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Assessment of genome and proteome profiles in cocaine abuse

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Abstract

Until recently, knowledge of the impact of abuse 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 non-human 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 chapter will review gene expression data from rodent, non-human primate and human post-mortem studies of cocaine abuse and will provide a preliminary proteomic profile of human cocaine abuse and explore how these studies have advanced our understanding of addiction.

Keywords

microarray; RNA amplification; gene expression; molecular fingerprint; qPCR; transcriptome; proteome; brain; post-mortem; monkey

Introduction

The efforts to complete sequencing of the human genome have enabled new endeavors into the function of these genes in human disorders and have provided a wealth of knowledge about the molecular underpinnings of behavior. 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. Microarray and proteomic analysis strategies allow the simultaneous assessment of thousands 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

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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 content of this chapter will include recent studies employing genomic and proteomic strategies to develop a comprehensive understanding of the changes induced by cocaine, a commonly abused stimulant. Furthermore, the chapter will focus on studies employing rodent and non-human primate models as well as studies examining the neuropathology identified in post-mortem human tissue of individuals with chronic histories of illicit substance abuse. The chapter is limited to studies on cocaine due to the fact that this is the most-studied abused drug with respect to genomic and proteomic strategies and thus may provide an investigative template for studying other abuse substances.

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. A recent report indicates that approximately 13.6 million Americans are current users of illicit drugs (e.g. marijuana, 11 million; cocaine, 1.8 million; heroin 130,000) and over 4 million Americans meet the diagnostic criteria for dependence on illicit drugs (SAMHSA, 2002). Despite intense behavioral and biological research, few effective pharmacotherapeutic strategies exist, with the arguable exception of methadone and LAAM treatment programs for opiate dependence. In order to devise effective treatment strategies, it is necessary to understand the interactions of behavioral, pharmacological and biochemical factors that underlie use and abuse. 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. The development of new and innovative medications for drug addiction requires multidisciplinary research approaches examining the spectrum of drug-induced effects from behavior to the biological and biochemical effects in discrete neuronal populations.

A generally accepted tenet in drug abuse research is that drugs can function as reinforcing stimuli. Hence, with respect to drug abuse, the reinforcing effects of certain drugs contribute largely to their abuse liability. A significant amount of research investigating the neurobiology of drug abuse is conducted in animal models which closely resemble characteristics of human drug intake l. Criteria should include, but not be limited to the following: (1) behaviors are contingent upon drug delivery, (2) behaviors are engendered and maintained by drug delivery, and (3) drug delivery increases the frequency of those behaviors. The self-administration paradigm meets these criteria, unlike the other procedures, and is widely accepted as an appropriate model for studying the reinforcing effects of drugs. Generally, the self-administration paradigm involves the emission of specific behavior(s) (e.g. lever-press; nose-poke) that is maintained by drug administration (e.g. intravenous, oral, or intracranial). Advantages of self-administration include the following: (1) substances abused by humans can function as positive reinforcing stimuli under laboratory conditions, (2) general concordance between substances abused by humans and those self-administered by laboratory animals, (3) a variety of species readily acquire and maintain self-administration under a number of operant schedules and (4) the ability to

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generate clear dose-effect curves using this procedure (Hemby et al., 1997b; Hemby, 1999). Procedures such as place conditioning are hindered by the lack of objectively quantifiable behaviors, lack of dose dependency and most importantly by the fact that drug administration is not contingent on the behavior of the animal.

The concept of the contingency is critical for researchers attempting to draw conclusions regarding the involvement of specific neural substrates in drug reinforcement. The majority of studies investigating the neurobiological basis of drug administration have used experimenter-controlled drug administration and extrapolated the relevance of those findings to reinforcement mechanisms (Di Chiara and Imperato, 1988). However, a growing body of literature has demonstrated pronounced neurochemical differences resulting from the context and contingency of drug administration (self-administered versus experimenter delivered) (Wilson et al., 1994; Hemby et al., 1995, 1997a,b). Neurobiological differences between rats self-administering drugs and rats receiving experimenter-administered infusions are based on the context of drug presentation and suggest inferences of reinforcement mechanisms drawn from studies using experimenter-drug administration protocols may be misleading. These studies clearly indicate a need for reliance on accepted behavioral models when asserting relevance of biological findings to behavioral phenomena such as reinforcement. While reinforcement does not solely explain drug abuse, it allows for the quantification of the initiation and maintenance of drug self-administration.

Neuroanatomy of cocaine addiction

Similar to other psychiatric illnesses, drug abuse is a heterogeneous disorder with multiple causes all of which can lead to the same functional endpoint — namely addiction. While the regulation of individual transcripts and proteins have been suggested as mediators of the addictive process, a more probable scenario is that the coordinate regulation of multiple genes and proteins in defined neuroanatomical loci are either the mediators of addictive behaviors or are modulated by chronic drug use. Over the past 20 years, the driving theoretical construct in drug abuse research has been the psychomotor-stimulant theory of addiction which attempts to provide a unifying theory for the neurobiological basis of all abused drugs (Wise and Bozarth, 1987). The theory indicates that both the stimulant and the reinforcing effects of all abused drugs are mediated by a common neural mechanism, the mesolimbic dopamine system. The pathway originates in the mesencephalon, ventral tegmental area (VTA) and projects to several basal forebrain regions including the nucleus accumbens (NAc), ventral caudate-putamen, bed nucleus of the stria terminalis, diagonal band of Broca, olfactory tubercles, prefrontal and anterior cingulate cortices. Administration of drugs that are abused by humans lead to activation of this pathway in humans, non-human primates and rodents (Porrino, 1993; Lyons et al., 1996; Volkow et al., 1997). Activation of this circuit has been correlated with subjective reports of craving and euphoria in cocaine addicts (Volkow et al., 1997; Childress et al., 1999).

