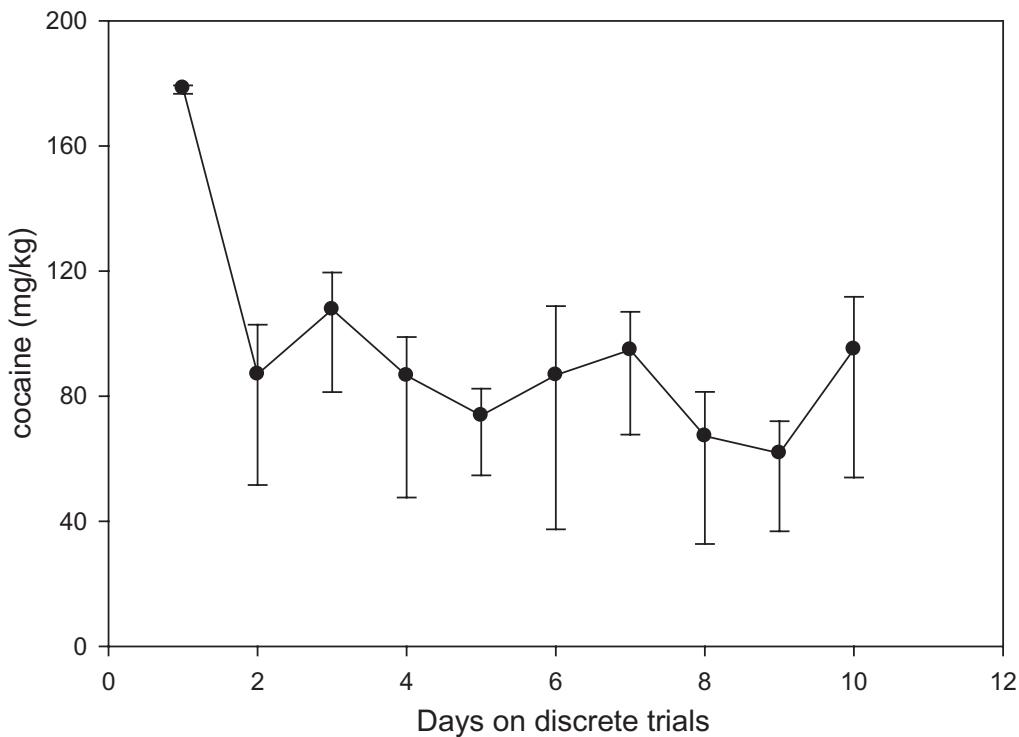


Figure 1. Average daily intakes of cocaine in 5 trial/hour, 24 hour access self-administration paradigm across 10 days. Data are presented as mean intake  $\pm$ S.E.M.

with signals at least 50% over background on one of the arrays (Figure 2). Five genes showed a greater than 50% induction on both replicate arrays. NMDAR1 showed cocaine-to-saline signal ratios of 1.54 and 3.01. GNG7 presented ratios of 1.9 and 2.45. SgV, also known as neuroendocrine protein 7B2 precursor, had ratios of 1.58 and 1.73 across the two replicates. JNK2, also known as stress-activated protein kinase alpha (SAPK) was induced with ratios of 1.51 and 2.98. Rab15 showed a ratio of 2.10 in one replicate and an undefined induction in the other. The undefined cocaine-to-saline ratio is the result of the cocaine array having a signal 50% above background and the saline array not having a signal 50%

above background. As well, two genes were seen to be downregulated across replicates. NHE1 had cocaine-to-saline ratios of 0.60 and 0.58, and ATP5B had ratios of 0.54 and 0.58.

Array analysis of mRNA from the NAcc of the self-administering animals also revealed a number of possible cocaine-responsive genes (Figure 3). A total of 335 genes had a signal 50% above background in at least one of the replicate arrays. Potentially induced genes included the synaptosomal associated protein of 25kDa (SNAP25) and the cocaine and amphetamine induced transcript (CART). SNAP25 had an average cocaine-to-saline signal ratio of 1.62 and CART showed a mean ratio of



Figure 2. Scatterplot of hybridization array data from NAcc of rats treated non-contingently with cocaine (45mg/kg/day/i.m.)

1.63 across the replicates. One potentially down-regulated gene was also identified, the sodium and chloride dependent GABA transporter 3 (GABT3), with an average expression ratio of 0.64.

While analysis using standard 50% induction, 33% reduction values did not produce the same set of genes in the NC and SA animals, closer examination of the expression data shows some similarities and some

differences. NMDAR1, seen to be induced on NC arrays showed an average expression ratio of 1.4 in the SA animals. SNAP25 which was consistently up-regulated in the SA animals showed an average expression ratio of 0.77 in the NC animals. The potassium channel Kv1.1, previously shown to be induced in the hippocampus of the NC animals, showed ratios of 0.46 and 0.84 in the NAcc of the NC animals and ratios of 1.24

NAcc cocaine i.v. 10 days

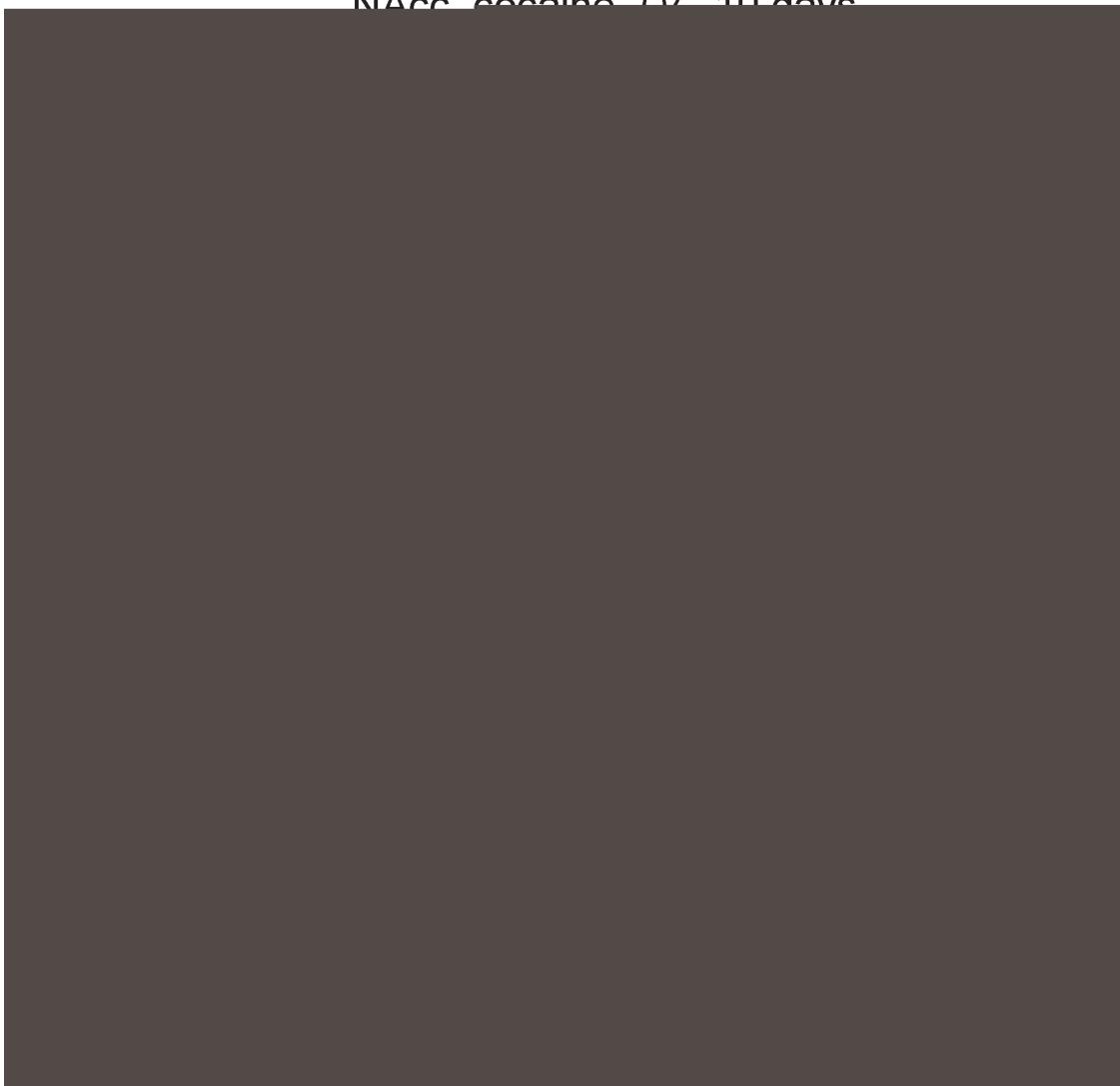


Figure 3. Scatterplot of hybridization array data from NAcc of rats in a 10 day discrete-trials cocaine self-administration paradigm.

and 1.56 in the SA animals. Protein analysis will confirm any of these differences between animal models or brain regions.

#### *Cocaine-responsive changes in protein expression*

To confirm the changes seen on the hybridization arrays and to make general comparisons between the NC and SA animals, specific immunoblots were used to

measure protein levels. Preliminary examination of the NC animal protein levels has been conducted. PYK2 is induced in the NAcc of NC-treated animals by 82% ( $p=0.05$ ) in a similar manner to previously seen in the non-human primate NAcc (Freeman et al. 2001b), in the hippocampus (Freeman et al. 2001a) and frontal cortex (Chapter 4) (Figure 4). No other changes observed in the NAcc of the non-human primate (Chap-

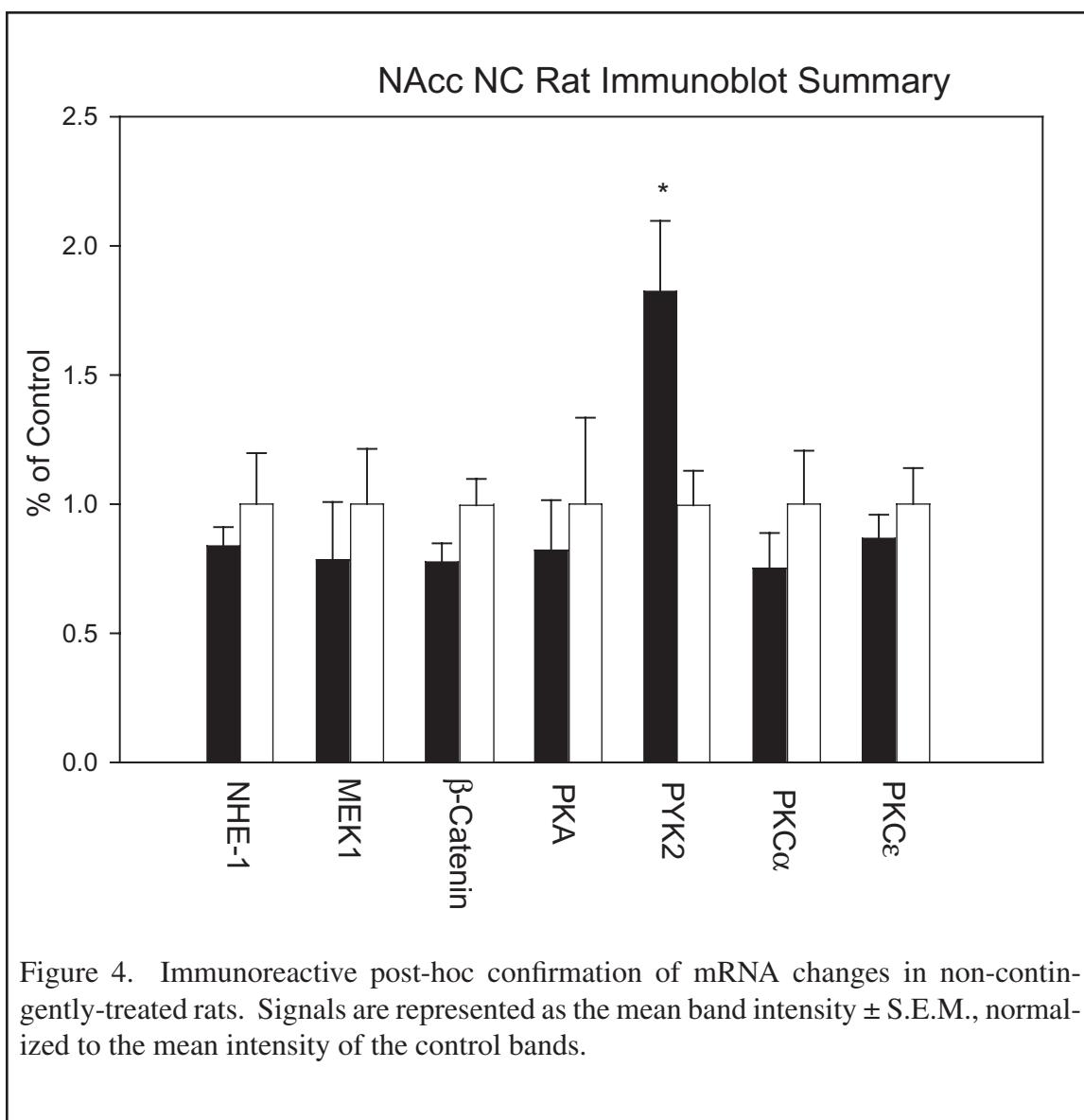


Figure 4. Immunoreactive post-hoc confirmation of mRNA changes in non-contingently-treated rats. Signals are represented as the mean band intensity  $\pm$  S.E.M., normalized to the mean intensity of the control bands.

ter 2) were confirmed for MEK1,  $\beta$ -Catenin, or PKA $\alpha$  in the NC rats. Of the changes on the hybridization arrays only NHE1 has been tested and no change was documented. Further immunoblot analysis is needed to test the other changes seen from the NC animal array analysis. Confirmation of changes seen by hybridization arrays and testing of changes observed in other animal models will have to be performed on SA animals to confirm changes in protein levels.

#### Discussion:

This study examined changes in gene expression in rats either treated non-contingently with cocaine or that had self-administered cocaine. The intent of the study was to screen for changes in gene expression after chronic cocaine and to examine previously described (Freeman et al. 2001b) changes in the non-human primate in a rodent model. While direct comparisons between the models of cocaine administra-

tion used in this study are not possible, differences in hybridization array data between these models are noteworthy in two respects.

