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Cocainomics: New Insights into the Molecular Basis of Cocaine Addiction

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Abstract

Until recently, knowledge of the impact of abused drugs on gene and protein expression in the brain was limited to less than 100 targets. With the advent of high-throughput genomic and proteomic techniques, investigators are now able to evaluate changes across the entire genome and across thousands of proteins in defined brain regions and generate expression profiles of vulnerable neuroanatomical substrates in rodent and nonhuman primate drug abuse models and in human post-mortem brain tissue from drug abuse victims. The availability of gene and protein expression profiles will continue to expand our understanding of the short- and long-term consequences of drug addiction and other addictive disorders and may provide new approaches or new targets for pharmacotherapeutic intervention. This review summarizes several important genomic and proteomic studies of cocaine abuse/addiction from rodent, nonhuman primate, and human postmortem studies of cocaine abuse and explores how these studies have advanced our understanding of addiction.

Keywords

cocaine; proteomic; genomic

Introduction

Completion of the sequencing of the human genome has provided new insights into the function of genes and gene products in human health and disease. The next challenge in addiction biology is the utilization of this information to determine the function of the genes and proteins in the context of human disease. The advent of high-throughput screening technologies has produced a paradigm shift in the manner in which scientists are able to detect and identify molecular mechanisms related to disease. Genomic and proteomic analysis strategies allow the assessment of coordinate expression of genes and proteins of known and unknown function—thereby enabling a global biological view of addictive disorders. Broad-scale evaluations of gene and protein expression are well suited to the study of drug abuse, particularly in light of the complexity of the brain compared with other tissues, the multigenic nature of drug addiction, the vast representation of expressed genes in the brain, and our relatively limited knowledge of the molecular pathology of this illness. The review will provide a review of recent genomic and proteomic studies employing rodent and nonhuman primate models as well as studies examining the neuropathology identified in postmortem human tissue of individuals with chronic histories of illicit substance abuse genomic and proteomic strategies.

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The use and abuse of illicit drugs has continued to increase and poses one of the most significant public health care concerns in American society. Cocaine is widely recognized as one of the most addictive and dangerous illicit drugs in use today, and abuse of cocaine in the USA remains a major public health problem (SAMHSA 2007). Of the over 2.4 million Americans who use cocaine, the rate of current drug use is similar for males and females ages 12–17 (SAMSHA 2006). Furthermore, cocaine was the most frequently mentioned drug in females (33.3%) and the second most frequently mentioned in males (44.8%) of all drug abuse-related decedent case counts in Medical Examiners reports in 2000 (SAMSHA 2001). The 2001 Household Survey on Drug Abuse estimated over 20 million Americans have used cocaine at least once in their lifetime and that 1.5 million are current users (SAMSHA 2002). Clearly, there is an urgent need to identify relevant behavioral and neurobiological mechanisms that mediate stimulant addiction as well as the ability of candidate medications to attenuate or reverse cocaine-induced adaptations in the brain. Substance abuse is the culmination of a number of contributing factors spanning scientific disciplines from behavior to molecular biology. As such, to understand the biology of addiction requires a multidisciplinary approach to identify the contributing factors, synthesize the information in the appropriate biological context, and eventually relate this context to the behavioral abnormality.

Neurobiological substrates of cocaine addiction

The majority of research designed to understand the addictive properties of cocaine have focused largely on the neural circuit that mediates sensorimotor integration and motivational processes implicated in a variety of psychiatric disorders, including drug abuse—the corticostriatal–pallidal–thalamic/mesencephalic circuit. An important component of this circuit is the mesocorticolimbic dopamine system (Lindvall and Bjorklund 1974a, b; Ungerstedt 1971). Cell bodies of the ventral tegmental area (VTA) project to several basal forebrain areas including the nucleus accumbens (NAc), bed nucleus of the stria terminalis, diagonal band of Broca and olfactory tubercles, the prefrontal and anterior cingulate cortices, hippocampus, and amygdala (Lindvall and Bjorklund 1974a, b; Lindvall et al. 1974; Ungerstedt 1971). Neuropharmacological studies in animal models and imaging studies in humans indicate that the mesocorticolimbic dopamine pathway is regulated by cocaine administration and that the functional integrity of this pathway is essential for the reinforcing and euphoric effects of cocaine (Koob 1999; Ritz et al. 1987; Volkow and Li 2004; Wise and Bozarth 1987). Furthermore, chronic cocaine administration induces neuroadaptive changes that lead to transient and perhaps persistent alterations in regional brain function that may underlie persistent drug taking behaviors, “craving”, and relapse (Nestler 1997; White and Kalivas 1998; White et al. 1995). Previously described neuroadaptations induced by chronic cocaine administration include a generalized upregulation of the cyclic adenosine 3′,5′-monophosphate (cAMP) pathway (Carlezon et al. 1998; Miserendino and Nestler 1995; Pliakas et al. 2001; Terwilliger et al. 1991), activation of members of the activator protein 1 (AP-1) family (Haile et al. 2001; Hope 1998; Mackler et al. 2003), and other potential transcriptional regulators. For example, chronic cocaine administration increased formation of cAMP-dependent protein kinase (PKA) and adenylate cyclase in the nucleus accumbens of rats (Terwilliger et al. 1991), as well as mRNA and protein levels of the α catalytic subunit of PKA and other potential transcriptional regulators in the NAc of rhesus monkeys following chronic cocaine administration (Freeman et al. 2001b). While such studies have employed serial analytical approaches to study one or a few genes or proteins at a time, the advent of genomic and proteomic analyses enables an unbiased, parallel assessments of thousands of genes and proteins simultaneously thereby providing a more comprehensive compendium of biochemical neuroadaptations induced by cocaine.