Dopaminergic projections from the VTA to the NAc have been implicated in the reinforcing effects of psychomotor stimulants (cocaine and amphetamine) and alcohol, whereas the role of this pathway in opiate reinforcement remains controversial (Hemby et al., 1997b). Previous studies have shown that rats will self-administer cocaine, amphetamine, opiates,

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and alcohol directly into regions of this pathway. Altering the functional integrity of the mesolimbic pathway by dopamine-selective neurotoxic lesions and dopamine D1 and D2 receptor blockade attenuate psychomotor stimulant self-administration. Similar manipulations of the other monoamines serotonin and norepinephrine fail to significantly influence drug intake. Thirdly, microdialysis studies indicate that extracellular dopamine concentrations are elevated during cocaine and amphetamine self-administration sessions (Hemby et al., 1997b). Taken together, the most recent research indicates that the neurobiological substrates of drug abuse are not the same across all dug classes and probably involve a myriad of neurotransmitter and receptor systems.

Functional genomics

Over the past 10 years, approximately 20 studies have employed various high-throughput gene expression strategies to examine stimulant-induced changes in various brain regions of animal models and humans. Several obstacles prevent the assimilation of the results from these studies into an overarching understanding of stimulant-induced transcriptional regulation such as species, brain regions, route and contingency of administration, dose and duration of drug administration, length of time since the final drug administration, experimental variables in microarray analysis, validation of findings with alternative techniques, etc. Although several studies have examined the effects of stimulants on gene expression, there is minimal literature on stimulant-induced proteomic analysis on a broad scale; however, preliminary data will be presented on proteomic analysis of human cocaine overdose victims.

Rodent studies: non-contingent administration

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 NAc (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 VTA (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) as an analogous “binge” paradigm, and gene expression was evaluated in the hippocampus using RNA pools. Using stringent inclusion criteria of 50% induction or 33% reduction, the authors noted only five transcripts were differentially regulated — all were upregulated in the cocaine-treated rats: protein kinase A alpha (PKAcα), metabotropic glutamate receptor 5 (mGluR5) and voltage-gated potassium channel 1.1 (Kv1.1), survival of motor neuron (SMN) and protein phosphatase 2A alpha subunit (PP2Aα). From this set, only mGluR5, PKCα, 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 cynomolgus monkeys, were also elevated in the hippocampus of cocaine-treated rats suggesting these changes are not region or treatment-specific regimen.

In a separate study, changes in gene expression in the PFC of the same subjects (Freeman et al., 2002) were examined by screening 588 rat genes (BD Bioscience Clonetech Atlas cNDA Expression Array). Cocaine administration induced the expression of activity-regulated

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cytoskeletal protein (ARC), NGFI-B and HMG-CoA synthase I and decreased the expression of casein kinase II alpha (CKIIa), glycogen synthase 3 alpha (GSK3α), and fos-related antigen (FRA1). The upregulation of NGFI-B was confirmed by quantitative 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 somatodendritic localization of ARC in neurons. The authors also examined proteins that had been shown to be upregulated in the hippocampus of rats and NAc of monkeys administered cocaine including PYK2, mitogen-activated kinase I (MEK), β-catenin, PKCα, PKCε, – of which only PYK2 was found to be upregulated in the frontal cortex of cocaine-reated 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 micro-array study which screened 1176 rat genes (BD Bioscience Clontech Atlas cNDA Expression Array) in samples of NAc core, NAc shell, striatum and dorsal PFC of rats following 3 weeks of withdrawal from 7 days of cocaine administration (intraperitoneal; 15 mg/kg on days 1 and 7, 30 mg/ kg on days 2–6) (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 (Ntrk2) in the PFC of cocaine-treated rats. Ntrk2 is the receptor for brain-derived neurotrophic factor (BDNF) previously shown to be involved in the behavioral effects of cocaine in the VTA and NAc (Berhow et al., 1996; Horger et al., 1999; Pierce and Bari, 2001; Freeman and Pierce, 2002). Though not significantly different at the protein level in the PFC, protein levels of the Ntrk2 truncated isoforms p95 and p145 were 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), 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 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.

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Kreek and colleagues further examined cocaine-induced gene expression in the striatum following acute (3 hourly injection of 15 mg/kg for 1 day) and chronic (3 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, disruption 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 possibly reflect a less-studied mechanism of cocaine-regulated dopamine release in the striatum as noted by the authors. Additional studies that examine the cellular origin and localization of the Per 2 transcript and protein and the role of SSTR2 in the behavioral effects of cocaine are warranted.