**References:**

- Bibb J. A., Chen J., Taylor J. R., Svenningsson P., Nishi A., Synder G. L., Nestler E. J., and Greengard P. (2001) Effects of chronic exposure to cocaine are regulated by the neuronal protein Cdk5. *Nature* 410, 376-380.
- Cha X. Y., Pierce R. C., Kalivas P. W., and Mackler S. A. (1997) NAC-1, a rat brain mRNA, is increased in the nucleus accumbens three weeks after chronic cocaine self-administration. *J Neurosci* 17, 6864-6871.
- Chomczynski P. and Sacchi N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate- phenol-chloroform extraction. *Anal Biochem* 162, 156-159.
- Douglass J., McKinzie A. A., and Couceyro P. (1995) PCR differential display identifies a rat brain mRNA that is transcriptionally regulated by cocaine and amphetamine. *J Neurosci* 15, 2471-2481.
- Fagergren P. and Hurd Y. L. (1999) Mesolimbic gender differences in peptide CART mRNA expression: effects of cocaine. *Neuroreport* 10, 3449-3452.
- Fitzgerald L. W., Ortiz J., Hamedani A. G., and Nestler E. J. (1996) Drugs of abuse and stress increase the expression of GluR1 and NMDAR1 glutamate receptor subunits in the rat ventral tegmental area: common adaptations among cross-sensitizing agents. *J Neurosci* 16, 274-282.
- Freeman, W. M., Brebner, K., Lynch, W., Robertson, D. J., Roberts, D. C. S., and Vrana, K. E. (2001a) Cocaine-responsive gene expression changes in rat hippocampus. Submitted to *Neuroscience*.
- Freeman, W. M., Nader, M. A., Nader, S. H., Robertson, D. J., Gioia, L., Mitchell, S. M., Porrino, L. J., Friedman, D. P., and Vrana, K. E. (2001b) Chronic Cocaine-Mediated Changes in Non-Human Primate Nucleus Accumbens Gene Expression. In press *J.Neurochem*.
- Freeman W. M., Robertson D. J., and Vrana K. E. (2000) Fundamentals of DNA hybridization arrays for gene expression analysis. *Biotechniques* 29, 1042-1055.
- Ghasemzadeh M. B., Nelson L. C., Lu X. Y., and Kalivas P. W. (1999) Neuroadaptations in ionotropic and metabotropic glutamate receptor mRNA produced by cocaine treatment. *J Neurochem* 72, 157-165.
- Hemby S. E., Co C., Koves T. R., Smith J. E., and Dworkin S. I. (1997) Differences in extracellular dopamine concentrations in the nucleus accumbens during response-dependent and response-independent cocaine administration in the rat. *Psychopharmacology* 133, 7-16.
- Huang Y., Lu W., Ali D. W., Pelkey K. A., Pitcher G. M., Lu Y. M., Aoto H., Roder J. C., Sasaki T., Salter M. W., and MacDonald J. F. (2001) CAKbeta/Pyk2 kinase is a signaling link for induction of long-term potentiation in CA1 hippocampus. *Neuron* 29, 485-496.
- Ivankovic-Dikic I., Gronroos E., Blaukat A., Barth B. U., and Dikic I. (2000) Pyk2 and FAK regulate neurite outgrowth induced by growth factors and integrins. *Nat Cell Biol* 2, 574-581.

- Kelz M. B., Chen J., Carlezon W. A., Whisler K., Gilden L., Beckmann A. M., Steffen C., Zhang Y. J., Marotti L., Self D. W., Tkatch T., Baranauskas G., Surmeier D. J., Neve R. L., Duman R. S., Picciotto M. R., and Nestler E. J. (1999) Expression of the transcription factor deltaFosB in the brain controls sensitivity to cocaine. *Nature* 401, 272-276.
- Kumer S. C., Mockus S. M., Rucker P. J., and Vrana K. E. (1997) Amino-terminal analysis of tryptophan hydroxylase: protein kinase phosphorylation occurs at serine-58. *J Neurochem* 69, 1738-1745.
- Kuzmin A. and Johansson B. (1999) Expression of c-fos, NGFI-A and secretogranin II mRNA in brain regions during initiation of cocaine self-administration in mice. *Eur J Neurosci* 11, 3694-3700.
- Laemmli U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685.
- Loftis J. M. and Janowsky A. (2000) Regulation of NMDA receptor subunits and nitric oxide synthase expression during cocaine withdrawal. *J Neurochem* 75, 2040-2050.
- Nestler E. J. (2001) Molecular basis of long-term plasticity underlying addiction. *Nature Neuroscience Reviews* 2, 119-128.
- Nestler E. J., Kelz M. B., and Chen J. (1999) DeltaFosB: a molecular mediator of long-term neural and behavioral plasticity. *Brain Res* 835, 10-17.
- Roberts D. C. and Goeders N. E. (1989) Drug self-administration: Experimental methods and determinants., in *Neuromethods: Psychopharmacology*, Vol.13 (Boulton A. A., Baker G. B., and Greenshaw A. J., eds.), pp. 349-398. Humana Press Inc., Clifton, N.J.
- Roberts, D. C. S., Brebner, K., Vincler, M., and Lynch, W. J. (2001) Binge Cocaine Self-administration in Rats Responding Under a Discrete Trials Procedure. Submitted to *Journal of Pharmacology and Experimental Therapeutics*.
- Smith P. K., Krohn R. I., Hermanson G. T., Mallia A. K., Gartner F. H., Provenzano M. D., Fujimoto E. K., Goeke N. M., Olson B. J., and Klenk D. C. (1985) Measurement of protein using bicinchoninic acid. *Anal Biochem* 150, 76-85.
- Spangler R., Unterwald E. M., and Kreek M. J. (1993) 'Binge' cocaine administration induces a sustained increase of prodynorphin mRNA in rat caudate-putamen. *Brain Res Mol Brain Res* 19, 323-327.
- Striplin C. D. and Kalivas P. W. (1993) Robustness of G protein changes in cocaine sensitization shown with immunoblotting. *Synapse* 14, 10-15.
- Todtenkopf M. S., De Leon K. R., Stellar J. R., Behavioral s., Dopamine, Striatum, Immunoreactivity, and Varicosities (2000) Repeated cocaine treatment alters tyrosine hydroxylase in the rat nucleus accumbens. *Brain Research Bulletin* 52, 407-411.
- Torres G. and Horowitz J. M. (1999) Drugs of abuse and brain gene expression. *Psychosom Med* 61, 630-650.
- Vrana SL, Vrana KE, Koves TR, Smith JE, Dworkin SI (1993) Chronic cocaine administration increases CNS tyrosine hydroxylase enzyme activity and mRNA levels and tryptophan hydroxylase enzyme activity levels. *J Neurochem* 61: 2262-2268.

# **Chapter 6: A cocaine analog, 2 $\beta$ -propanoyl-3 $\beta$ -(4-tolyl)-tropane (PTT), reduces tyrosine hydroxylase in the mesolimbic dopamine pathway.**

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## **ABSTRACT:**

Tyrosine hydroxylase (TH, EC 1.14.16.2) is the rate-limiting enzyme in catecholamine biosynthesis. Previously published results have established that chronic cocaine administration (30 to 45 mg/kg/day, 10 to 14 days) resulted in an upregulation of TH gene expression in dopaminergic pathways of rats. The present studies tested the effects of a tropane analog, PTT (2 $\beta$ -propanoyl-3 $\beta$ -(4-tolyl)-tropane), on TH expression. This drug has similar actions to cocaine, but possesses markedly different pharmacokinetics (20 times more potent at binding the dopamine transporter, markedly increased metabolic stability, and 10-20 times more potent in behavioral measures). Moreover, this compound demonstrates an increased selectivity for the dopamine (DA) and norepinephrine (NE) transporters compared with cocaine. In direct contrast to the previously reported effects of cocaine, PTT administration (3.0 mg/kg/day, i.p.; racemic mixture) produced a uniform down-regulation of TH protein and activity gene expression. TH activity and immunoreactive protein were decreased by 54% and 69% respectively in the nucleus accumbens. Within the ventral tegmental area, TH activity and protein were decreased by 33% and 19% respectively. The underlying mechanisms for this fundamental difference are unclear, but likely reflect varying and selective affinities and lengths of occupancy at biogenic amine transporters.

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The following chapter was published in *Drug and Alcohol Dependence* (2000), 61: 15-21. The materials have been reprinted with permission of the publisher. Stylistic variations are due to the requirements of the journal and publisher. Linda J. Porrino, James B. Daunais, and Stephanie L. Hart were responsible for the design and execution of the animal treatments. Huw M.L. Davies prepared the tropane analog (PTT). Lynda Gioia and George J. Yohrling IV assisted in the development and execution of the activity assay and immunoblot protocols. Willard M. Freeman performed all other experimental work. Kent E. Vrana served in an advisory position on experimental design and manuscript preparation.

## 1. INTRODUCTION

The primary action of cocaine in the brain is the blockade of the reuptake of the monoamines, dopamine (Moore et al., 1977; Heikkila et al., 1979), norepinephrine (Herrting et al., 1961; Moore et al. 1977) and serotonin (Ross and Renyi, 1967). Cocaine binds with relatively equal potency to all three transporters. Its behavioral effects have been largely attributed to its actions at dopamine transporters (cf. DeWit and Wise, 1977; Colpaert et al., 1978; Miczek and Yoshimura, 1982; Ritz et al., 1987; Wilcox et al., 1999). One important characteristic of cocaine is its rapid onset of action when given by the routes of administration used in abuse. Peak plasma concentrations have been reported to be achieved within the first 30 minutes when cocaine is administered either intranasally or intravenously (Shuster, 1992). In addition to its rapid onset, the duration of action of cocaine is also relatively short. Estimates of its half-life in rats vary with route of administration

from between 15 minutes to 1 hour (Nayak et al., 1976). These pharmacokinetic characteristics have been hypothesized to contribute to the intense reinforcing effects of cocaine and the frequency of its repeated use.

It has been well-established that the reinforcing properties of cocaine are mediated largely by the mesolimbic dopaminergic system (Roberts et al., 1977; Koob, 1992). Specifically, the ventral tegmental area (VTA) (Roberts and Koob, 1982) and its terminal field, the nucleus accumbens (NAcc), have been implicated as areas of a drug abuse reward pathway (Koob and Bloom, 1988; Koob, 1992; Robledo et al., 1992; Samson et al., 1992; Grant, 1995). Changes in dopaminergic systems have a regulatory effect on the rate-limiting enzyme in catecholamine biosynthesis, tyrosine hydroxylase (TH, E.C. 1.14.16.2; see Kumer and Vrana, 1996 for review). The changes in TH activity, immunoreactive protein, and mRNA levels in the VTA and NAcc following cocaine administration, are sum-

Table 1  
Summary of cocaine and PTT effects on TH expression in the mesolimbic dopamine pathway

Cocaine effect	Reference	PTT effect
Ventral tegmental area		
↑ TH protein	Beitner-Johnson and Nestler, 1991; Sorg et al., 1993	↔ TH protein
↑ TH activity	↔ Vrana et al., 1993; Masserano et al., 1996	↔ TH activity
↑ TH mRNA	Vrana et al., 1993	
↑ TH phosphorylation	Beitner-Johnson and Nestler, 1991	
Nucleus accumbens		
↔ TH protein	Beitner-Johnson and Nestler, 1991	↓ TH protein
↔ TH activity	Vrana et al., 1993	↓ TH activity
↑ TH phosphorylation	Beitner-Johnson and Nestler, 1991	

marized in Table 1 (Beitner-Johnson and Nestler, 1991; Sorg et al., 1993; Vrana et al., 1993; Masserano et al., 1996). The common effect found in all of these studies is an upregulation of TH in the VTA and a trend towards upregulation (non-significant) in the NAcc. It remains unclear precisely how the up-regulation occurs and why it is limited to the VTA. One possible explanation is that expression of neurofilament proteins is down-regulated as a result of chronic cocaine administration (Beitner-Johnson et al., 1992) and that this leads to decreased transport of TH from the VTA cell body to its projections in the NAcc.

One recent strategy for investigation of the neurobiological actions of cocaine has been the use of cocaine derivatives that bind to dopamine and serotonin transporters with greater affinity and slower dissociation rates than cocaine. Several groups have synthesized varying tropane analogs (Abraham et al., 1992; Carroll et al., 1992a, 1992b, 1993 Kozikowski et al., 1992; Lewin et al., 1992; Meltzer et al., 1993, 1994, 1996; Boja et al., 1994) including Davies and colleagues (Davies et al., 1993, 1994), who have prepared a series of novel analogs utilizing a unique synthetic scheme based

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Figure 1: The comparison of the chemical structures for cocaine and its analog,  $2\beta$ -propanoyl- $3\beta$ -(4-tolyl)-tropane (PTT). To create PTT, the methyl ester in cocaine has been replaced with a  $2\beta$ -ethyl ketone moiety. Additionally, the benzoate ester group in cocaine has been replaced with a  $3\beta$ -(4-methylphenyl) group.

a therapeutic standpoint, synthesis of compounds with slow onsets and long durations of action is a potential strategy for the development of pharmacological interventions in cocaine abuse. The use of such long-acting compounds could result in reduced drug-seeking behavior and they might, therefore, serve as substitutes for cocaine in much the same way that methadone is utilized in the treatment of opioid dependence.

The working hypothesis of this study was that PTT, by virtue of its enhanced potency and metabolic half-life, would have more pronounced effects on TH levels than cocaine. The results, however, demonstrated that PTT had an opposite effect than cocaine. That is, TH activity and protein were decreased following chronic administration of PTT, in contrast to previous reports of cocaine-responsive induction.

## **2. MATERIALS AND METHODS**

### *2.1. Subjects*

Male Sprague-Dawley rats weighing 250 g at the start of the experiment, were housed in a climate-controlled room on a reverse 12-hr light/dark cycle, with food and water available ad libitum. All rats were adapted to vivarium conditions prior to drug administration, which was conducted during the light phase of the cycle. All experiments were conducted with approval of the institutional animal care and use committee and performed in AAALAC-approved facilities.

### *2.2. Drugs*

PTT was synthesized as previously described (Davies et al., 1994), and was dissolved in 50 mM TRIS, at pH 7.4, which served as the vehicle. Doses of PTT were selected based on results of in vitro binding

studies in which PTT was shown to be 10 to 20 times more potent than cocaine at binding to the dopamine transporter (Davies et al., 1993). Previous in vivo studies with PTT demonstrated that in vitro comparisons closely paralleled potency differences in vivo (Porrino et al., 1994). Animals were therefore injected i.p. with vehicle or PTT (3.0 mg/kg); (1/10th to 1/18th the cocaine dose employed in previous rat studies that had characterized TH activity and gene expression) once daily for 10 days. This dose has also been previously established to be behaviorally active (Porrino et al., 1994, 1995).