Functional genomics

Rodent studies

Several studies have examined the effects of cocaine administration on the coordinate expression of genes in rodent brain regions associated with the mesocorticolimbic pathway, including the nucleus accumbens (Toda et al. 2002), prefrontal cortex (PFC; Freeman et al. 2002; Toda et al. 2002), hippocampus (Freeman et al. 2001a), lateral hypothalamus (Ahmed et al. 2005), and ventral tegmental area (Backes and Hemby 2003). In the one study, rats were administered cocaine three times per day (15 mg/kg; intraperitoneal) for 14 days (Freeman et al. 2002) to simulate uncontrolled or "binge" intake of the drug. Thirty minutes following the last administration of cocaine, rats were sacrificed, the hippocampus was dissected, and gene expression was evaluated using RNA "pooled" by group. Using stringent inclusion criteria of 50% induction or 33% reduction, the authors noted that only five transcripts were differentially regulated—protein kinase A alpha (PKAc*α*), metabotropic glutamate receptor 5 (mGluR5), and voltage-gated potassium channel 1.1 (Kv1.1), survival of motor neuron, and protein phosphatase 2A alpha subunit—all of which were upregulated in the cocaine-treated rats. From this set, only mGluR5, PKAc*α*, and Kv1.1 showed analogous changes in protein levels in this region. Interestingly, the authors note that protein tyrosine kinase 2 (PYK2), protein kinase C epsilon (PKCε), and β catenin, proteins found to be elevated in the NAc of cocaine-treated cynomolgus monkeys (Freeman et al. 2001b), were also elevated in the hippocampus of cocaine treated rats suggesting these changes are not region or treatment regimen specific.

In a separate study, changes in gene expression in the PFC of the same subjects (Freeman et al. 2002) were examined by screening 588 genes (BD Bioscience Clontech Atlas cDNA Expression Array). Cocaine administration increased the expression of activity-regulated cytoskeletal protein (ARC), nerve growth factor-induced clone B (NGFI-B), and 3-hydroxy-3-methylglutaryl-CoA synthase I and decreased the expression of casein kinase II alpha, glycogen synthase 3 alpha, and fos-related antigen 1. The upregulation of NGFI-B was confirmed by quantitative polymerase chain reaction (PCR); however, the remaining encoded proteins of the differentially expressed transcripts were assessed by Western blot analysis. Interestingly, only ARC protein levels were increased in the PFC similar to the mRNA levels—which may be due in part to the somato-dendritic localization of ARC in neurons. The authors also examined proteins that had been shown to be upregulated in the hippocampus of rats and nucleus accumbens of monkeys administered cocaine including PYK2, mitogen-activated protein kinase I (MEK), β catenin, protein kinase C alpha (PKC*α*), and PKCε—of which only PYK2 was found to be upregulated in the frontal cortex of cocaine-treated rats. The study provides confirmatory data from previous studies showing increased arc mRNA expression following cocaine administration (Fosnaugh et al. 1995; Tan et al. 2000; Ujike et al. 2002) as well as extending current knowledge on the ability of cocaine to induce genes and protein involved in neuroplasticity.

Additional insight into prefrontal and striatal synaptic dysfunction came from a cDNA microarray study which screened 1,176 rat genes (BD Bioscience Clontech Atlas cDNA Expression Array) in samples of NAc core, NAc shell, striatum, and dorsal prefrontal cortex of rats following 3 weeks of withdrawal from 7 days of cocaine administration (intraperitoneal; 15 mg/kg on days1 and 7, 30 mg/kg on days2–6; Toda et al. 2002). Nine genes were identified with at least 40% increase or 29% decrease relative to controls in one of the four brain regions studied. In the PFC, the authors noted a significant downregulation of the neurotrophic tyrosine kinase receptor type 2 (TrkB) in the PFC of cocaine-treated rats. TrkB, the high-affinity receptor for brain-derived neurotrophic factor, is well known for conferring neuroplastic responses in the cortex and hippocampus and has been shown to be involved in the behavioral effects of cocaine in the VTA and NAc (Berhow et al. 1996;

Freeman and Pierce 2002; Horger et al. 1999; Pierce and Bari 2001). Though not significantly different at the protein level in the PFC, protein levels of the active TrkB-truncated isoform was upregulated in the core of the NAc—a region receiving inputs from the distal regions such as the VTA, hippocampus, etc. Interestingly, the NAc core region exhibited changes in the expression of five transcripts: mitochondrial ATP synthase subunit D (Atp5h), adenosine receptor 1 (Adora1/A1), leukocyte common antigen-related tyrosine phosphatase (LAR), and RET ligand 2 (Retl2; also known as glial cell line-derived neurotrophic factor family receptor alpha 2; Gfra2). The authors also identified a cocaine-induced downregulation of gastric inhibitory peptide (Gip) mRNA (also known as glucose-dependent insulinotropic polypeptide)—recently shown to be upregulated by chronic clozapine administration in the striatum (Sondhi et al. 2006) suggesting mediation of this transcript by dopamine given the reciprocal regulation by cocaine and clozapine. More recently, Gip was shown to be expressed in rat hippocampus and involved in a regulatory function in progenitor cell proliferation in the dentate gyrus (Nyberg et al. 2005). Examination of transcript encoded transcripts showed significantly elevated levels of adenosine 1 receptor protein in the NAc core which may represent a compensatory response to the cocaine-induced upregulation of the D1/Gs protein signaling cascade documented previously (Nestler 2001; Scheggi et al. 2004; Zhang et al. 2005), a decreased Gi/Go function (Nestler et al. 1990), elevated adenosine levels (Manzoni et al. 1998), or some combination thereof.