Rodent studies: self-administration

The previous studies have expanded the knowledge base of the cocaine's effects in the brain and provided novel insights into the pharmacological effects of cocaine in various brain regions; however, all used the non-contingent administration of cocaine and thus may have limited applicability to understanding the abuse liability/reinforcing effects of cocaine. As discussed in the Introduction, inferences of reinforcement mechanisms drawn from studies using experimenter drug administration protocols may be misleading as several studies have shown significant differences between experimenter- and self-administered drugs of abuse (Wilson et al., 1994; Hemby et al., 1995, 1997a, b; Hemby, 1999). To date, two studies have combined rodent intravenous self-administration procedures with functional genomics procedures. Ahmed and colleagues examined gene expression profiles in samples of NAc, lateral hypothalamus, septum, VTA, medial PFC 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) in which one press of a level resulted in the delivery of the dose of cocaine through the intravenous catheter. 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 and Koob, 1998, 1999; Ahmed et al., 2002). 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) when compared to the other brain regions studied and differential expression of select transcripts were confirmed by qPCR. Transcripts altered by the escalation paradigm were members of several functional classes including functional and

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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 (Ginsberg et al., 1999, 2000, 2004; Hemby et al., 2002; Fasulo and Hemby, 2003). 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 (Van Gelder et al., 1990; Eberwine et al., 1992; Eberwine, 2001; Hemby et al., 2002). 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 subunit of protein phosphatase 2A (PP2α), GABA-A α1 and $G\alpha_{i2}$ were significantly increased at both time points. Additionally, calcium/calmodulin-dependent protein kinase IIα (CaMKIIα) mRNA levels were increased initially followed by a slight decrease after 20 days, whereas neuronal nitric oxide synthase (nNOS) mRNA levels were initially decreased but returned to near control levels by day 20. These results indicate that alterations of specific GABA-A receptor subtypes and other signal transduction transcripts appear to be specific neuroadaptations 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 FosB and CREB-inducible transgenic mice with targets know 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 FosB levels in brain regions associated with the behavioral effects of cocaine (Hope et al., 1994; Moratalla et al., 1996; Nestler, 2001; Nestler et al., 2001; McClung and Nestler, 2003; Perrotti et al., 2005; Brenhouse and Stellar, 2006). Using the FosB-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 cNDA Expression Array). More importantly, a functional role of cdk5 in cocaine-mediated behaviors was shown by antagonism of cdk5 in the striatum and

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attenuation of kainate peak currents in the striatum following cocaine administration (Bibb et al., 2001). In a separate study using the FosB-inducible transgenic mouse model, the authors employed the higher density Affymetrix DNA mouse array and found significantly higher levels of NFκB mRNA and protein in the transgenic mice and similar elevations in NFκB protein levels in wild-type mice administered cocaine (20 mg/kg; 14 days) (Ang et al., 2001).

Comparison of the effects of FosB- 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, FosB expression generated dichotomous patterns of gene expression at 2 and 8 weeks with the 2-week expression pattern for FosB similar to CREB expression. The longer FosB 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/g; 20 days) induced 27% of the genes induce by FosB 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 FosB. The list of genes attributable to the induction of CREB and FosB is lengthy and will not be reviewed in here 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 transcription 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 per 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 post-synaptic density protein 95 kDa (PSD-95; involved in scaffolding of 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 whole 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.

Non-human 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 Clonetech consisting of 588 probes (Freeman et al., 2001b). Pools of mRNA from each group were hybridized to two separate arrays

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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 PKAα 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 post-transcriptional 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 non-human primate cocaine self-administration model to validate protein and mRNA changes observed in human post-mortem tissue of cocaine-overdose victims (Hemby et al., 2005b). Unfortunately, attempts to recapitulate changes observed in cocaine overdose victims and non-human primate models in rodent self-administration models have not succeeded (Tang et al., 2004; Hemby et al., 2005a). 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, non-human 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, mid-brain 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 non-human primates may allow the development of a more clear and clinically relevant characterization of the biochemical changes associated with cocaine use.

Human post-mortem studies

Understanding the consequences of long-term cocaine abuse on post-mortem brain tissues requires vigorous investigation with the benefit of revealing whether the adaptations

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observed in rodent and non-human 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 post-mortem 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 post-mortem 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 post-mortem 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 et al., 1995; Douglass and Daoud, 1996). In addition, several myelin-associated transcripts were significantly decreased in the NAc of cocaine abusers including myelin basic protein (MBP), proteolipid protein 1 (PLP) and myelin-associated oligodendrocyte basic protein (MOBP) and a significant increase in T-cell differentiation protein (MAL2) — which were confirmed by qPCR. Immunohistochemistry 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 (Volkow et al., 1988; Wiggins and Ruiz, 1990; Lim et al., 2002).

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., 2003). Evaluated transcripts included ionotropic glutamate receptor (iGluR) 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 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 cocaine-overdose victims (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) were 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 these alterations are pathway specific and likely mediate in part the persistent drug intake and craving in the human cocaine abuser.