### *2.3. Tissue Dissection*

Animals were anesthetized with sodium pentobarbital followed by decapitation, and the brains were placed in a ASI brain slicer (ASI Instruments, Warren MI). The cell body (SN,VTA) and terminal field regions (Striatum, NAcc) of the nigrostriatal and mesolimbic dopamine pathways, respectively, were block dissected and isolated from brain slices using coordinates described by Paxinos and Watson (Paxinos and Watson, 1986).

### *2.4. Homogenate preparation*

The various brain regions analyzed were resuspended in 10 ml of homogenation buffer per 1 mg of tissue. The buffer contained 50 mM PIPES (pH 6.0), 50 mM EDTA (pH 8.0), 250 mM sucrose, 1 mM PMSF, 2 mM leupeptin, 0.5 mM pepstatin A, 1 mM dithiothreitol, 10 mM Fe(NH<sub>4</sub>)<sub>2</sub>(SO<sub>4</sub>)<sub>2</sub>, and 0.2% Triton X-100. Tissue samples were sonicated for 10 seconds with a Vibra Cell sonicator (Sonics and Materials, Inc., Danbury, CT) set at 40% maximal power. Homogenates, fol-

lowing protein determinations (Bradford, 1976)(BioRad Protein Assay, Bio-Rad Laboratories, Inc., Hercules, CT), were then subjected to denaturing gel electrophoresis, western blot characterization, and radioenzymatic enzyme assay analysis.

### *2.5. Polyacrylamide gel electrophoresis and western blot analysis*

Proteins from the brain homogenates (50 g total protein) were resolved by denaturing polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). The proteins were transferred to reinforced nitrocellulose membrane (Duralose; Stratagene, LaJolla, CA) using a semi-dry electroblot apparatus (Owl Scientific; Cambridge, MA). After transfer, the membrane was placed in blocking buffer (5% dry, non-fat milk in 10mM Tris-HCl (pH 7.5), 0.05% Tween 20, and 0.9% NaCl) overnight. TH immunoreactive protein was detected using an immunopurified rabbit anti-rat TH polyclonal antibody (a 1:1,500 dilution in blocking buffer; Pel-Freeze Biologicals, Rogers, AR) for one hour. This was followed by a 1:1,000 dilution of a secondary donkey anti-rabbit Ig antibody coupled to horseradish peroxidase (Amersham Life Sciences, Arlington Heights, IL) for 30 minutes. Immunoreactive TH bands were visualized by enhanced chemiluminescence (ECL Western Blotting Analysis Systems; Amersham, Arlington Heights, IL) and exposed to x-ray film (Kodak Biomax MR2; Eastman Kodak Inc., Rochester, NY). In all cases, TH protein migrated with a molecular weight of approximately 61 kDa, in accordance with previously determined values (rev. in Kumer and Vrana, 1996). The TH bands were then quantified by densitometry according to previously described methods (O'Neill et al., 1989) using a flatbed scanner

(UMAX Technologies, Inc., Fremont, CA) and TINA software (Fuji Medical Systems Inc., Stamford, CT). The amount of immunoreactive TH protein is reported in relative densitometric units.

### *2.6. TH enzyme activity analysis*

TH activity was determined utilizing the  $^3\text{H}_2\text{O}$  release assay of Reinhard et al. (1986) at 100 mM tyrosine and 100 mM tetrahydrobiopterin at 37°C. L-[3,5- $^3\text{H}$ ] tyrosine with a specific activity of 40 Ci/mmol (NEN, Boston, MA.) as the radioactive substrate in the TH activity assays. Non-enzymatic  $[^3\text{H}]\text{-H}_2\text{O}$  formation was determined in a reaction lacking homogenate (buffer blank) and was subtracted from all raw radioactivity values prior to the calculation of specific activities. Individual samples were analyzed in duplicate and the mean considered an 'n' of 1. Results were normalized to the amount of protein (Bradford, 1976) and expressed as nmol product  $\text{hr}^{-1}$  mg total protein $^{-1}$ .

### *2.7. Statistics*

In each experiment, enzymatic assay and immunoreactive protein results were subjected to student T-test analysis comparing control and PTT-treated groups ( $p < 0.05$  was considered significant).

## **3. RESULTS**

### *3.1. NAcc*

Following 10 days of PTT administration, rats were killed and the VTA, NAcc, SN and striatum were dissected. Brain homogenates were subjected to western blot analysis and a radioenzymatic TH activity assay.

The western blots were quantified by obtaining their relative optical densities. In the NAcc, chronic PTT administration resulted in a 69% decrease in the mean amount of immunoreactive protein, according to densitometry analysis (Fig. 2A). Additionally, a 54% decrease in TH activity was observed in the NAcc following PTT exposure (Fig. 2B). Statistical analysis (student t-test) of the NAcc protein and TH activity demonstrated that these PTT-dependent decreases were significant at the  $P<0.02$  level.

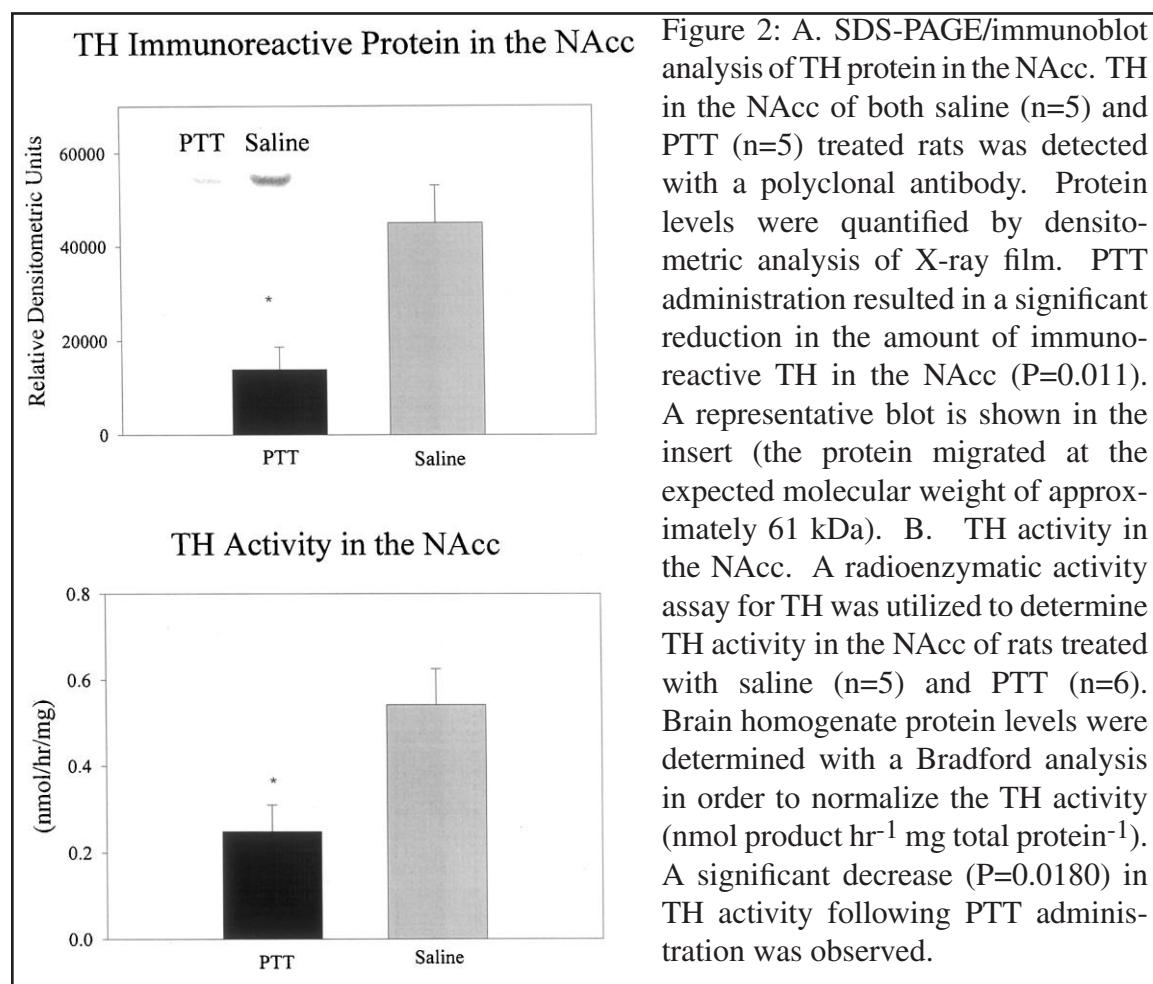
### 3.2. VTA

In the VTA, PTT administration resulted in a 19% decrease in the mean relative densitometric units when compared to

the saline treated animals (Fig. 3a). This was accompanied by a 33% decrease in TH specific activity (Fig. 3b). Neither of the decreases, however, reached statistical significance.

### 3.3. SN and Striatum

Brain homogenates from both the substantia nigra (SN) and the striatum were subjected to western analysis and TH enzymatic activity assay. Protein levels, as well as TH activity, remained unchanged in both the SN and striatum following PTT exposure (data not shown).

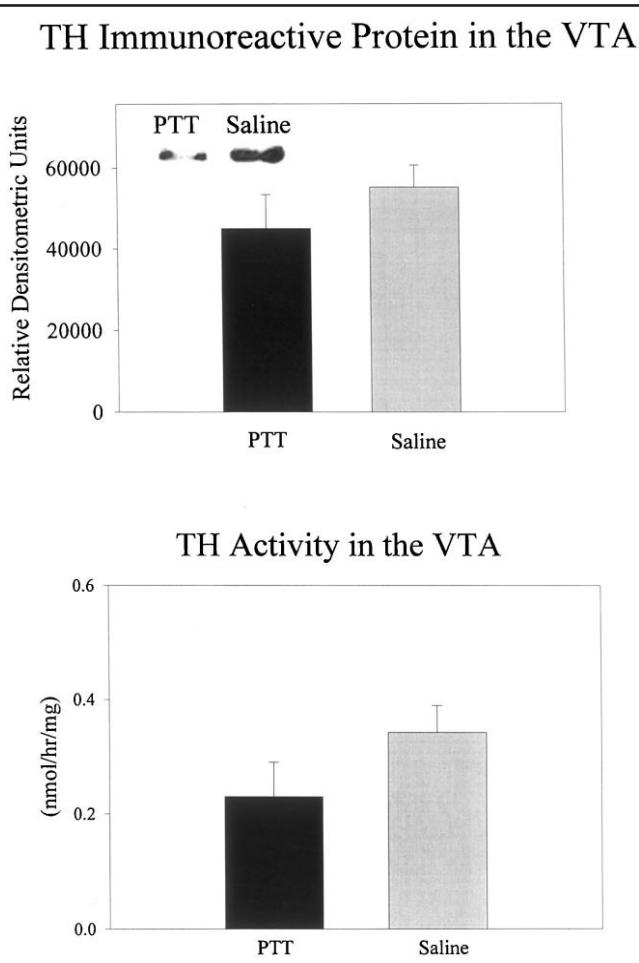


#### 4. DISCUSSION

The present study demonstrated that PTT reduced TH expression in the mesolimbic DA system. This is in contrast to previously reported increases in TH following repeated cocaine administration; see Table 1 (Beitner-Johnson and Nestler, 1991; Sorg et al., 1993, Vrana et al., 1993; Masserano et al., 1996). The reasons for this difference remain unclear, but might be in part due to the differing pharmacokinetic profile of PTT when compared to cocaine. Whereas cocaine is relatively equipotent at dopamine and norepinephrine transporters and slightly less potent at the serotonin uptake sites, PTT has some notable differences. First,

it is approximately 20- to 40-times more potent at the dopamine and norepinephrine uptake sites (e.g., IC<sub>50</sub> for uptake inhibition of approximately 3 nM for PTT and approximately 125 nM for cocaine; Bennett et al., 1998). Second, because PTT and cocaine are relatively equipotent at the serotonin transporter (approximately 450 nM), PTT therefore shows a more pronounced selectivity for dopamine and norepinephrine transport than serotonin. (Bennett et al., 1998). Additionally, PTT exhibits a longer duration of action than cocaine. In contrast to the behavioral half-life of 45 min exhibited by cocaine, PTT possesses a half-life on the order of 4 hours (Porrino et al., 1995). The increased potency and long duration

**Figure 3:** A. SDS-PAGE/immuno blot analysis of TH protein in the VTA. TH in the VTA of both saline ( $n=6$ ) and PTT ( $n=5$ ) treated rats was detected with a polyclonal antibody. Protein levels were quantified by densitometric analysis of X-ray film. Although not statistically significant ( $P=0.319$ ), there was a trend toward decreasing protein levels (19% reduction) following chronic PTT administration (see insert for typical examples of the 61 kDa TH signal). B. TH activity in the VTA. A radioenzymatic activity assay for TH was utilized to determine TH activity in the VTA of rats treated with saline ( $n=6$ ) and PTT ( $n=5$ ). Brain homogenate protein levels were determined with a Bradford assay in order to normalize TH activity (nmol product hr<sup>-1</sup> mg total protein<sup>-1</sup>). A non-significant (33%) decrease ( $P=0.170$ ) in TH activity following PTT administration was observed.



of action of PTT at DA transporters should result in a prolonged increase in synaptic DA levels within aspects of the mesolimbic pathway. It is hypothesized that this sustained increase in synaptic DA, as opposed to the episodic increases in synaptic DA that result from repeated cocaine, activates a negative feedback mechanism. The prolonged increases in synaptic DA levels would activate DA D2 receptors presynaptically (Kohl et al., 1998), which in turn signal the neuron to decrease DA output and decrease the need for catecholamine biosynthesis. In a similar fashion, chronic stimulation of postsynaptic D1 and D2 receptors could engage anatomical feedback circuitries. In the short-term, these responses are known to decrease the posttranslational phosphorylation of TH thus decreasing the enzyme's activity. However, in the present case (following chronic and excessive stimulation of autoreceptors and anatomical feedback circuitry), these same systems may decrease TH gene expression perhaps through down-regulation of PKA-mediated phosphorylation and subsequent decreased stimulation of CREB activity. Such long-term responses would serve to decrease TH protein and activity. Paradoxically, this is precisely what was originally predicted to occur with cocaine (i.e., increased synaptic dopamine decreasing TH expression). While that logic did not prove to be the case in those cocaine experiments, it may apply in the present circumstance with a long-acting cocaine analog.