Kreek and colleagues further examined cocaine-induced gene expression in the striatum following acute (three hourly injection of 15 mg/kg for 1 day) and chronic (three hourly injections of 15 mg/kg for 3 days) “binge” administration using the Affymetrix rat genome U34A containing approximately 8000 gene/EST clusters (Yuferov et al. 2003). The authors noted 117 upregulated and 22 downregulated transcripts as a result of cocaine administration. Upregulated transcripts included immediate early genes, “effector” and scaffolding proteins and receptors, and signal transduction proteins, while downregulated transcripts was comprised primarily of transcripts related to mitochondrial function along with transcripts encoding signal transduction proteins. RNAse protection assays were used to confirm differential expression as noted by array analysis. In addition to expanding our understanding of cocaine-induced regulation of several gene families and pathways, the authors revealed upregulation of the Per2 clock gene and the somatostatin receptor 2 following “binge” cocaine administration. Previously, disruptions of Per genes have been shown to block cocaine-induced sensitization in *Drosophila* (Andretic et al. 1999) and mice (Abarca et al. 2002); however, the localization to the striatum is interesting in that previous studies have found expression limited to the suprachiasmatic nucleus (Masubuchi et al. 2000). The elevated expression of SSTR2 may reflect a less studied mechanism of cocaine-regulated dopamine release in the striatum as noted by the authors. Additional studies which examine the cellular origin and localization of the Per2 transcript and protein and the role of SSTR2 in the behavioral effects of cocaine are warranted.

As noted in the aforementioned study by Yuferov et al. (2003), circadian or clock genes in the dorsal striatum are significantly regulated by chronic administration of cocaine. Previous studies have revealed significant alterations in cocaine sensitization and place conditioning Per1, Per2, and Clock knockout mice (Abarca et al. 2002;McClung et al. 2005) and blockade of cocaine-induced place conditioning in Zif268 mutant mice (Valjent et al. 2006). Based on these studies, (Lynch et al. 2008) examined gene expression in the dorsal striatum following 7 days of intravenous cocaine self-administration using a 24-h/day discrete trial procedure (FR5; four 10 min trials per hour; 1.5 mg/kg cocaine; n=5 for cocaine and saline groups). Microarray analysis, conducted 1 day following the last self-administration session, was conducted between cocaine and saline groups using a laboratory generated array consisting of 645 cDNAs including genes related to circadian rhythm, growth factors,

receptors, cAMP response element (CRE) sequence-containing genes, antidepressant-regulated genes, and cytokines. One hundred and thirty-nine transcripts were found to be significantly upregulated by cocaine, 29 of which were associated with circadian signaling. Reverse transcriptase (RT)-PCR revealed three additional transcripts, Per2, Clock, and Cry1, to be upregulated in the dorsal striatum by cocaine self-administration. Results of the study contribute to a growing body of literature associating circadian signaling with the reinforcing effects of cocaine (Malison et al. 2006; Shang and Zhdanova 2007); however, examinations of the direct manipulation of circadian genes on cocaine intake are needed to establish causal roles of these molecules in the addictive process.

In addition to increasing locomotor activity and inducing sensitization to the locomotor effects, cocaine can also induce a preference for an environment that has been previously associated with the drug, generally considered a reflection of the conditioned rewarding effects of the drug. Krasnova et al. examined the effects of cocaine-induced place conditioning on gene expression in the hippocampus and prefrontal cortex (medial prefrontal, anterior cingulate, and orbitofrontal cortices) using two sets of mammalian genome arrays consisting of cDNAs representing approximately 26,100 transcripts (Krasnova et al. 2008). Rats were administered either cocaine (10 mg/kg; i.p.) or saline on alternate days and confined to one of two compartments in the place conditioning. The study also included cocaine and saline nonpaired control groups for which administration was not paired with a specific compartment. Rats in the cocaine-paired group exhibited clear preferences for the drug-paired compartment while rats in the saline and cocaine-nonpaired groups did not. For microarray analysis, rats were decapitated 24 h after the last conditioning phase. Principal component analysis revealed clear separation among the cocaine-paired, cocaine-nonpaired, and saline groups for the hippocampus but not for the prefrontal cortex. In the cocaine paired group, 151 transcripts were increased and 63 transcripts decreased in the hippocampus compared with 22 increased and 17 decreased in the prefrontal cortex. The surprisingly few number of transcripts found to be differentially expressed in the prefrontal cortex could be due in part to the effect of combining three anatomically similar but biochemically and functionally distinct regions—thereby potentially masking changes occurring within each cortical region. The study reports marked upregulation of transcriptional and translational proteins, signal transduction metabolic enzymes, and cytoskeletal proteins. The prevalence of transcripts that coincide with those involved in long-term potentiation and cocaine-induced synaptic plasticity suggest that the transcriptional profile in the hippocampus may be involved in long-term biochemical responses associated with distinct environmental cues related to cocaine.

The majority of the aforementioned studies, with the exception of Lynch et al. (2008), have utilized experimenter administered cocaine to study the effects on gene expression, while these studies have expanded our knowledge of cocaine's effects in the brain and provided novel insights into the pharmacological effects of cocaine in various brain regions but may not reflect neurobiological mechanisms related to the abuse/liability/reinforcement of cocaine. A growing body of literature indicates significant neurochemical differences between *experimenter*-administered and *self*-administered drugs of abuse (Hemby 1999; Hemby et al. 1997a, b; 1995; Wilson et al. 1994). To date, few studies have combined rodent intravenous self-administration with functional genomics procedures. In one such study, Ahmed and colleagues examined gene expression profiles in samples of nucleus accumbens, lateral hypothalamus, septum, ventral tegmental area, medial prefrontal cortex, and amygdala from rats self-administering cocaine or serving as controls using pooled samples of RNA on the Affymetrix Neurobiology RNU434 chips (Ahmed et al. 2005). The cocaine self-administration group was divided into two subgroups: short access (ShA; 1 h/day; 250 mg/infusion) and long access (LhA; 6 h/day; 250 mg/infusion access). This procedure results in a marked escalation of cocaine intake within the first hour of access and

has been proposed as a model of compulsive drug intake (Ahmed et al. 2002; Ahmed and Koob 1998, 1999). Interestingly, the lateral hypothalamus exhibited the greatest number of genes that were regulated by cocaine self-administration access (ShA and LhA) and by the escalation paradigm (LhA versus ShA) by comparing to the other brain regions studied, and differential expressions of select transcripts were confirmed by qPCR. Transcripts altered by the escalation paradigm were members of several functional classes including functional and structural plasticity, receptors, synthetic and metabolic enzymes, neurotransmitter release, and proteins coding for neuronal growth and survival.