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; Freeman et al., 2005; Kim et al., 2005). In order to begin to fill this void in the field of the neurobiology of cocaine addiction, our lab has embarked on several studies in rhesus monkey cocaine self-administration models and in human post-mortem tissue from COD victims. Initial efforts have focused on changes in the NAc given the role of this brain region in the addictive processes of cocaine and the growing gene expression databases. In a preliminary study, cytosolic fractions of NAc proteins from human COD and controls ($n = 5$/group) were separated and quantified by two-dimensional difference gel electrophoresis (2-DIGE) and identified by matrix-assisted laser desorption/ionization time-of-flight (MALDI ToF/ToF) mass spectroscopy (see Chapter 4 for detailed explanation of procedures). Greater than 1000 spots were detected across the five pairs (COD and controls) of which 340 spots were excised, digested in-gel with trypsin, and subsequently analyzed by MALDI ToF/ToF (see Supplemental Table I). Fifty-two percent of the spots were positively identified including 11 upregulated proteins including DJ1 (Parkinson’s disease 7 (PARK7; autosomal recessive, early onset)), ubiquitin carboxyl-terminal esterase L1 (UCHL1; PARK5), lamda crystallin, endothelial monocyte-activating polypeptide 2 (EMAP-II) and others (Fig. 1). DJ1, a redox-sensitive chaperone that protects neurons against oxidative stress and cell death, and UCHL1, a neuronal de-ubiquitinating enzyme, are both associated with Parkinson Disease (Abou-Sleiman et al., 2006). Combined with elevated a-synuclein levels in human COD victims (Qin et al., 2005), these data support the suggestion by Deborah Mash and colleagues that chronic cocaine use induce Parkinson-like pathology in striatal regions. Eighteen-positively identified proteins were found to be downregulated in the NAc of COD victims including gelsolin, ATP5b, dihydropyrimidinase-like 3 (DRP3/TUC-4) and dihydropyrimidinase-like 2 (DRP2) (Fig. 2). Decreased expression of gelsolin and DRP3/TUC-4 and gelsolin was confirmed by

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immunoblotting (Fig. 3) Gelsolin has been reported to exhibit antiapoptotic properties in neurons (Harms et al., 2004) and fibroblasts (Ahn et al., 2003a, b) such that decreased gelsolin expression may render NAc cells more susceptible to apoptosis and oxidative stress due to cocaine exposure. DRP2 and 3 are generally associated with nerve terminal activity, more specifically axonal restructuring and decreased expression may imply decreased plasticity of NAc cells with chronic cocaine exposure. Efforts are underway to assimilate genomic and proteomic databases in a more systematic manner. The application of proteomics holds great promise to understanding the biology of psychiatric diseases, including substance abuse disorders. Further investigation of the changes found and a more comprehensive examination of the human proteome, which may provide the biological understanding and identification of novel therapeutic targets for treatment of cocaine dependence.

Conclusion

In conclusion, relevant gene 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, non-human primate and human post-mortem 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 non-human 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 neurobiological and molecular characteristics of cocaine addiction, although specific to cocaine, may generalize to other drug dependencies. Understanding the coordinated involvement of multiple proteins with chronic cocaine abuse provides insight into the molecular basis of drug dependence 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 gene and protein expression remains in its infancy. The relationship between altered gene and protein expression and the addictive effects of specific drugs remains understudied. 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 has proceeded in relative isolation of each other. To date, there have been few published studies combining models of self-administration with genomic and proteomic approaches. Other possible explanations include (1) the inappropriate use of experimental models, (2) reliance on non-neuronal 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

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the molecular basis of drug reinforcement and for potentially establishing novel targets for pharmacotherapeutic intervention.

A more detailed understanding of the molecular and biochemical cascades in specific neuronal populations and the interactions between well-defined neuronal populations within discrete brain regions could lead to a greater knowledge of the basic neurobiological processes involved in drug reinforcement. Future efforts investigating the biological basis of drug reinforcement should be directed at specific cellular targets in brain regions considered to be involved in drug reinforcement. The integration of basic neuroscience and behavior offers the most productive avenue for delineating the complexity of the neurobiological underpinnings of drug reinforcement and the subsequent development of effective pharmacotherapies to treat addiction.

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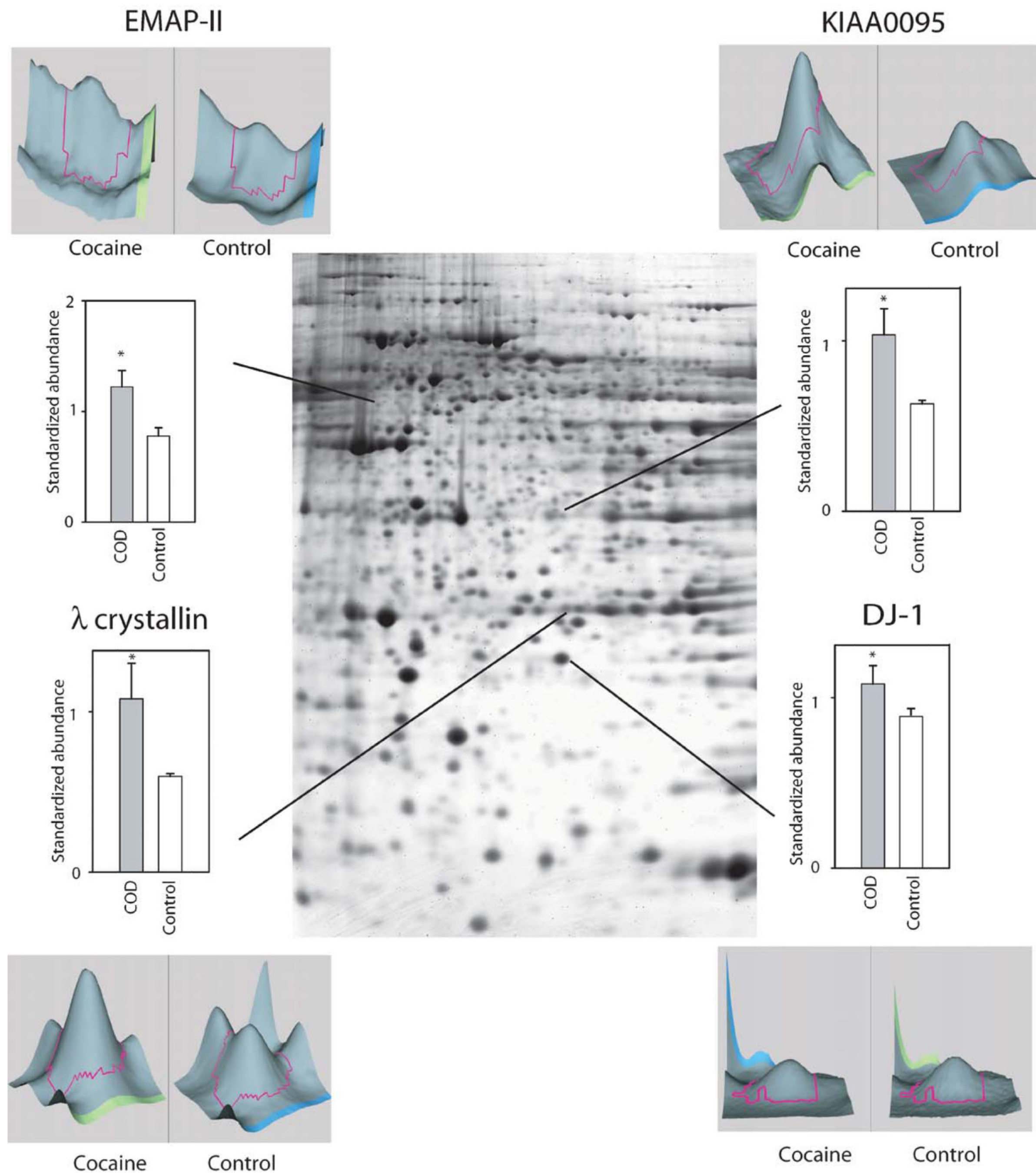