The observed decreases in TH protein and activity are quite dramatic. TH levels for saline treated animals are approximately 2- and 3-times the values observed following PTT treatment for activity (54% decrease) and for immunoreactive protein (69% decrease), respectively. This is compared with the 20% to 50% increases observed following chronic cocaine admin-

istration (Beitner-Johnson and Nestler, 1991; Sorg et al., 1993; Vrana et al., 1993; Masserano et al., 1996). Moreover, these types of decreases are comparable to, or exceed, the decreases in TH observed following methamphetamine administration (Fukamauchi et al., 1996; Haughey et al., 1999). Functionally, the observed decreases in TH could have similarly robust effects on catecholamine levels. However, we failed to observe overt behavioral changes (e.g., akinesia, bradykinesia) in response to the chronic administration of PTT. This could be explained by the well-documented clinical observation that symptoms of Parkinson's disease are not manifested until >90% of the nigrostriatal dopaminergic neurons and DA are depleted. Nevertheless, such changes could compromise the animal in times of increased requirements for DA. Alternatively, TH activity and protein may be down-regulated precisely because stores of DA have not been utilized (due to decreased stimulated release in the face of reuptake blockade). While both TH activity and immunoreactive protein were decreased to comparable extents, there may also be differences in the post-translational phosphorylation state of the enzyme that were not addressed in the present studies. In fact, it would be predicted that overstimulation of the system should produce differences in the regulation of the enzyme. Further studies will be required to resolve these issues.

The geographic differences seen in TH protein and activity with PTT, compared to cocaine are also difficult to reconcile (Table 1). That is, in the case of cocaine, all studies have suggested significant increases in mRNA, protein, and activity in cell bodies with non-significant changes in the terminal fields (Beitner-Johnson and Nestler, 1991; Sorg et al., 1993; Vrana et al., 1993; Masserano et al., 1996). This phenomenon

has been attributed to a downregulation of neurofilaments seen with cocaine and morphine administration (Beitner-Johnson et al., 1992). This downregulation in cytoskeletal proteins is hypothesized to produce decreased transport from the cell body (VTA) to the terminal fields (NAcc); hence, TH is increased in the cell bodies but cannot be transported to the terminal fields. In the present case, there are decreases in TH protein and activity in the VTA (Fig. 3a and 3b) that do not achieve statistical significance. The decreased expression of TH in the VTA would be exacerbated by decreased transport to the NAcc (should the neurofilaments prove to also be downregulated), resulting in the further decreases in TH protein and activity observed in the NAcc (Fig. 2a and 2b). Thus, the downregulation of TH in the VTA might be mitigated somewhat by decreased TH transport from the cell bodies to the terminal fields.

These findings identify biochemical correlates for the differential behavioral results seen with cocaine analogs. For example, when PTT was substituted for cocaine in non-human primates, it did not function as a reinforcer and yet produced discriminative stimulus effects similar to cocaine (Nader et al., 1997). As discussed above, while both PTT and cocaine act to block biogenic amine reuptake, differences in their selectivity and pharmacokinetics may produce dramatically different cellular and behavioral responses. Further understanding of these differences will be crucial for the formulation of cocaine abuse pharamcotherapies.

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**References:**

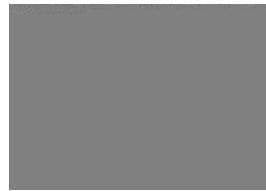
- Abraham, P., Pitner, J.B., Lewin, A.H., Boja, J.W., Kuhar, M.J., Carroll, F.I., 1992. N-modified analogues of cocaine: synthesis and inhibition of binding to the cocaine receptor. *J. Med. Chem.* 35, 141-144.
- Beitner-Johnson, D., Nestler, E.J., 1991. Morphine and cocaine exert common chronic actions on tyrosine hydroxylase in dopaminergic brain reward regions. *J. Neurochem.* 57, 344-347.
- Beitner-Johnson, D., Guitart, X., Nestler, E.J., 1992. Neurofilament proteins and the mesolimbic dopamine system: common regulation by chronic morphine and chronic cocaine in the rat ventral tegmental area. *J. Neurosci.* 12, 2165-2176.
- Bennett, B.A., Hollingsworth, C.K., Martin, R.S., Childers, S.R., Ehrenkaufer, R.E., Porriño, L.J., Davies, H.M.L., 1998. Prolonged dopamine and serotonin transporter inhibition after exposure to tropanes. *Neuropharmacology.* 37, 123-130.
- Boja, J.W., Kuhar, M.J., Kopajtic, T., Yang, E., Abraham, P., Lewin, A.H., Carroll, F.I., 1994. Secondary amine analogues of 3 beta-(4'-substituted phenyl)tropane-2 beta-carboxylic acid esters and N-norcocaine exhibit enhanced affinity for serotonin and norepinephrine transporters. *J. Med. Chem.* 37, 1220-1223.
- Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248-254.
- Carroll, F.I., Gao, Y., Abraham, P., Lewin, A.H., Lew, R., Patel, A., Boja, J.W., Kuhar, M.J., 1992a. Probes for the cocaine receptor. Potentially irreversible ligands for the dopamine transporter. *J. Med. Chem.* 35, 1813-1817.
- Carroll, F.I., Lewin, A.H., Boja, J.W., Kuhar, M.J., 1992b. Cocaine receptor: biochemical characterization and structure-activity relationships of cocaine analogues at the dopamine transporter. *J. Med. Chem.* 35, 969-981.
- Carroll, F.I., Gray, J.L., Abraham, P., Kuzemko, M.A., Lewin, A.H., Boja, J.W., Kuhar, M.J., 1993. 3-Aryl-2-(3'-substituted-1',2',4'-oxadiazol-5'-yl) tropane analogues of cocaine: affinities at the cocaine binding site at the dopamine, serotonin, and norepinephrine transporters. *J. Med. Chem.* 36, 2886-2890.
- Colpaert, F.C., Niemegeers, C.J., Janssen, P.A., 1978. Neuroleptic interference with the cocaine cue: internal stimulus control of behavior and psychosis. *Psychopharmacology.* 58, 247-255.
- Davies, H.M.L., Saikali, E., Sexton, T., Childers, S.R., 1993. Novel 2-substituted cocaine analogs: binding properties at dopamine transport sites in rat striatum. *Eur. J. Pharmacol.* 244, 93-97.
- Davies, H.M.L., Saikali, E., Huby, N.J., Gilliatt, V.J., Matasi, J.J., Sexton, T., Childers, S.R., 1994. Synthesis of 2 beta-acyl-3 beta-aryl-8-azabicyclo[3.2.1]octanes and their binding affinities at dopamine and serotonin transport sites in rat striatum and frontal cortex. *J. Med. Chem.* 37, 1262-1268.
- DeWit, H., Wise, R., 1977. Blockade of cocaine reinforcement in rats with the dop-

- maine blokcer, pimozide, but not with the noradrenergic blockers phentolamine or phenoxybenzamine. *Can. J. Psych.* 31, 195-303.
- Freedland, C.S., Daunais, J.B., Hart, S.L., Smith H.R., Davies, H.M.L., Porrino, L.J., 2000. A comparison of the behavioral and neurobiological effects of the repeated administration of PTT (2-propanoyl-3(4-tolyl)-tropane) and cocaine. *Brain Res.* 869, 98-104.
- Fukamauchi, F., Ishimaru, M., Hashimoto, T., Obata, K., 1996. Neurochemical analysis of the tyrosine hydroxylase expression in methamphetamine-sensitized rats. *Annals New York Acad. Sci.* 801, 371-6.
- Grant, K.A., 1995. The role of 5-HT3 receptors in drug dependence. *Drug and Alcohol Depend.* 38, 155-171.
- Haughey, H.M., Fleckenstein, A.E., Hanson, G.R., 1999. Differential regional effects of methamphetamine on the activities of tryptophan and tyrosine hydroxylase. *J. Neurochem.* 72, 661-8.
- Heikkila, R.E., Cabbat, F.S., Manzino, L., Duvoisin, R.C., 1979. Rotational behavior induced by cocaine analogs in rats with unilateral 6-hydroxydopamine lesions of the substantia nigra: dependence upon dopamine uptake inhibition. *J. Pharmacol. Exp. Ther.* 211, 189-194.
- Hemby, S.E., Co, C., Reboussin, D., Davies, H.M.L., Dworkin, S.I., Smith, J.E., 1995. Comparison of a novel tropane analog of cocaine, 2 beta- propanoyl-3 beta-(4-tolyl)-tropane with cocaine HCl in rats: nucleus accumbens extracellular dopamine concentration and motor activity. *J. Pharmacol. Exp. Ther.* 273, 656-666.
- Herrting, G., Axelrod, J., Whitby, L., 1961. Effect of drugs on the uptake and metabolism of 3H-norepinephrine. *J. Pharmacol. Exp. Ther.* 134, 146-153.
- Kohl, R.R., Katner, J.S., Chernet, E., McBride, W.J., 1998. Ethanol and negative feedback regulation of mesolimbic dopamine release in rats. *Psychopharmacology.* 139, 79-85.
- Koob, G.F., Bloom, F.E., 1988. Cellular and molecular mechanisms of drug dependence. *Science* 242, 715-723.
- Koob, G.F., 1992. Neural mechanisms of drug reinforcement. *Annals New York Acad. Sci.* 654, 171-191.
- Kozikowski, A.P., Roberti, M., Xiang, L., Bergmann, J.S., Callahan, P.M., Cunningham, K.A., Johnson, K.M., 1992. Structure-activity relationship studies of cocaine: replacement of the C-2 ester group by vinyl argues against H-bonding and provides an esterase-resistant, high-affinity cocaine analogue. *J. Med. Chem.* 35, 4764-4766.
- Kumer, S.C., Vrana, K.E., 1996. Intricate regulation of tyrosine hydroxylase activity and gene expression. *J. Neurochem.* 67, 443-462.
- Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685.
- Lewin, A.H., Gao, Y.G., Abraham, P., Boja, J.W., Kuhar, M.J., Carroll, F.I., 1992. 2 beta-substituted analogues of cocaine. Synthesis and inhibition of binding to the cocaine receptor. *J. Med. Chem.* 35, 135-140.

- Masserano, J.M., Baker, I., Natsukari, N., Wyatt, R.J., 1996. Chronic cocaine administration increases tyrosine hydroxylase activity in the ventral tegmental area through glutaminergic- and dopaminergic D2-receptor mechanisms. *Neurosci. Letters* 217, 73-76.
- Meltzer, P.C., Liang, A.Y., Brownell, A.L., Elmaleh, D.R., Madras, B.K., 1993. Substituted 3-phenyltropane analogs of cocaine: synthesis, inhibition of binding at cocaine recognition sites, and positron emission tomography imaging. *J. Med. Chem.* 36, 855-862.
- Meltzer, P.C., Liang, A.Y., Madras, B.K., 1994. The discovery of an unusually selective and novel cocaine analog: difluoropine. Synthesis and inhibition of binding at cocaine recognition sites. *J. Med. Chem.* 37, 2001-2010.
- Meltzer, P.C., Liang, A.Y., Madras, B.K., 1996. 2-Carbomethoxy-3-( diarylmethoxy)-1 alpha H, 5 alpha H-tropane analogs: synthesis and inhibition of binding at the dopamine transporter and comparison with piperazines of the GBR series. *J. Med. Chem.* 39, 371-379.
- Miczek, K.A., Yoshimura, H., 1982. Disruption of primate social behavior by d-amphetamine and cocaine: differential antagonism by antipsychotics. *Psychopharmacology* 76, 163-171.
- Moore, K., Chiueh, C., Zeldes, G., 1977. Release of neurotransmitters in the brain in vivo by amphetamine, methylphenidate and cocaine. In: Ellinwood E., Kilbey M., (Eds.), *Cocaine and Other Stimulants*. Plenum Press, New York, 143-160.
- Nader, M.A., Grant, K.A., Davies, H.M.L., Mach, R.H., Childers, S.R., 1997. The reinforcing and discriminative stimulus effects of the novel cocaine analog 2beta-propanoyl-3beta-(4-tolyl)-tropane in rhesus monkeys. *J. Pharmacol. Exp. Ther.* 280, 541-550.
- Nayak, P.K., Misra, A.L., Mule, S.J., 1976. Physiological disposition and biotransformation of (3H) cocaine in acutely and chronically treated rats. *J. Pharmacol. Exp. Ther.* 196, 556-569.
- O'Neill, R.R., Mitchell, L.G., Merril, C.R., Rasband, W.S., 1989. Use of image analysis to quantitate changes in form of mitochondrial DNA after x-irradiation. *Applied Theor. Electrophor.* 1, 163-167.
- Paxinos, G., Watson, C., 1986. *The Rat Brain in Stereotaxic Coordinates*. 2nd Ed. Academic Press, San Diego.
- Porrino, L.J., Migliarese, K., Davies, H.M.L., Saikali, E., Childers, S.R., 1994. Behavioral effects of the novel tropane analog, 2 beta-propanoyl- 3 beta-(4-tolyl)-tropane (PTT). *Life Sci.* 54, PL511-7.
- Porrino, L.J., Davies, H.M.L., Childers, S.R., 1995. Behavioral and local cerebral metabolic effects of the novel tropane analog, 2 beta-propanoyl-3 beta-(4-tolyl)-tropane. *J. Pharmacol. Exp. Ther.* 272, 901-910.
- Ritz, M.C., Lamb, R.J., Goldberg, S.R., Kuhar, M.J., 1987. Cocaine receptors on dopamine transporters are related to self-administration of cocaine. *Science* 237, 1219-1223.
- Roberts, D.C.S., Corcoran, M.E., Fibiger, H.C., 1977. On the role of ascending catecholaminergic systems in intravenous

- self-administration of cocaine. *Pharmacol. Biochem. Behav.* 6, 615-620.
- Roberts, D.C.S., Koob, G.F., 1982. Disruption of cocaine self-administration following 6-hydroxydopamine lesions of the ventral tegmental area in rats. *Pharmacol. Biochem. Behav.* 17, 901-904.
- Robledo, P., Maldonado-Lopez, R., Koob, G.F., 1992. Role of dopamine receptors in the nucleus accumbens in the rewarding properties of cocaine. *Annals New York Acad. Sci.* 654, 509-512.
- Ross, S.B., Renyi, A.L., 1967. Inhibition of the uptake of tritiated catecholamines by antidepressant and related agents. *Eur. J. Pharmacol.* 2, 181-186.
- Samson, H.H., Tolliver, G.A., Haraguchi, M., Hodge, C.W., 1992. Alcohol self-administration: role of mesolimbic dopamine. *Annals New York Acad. Sci.* 654, 242-253.
- Shuster, L., 1992. Pharmacokinetics, metabolism, and disposition of cocaine. In: Lakowski, J.M., Galloway, M.P., White, F.J., (Eds.), *Cocaine: Pharmacology, Physiology and Clinical Strategies*. CRC Press, Boca Raton, FL, 1-14.
- Sorg, B.A., Chen, S.Y., Kalivas, P.W., 1993. Time course of tyrosine hydroxylase expression after behavioral sensitization to cocaine. *J. Pharmacol. Exp. Ther.* 266, 424-430.
- Vrana, S.L., Vrana, K.E., Koves, T.R., Smith, J.E., Dworkin, S.I., 1993. Chronic cocaine administration increases CNS tyrosine hydroxylase enzyme activity and mRNA levels and tryptophan hydroxylase enzyme activity levels. *J. Neurochem.* 61, 2262-2268.
- Wilcox, K.M., Paul, I.A., Woolverton, W.L., 1999. Comparison between dopamine transporter affinity and self-administration potency of local anesthetics in rhesus monkeys. *Eur. J. Pharm.* 367, 175-181.