The aforementioned studies utilized dissected brain regions from rats to generate molecular profiles of cocaine administration. As noted in the previous section on the neuroanatomical basis of reinforcement, the circuitry that mediates the reinforcing effects of cocaine and others drugs of abuse is well defined and includes dopaminergic cell bodies in the VTA that projects to several forebrain and cortical regions. The advent of discrete cell microdissection and laser capture microdissection (LCM) combined with RNA amplification strategies makes it possible to evaluate expression patterns in defined cell populations in the brain (Fasulo and Hemby 2003; Ginsberg et al. 1999, 2004, 2000; Hemby et al. 2002). Whereas previous studies have examined regional gene expression profiles in the VTA as a function of cocaine administration, the effects of cocaine self-administration on VTA dopamine neurons remain largely unknown even though these cells are a critical substrate of drug reinforcement. To this end, the expression profile of 95 transcripts following 1 or 20 days of intravenous cocaine self-administration was assessed in dopamine neurons of the VTA in rats (Backes and Hemby 2003). Tyrosine hydroxylase immunopositive cells were microdissected from the VTA using LCM microdissection, and aRNA amplification was used to provide a linear amplification of the mRNA from each rat (Eberwine 2001; Eberwine et al. 1992; Hemby et al. 2002; Van Gelder et al. 1990). Five GABA-A receptor subunit mRNAs (α4, α6, β2, γ2, and δ) were downregulated at both 1 and 20 days of cocaine self-administration. In contrast, the catalytic subunits of protein phosphatase 2A, GABA-A α1, and Gα_{i2} were significantly increased at both time points. Additionally, calcium/calmodulin-dependent protein kinase IIα mRNA levels were increased initially followed by a slight decrease after 20 days, whereas neuronal nitric oxide synthase mRNA levels were initially decreased but returned to near control levels by day20. These results indicate that alterations of specific GABA-A receptor subtypes and other signal transduction transcripts appear to be specific neuro-adaptations associated with cocaine self-administration. Moreover, as subunit composition determines the functional properties of GABA-A receptors, the observed changes may indicate alterations in the excitability of dopamine transmission underlying long-term biochemical and behavioral effects of cocaine.

Transgenic mouse studies—In an elegant series of experiments, Nestler and colleagues utilized ΔFosB and cAMP response element binding (CREB) inducible transgenic mice with targets known to be involved in the behavioral effects of cocaine to ascertain their effects on the down-stream regulation of gene expression. Previous studies have shown that repeated cocaine administration leads to sustained elevation of ΔFosB levels in brain regions associated with the behavioral effects of cocaine (Brenhouse and Stellar 2006; Hope et al. 1994; McClung and Nestler 2003; Moratalla et al. 1996; Nestler 2001; Nestler et al. 2001; Perrotti et al. 2005). Using the ΔFosB inducible transgenic mouse model, the investigators were able to demonstrate increased levels of cyclin-dependent kinase 5 (cdk5) mRNA following induction and similarly increased following chronic cocaine administration (Bibb et al. 2001) using a 588 cDNA mouse array (BD Bioscience Clontech Atlas cDNA Expression Array). More importantly, a functional role of cdk5 in cocaine-mediated behaviors was shown by antagonism of cdk5 in the striatum and attenuation of kainate peak currents in the striatum following cocaine administration (Bibb et al. 2001). In a separate study using the ΔFosB inducible transgenic mouse model, the authors employed the higher

density Affymetrix DNA mouse array and found significantly increased levels of NFκB mRNA and protein in the transgenic mice and similar elevations in NFκBprote in levels in wild-type mice administered cocaine (20 mg/kg; 14 days; Ang et al. 2001b).

Comparison of the effects of ΔFosB and CREB inducible transgenic mouse models on transcription in the NAc revealed that the majority of transcripts induced by CREB occurred after 2 weeks of expression and were sustained at 8 weeks of expression (McClung and Nestler 2003). Conversely, ΔFosB expression generated dichotomous patterns of gene expression at 2 and 8 weeks with the 2-week expression pattern for ΔFosB similar to CREB expression. The longer ΔFosB expression was similar to effects observed following expression of the dominant negative CREB. Interestingly, acute cocaine administration (5 days; 10 mg/kg) induced 21% of the genes induced by CREB expression alone whereas chronic cocaine administration (15 mg/kg; 20 days) induced 27% of the genes induced by ΔFosB expression alone, leading the authors to conclude that the effects of short-term cocaine administration are more dependent on CREB whereas chronic administration is dependent on ΔFosB. The list of genes attributable to the induction of CREB and ΔFosB is lengthy and will not be reviewed in its entirely for the sake of brevity; however, it is important to note that these studies have significantly expanded the knowledge of transcriptional regulation by these factors and the understanding of the neuroadaptive effects of cocaine administration.