Fig. 1.

Preliminary data of representative proteins exhibiting increased abundance in COD victims. Signal intensities for specific gel spots from COD victims and control subjects were compared. Included in the figure are the proteins quantified by the 2DIGE technique using the normalization by Cy2-labeled pool sample and have statistical significance difference in expression profiles between the two groups (*p<0.05, *t*-test). Examples of proteins are provided with representative 3-D plots of individual COD and control spots.

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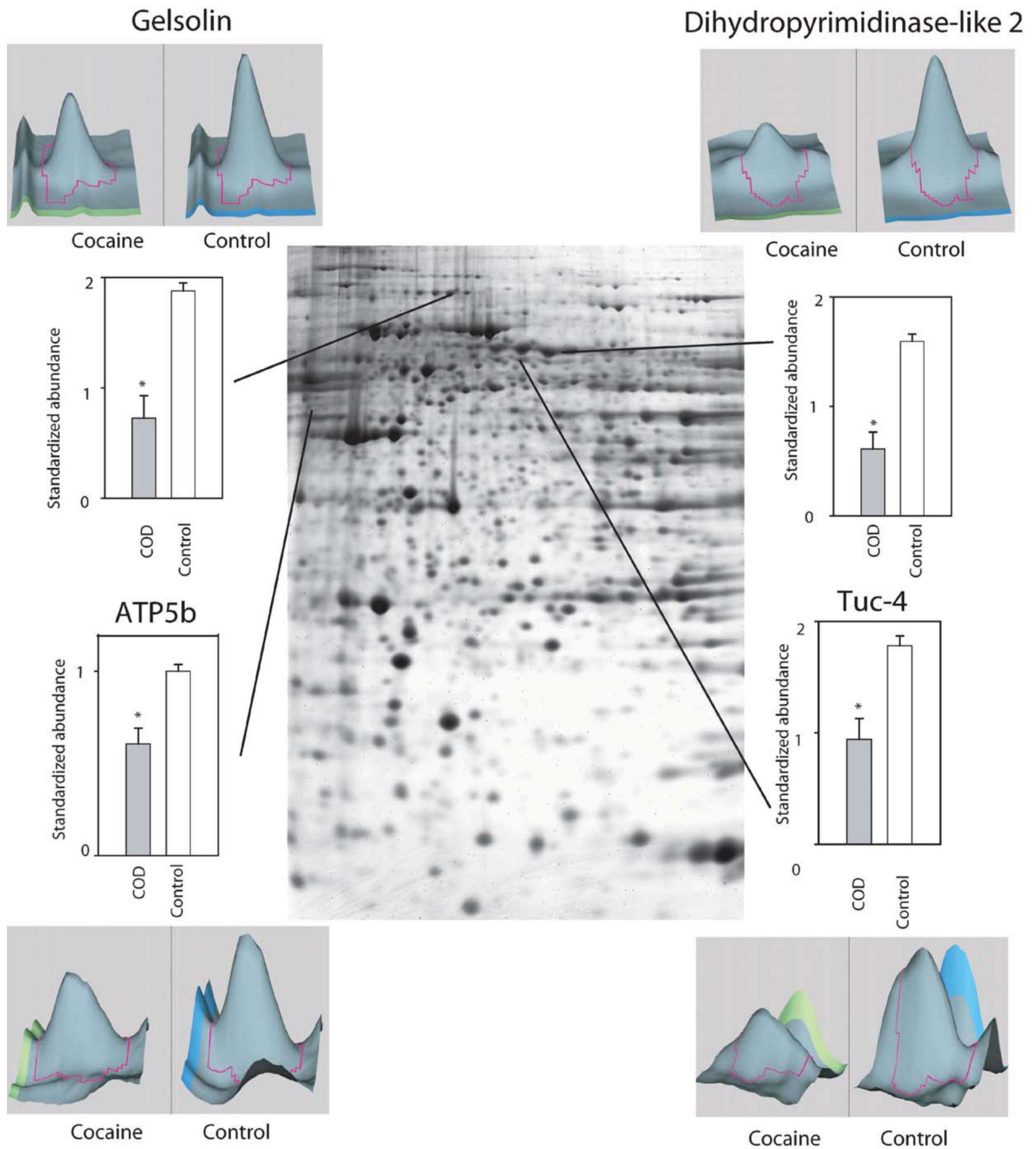