# Chapter 7: Conclusion



Drug abuse remains a major societal problem with a direct and indirect toll of billions of dollars and immeasurable suffering. Additionally, the large scale criminal enterprises associated with supplying drugs of abuse undermines stability and peace across the world (Pardo 2000). As one of the many abused drugs, cocaine remains a major problem, with millions of regular users. While much progress has been made on understanding cocaine's actions on the brain, much remains to be learned. A greater understanding of cocaine's molecular biological actions will aid in developing pharmacotherapies (Leshner and Koob 1999; Leshner 1997).

One of the primary actions of cocaine on the brain is to increase synaptic dopamine levels through blockade of dopamine reuptake by the presynaptic terminal (Moore et al. 1977; Heikkila et al. 1979; Ritz et al. 1987). This action has been shown to mediate many of the behavioral (DeWit and Wise 1977; Colpaert et al. 1978; Miczek and Yoshimura 1982; Ritz et al. 1987) and locomotor activating effects (Kelly and Iverson, 1976; Giros et al., 1996) of cocaine. Many research efforts have, therefore, focused on dopaminergic brain regions for the study cocaine's behavioral and physiological effects. Roberts et al. (1977) demonstrated through lesion studies that the nucleus accumbens (NAcc), a terminal projection of the dopaminergic ventral tegmental area (VTA) produced an inhibition of the rewarding aspects of cocaine. Another projection region of the VTA, the prefrontal cortex has also been implicated in the

behavioral effects of cocaine through the demonstration that rats will self-administer microinjections of cocaine into that region (Goeders and Smith 1983). Other pathways, like the nigrostriatal pathway, or other regions receiving dopaminergic projections, such as the hippocampus (Gasbarri et al. 1997), are likely to play important roles in cocaine's behavioral effects (Bardo 1998). The mesohippocampal pathway is one such pathway as it involves both the dopaminergic synapses and hippocampal learning and memory functions (Gasbarri et al. 1996).

Before examining the changes in physiology or gene expression in any of the areas described, it is critical to begin with a framework for understanding behavior. These behaviors include the phenomena of sensitization, tolerance, psychological dependence, compulsive drug taking, and drug seeking. The focus of this dissertation is on chronic cocaine intake and, by proxy, the behaviors associated with this chronic state. One model for understanding behavior after chronic cocaine abuse is that of an allostatic state in which the behavior phenotype has been altered to a new baseline as compared to the 'normal' baseline of the naive state (Koob and Le Moal 2001). In this allostatic state there is increased difficulty in drug abuse cessation, loss of control, and increased vulnerability to relapse. If this model is indeed correct, then the next step in understanding this state is to identify the underlying molecular causes.

Changes in behavior may have their roots in physiological and molecular mech-

anisms (Koob and Le Moal 1997). As the focus of this dissertation is molecular adaptations to chronic cocaine, these changes deserve the most attention, but recent studies on morphological changes in neurons after chronic cocaine are worth describing first. Robinson and Kolb, (Robinson and Kolb 1999; Robison et al. 2001) have described increases in dendritic branching and density in the NAcc and prefrontal cortex. This increased branching and density has been shown in both non-contingent administration and self-administration of cocaine. Control animals which performed an operant task for a food reinforcer demonstrated no change in dendritic morphology, suggesting that these changes are specific to self-administration of cocaine and not the performance of an operant task for an alternate reinforcer.

With these well documented changes in behavior and physiology, the next step in understanding drug abuse is again to go a step deeper and look for the causes of changes in physiology and behavior. Changes in gene expression are thought to be one of the primary mechanisms by which the brain could adapt to chronic cocaine, producing an altered physiology and an allostatic behavioral state (Koob and Le Moal 2001; Nestler 2001) (see Figure 1, Chapter 1; page 2). While a number of gene expression changes have been described in response to chronic cocaine (Harlan and Garcia 1998; Torres and Horowitz 1999), the series of studies described here attempted to expand the knowledge of functional neurogenomic changes through the use of hybridization arrays.

The initial study on cocaine-responsive gene expression in the primate NAcc, showed that after a year of non-contingent cocaine, 4 genes: Protein Kinase A catalytic alpha subunit (PKA<sub>cα</sub>), mitogen activated

protein kinase kinase 1 (MEK1), β-Catenin, and protein tyrosine kinase 2 (PYK2) were significantly induced at the level of protein. None of these changes had been previously described in the non-human primate NAcc. Individually, however, all of these proteins (MEK1, PKA<sub>cα</sub>, β-Catenin, and PYK2) could affect a number of cellular processes ranging from receptor activity (Huang et al. 2001), to transcriptional activation (Berhow et al. 1996). In addition to these individual actions, and perhaps most importantly, these changes dovetail with known changes in activator protein 1 (AP-1) and cyclic AMP response element binding (CREB) activities following chronic cocaine which have been demonstrated to mediate some cocaine-responsive behaviors (Hope 1998; Hope et al. 1992; Self et al. 1998; Carlezon et al. 1998). While this study does not draw causal links between the observed changes in gene expression and these transcription factor complexes or behavior, it does provide new hypotheses for testing in future studies (see Figure 5, Chapter 2; page 57).

Using a non-contingent model of chronic cocaine administration in rats, the hippocampus, frontal cortex, and NAcc were examined for cocaine-responsive gene expression. In the hippocampus, a number of genes were found to be induced. Protein kinase C α (PKCα), Protein kinase C ε (PKCε), metabotropic glutamate receptor 5 (mGluR5), shaker potassium channel Kv1.1 (Kv1.1), PYK2, and β-Catenin protein expression were all found to be induced by chronic cocaine. These results individually point towards a number of possible cellular adaptations to cocaine. Interestingly, PYK2 and β-Catenin were seen once again to be induced. In respect to each other, the changes seen could represent both initial changes and compensatory responses due to the seemingly antagonistic interactions

between these genes (see figure 5, Chapter 3; page 75). For example, PKC $\alpha$  may help increase  $\beta$ -Catenin accumulation (Cook et al. 1996), while PKC $\epsilon$  may target  $\beta$ -Catenin for degradation (Orford et al. 1997). As well, the induction of PKC isoforms may inhibit the induced Kv1.1 channels (Boland and Jackson 1999). This study has illuminated changes in gene expression within a dopaminergic projection region highly associated with learning and memory and that is not typically examined for cocaine-responsive gene expression. The behavioral and physiological ramifications of these changes will have to be examined.

In the frontal cortex of chronically treated rats, hybridization array analysis and immunoblotting found increased expression of PYK2, activity-regulated cytoskeletal protein (ARC), and an antigen related to nerve-growth factor 1-B (NGF1-B). These changes are more difficult to ascribe functional properties to, but represent confirmation at the level of protein of previously described changes in gene expression (ARC) (Fosnaugh et al. 1995; Tan et al. 2000) and another brain region in which PYK2 is induced. The identity of the high molecular weight antigen related to NGF1-B will have to be identified. Immunoprecipitation and time-of-flight mass spectrometry analysis of the isolated protein would determine if this is a known protein or a novel entity. The fact that NGF1-B mRNA has been shown to increase in cortical areas after cocaine administration, (Werme et al. 2000) along with the large number of uncharacterized nuclear receptors related to NGF1-B, makes this a compelling avenue for future research.

In the NAcc of these same animals, hybridization array analysis has highlighted a number of cocaine-responsive genes. Immunoblot assays of these changes will

have to be performed to determine which change at the level of protein. Examination of genes found to be changed in the NAcc of the non-human primate showed that PYK2, is induced. This widespread activation of PYK2 expression makes investigation of PYK2 an important focus for future work. While the exact role of PYK2 in cocaine-responsive neuronal adaptation remains to be determined, the fact that it is induced in a fairly ubiquitous manner points towards a general role for PYK2 rather than one that represents a response of a particular brain region. This generalized induction could be a potentially important mechanism for any number of plastic actions and might represent a promising avenue for pharmacotherapeutic treatment development.

To expand the results previously described to a behaviorally-relevant paradigm, gene expression changes have begun to be investigated through hybridization array analysis of the NAcc of animals in a binge-abstinence cocaine self-administration paradigm (Roberts et al. 2001). Confirmation of changes seen by hybridization array analysis will have to be made to see which of these changes is recapitulated at the level of protein. Indirect comparisons with changes seen in non-contingent models will help generate hypotheses on the role of changes observed in the non-contingent animals.

Analysis of tyrosine hydroxylase (TH) protein expression and enzymatic activity in response to administration of the tropane analog, 2 $\beta$ -propanoyl-3 $\beta$ -(4-tolyl)-tropane (PTT or WF11) produced findings the opposite of what had been anticipated. Chronic administration of cocaine increases TH protein and enzymatic activity in the ventral tegmental area (VTA) and does not change either of these in the NAcc (Beitner-Johnson and Nestler 1991; Sorg et al.

1993; Vrana et al. 1993). PTT, a long-acting cocaine analog, did not affect TH protein or activity in the VTA, but decreased TH protein and activity in the NAcc. This finding speaks directly to the role of pharmacokinetics in differential gene expression. As has been stated previously, changes in gene expression accompanying cocaine administration are dependent of dose, duration of administration, length of withdrawal, brain region and contingency. This study demonstrated that an analog of cocaine that differs only in that two ester linkages have been removed (Bennett et al. 1995; Davies et al. 1993), produces a completely different effect on gene expression with the mesolimbic pathway. The extremely long-lasting nature of PTT makes these findings similar to the report that mice, lacking the dopamine transporter, have decreased levels of ventral midbrain TH protein (Jaber et al. 1999).

Taken as a whole, these studies provide a great deal of information on the use of hybridization arrays for functional neurogenomics research. Several issues regarding hybridization arrays remain to be answered, but on-going and future studies should answer these questions. First, the sources of variability in each step of hybridization array experiments need to be quantified. A study is underway in which the variability associated with the labeling and hybridization steps are being determined. This study uses equal amounts of the same labeling reaction hybridized to separate arrays and the same RNA labeled and hybridized to separate arrays. Determination of these factors will aid in creating a method for determining what magnitude of change can be reliably detected with microarrays.

Second, the reproducibility of results across laboratories and array platforms

remains unknown. Determination of these parameters is critical for the field of functional genomics and the creation of public gene expression databases. To study this, we have engaged a number of functional genomic and bioinformatic researchers in a project called MicroArray Research on Cocaine Self-Administration (MARCSA). Laboratories at the Medical College of South Carolina, the Vollum Institute, the Scripps Research Institute, University of Texas Southwestern, and the Medical College of Wisconsin will perform hybridization array experiments on the same samples. This project will allow rigorous analysis of the reproducibility and greater insight into the variability of hybridization array experiments.

Third, the large amounts of data generated by functional genomic research must be shared with the larger research community. While the previous two points illustrate the need for efforts underway to understand variability among hybridization array experiments, the mechanism for data-sharing still needs perfecting. To contribute to this effort the Drug Abuse & Alcohol Abuse Microarray Data Consortium (DAMDC) has been created here at Wake Forest University ([www.arraydata.org](http://www.arraydata.org)). This database provides a mechanism by which data from experiments performed here can be downloaded by any interested party once the original research paper has been published. Moreover, data sets from the research studies described in this dissertation are being submitted to the Gene Expression Omnibus (GEO) ([www.ncbi.nlm.nih.gov/geo/](http://www.ncbi.nlm.nih.gov/geo/)) a National Library of Medicine data-sharing project.

The results of these studies do not conclusively show what role these gene expression changes have on physiology and/or behavior. However, the cocaine-

responsive changes in gene expression found through this research provide the basis for a number of studies to determine the end result of these changes. Understanding the role of these changes involves two steps. The first step is to interfere with the action of these genes seen through inhibitors and to analyze molecular, physiological and behavioral effects. For example, analyzing the effects of isoform specific inhibition of PKC activity in the hippocampus on cocaine self-administration, changes in gene expression, and morphological changes (Robinson and Kolb 1999; Robinson et al. 2001) will show the role of PKC. If PKC is shown to be crucial in mediating any of these changes, inducible, temporally specific transgenic animals (Kelz et al. 1999; Bibb et al. 2001), could then be used to examine the specific effects of increased expression of a PKC isoform.

In conclusion, the many efforts on functional genomic research (Lockhart and Winzeler 2000) are beginning to expand our understanding of molecular biology and its function in physiology and psychology. For molecular neurobiology and pharmacology, the greatest benefit will come when targets identified through functional neurogenomic studies such as those presented here can be tested with the many experimental tools available.