Using a similar approach, Caron and colleagues examined the striatal transcriptomes of three transgenic mouse models, dopamine, norepinephrine, and vesicular monoamine 2 transporter knockouts and a cocaine-treated mouse model using the Affymetrix mouse Genechips (MG U74v2 Set) containing approximately 36,000 gene clusters (Yao et al. 2004). Twenty-six transcripts were altered in all three knockouts, and six genes were also found to be altered following chronic cocaine administration (20 mg/kg/day for 5 days followed by 14 days of withdrawal)—adenylate cyclase 1 (signal transduction and plasticity), Pin/Dic-2 (involved in NOS activity and signaling), and postsynaptic density protein 95 kDa (PSD-95; involved in scaffolding of *N*-methyl-D-aspartate (NMDA) receptors and plasticity). In situ hybridization indicated a significant decrease in PSD-95 levels in the NAc and striatum of all knockdowns and the cocaine-treated groups and qPCR confirmed similar decreases in the entire striatum—separate qPCR assessments in NAc and caudate–putamen were not performed. Similarly, PSD-95 protein levels were decreased in the NAc, caudate–putamen, and in whole striatum of all three knockouts and the cocaine-treated mice. In addition, all four groups exhibited altered synaptic plasticity of cortical accumbal plasticity.

Nonhuman primates—One of the first published studies to utilize array technology examined the effects of chronic intramuscular injections of cocaine in cynomolgus monkeys on gene expression in the NAc using a low density human macroarray from Clontech consisting of 588 probes (Freeman et al. 2001b). Pools of mRNA from each group were hybridized to two separate arrays leading to the identification of 18 transcripts designated as differentially expressed and included. Unfortunately, the complete list of differentially expressed transcripts is not provided in the manuscript, and the website containing the complete dataset is no longer functional. Of the 18 differentially expressed transcripts, eight were selected for post hoc analysis using Western blot procedures. Four of the eight selected encoded proteins exhibited significant increases in abundance (as hypothesized from the array data) and included protein kinase alpha subunit (catalytic; PKAα), the beta subunit of cell adhesion tyrosine kinase, MEK1, and β-catenin. Differences in the protein expression of the remaining four targets did not agree with the array data which could be due to several factors including posttranscriptional degradation, differences in spatial trafficking of mRNA and protein in neurons, or more practical factors such as the extrapolation of data from

pooled RNA samples. An additional limitation of this study is the cross-species hybridization of monkey cDNA (generated using human PCR primers) with human extended oligo probes. The generation of targets for the Clontech assay is a PCR-based method in which primers are used which correspond to the *human* cDNA sequence. In this case, the overriding assumption is that the *Macaca fascicularis* cDNA is identical to the human cDNA sequence for the transcripts of interest such that the primers would readily anneal to the monkey cDNA and prime the PCR reaction. The lack of specificity of the human primers for cynomolgus cDNA may lead to an underestimation of the abundance of target transcripts and/or may represent the amplification of multiple transcripts in the cynomolgus monkeys. Nonetheless, the authors aptly point out that the confirmed targets are members of a common biochemical pathway that interact with CREB and AP-1 proteins shown previously to be regulated in rodent models following cocaine administration.

More recently, Hemby and colleagues have used a nonhuman primate cocaine self-administration model to validate protein and mRNA changes observed in human postmortem tissue of cocaine-overdose (COD) victims (Hemby et al. 2005b). Unfortunately, attempts to recapitulate changes observed in cocaine-overdose victims and nonhuman primate models in rodent self-administration models have not succeeded (Hemby et al. 2005a; Tang et al. 2004). Additional studies are needed to specifically address the ability of the rodent model to recapitulate biochemical changes observed in the primate brain. Whereas rodent models have provided significant information on drug-induced alterations, nonhuman primate models more closely approximate the anatomy and biochemical milieu of the human brain. For instance, differences between rodents and primates in frontal lobe anatomy (Preuss 1995) are likely to be reflected in prefrontal–accumbal glutamatergic neurotransmission. In addition, midbrain dopamine projections in rodents have been ascribed to different midbrain nuclei; however, studies in primates suggest a more complex pattern (Lynd-Balta and Haber 1994; Williams and Goldman-Rakic 1998). The use of nonhuman primates may allow the development of a more clear and clinically relevant characterization of the biochemical changes associated with cocaine use.

Human postmortem studies

Understanding the consequences of long-term cocaine abuse on postmortem brain tissues requires vigorous investigation with the benefit of revealing whether the adaptations observed in rodent and nonhuman primates are applicable to human brain and which changes are state or trait markers in human drug abusers. Findings in postmortem brains often provide the first leads that can be investigated in living brain, for example, the loss of dopamine in Parkinson's disease (Kish et al. 1988), changes in the levels of the dopamine transporter (Little et al. 1993a; b; Staley et al. 1994a; b) or opiate system (Hurd and Herkenham 1993;Staley et al. 1997) with chronic cocaine exposure, and the downregulation of the nicotinic ACh receptor after chronic nicotine (Breese et al. 1997). Although there are many difficulties with postmortem brain studies, this approach is one of the most promising ways to view biochemical changes relevant to human drug abusers and to educate the public about the consequences of cocaine abuse. Whereas animal studies have advanced our understanding of the neurobiological basis of drug addiction, the evaluation of similar questions in human tissue are few, yet are essential. By assessing changes in defined biochemical pathways in human postmortem tissue, the fundamental molecular and biochemical processes associated with long-term cocaine use can be ascertained.