Fig. 2.

Preliminary data of representative proteins exhibiting decreased abundance in COD victims. Signal intensities for specific gel spots from COD victims and control subjects were compared. Included in the figure are the proteins quantified by the 2DIGE technique using the normalization by Cy2-labeled pool sample and have statistical significant difference in expression profiles between the two groups (*$p<0.05$, *t*-test). Examples of proteins are provided with representative 3-D plots of individual COD and control spots.

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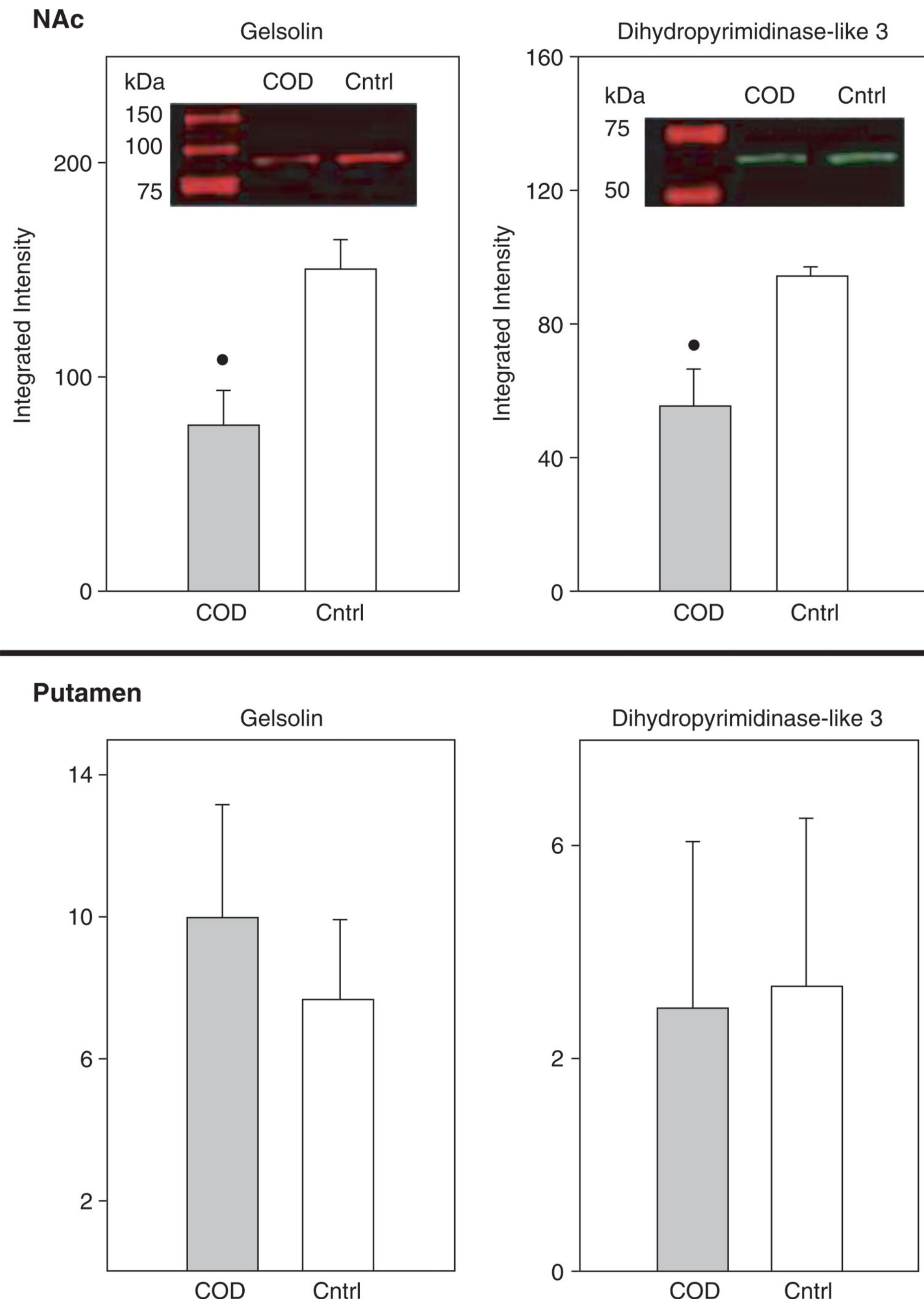

Fig. 3.

Western blot analysis of gelsolin and DRP3. Assessment of protein levels from the samples used for 2DIGE revealed significant decreases in gelsolin and DRP3 in agreement with the 2DIGE analyis. Moreover, these changes were specific to the NAc and not observed in the putamen. (*p<0.05, t-test). β tubulin was used as a loading control and no differences were observed for this protein.