## References:

- Bardo M. T. (1998) Neuropharmacological mechanisms of drug reward: beyond dopamine in the nucleus accumbens. *Crit Rev Neurobiol* 12, 37-67.
- Beitner-Johnson D. and Nestler E. J. (1991) Morphine and cocaine exert common chronic actions on tyrosine hydroxylase in dopaminergic brain reward regions. *J Neurochem* 57, 344-347.
- Bennett B. A., Wichems C. H., Hollingsworth C. K., Davies H. M., Thornley C., Sexton T., and Childers S. R. (1995) Novel 2-substituted cocaine analogs: uptake and ligand binding studies at dopamine, serotonin and norepinephrine transport sites in the rat brain. *Journal of Pharmacology & Experimental Therapeutics* 272, 1176-1186.
- Berhow M. T., Hiroi N., and Nestler E. J. (1996) Regulation of ERK (extracellular signal regulated kinase), part of the neurotrophin signal transduction cascade, in the rat mesolimbic dopamine system by chronic exposure to morphine or cocaine. *J Neurosci* 16, 4707-4715.
- Bibb J. A., Chen J., Taylor J. R., Svartengrensson P., Nishi A., Synder G. L., Nestler E. J., and Greengard P. (2001) Effects of chronic exposure to cocaine are regulated by the neuronal protein Cdk5. *Nature* 410, 376-380.
- Boland L. M. and Jackson K. A. (1999) Protein kinase C inhibits Kv1.1 potassium channel function. *Am J Physiol* 277, C100-C110.
- Carlezon W. A., Thome J., Olson V. G., Lane-Ladd S. B., Brodkin E. S., Hiroi N., Duman R. S., Neve R. L., and Nestler E. J. (1998) Regulation of cocaine reward by CREB. *Science* 282, 2272-2275.
- Colpaert F. C., Niemegeers C. J., and Janssen P. A. (1978) Neuroleptic interference with the cocaine cue: internal stimulus control of behavior and psychosis. *Psychopharmacology* 58, 247-255.
- Cook D., Fry M. J., Hughes K., Sumathipala R., Woodgett J. R., and Dale T. C. (1996) Wingless inactivates glycogen synthase kinase-3 via an intracellular signalling pathway which involves a protein kinase C. *EMBO J* 15, 4526-4536.
- Davies H. M., Saikali E., Sexton T., and Childers S. R. (1993) Novel 2-substituted cocaine analogs: binding properties at dopamine transport sites in rat striatum. *European Journal of Pharmacology* 244, 93-97.
- DeWit H. and Wise R. (1977) Blockade of cocaine reinforcement in rats with the dopamine blocker, pimozide, but not with the noradrenergic blockers phentolamine or phenoxybenzamine. *Canadian Journal of Psychology* 31, 195-303.
- Fosnaugh J. S., Bhat R. V., Yamagata K., Worley P. F., and Baraban J. M. (1995) Activation of arc, a putative "effector" immediate early gene, by cocaine in rat brain. *J Neurochem* 64, 2377-2380.
- Gasbarri A., Sulli A., Innocenzi R., Pacitti C., and Brioni J. D. (1996) Spatial memory impairment induced by lesion of the mesohippocampal dopaminergic system in the rat. *Neuroscience* 74, 1037-1044.
- Gasbarri A., Sulli A., and Packard M. G. (1997) The dopaminergic mesencephalic projections to the hippocampal formation

- the rat. *Prog Neuropsychopharmacol Biol Psychiatry* 21, 1-22.
- Goeders N. E. and Smith J. E. (1983) Cortical dopaminergic involvement in cocaine reinforcement. *Science* 221, 773-775.
- Harlan R. E. and Garcia M. M. (1998) Drugs of abuse and immediate-early genes in the forebrain. *Mol Neurobiol* 16, 221-267.
- Heikkila R. E., Cabbat F. S., Manzino L., and Duvoisin R. C. (1979) Rotational behavior induced by cocaine analogs in rats with unilateral 6-hydroxydopamine lesions of the substantia nigra: dependence upon dopamine uptake inhibition. *Journal of Pharmacology & Experimental Therapeutics* 211, 189-194.
- Hope B., Kosofsky B., Hyman S. E., and Nestler E. J. (1992) Regulation of immediate early gene expression and AP-1 binding in the rat nucleus accumbens by chronic cocaine. *Proc Natl Acad Sci U S A* 89, 5764-5768.
- Hope B. T. (1998) Cocaine and the AP-1 transcription factor complex. *Ann NY Acad Sci* 844, 1-6.
- Huang Y., Lu W., Ali D. W., Pelkey K. A., Pitcher G. M., Lu Y. M., Aoto H., Roder J. C., Sasaki T., Salter M. W., and MacDonald J. F. (2001) CAKbeta/Pyk2 kinase is a signaling link for induction of long-term potentiation in CA1 hippocampus. *Neuron* 29, 485-496.
- Jaber M., Dumartin B., Sagne C., Haycock J. W., Roubert C., Giros B., Bloch B., and Caron M. G. (1999) Differential regulation of tyrosine hydroxylase in the basal ganglia of mice lacking the dopamine transporter. *Eur J Neurosci* 11, 3499-3511.
- Kelz M. B., Chen J., Carlezon W. A., Whisler K., Gilden L., Beckmann A. M., Steffen C., Zhang Y. J., Marotti L., Self D. W., Tkatch T., Baranauskas G., Surmeier D. J., Neve R. L., Duman R. S., Picciotto M. R., and Nestler E. J. (1999) Expression of the transcription factor deltaFosB in the brain controls sensitivity to cocaine. *Nature* 401, 272-276.
- Koob G. F. and Le Moal M. (1997) Drug abuse: hedonic homeostatic dysregulation. *Science* 278, 52-58.
- Koob G. F. and Le Moal M. (2001) Drug addiction, dysregulation of reward, and allostasis. *Neuropsychopharmacology* 24, 97-129.
- Leshner A. I. (1997) Addiction is a brain disease, and it matters. *Science* 278, 45-47.
- Leshner A. I. and Koob G. F. (1999) Drugs of abuse and the brain. *Proc Assoc Am Physicians* 111, 99-108.
- Lockhart D. J. and Winzeler E. A. (2000) Genomics, gene expression and DNA arrays. *Nature* 405, 827-836.
- Miczek K. A. and Yoshimura H. (1982) Disruption of primate social behavior by d-amphetamine and cocaine: differential antagonism by antipsychotics. *Psychopharmacology* 76, 163-171.
- Moore K., Chiueh C., and Zeldes G. (1977) Release of neurotransmitters in the brain in vivo by amphetamine, methylphenidate and cocaine., in *Cocaine and other stimulants* (Ellinwood E. and Kilbey M., eds.), pp. 143-160. Plenum, New York.

Nestler E. J. (2001) Molecular basis of long-term plasticity underlying addiction. *Nature Neuroscience Reviews* 2, 119-128.

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# **Appendix A: Experimental Design for Hybridization Array Analysis of Gene Expression**



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## **ABSTRACT:**

Given the explosion in genomic information, the historical “one-gene-at-a-time” approach to gene expression analysis is no longer adequate. Instead, large-scale multiplex methods for analyzing gene expression patterns are needed. Several technologies have been developed to serve this function, including differential display, serial analysis of gene expression (SAGE), total gene expression analysis (TOGA), subtraction cloning, and DNA hybridization arrays (microarrays). This last approach, which is rapidly becoming the dominant technology in the gene expression field, is the subject of the present text. However, this powerful new technology also comes with a unique set of considerations when it comes to designing and executing experiments. In this chapter, experimental design will be considered from both strategic and tactical standpoints.

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### **3.1. Introduction**

Given the explosion in genomic information, the historical “one-gene-at-a-time” approach to gene expression analysis is no longer adequate. Instead, large-scale multiplex methods for analyzing gene expression patterns are needed. Several technologies have been developed to serve this function, including differential display, serial analysis of gene expression (SAGE), total gene expression analysis (TOGA), subtraction cloning, and DNA hybridization arrays (microarrays)(1). This last approach, which is rapidly becoming the dominant technology in the gene expression field, is the subject of the present text. However, this powerful new technology also comes with a unique set of considerations when it comes to designing and executing experiments. In this chapter, experimental design will be considered from both strategic and tactical standpoints.

In the three decades since the first recombinant DNA technologies were introduced, the standard paradigm has been to examine and characterize the sequence and expression of one or two genes at a time. At best, this approach involved the time- and labor-intensive sequential analysis of gene products in a given pathway. At worst, in the case of complex polygenic phenotypes or diseases, this time-consuming process has severely limited the ability of the molecular biology research community to move scientific understanding forward. The vast amounts of genomic data being generated by the Human Genome Project are

exacerbating this problem. In June 2000, researchers announced the completion of a rough draft of the human genome - the beginning of what some are calling the postgenomic era (a period of research in which the question is not how to sequence the genome, but what to do with the complete sequence). By 2001/2002, a high-fidelity sequence for all human genetic material will be available, providing detailed information on the estimated 100,000 genes required to encode a human being. In this postgenomic era of research, the old practices of “one gene at a time” will be inefficient and unproductive. Such approaches would not only be inefficient but would not sufficiently illuminate patterns of gene expression; therefore, they will be inappropriate for analyzing complex diseases or physiological/behavioral/pharmacological states.

### **3.2. Role of Hybridization Arrays in Functional Genomics**

The current challenge, therefore, is to develop/optimize methods for monitoring thousands of gene products simultaneously (genomic-scale analysis of gene expression). To this end, functional genomics is becoming a dominant feature of the molecular biology landscape (Figure 1 shows the various types of genetic information that can be mined). For the purpose of this chapter, “functional genomics” is defined as the study of all the genes expressed by a specific cell or group of cells and the changes in their expression pattern during development, disease, or environmental exposure. DNA polymorphism analysis is sometimes included under functional genomics, but for this chapter it is included under Genomics. With this definition in mind, we can say that functional genomics is simply large-scale

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#### Abbreviations:

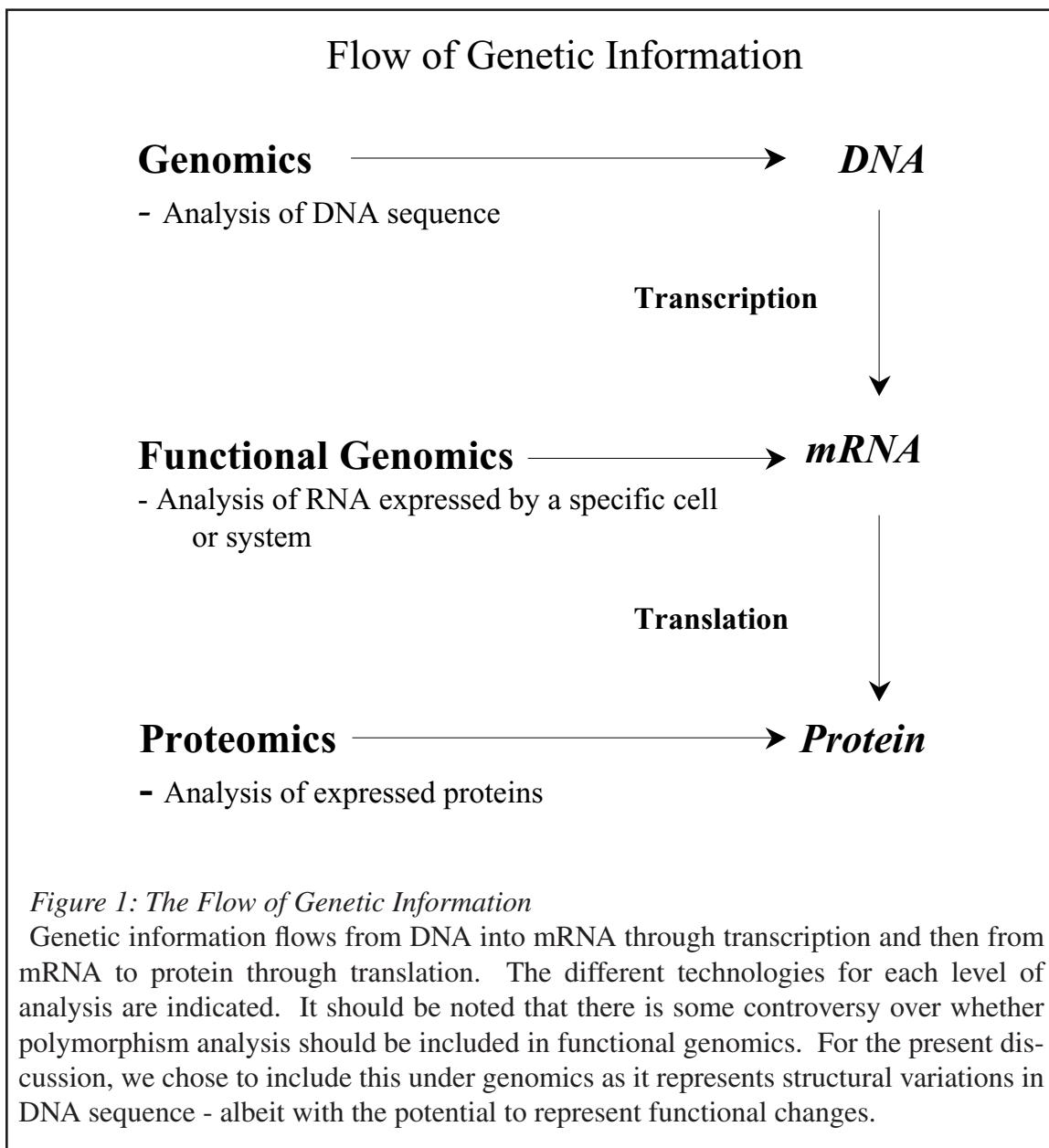
RT-PCR, reverse transcriptase-polymerase chain reaction; SAGE, serial analysis of gene expression; SNP, single nucleotide polymorphism; TOGA, total gene expression analysis

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gene expression analysis at the RNA level. Given that each cell in an organism inherits a constant genetic legacy (the DNA contained within the nucleus), it is the pattern of specific genes that is expressed that establishes the identity of a given cell or tissue. Analysis of these patterns in the context of the administration of drugs, in various disease process, or following exposure to toxins, will be central to understanding the biology and how humans respond, on a

molecular level, to these conditions.

Biological research and discovery in the postgenomic era will require management of an incredible wealth of information. The question is no longer one of being able to sequence genomes but what to do with the sequences. The vast amount of genetic information being generated by sequencing projects will not only tax our existing methods of data collection and management but will require us to change our fundamental



*Figure 1: The Flow of Genetic Information*

Genetic information flows from DNA into mRNA through transcription and then from mRNA to protein through translation. The different technologies for each level of analysis are indicated. It should be noted that there is some controversy over whether polymorphism analysis should be included in functional genomics. For the present discussion, we chose to include this under genomics as it represents structural variations in DNA sequence - albeit with the potential to represent functional changes.



*Figure 2: The Conceptual Flow of Functional Genomics Analysis*

Functional genomic analysis is designed to gain a global perspective of gene expression in a particular experimental state. Functional genomic analysis begins with the screening of as many genes as possible to see what genes are expressed in the cells of interest in a particular condition and what differences in gene expression may be of importance. To overcome the lack statistical power and the large possibility of false positives with arrays some form of post-hoc testing is needed. Changes seen with the hybridization array and confirmed then need to be incorporated into the existing knowledge of the question at hand. Finally, to show direct causative links, interference or manipulation studies are needed.

experimental mind-set. We will no longer be interested in individual genes; rather, the emphasis will be the analysis of patterns of gene expression.