Bannon and colleagues examined gene expression in the NAc of postmortem brain tissue of human cocaine abusers and controls using Affymetrix Human U133A and U133B arrays with represent over 39,000 transcripts (Albertson et al. 2004). Forty-nine transcripts were present in all pairs (n=10) of cocaine and control cases and were differentially expressed in the NAc of cocaine abusers. Transcripts were members of several functional classes

including signal transduction, transcriptional and translational processing, neurotransmission and synaptic function, glia, structural and cell adhesion, receptors/transporters/ion channels, cell cycle and growth, and lipid and protein processing. The authors noted a significant upregulation of cocaine and amphetamine related transcript (CART), a transcript previously discovered following cocaine administration in rats (Douglass and Daoud 1996; Douglass et al. 1995). In addition, several myelin-associated transcripts were significantly decreased in the NAc of cocaine abusers including myelin basic protein (MBP), proteolipid protein 1, and myelin-associated oligodendrocyte basic protein and a significant increase in T cell differentiation protein (MAL2)—which were confirmed by qPCR. Immunohisto-chemistry revealed a similar decrease in MBP immunoreactivity in the NAc of these subjects as well. These data provide molecular basis of previous studies which suggested altered white matter density and myelin expression in cocaine abusers (Lim et al. 2002; Volkow et al. 1988; Wiggins and Ruiz 1990).

In a separate cohort, Hemby and colleagues used targeted macroarrays consisting of 96 cDNAs to compare gene and protein expression patterns between cocaine-overdose victims and age-matched controls in the VTA and lateral substantia nigra (l-SN; Tang et al. 2003a). Evaluated transcripts included ionotropic glutamate receptor subunits, GABAA receptor subunits, dopamine receptors, G protein subunits, regulators of G protein signaling and other GTPases, transcriptional regulation, cell growth and death, and others (CART, cannabinoid receptor 1, and serotonin receptors 2A, 2C, and 3). Array analysis revealed significant upregulation of numerous transcripts in the VTA, but not l-SN, of cocaine-overdose victims including NMDAR1, GluR2, GluR5, and KA2 receptor mRNAs. Corresponding Western blot analysis revealed VTA-selective upregulation of CREB, NR1, GluR2, GluR5, and KA2 protein levels in cocaine-overdose victims. These results indicate that selective alterations of CREB and certain ionotropic glutamate receptor (iGluR) subunits appear to be associated with chronic cocaine use in humans in a region specific manner. Extending these studies, we recently examined the extent of altered iGluR subunit expression in the NAc and putamen in cocaine-overdose victims (Hemby et al. 2005b). Results revealed statistically significant increases in the NAc, but not in the putamen, of NR1 and GluR2/3 with trends in GluR1 and GluR5 in COD. In order to determine that changes were related to cocaine intake and not to other factors in the COD victims, the effects of cocaine intravenous self-administration in rhesus monkeys for 18 months (unit dose of 0.1 mg/kg/injection and daily drug intake of 0.5 mg/kg/session) examined. Statistically significant elevations were observed for NR1, GluR1, GluR2/3, and GluR5 ($P<0.05$) and a trend toward increased NR1 phosphorylated at Serine 896 ($P=0.07$) in the NAc but not putamen of monkeys self-administering cocaine compared to controls (Hemby et al. 2005b). These results extend previous results by demonstrating an upregulation of NR1, GluR2/3, and GluR5 in the NAc and suggest that these alterations are pathway specific and likely mediate in part the persistent drug intake and craving in the human cocaine abuser.

In addition to the nucleus accumbens, other studies have assessed cocaine-induced gene expression changes in hippocampus and prefrontal cortex of cocaine-overdose victims. The hippocampus is involved in memory processing and chronic cocaine administration though to induce biochemical adaptations that underlie persistent memory and recall of drug-induced euphoric events that influence drug craving and relapse. Mash and colleagues compared hippocampal gene expression in cocaine-overdose victims ($n=10$) and controls ($n=11$) using the Affymetrix Human Genome U133AB set (>39,000 transcripts; Mash et al. 2007). Analysis revealed upregulation of 151 mRNAs and decreased expression of 91 mRNAs, and biopathway analysis identified several molecular functions disrupted in the cocaine-overdose groups including cell adhesion, neurogenesis and axon guidance, receptors and signal transduction, and ion channels and transport. Differential expression of several transcripts was validated by RT-PCR as well as Western blot analysis. Among the

transcripts belonging to the cell adhesion pathway, reversion-inducing cystine-rich protein with kazal motifs (RECK) exhibited the greatest degree of upregulation along with three protocadherin-related transcripts and others. The authors utilized the gene expression data and subsequent pathway analysis as an initial step in the identification of potential mechanisms of dysregulation in the hippocampus. For example, RECK is known to regulate expression of matrix metalloproteinases, endopeptidases that regulated proteins associated with the extracellular matrix and cellular adhesion, thereby influencing the structural synaptic remodeling known to occur as a result of cocaine administration.

A critical issue in the analysis of neurobiological substrates of addiction is the preference and history of drug use of human drug abusers—with abuse of more than one substance (polydrug abuse) relatively common (Rounsaville et al. 2003). Results from rodent studies have demonstrated that while different classes of abused drugs exert their effects via unique mechanisms of action, common brain regions and biochemical pathways appear to be activated by most of these substances. Using prefrontal cortical tissue from acquired postmortem from drug abusers, Lehrmann and colleagues hypothesized that common molecular pathways would be activated that were independent of the individuals' drug history (Lehrmann et al. 2006). To this end, comparisons were made between individuals with cocaine, cannabis, and/or phencyclidine (PCP) abuse histories (n=42) and controls (n=30) using Mammalian Gene Collection cDNA arrays (9,216 transcripts represented). Interestingly, 160 transcripts were shared by cocaine, cannabis, and PCP groups, 474 shared transcripts between cocaine and PCP, 83 transcripts between cocaine and cannabis, and 146 shared transcripts between cannabis and PCP. In contrast, 964, 299, and 1,152 transcripts were exclusively expressed in the cocaine, cannabis, and PCP groups, respectively, suggesting greater dissimilarities than similarities between groups. Analysis of the transcripts exhibiting differential expression irrespective of drug use resulted in the classification of 39 transcripts to three major functional classes: calmodulin-related signaling, Golgi/ER-related transcripts, and lipid/ cholesterol metabolism.