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Table 1

Identified and matched proteins

Spot #	Protein GI #	Protein name	Theoretical MW	Theoretical pI	Peptide count	Mascot score	Confidence interval	*t*-test value	Average ratio	Protein homolgues/other protein names
526	28595	Aldolase A; fructose-bisphosphate aldolase	39851.5	8.3	15	196	100		−1.1286	ALDOA
769	136066	Triosephosphate isomerase	26909.8	6.45	4	74	99.944	0.05221	−1.3549	TIM
772	136066	Triosephosphate isomerase	26909.8	6.45	4	74	99.944	0.1059	1.3564	TIM
775	136066	Triosephosphate isomerase	26909.8	6.45	4	74	99.944	0.111	−1.1886	TIM
459	180570	Creatine kinase	42876.4	5.3	17	571	100	0.5089	−1.0726	CKB
401	285975	Rab GDI	51088	5.94	13	200	100	0.654	−1.0426	GDP dissociation inhibitor 2, GDI2
259	334284	GP120	58060.7	5.35	7	87	99.997	0.2132	−1.3476	
660	387016	Phosphoglycerate mutase	28867.8	8.77	2	62	99.176	0.09345	−1.1645	PGAM2, phosphoglycerate mutase 2 (muscle)
450	423123	Tpr protein	238769.7	5.05	20	63	99.315	0.1194	−1.2695	
951	494781	Fatty acid-binding protein	14774.7	6.34	6	236	100	0.4423	1.0726	
162	763431	Albumin	52047.8	5.69	7	80	99.986	0.06642	−1.8004	
639	999892	Chain A, triosephosphate isomerase	26806.8	6.51	5	274	100		2.9063	
221	1465733	Cytosolic NADP(+)-dependent malic enzyme	63858.9	5.88	4	59	98.318	0.4025	− 1.3355	ME1
531	2118269	Zebrin II	39797.4	6.67	8	198	100		1.4454	Similar to human Aldolase C
322	2183299	Aldehyde dehydrogenase 1	55427.2	6.3	12	219	100	0.6272	1.0482	ALDH1A1
741	2737906	Plasminogen-related protein A	7983.9	8.44	5	61	99.01	0.2901	1.8309	LOC285189
705	2914390	Chain B, hemoglobin mutant	15834.2	6.76	4	84	99.995	0.4753	−1.1197	
953	2981643	Chain B, hemoglobin	15980.2	6.75	4	93	99.999	0.6047	−1.0872	
864	2982080	Familial als mutant G37r, chain A	16122	5.87	2	133	100	0.158	1.7454	
861	2982080	Familial als mutant G37r, chain A	16122	5.87	2	133	100	0.9453	−1.0013	
797	3205211	Non-muscle myosin heavy chain	72555.1	5.18	9	62	99.117	0.05802	1.4379	
413	3766197	ATP-specific succinyl-CoA synthetase beta subunit	46732.3	5.84	4	62	99.117	0.3475	1.5502	Succinate-CoA ligase, ADP-forming, beta subunit; SUCLA2
112	3811317	Tryptophan hydroxylase isoform 1	6476.5	9.7	4	56	96.486	0.8722	1.3864	TPH
150	4389275	Serum albumin	67988.5	5.69	25	679	100	0.1761	2.0119	
161	4389275	Serum albumin	68424.7	5.67	35	975	100	0.4513	1.597	

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523	4502561	Capping protein (actin filament), gelsolin-like	38778.6	5.88	3	67	99.727	0.2784	−1.2687	CAPG
280	4503377	Hydropyrimidinase-like 2; collapsin response mediator	62710.7	5.95	14	325	100	0.3267	−1.3266	CRMP2; DRP2; DPYSL2
282	4503377	Hydropyrimidinase-like 2; collapsin response mediator	62710.7	5.95	14	380	100	0.6647	−1.0535	CRMP2; DRP2; DPYSL2
272	4503971	Rab GDI-alpha	51177.4	5	8	131	100	0.2966	1.5102	GDP Dissociation Inhibitor 1; GDI1; oligophrenin 2; OPHN2; RHOGDI
261	4503971	GDP Dissociation inhibitor 1	51177.4	5	9	112	100	0.3715	1.3108	GDI1
58	4504165	Gelsolin	86043.3	5.9	15	258	100	0.2562	−1.6123	GSN
385	4504169	Glutathione synthetase	52523.3	5.67	17	346	100	0.06022	−2.0733	GSS; GSHS; MGC14098
391	4504169	Glutathione synthetase	52523.3	5.67	13	227	100	0.4307	−1.1292	GSS; GSHS; MGC14098
667	4505585	Platelet-activating factor acetylhydrolase	25724.2	5.57	2	60	98.724	0.8345	1.0289	PAFAH1B2; platelet-activating factor acetylhydrolase, isoform Ib, beta subunit 30 kDa
366	4506019	Protein phosphatase 3, catalytic subunit, alpha isoform	52172.7	5.82	7	72	99.912	0.9398	−1.0099	Calcineurin A alpha, PPP3CA, PP2BCA
275	4506089	Mitogen-activated protein kinase 4	63039.9	6.05	8	64	99.417	0.06896	−1.9117	MAPK4, p63MAPK
314	4506089	Mitogen-activated protein kinase 4	63039.9	6.05	11	72	99.916	0.3501	−1.2684	MAPK4, p63MAPK
920	4507793	Ubiquitin-conjugating enzyme E2N	17184	6.13	2	66	99.694	0.5976	−1.0522	Uniquitin-conjugating enzyme 13, UBC13; bendless, ubchen
633	4557032	Lactate dehydrogenase B	36900.2	5.71	13	559	100	0.1127	−1.4306	LDHB
783	4557032	Lactate dehydrogenase B	36900.2	5.71	13	559	100	0.5403	−1.1423	LDHB
636	4557032	Lactate dehydrogenase B	36900.2	5.71	8	219	100	0.8655	1.0184	LDHB
862	4557797	Nucleoside-diphosphate kinase 1 isoform b	17308.7	5.83	8	274	100		1.1544	Non-metastatic cells 1; NME1; NM23A
95	4557871	Transferrin	79280.5	6.81	9	105	100	0.1594	2.0797	TF
97	4557871	Transferrin	79280.5	6.81	9	105	100	0.8981	1.443	TF
418	4758426	Guanine deaminase	51483.8	5.44	11	323	100	0.2742	−1.1109	GDA
684	4758484	Glutathione S-transferase omega 1	27833.1	6.23	7	132	100	0.434	−1.0991	GSTO1
708	4758484	Glutathione S-transferase omega 1	27833.1	6.23	7	132	100	0.4908	1.1479	
647	4758638	Peroxiredoxin 6	25133.2	6	9	326	100	0.1888	1.2268	PRDX6
764	4758638	Peroxiredoxin 6	25133.2	6	9	326	100	0.6421	1.0663	
567	4759036	Regucalcin;senescence marker protein-30	33801.7	5.89	5	64	99.48	0.3902	1.2342	RGN, SMP30