Returning to Figure 1, note that molecular-biological analysis can occur at three different levels. Most of the previous work has focused on the genomic - or DNA - level. Diseases have traditionally been examined by mapping inherited disorders with traditional genetic methods. Alternatively, individual genes were cloned (based on rational biochemical insights) and characterized relative to a disease or physiological response. Now, a new generation of genomic technologies will take the dominant position. These technologies allow rapid sequencing of DNA for diagnostic and research purposes and genome scans for single nucleotide polymorphisms (SNPs). SNPs are single base-pair variations in DNA that may cause disease or be useful as markers of disease. While extremely important, work at the DNA level does not answer all questions associated with the transcription of RNA and the translation of protein - gene expression. For example, exposure to a neurotoxin may induce the expression of a programmed cell death (apoptosis) pathway, leading to neurodegeneration. Such a change in gene expression in response to an environmental insult might be unrelated to a specific sequence polymorphism and yet still represent a valuable therapeutic target for drug design. None of the traditional genomic approaches - nor most of the new SNP analysis methods - is well suited to broad-based gene expression studies.

One of the best ways (if not theoretically the best way) to study gene expression is to examine the proteins encoded by genes. Studying all the proteins expressed in a cell is known as proteomics (2). By comparing protein patterns in treated versus

untreated tissues or in diseased versus non-diseased tissues or cells, researchers can pinpoint the proteins involved in disease processes-proteins that could be targets of novel therapies. The proteins, after all, are the key to realizing the potential encoded in the genome. Unfortunately, proteomic analysis - although clearly the best choice - is technically tedious (involving two-dimensional protein electrophoresis), requires sophisticated infrastructure (mass spectrometry), and is not necessarily high-throughput in nature. These characteristics have placed this approach beyond the reach of most investigators outside of the large pharmaceutical companies and have made companies that have improved the technology unwilling to publicize their progress for proprietary reasons.

The other means of gene expression analysis is functional genomics, which, on the surface, is not the stage-of-choice for analyzing gene expression because RNA is a transitional step from DNA to protein. Indeed, RNA has limited value except as a protein precursor. However, functional genomics can build upon the base of knowledge generated by the Human Genome Project to simultaneously examine the expression of thousands of genes. This large-scale expression analysis is possible because gene-specific probes for mRNA can be generated from DNA sequence information. Once identified at the level of mRNA, alterations in gene expression can be extended to protein. The functional genomic analysis therefore helps to identify target proteins for additional study.

The limitations of examining mRNA levels is that it does not provide direct insight into underlying polymorphisms (SNPs) that could be basis of disease, and that just because an mRNA level changes does not mean the corresponding protein levels must

# Technical Flow of Functional Genomic Experiments

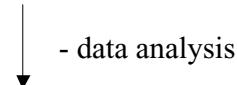
## Experimental Design

- sample collection
- detection sensitivity
- array format choice

## Hypothesis generation

- large scale screening of thousands of genes

## Targets



## Validation



## Hypothesis testing

- custom small scale arrays

## Biological narrative

*Figure 3: Technical Flow of Functional Genomics Experiments*

Technically, functional genomic analysis goes through three stages: hypothesis generation, target validation, and hypothesis testing to arrive at the endpoint of all functional genomic research, the biological narrative. Large scale arrays (thousands of genes) are useful for initial screens of gene transcription. Changes seen on the hybridization array need to be validated by either nucleic acid or protein methodologies. To further investigate the question, custom or small scale arrays can be constructed that contain the genes initially identified to be changed as well as related genes. Ultimately, these gene expression changes can be incorporated into existing knowledge about the individual genes and the experimental question.

change(3). In addition, mRNA measurements do not account for changes that a protein may undergo (glycosylation, phosphorylation, subcellular targeting, etc.) after it is produced. However, hybridization array technology is readily available and can be accessed by nearly any laboratory to provide valuable insights into functional genomics. The key point is that these are unique problems associated with this technology that must be taken into account.

### ***3.3. Strategic Considerations in Array Experimental Design***

The main reason for undertaking DNA hybridization analysis is to accomplish two important goals. The first is to provide a broad-based screen of gene expression. The desire is to effectively and economically filter through thousands of genes to identify those that are regulated by a physiological or pharmacological intervention. As the field rapidly accumulates knowledge on the 100,000 or so distinct genes, this will prove to be the only way to effectively study biological processes. A second goal is actually to understand patterns of gene expression. We will soon be in a position to understand not only how genes are regulated in isolation, but how families of genes or members of common regulatory pathways are coordinately regulated. Therefore, the strategic implications of how we recognize and analyze patterns of gene expression will be at least as important as the array technology itself.

#### ***3.3.1. Large Scale Functional Genomic Screening***

Initial functional genomic screens seek to establish what genes are expressed in a given cellular population and what

genes appear to be regulated by experimental conditions as compared to control conditions. Large scale screens are initially needed because the full complement of genes expressed in different tissues and cells is usually unknown. While much may be known about the genes expressed in a particular cell, this set of genes may change under the experimental condition. Though the genes contained on the arrays used for this initial screen may be very large the array will most likely be incomplete. The overriding principle of this step in the process is ‘hypothesis generation’(4). That is, large-scale DNA arrays should be considered a means for creating testable hypotheses.

There are three main platforms available for large scale gene expression scans: macroarrays, microarrays and high-density oligonucleotide arrays. The nomenclature of the field sometimes uses these terms interchangeably, but for the purposes of this discussion these terms refer to specific types of hybridization arrays(5). Macroarrays use a membrane array matrix, radioactively labeled targets for detection, and the samples are hybridized to separate arrays. This form of array generally contains between 1,000 and 5,000 genes. Several different arrays can be used to give even broader coverage. Microarrays use a glass or plastic matrix with fluorogenically-labeled targets and the targets are competitively hybridized to the same array. These arrays can contain up to tens of thousands of genes. Finally, high-density oligonucleotide chips use *in situ* constructed oligonucleotides for probes. Samples are hybridized to separate arrays and a fluoroprobe is used for detection. These arrays also contain up to tens of thousands of genes. Each of these formats has different advantages and limitations in terms of number of genes, model organisms avail-

able, sensitivity, and cost.

### ***3.3.2. Post hoc Confirmation of Changes***

Post hoc confirmation is a critical step in functional genomic research and yet it is often under represented in the literature. While initial large scale screening can produce a number of targets, that screen is not the final experiment. The targets generated from the large scale screening are like suspects in a police line up and the post hoc confirmation is the beginning of proving a scientific case for which gene(s) are responsible for the biological phenomenon being studied. Confirmation can be achieved at the level of nucleic acids (northern blotting or QRT-PCR(6)) or at the level of protein (immunoblotting and other proteomic approaches). These are discussed further in 3.4.3.

### ***3.3.3. Custom Arrays***

Custom arrays serve as a form of hypothesis-testing in functional genomic experiments. These arrays contain a smaller set of genes than the large scale screening arrays and are focused on genes and gene families highlighted in large scale screens. The advantage of custom arrays is that they can exhaustively examine a smaller set of genes. This is an advantage both scientifically and practically. Because large arrays often contain only a few members/isoforms of specific gene families, custom arrays can be constructed which contain all of the subtypes and splice variants. As well, because the cost of custom hybridization arrays is often less when measured on a per gene basis.

There are a number of technical considerations with generating custom

arrays(7,8). The key is in selection of the probes placed on the array. Probes must be carefully designed to discriminate between highly homologous genes. In addition, multiple spots of the same gene per array increases confidence intervals. Finally, with the low cost per custom array (after initial start up), more replicates of the experiment can be performed, and arrays can be applied to individual animals/samples. All of these steps combine to allow detailed investigation of the hypothesis generated from the initial large scale screen and post hoc confirmation.

### ***3.3.4. Bioinformatics***

Within the flow of functional genomic research (figure 2), bioinformatics is where targets from the initial large scale screen that have been validated post hoc and tested on custom arrays begin to form a biological narrative. While the amount of data generated from functional genomic research is amazing, databases and clustering charts are not the ultimate goal of this research. Combining the existing knowledge of specific gene functions, the previous work on the subject, and the gene expression array data should result in a descriptive biological story. This may seem to be an obvious point, but in the excitement to use this new technology, the old rules of research should not be forgotten. To this end, new technologies and databases are currently being developed that will permit integration and mining of biological data for all genes, gene families, chromosome locations, and ESTs.

### ***3.3.5. Dynamic Intervention/ Target Validation***

Traditionally the gold-standard for biological research has been to interfere

with a biological phenomenon to show causative nature. Approaches used in this manner include, gene knockout mice, anti-sense knockout approaches, specific protein inhibitors, and antagonists. Therefore, a key consideration is that once a gene has been illuminated by array analysis and its change confirmed by post hoc methods, a dynamic intervention should be conducted to confirm the direct involvement of the gene in the biology under study.

### ***3.4. Technical Considerations in Array Experimental Design***

All successful science is based on sound experimental design. From a practical standpoint, this is especially true of hybridization array experiments because the time and resources that can be wasted on poorly designed functional genomic research are staggering. For both the beginning researcher and those already conducting experiments using hybridization arrays, it is worth examining the concerns of sample collection, sensitivity, post-hoc confirmation and data analysis (figure 3).

#### ***3.4.1. Sample Collection***

Sample collection is a basic element of experimental design for many molecular biological experiments, but it is worth reiterating. Specifically, given the expense of array analysis (in both time, money, and energy), it is wise to invest considerable effort in determining that: (a) the key experiment is well-conceived; and (b) that the input samples are intact and appropriately prepared. Depending on the cells or tissue being examined, it is often unavoidable that a sample will contain multiple cell types. In complex samples, such as brain tissue, there

is routinely a heterogeneous cell population. Therefore, observed changes may represent a change in one cell type or all cell types. Similarly, smaller changes occurring in only one type of cell may be hidden. Thus, researchers must be mindful of heterogeneous cell populations when drawing conclusions. Similarly, in comparing normal and cancer samples, there will be obvious differences in the proportion of the cell types (i.e., cancer cells will be over-represented). Therefore, interpretations of differences in gene expression may be complicated by the sheer mass of one cell over another. A promising technological solution to this problem is laser capture microdissection which allows very small and identified cellular populations to be dissected (9). The amount of sample and RNA collected in this manner is so small, however, that either target or signal amplification steps must be used(10,11).

The timing of tissue collection goes hand-in-hand with the nature of the collected tissue and therefore sample collection times will be important. For example, in an experiment in which cells undergo programmed cell death, the collection time point will determine if causative changes or end-point changes are to be observed. If a late time point is chosen, it becomes increasingly difficult to distinguish changes due to the general breakdown of cellular processes from those which have triggered the cell death.

An important issue in DNA array analysis is the use of individual samples or pooled RNA preparations from a number of samples. The pooling of equal amounts of RNA from all of the representatives of an experimental or control group (whether cells or animals) produces what can be termed an expression mean. The alterations in gene expression, illuminated by the result

ing array analysis, reflect changes that are common to most/all animals or samples in a group. The outlier expression of one gene in a given animal/sample is therefore averaged towards unity. Of course on the converse side, unique responses that appear in a given animal and that might be quite relevant to the specific response of that animal, are also lost. This aspect of experimental design, however, is intended to maximize the chances of a legitimate “hit” in the initial analysis. The result of this approach is that more of the target genes generated from the initial screen are statistically confirmed in post hoc testing. Finally, the cost of the array technology also necessitates consideration of pooled analysis, because it is often prohibitively expensive to perform experiments on individual animals/samples.

The most important component of a successful array experiment is the isolation and characterization of intact RNA. The common method for RNA isolation is the guanidinium thiocyanate procedure(12). Modifications of this protocol have been developed(13), and the relative merits of this and other techniques have been reported(14). RNA should always be subjected to denaturing gel electrophoresis to visually verify the integrity of the RNA by 28S and 18S ribosomal RNA bands and spectrophotometric measurements of RNA concentration have been reported to be sensitive to pH(15). The same denaturing gel used to confirm integrity can also be used visually verify the spectrophotometric quantification. Although this is such a basic aspect of all functional genomic analysis, it is worth reiterating the importance of careful sample preparation. RNA degradation is a serious technical problem and can lead to variable results. Though ribonuclease levels vary by organism and tissue, careful RNA isolation will enhance the subsequent output from the

array.

### ***3.4.2. Detection Sensitivity***

There are two key detection issues when thinking about DNA hybridization arrays. The first is whether or not an mRNA can be detected (threshold sensitivity), and the second is whether or not changes in mRNA level will be large enough to be detected (fold-change sensitivity). These considerations will determine decisions about what platform to use, the use of poly (A+) or total RNA, and detection methods (radioactivity or fluorescence).

#### ***3.4.2.1. Threshold Sensitivity***

Detection sensitivity in array research takes two very distinct forms. The first, termed threshold sensitivity, is the ability to detect one RNA species out of a population and is a concern for rarely expressed messages, for small sample sizes, and is the traditional issue of sensitivity common to other techniques. Array analysis, when it does not involve signal amplification, is not the most sensitive method. This is in contrast to transcription-based aRNA amplification, or PCR-based differential display or quantitative RT-PCR. Therefore, levels of detection (the number of copies of a specific gene needed per unit of RNA in order to yield a signal) are not particularly sensitive. A number of approaches have been developed to increase signal output (RNA amplification, poly (A+) RNA isolation, output signal amplification [sandwich detection methodologies]). However, every amplification procedure comes at the cost of variable amplification efficiencies and so extreme care must be taken in adopting these approaches. Unfortunately, there has been very little systematic comparison of

array platforms and detection methods (16). There is anecdotal evidence that membrane- and radioactivity-based macroarrays are more sensitive. However, there are unique concerns with the use of radioactivity and the macroarrays are generally perceived as less valuable because they screen fewer genes and generally do not provide widespread EST arrays for gene discovery.

### ***3.4.2.2. Fold-Change Sensitivity***

The second sensitivity parameter is ‘fold-change sensitivity’, or the ability of hybridization arrays to reliably determine a certain magnitude difference in expression. The claimed fold-change sensitivity of different platforms varies. Determination of this parameter is crucial to characterizing the technology and ensuring that researchers choose the technology most appropriate to their goals. For research involving systems that undergo large gene expression changes (e.g. yeast cell-cycle regulation, or organ developmental processes where 10-fold changes are expected), one can detect such changes with fluorescent protocols. Other research efforts, for example in neuroscience, where gene changes are less dramatic, may find radioactivity-based methods more applicable.