Proteomics

Whereas several studies have assessed gene and subsequent protein expression as a function of cocaine administration in humans and animal models, to date, there are few studies using high-throughput proteomic technologies to examine drug-induced global protein expression patterns in brain regions (Freeman and Hemby 2004;Hemby 2006;Kim et al. 2005). Genomics based studies have revealed novel mechanisms of drug-induced neuronal and nonneuronal dysregulation in human postmortem brain tissue (Albertson et al. 2004;Tang et al. 2003b) and rodent models (Ahmed et al. 2005; Ang et al. 2001a; Backes and Hemby 2003;Hemby 2004; Yuferov et al. 2005). While these studies have been highly informative in furthering our understanding of drug-induced transcriptional regulation contributing to long-term changes in cellular function, research determining coordinate changes in the expression of multiple proteins following cocaine exposure has been scarce (Hemby 2006). To comprehend the intricate neuroadaptive machinery implicated in the development and expression of cocaine abuse, it is desirable to complement the global gene expression analyses with studies examining the corresponding proteomes. Historically, research examining the abundance of proteins and posttranslational modifications as a function of cocaine abuse has been restricted to the serial analysis of individual proteins. With the advent of high-throughput separation and mass spectrometry-based analysis strategies, it is possible to provide a broad and unbiased coverage of the proteome to delineate the multitude of neurobiological effects of abused drugs (Hemby 2006; Matsumoto et al. 2007). Several recent studies in animal models and human postmortem tissue have utilized the emergent technologies in proteomics to broaden our understanding of protein changes in defined brain regions as a result of chronic cocaine intake.

Rodents

To date, relatively few studies have utilized proteomic technologies to examine protein changes in rodent models. One study compared the nucleus accumbens proteomes from rats undergoing different rates of extinction from cocaine-induced place conditioning (del Castillo et al. 2009). Following testing of place conditioning, rats remained abstinent for 5 days and subsequently were retested for place conditioning. Rats were subsequently divided into two groups—those that did not extinguish (NE) and those that did extinguish (E) conditioning and administered either cocaine (NE COC and E COC) or saline (NE SAL and E SAL). Using 2D gel electrophoresis (2DGE) and matrix-assisted laser desorption/ ionization time-of-flight (MALDI ToF) mass spectrometry, analysis revealed 18 proteins that were differentially expressed between the four groups. Comparison of E SAL and NE SAL revealed five proteins including four mitochondrial proteins and a nucleoside kinase—likely changes that are associated with extinction to cocaine-associated environmental cues. Additional comparisons were made between groups (NE COC vs. E COC; NE COC vs. NE SAL; E COC vs. E SAL) with several proteins found to be differentially expressed between groups; however, the interpretation of these comparisons is less clear as no behavioral assessments were conducted to substantiate the claims of vulnerability to cocaine-seeking behaviors.

Nonhuman primate

To date, two studies have been published examining the effects of cocaine in nonhuman primate brain. The first study demonstrated that administration of cocaine to juvenile vervet monkeys resulted in cognitive deficits that are dependent on the functional integrity of the orbitofrontal cortex (OFC) and that these changes occurred concurrently with enhancements in stimuli previously associate with cocaine (Olausson et al. 2007). Following behavioral analysis, differential expression of proteins in the OFC of these monkeys was compared with controls 3 to 4 weeks following the last cocaine administration using two complementary proteomic approaches: 2D difference in gel electrophoresis (2D-DIGE) and isobaric tagging for relative and absolute quantitation (iTRAQ). Synaptoneurosomes were isolated from OFC tissue punches in order to reduce the complexity of the proteome and target a specific cellular domain. Both 2D-DIGE and iTRAQ revealed significant differences in proteins involved in metabolism/mitochondrial function and signal transduction as well as cytoskeletal proteins, although information on specific proteins was not provided.

Tannu et al. (2008) examined the effects chronic intravenous cocaine self-administration on protein abundance and phosphorylation in the NAc of rhesus monkeys using 2D-DIGE and 2DGE followed by gel staining with Pro-Q® Diamond phosphoprotein gel stain, respectively. As detailed for the aforementioned studies in human postmortem tissue, gel images were normalized for each set of experiments, and spots with significantly differential image intensities ($P<0.05$) were identified, excised, and trypsin digested and analyzed by MALDI ToF/ToF mass spectrometry. Eighteen positively identified were found to be differentially expressed in the accumbens between the groups—a significant number of which were either directly or indirectly related to the hyperglutamatergia identified in both COD and rhesus monkeys self-administering cocaine (Hemby et al. 2005b; Tang et al. 2003b). Interestingly, the study identified several proteins which compliment/supplement the results of the study in COD including proteins involved in cell structure, synaptic plasticity/signal transduction, metabolism, and mitochondrial function. Specifically, glial fibrillary acidic protein, syntaxin binding protein 3, protein kinase C isoform, adenylate kinase isoenzyme 5, and mitochondrial-related proteins were increased in monkeys self-administering cocaine while beta-soluble *N*-ethylmaleimide-sensitive factor attachment protein and neural and nonneural enolase were decreased. In addition to determination of

overall protein abundance, the study also explored the "functional" proteome of the accumbens, in this case by evaluating the expression of phosphorylated proteins. Of the identified spots on the gel, 15 phosphoproteins were positively identified including increased levels of GABA-A receptor associated protein 1, 14-3-3 gamma protein, glutathione *S*-transferase, and brain-type aldolase and decreased levels of beta-actin, Rab GDP dissociation inhibitor, guanine deaminase, peroxiredoxin 2 isoform b, and several mitochondrial proteins. Results from this study compliment previous studies of cocaine-induced biochemical alterations in COD using an animal model that closely recapitulates the human condition. The findings suggest a coordinated dysregulation of proteins related to cell structure, signaling, metabolism, and mitochondrial function that likely indicate long-term compromised cellular function. The reversal or attenuations of these biochemical alterations are important targets for addressing the neuropathology associate with drug abuse.