### ***3.4.3. Post hoc Confirmation***

One of the most common criticisms of hybridization arrays is that when hundreds or thousands of gene are examined at once, some apparent changes are the result of random chance. This is because a single array experiment, representing an  $n$  of one, lacks the sample size needed for statistical analysis. Indeed, at their core, most arrays essentially represent one- to ten-thousand t-tests. As such, one is likely to find small

magnitude changes (less than 2-fold) in signals that are not reflective of actual changes in mRNA levels. This is a statistical reality and highlights the requirement for post hoc confirmation of changes seen with arrays. So, how does one separate bona fide changes from type II statistical errors, (false positives). Tests on individual samples themselves are necessary to produce statistical significance. Such corroborating experiments can examine the gene changes at the level of mRNA (northern blot, QRT-PCR), protein (immunoblot), or activity (enzymatic activity, DNA binding, or other measures). The protein and activity tests are recommended because they assess the gene of interest at a level closer to the function of the protein or actually address the function itself. Protein analysis is important because increased levels of transcription do not always translate into increased levels of protein(3). In addition, protein assessment is achieved with fundamentally different experimental techniques and may not therefore be subject to the same sources of error as the array. Unfortunately, immunoblotting and activity assays would appear to return researchers to the single gene assay that hybridization arrays were intended to avoid. This is not true in practice, however, because large numbers of genes have already been screened by the array (see Figure 2). The optimal solution to ascribing relevance to the data is to develop techniques by which confidence intervals for individual genes can be generated from arrays and these results can be combined with proteomic techniques under development(2). Alternatively, as costs are decreased, individual hybridization array experiments will be performed for each sample. As well, many researchers are exploring the use of small (in the number of genes) arrays that focus on a specific gene family or pathway.

Hybridization array technology has opened exciting new avenues of biomedical research. With this excitement a sober view of experimental design is required. Truly, groundbreaking research will require the same, if not greater, attention to experimental design than required in the past. Because of the large effort and investment required for functional genomic research poorly conceived experiments can squander, it is worth considering these issues before undertaking major investments of time and resources.

#### ***3.4.4. Data Analysis***

The creation, hybridization, and detection of microarrays can seem like a daunting task. It would appear that once an image of the array, with relative densities for each sample, has been generated, the experiment would nearly be finished. Unfortunately, this is not the case as scientists are now learning that the massive amounts of data generated by arrays pose a new challenge(17,18,19). In this section, basics data analysis, computational models and integration of data with existing biological knowledge will be examined.

##### ***3.4.4.1. Data analysis basics***

The first steps in data analysis are background subtraction and normalization. The principals of both are similar to the techniques used with conventional nucleic acid or protein blotting. Background subtraction pulls the non-specific background noise out of the signal detected for each spot and allows comparison of specific signals. For illustration, if the signal intensities for the control and experimental spots are 4 and 6, respectively, it would appear that the experimental is 50% higher. However, if a background of 2 is subtracted from both

signal intensities, the experimental value is actually 100% higher than control. A complication to background subtraction is that differences in background across the array can affect some spots more than others and therefore a local background from the area around each spot is often used.

Normalization is the process by which differences between separate arrays are accounted for. All macroarray (membrane based-radioactively detected arrays) experiments and any other multiple array experiments may require the use of normalization for consistent comparisons. For example, when a pair of macroarrays representing control and treated samples show a difference in overall or total signal intensity, such differences can arise from unequal starting amounts of RNA or cDNAs, from different efficiencies of labeling reactions, or from differences in hybridization. Any of these factors can skew the results. Common methods of normalization include: a housekeeping gene(s), a gene thought to be invariant under experimental conditions; using the sum of all signal intensities; or a median of signal intensities. Housekeeping genes do in fact vary under some experimental conditions and are problematic for many experiments. All of these approaches have limitations and exogenous synthetic RNA standards have been used for normalization(20).

##### ***3.4.4.2. Computational Methods***

The sheer quantity of data generated by arrays exceeds the ability of manual human assessment. Advances in computational biology and bioinformatics are being used to effectively and exhaustively explore hybridization array results and create a biological story out of the databases generated by hybridization array data(21,22).

Binary experiments where only one control and one experimental sample are being compared the data analysis requires only a ratio of control to treated. For more involved experimental designs where there are two or more experimental conditions (typically, an experiment looking at multiple time points, doses, or groups), the computational requirements are much greater. The question is not one of a simple change under one condition, but becomes how does one gene (out of thousands) change over multiple conditions. With large experiments analyzing thousands of genes, the data increases dramatically and as a result it can be difficult to find patterns in the data. To this end, computational algorithms are used. These approaches seek to find groups of genes, clusters, that behave similarly across the experimental conditions. Clusters, and the genes within them, can subsequently be examined for commonalities in function or sequence to better understand how and why they behave similarly. A number of different methods: k-means, self-organizing maps, hierarchical clustering, and Bayesian statistics are employed for clustering analysis(23,24,25). Clustering analyses will be critical for the mining of public expression databases that are being generated(26).

#### ***3.4.4.3. Integration with other biological knowledge***

In the excitement of using functional genomic technology it is important to not forget what we already know and other biological measures. This is accomplished by using the existing knowledge of genes and their functions and to combine gene expression data with chemical, biochemical and clinical measures. One example of combining gene expression data with other measures comes from the cancer field in the

recent work by Alizadeh et al.(27) In this work large B-cell lymphomas were put into subtypes by their gene expression profile and these subtypes were found to have significantly different reactions to therapy.

#### ***3.5. Conclusion and Future Directions***

Undeniably, functional genomics is opening new avenues of research. The advances in technology that have made this possible are exciting in themselves and require a great deal of effort to perfect. In this climate, it is easy to succumb to technical showmanship and produce complex works that highlight the technology. While these are interesting works, the goal of most researchers is to increase biological knowledge for humanity. The fruits of functional genomic research will go to those who not only master the new technology, but integrate these tools into well-designed experimental projects.

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## References:

1. Lockhart, D.J., and Winzeler, E.A., Genomics, gene expression and DNA arrays; Nature 405, 827, 2000.
2. Pandey, A., and Mann, M., Proteomics to study genes and genomes, Nature 405, 837, 2000.
3. Anderson, L., and Seilhamer, J., A comparison of selected mRNA and protein abundances in human liver, Electrophoresis, 18, 533, 1997.
4. Mir, K.U., The hypothesis is there is no hypothesis, Trends in Genetics, 16, 63, 2000.
5. Freeman, W.M., Robertson, D.J., and Vrana, K.E., Fundamentals of DNA hybridization arrays for gene expression analysis, BioTechniques, 29, 1042, 2000
6. Freeman, W.M., Walker, S.J., and Vrana, K.E., Quantitative RT-PCR: pitfalls and potential, Biotechniques, 26, 112, 1999.
7. Cheung, V.G., Morley, M., Aguilar, F., Massimi, A., Kucherlapati, R., and Childs, G. Making and reading microarrays, Nat. Genetics, 21, 15, 1999.
8. Schena, M., Ed., DNA Microarrays : A Practical Approach (Practical Approach Series), Oxford University Press, 1999.
9. Luo, L., et al., Gene expression profiles of laser-captured adjacent neuronal subtypes, Nat. Medicine, 5, 117, 1999.
10. Van Gelder, R.N., von Zastrow, M.E., Yool, A., Dement, W.C., Barchas, J.D., and Eberwine, J.H., Amplified RNA synthesized from limited quantities of heterogeneous cDNA. PNAS USA 87, 1663, 1990.
11. Wang, E., Miller, L.D., Ohnmacht, G.A., Liu, E.T., and Marincola, F.M., High-fidelity mRNA amplification for gene profiling, Nat. Biotech., 18, 457, 2000.
12. Chomczynski, P., and Sacchi, N., Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform

18. Brent, R., Functional genomics: learning to think about gene expression data, *Current Biol.*, 9, R338, 1999.
19. Vingron, M., and Hoheisel, J., Computational aspects of expression data, *J. Mol. Med.* 77, 3, 1999.
20. Eickhoff, B., Korn, B., Schick, M., Poustka, A., and van der Bosch, J., Normalization of array hybridization experiments in differential gene expression analysis, *Nucleic Acids Res.*, 27, e33, 1999.
21. Claverie, J.M., Computational methods for the identification of differential and coordinated gene expression, *Human Mol. Gen.*, 8, 1821, 1999.
22. Zhang, M.Q., Large-scale gene expression data analysis: a new challenge to computational biologists, *Genome Res.*, 9, 681, 1999.
23. Ben-Dor, A., Shamir, R., and Yakhini, Z. Clustering gene expression patterns, *J. Computational Bio.*, 6, 281, 1999.
24. Hilsenbeck, S.G., Friedrichs, W.E., Schiff, R., O'Connell, P., Hansen, R.K., Osborne, C.K., and Fuqua, S.A.W., Statistical analysis of array expression data as applied to the problem of tamoxifen resistance, *J. Natl. Cancer Inst.*, 91, 453, 1999.
25. Tamayo, P., Slonim, D., Mesirov, J., Zhu, Q., Kitareewan, S., Dmitrovsky, E., Lander, E.S., and Golub, T.R., Interpreting patterns of gene expression with self-organizing maps: Methods and application to hematopoietic differentiation, *PNAS USA*, 96, 2907, 1999.
26. Claverie, J.M., Do we need a huge new centre to annotate the human genome?, *Nature*, 403, 12, 2000.
27. Alizadeh, A.A., et al., Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling, *Nature*, 403, 503, 2000.

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**BIBLIOGRAPHY:**

**Journal Articles:**

**Freeman WM**, Vrana SL, Vrana KE. The use of elevated reverse transcriptase reaction temperatures in RT-PCR reactions. *BioTechniques* 1996; 20:782-783.

Gioia L, Vogt LJ, **Freeman WM**, Flood A, Vogt BA, Vrana KE. PCR-based apolipoprotein E genotype analysis from archival fixed brain. *Journal of Neuroscience Methods* 1998; 80: 209-214.

Xu Y, Stokes AH, **Freeman WM**, Kumer SC, Vogt BA, Vrana KE. Tyrosinase mRNA is expressed in the substantia nigra of humans. *Mol Brain Res* 1997; 45:159-162.

**Freeman WM**, Walker SJ, Vrana KE. Quantitative RT-PCR: Pitfalls and potential. *BioTechniques* 1999; 26: 112-125.

**Freeman WM**, Gioia L. The maturation of nucleic acid technologies. *Trends in Biotechnology* 1999; 17: 44-45.

**Freeman WM**, Vrana K.E. Panning for molecular gold. *Trends in Biotechnology* 1999; 17: 258.

**Freeman WM**, Robertson DJ, Vrana KE. Fundamentals of DNA hybridization arrays for gene expression analysis. *BioTechniques* 2000; 29: 1042-1055.

**Freeman WM**, Yohrling GJ, Gioia L, Daunais J, Porrino L, Vrana KE. A novel cocaine analog 2 $\beta$ -propanoyl-3 $\beta$ -(4-tolyl)-tropane reduces tyrosine hydroxylase gene expression in the mesolimbic dopamine pathway. *Drug and Alcohol Dependence* 2000, 61: 15-21.

**Freeman WM**, Nader MA, Nader SH, Robertson DJ, Gioia L, Mitchell SM, Daunais JB, Porrino LJ, Friedman DP, Vrana KE. Chronic cocaine-mediated changes in non-human primate nucleus accumbens gene expression. *Journal of Neurochemistry* 2001; 77:542-849.

Stokes, AH, **Freeman WM**, Mitchell SG, Burnette TA, Hellmann GM, Vrana KE. Induction of GADD45 and GADD153 in neuroblastoma cells by dopamine induced neurotoxicity. Molecular Brain Research, Submitted.

**Freeman WM**, Brebner K, Lynch WJ, Robertson DJ, Roberts DCS, Vrana KE. Cocaine-Responsive gene expression changes in rat hippocampus. Neuroscience, In Press.

**Freeman WM**, Brebner K, Lynch WJ, Robertson DJ, Roberts DCS, Vrana KE. Changes in rat frontal cortex gene expression following chronic cocaine. In preparation of Neuroreport.

**Freeman WM**, Brebner K, Lynch WJ, Robertson DJ, Roberts DCS, Vrana KE. Chronic cocaine self-administration and non-contingent cocaine administration changes nucleus accumbens gene expression. In preparation for Journal of Neurochemistry.

**Freeman WM**, Worst TJ, Vrana KE. Variability associated with membrane based hybridization arrays. In preparation for Nucleic Acids Research.

**Freeman WM**, Dougherty K, Vrana KE. An interactive database of cocaine-responsive neuronal gene expression. In preparation for The Scientific World.

**Freeman WM**, Dougherty K, Vrana KE. Functional neurogenomics and cocaine abuse. In preparation for the Journal of Neurobiology.

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Chemei Bir. Sürat Ticaret, İstanbul, Türkei, 1998 (**Freeman WM**, Technical/Linguistic Editor.)

**Freeman WM**, Vrana KE. Genomic scale gene expression analysis: Advancing from DNA to disease. Genomic Pathways Series 2000; 6 Cambridge Healthtech Institute, 116 pp.

**Freeman WM**, Vaccaro D, Vrana KE. Microarrays. Genomic Pathways Series, Cambridge Healthtech Institute 2001; In press.

Robertson DJ, **Freeman WM**, Vrana KE. Phosphorimaging. In: Encyclopedia of Life Sciences, Macmillan Publishers Ltd. 2001; In press.

**Freeman WM**, Vrana KE. Experimental design for hybridization array analysis of gene expression. In: DNA Arrays: Technologies and Experimental Strategies, Grigorenko, EV (ed.), CRC Press 2001; In Press.

Worst T, **Freeman WM**, Walker SJ, Vrana KE. Functional genomics analysis of neurological gene expression with cDNA macroarrays. In:Drugs of Abuse: Analysis of Neurological Effects (Methods in Molecular Medicine), Wang JQ (ed.), Humana Press. In preparation.

Walker SJ, **Freeman WM**, Vrana KE. Use of quk vt neue evt

ety on Alcoholism.

**Freeman WM.** Functional genomics of drug abuse. 2001, Functional genomics: Defining gene function for drug discovery, Barnett International.

**Freeman WM**, Brebner K, Lynch WJ, Robertson DJ, Roberts DCS, Vrana KE. Cocaine-Responsive Gene Expression in the Rat. 2001, Society for Neuroscience.

Jiang GC-T, **Freeman WM**, Vrana KE, Caron MG, Jones SR Functional neurogenomic and biochemical analysis of enzymatic differences in dopamine transporter knockout mice. 2001 Society for Neuroscience.

**Freeman WM**, Brebner K, Lynch WJ, Robertson DJ, Roberts DCS, Vrana KE. Functional Neurogenomics of Cocaine in Rat. 2001, College on Problems of Drug Dependence.