Human postmortem

To examine the neuropathological consequences of chronic cocaine abuse in the human brain, 2D-DIGE was used to compare protein alterations in the NAc between COD victims and controls (Tannu et al. 2007). The NAc was dissected from coronal blocks of frozen brain tissue that had been obtained previously from subjects that were matched on a number of demographic and pathological indices. Tissue was fractionated into membrane, nuclear, and cytoplasmic fractions as previously described (Hemby et al. 2005b; Tang et al. 2003a), with only cytosolic fractions used for this study. Differentially expressed proteins were identified by MALDI ToF/ToF mass spectrometry; 1,407 spots were found to be present in a minimum of five subjects per group, and the intensity of 18 spots was found to be differentially abundant between the groups leading to the eventual positive identification of 15 proteins by peptide mass fingerprinting (PMF). In addition, 30 two spots that were constitutively expressed were positively identified by PMF. The identified proteins are categorized as cell structure, synaptic plasticity/signal transduction, mitochondria, and metabolism and are representative of functional classes which have been shown to be affected either directly or indirectly by cocaine administration. Previous studies in human COD have reported significant dysregulation of ionotropic glutamate receptors in mesolimbic brain areas (VTA and NAc)—an effect that likely has far-reaching implications in terms of the mechanisms that support increased expression as well as the physiological implications of these upregulated proteins. For example, liprin α3 (upregulated over 2.5-fold in COD) belongs to a family of proteins whose postsynaptic expression is involved in the transport of NMDA receptor vesicles along microtubules. Along with increased beta tubulin (2.72-fold in COD), these results begin to provide a framework that could mediate the increased levels of iGluR subunits at the membrane surface in COD (Hemby et al. 2005b).

In addition to protein alterations that likely are involved in the maintenance iGluR expression, the abundance of several metabolic proteins were altered in COD that may be related to the consequence of increased iGluR expression—such as increased calcium flux and resulting oxidative stress. For example, peroxiredoxin 2, a neuronal protein involved in redox regulation, was decreased in COD, based on previous studies that cocaine administration increases lipid peroxidation (Kloss et al. 1984), alters antioxidant enzyme activity, and elevates reactive oxygen species in dopaminergic projection areas (Dietrich et al. 2005; Macedo et al. 2005). The mitochondrial protein ATP synthase beta chain, a protein that produces ATP from ADP that is generated from electron transport complexes involved in mitochondrial respiration, was also decreased in COD. These data provide but two examples by which chronic cocaine profoundly affects processes that are integral to normal neuronal function (i.e., decreased ability to reduce reactive oxygen species and improper functioning of energy metabolism). Such changes are likely reflected in changes in glucose metabolism and utilization following cocaine administration in rats (Porrino 1993), monkeys

(Lyons et al. 1996; Porrino et al. 2004), and humans (Breiter et al. 1997;Risinger et al. 2005). Understanding the coordinated involvement of multiple proteins in human brain as a function of cocaine abuse provides unique insight into the molecular basis of the disease offers new targets for pharmacotherapeutic intervention for drug abuse-related disorders and has the potential to reshape the debate on which biochemical indices are most relevant to the human condition.

Conclusion

In conclusion, relevant gene and protein expression profiles for cocaine abuse and other substance abuse disorders are being generated expanding our knowledge of drug-induced changes in the brain that may underlie persistent drug taking and relapse. Results from rodent, nonhuman primate, and human postmortem studies indicate significant impairments in neuronal function and plasticity in several brain regions. To date, the majority of studies have utilized rodents to model human cocaine intake; however, growing evidence indicates the need to refine rodent and nonhuman primate models to better recapitulate human drug intake and associated neuropathologies. As in other psychiatric and neurological illnesses, researchers should identify the molecular pathologies associated with cocaine addiction in humans and attempt to recapitulate such biological alterations in animal models.

The advent of genomic and proteomic technologies provides a unique opportunity to discover and explore biochemical substrates and consequences associated with abused substances. Results from rodent, nonhuman primate, and human postmortem studies indicate significant impairments in neuronal function and plasticity in several brain regions. To date, the majority of studies have utilized rodents to model human cocaine intake; however, growing evidence indicates the need to refine rodent and nonhuman primate models to better recapitulate human drug intake and associated neuropathologies. As in other psychiatric and neurological illnesses, researchers should identify the molecular pathologies associated with cocaine addiction in humans and attempt to recapitulate such biological alterations in animal models.

Understanding the coordinated involvement of multiple proteins with chronic cocaine and alcohol addiction provides insight into the molecular basis of drug dependence in general and may offer novel targets for pharmacotherapeutic intervention. Although significant advances have been made in the identification of neurochemical and neurobiological substrates involved in the behavioral effects of abused drugs, the relationship between these effects and resultant alterations in protein expression remains in its infancy and the application of this information to the development of treatment strategies has not been fruitful for several reasons. One explanation is that research in the areas of neurobehavioral pharmacology and molecular biology have proceeded in relative isolation of each other. To date, there have been few published studies combining models of self-administration with proteomic approaches. Other possible explanations include (1) the inappropriate use of experimental models, (2) reliance on nonneuronal systems or neuronal tissue not directly involved in the reinforcing effects of the drug, and (3) the lack of definable neural substrates at the cellular or biochemical level. The combination of appropriate behavioral models of drug reinforcement, specific neurobiological systems, and state-of-the-art molecular techniques will provide the most pertinent data for understanding the molecular basis of drug reinforcement and for potentially establishing novel targets for treatment.

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