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168	4827056	WD repeat-containing protein 1 isoform 2	58593.2	6.41	3	59	98.279	0.09829	−1.8217	WDR1
514	4885063	Aldolase C, fructose-bisphosphate;	39830.4	6.41	15	422	100	0.3166	−1.1063	ALDOC
557	5031777	Isocitrate dehydrogenase 3 (NAD+) alpha precursor	40022.2	6.47	6	95	100	0.7043	−1.0422	IDH3A
873	5031851	Stathmin 1; metablastin;	17291.9	5.76	5	147	100	0.547	1.0666	Leukemia-associated phosphoprotein p18; LAP18
549	5174391	Aldo-keto reductase family 1, member A1	36892	6.32	12	263	100	0.08951	−1.492	ALDR1
644	5174539	Cytosolic malate dehydrogenase	36631.1	6.91	8	206	100	0.06213	−1.639	MDH1
768	5174539	Cytosolic malate dehydrogenase	36631.1	6.91	5	159	100	0.6754	1.1273	MDH1
762	5174539	Cytosolic malate dehydrogenase	36631.1	6.91	5	159	100	0.8077	1.0359	MDH1
170	5729877	HSP70 protein 8 isoform 1	71082.3	5.37	23	437	100	0.1846	−1.8623	HSPA8
561	5803187	Transaldolase 1; dihydroxyacetone transferase; glycerone transferase	37687.5	6.36	11	178	100	0.4273	1.3777	TALDO1
189	6005938	Utrophin, dystrophin-related protein	396472.1	5.21	22	60	98.809	0.07498	−1.7499	Dystrophin-like protein, DMDL, DRP1
354	6137677	Mitochondrial aldehyde dehydrogenase	54394.4	5.7	7	111	100	0.2883	−1.1245	
121	6470150	BiP protein	71001.6	5.23	15	90	99.999	0.06848	−1.4204	HSPA5; heat shock 70 kDa protein 5 (glucose-regulated protein, 78kDa)
576	6688197	PAP-inositol-1,4-phosphatase	33743.3	5.46	9	243	100	0.1324	1.1991	3′(2′), 5′-bisphosphate nucleotidase 1; BPNT1
892	6806898	Synuclein, alpha	11365	7.88	3	67	99.727	0.9546	1.0552	SNCA
99	6912526	Nasopharyngeal epithelium-specific protein 1	46224	9.99	9	64	99.526	0.1088	1.6362	NESG1
441	7670399	Unnamed protein product	43689.4	6.1	10	149	100	0.09942	1.6662	MEK1
637	7677074	Lamda crystallin	33793.2	5.68	6	171	100	0.7142	−1.0275	CRYL1
755	8393948	Phosphoglycerate mutase 2	28907.9	8.85	2	70	99.857	0.1175	1.3015	Pgam2; Pgmut; PGAM-M; D14Mgh1
402	9966913	Actin-related protein 3-beta	40185.1	5	6	126	100	0.5354	1.0727	ARP11
638	10092677	Hypothetical protein	32077.4	6.12	6	81	99.99	0.06068	1.5844	
724	10092677	Hypothetical protein dJ37E16.5	32077.4	6.12	8	261	100	0.897	1.1513	Pyridoxal phosphate phosphatase, PLP
725	10092677	Hypothetical protein dJ37E16.5	32077.4	6.12	6	81	99.99		1.7072	Pyridoxal phosphate phosphatase, PLP
556	10241724	Hypothetical protein	31816.9	5.84	7	14	100	0.09672	1.3355	Isocitrate/isopropylmalate dehydrogenase
116	10433666	Unnamed protein product	88418.5	6.68	9	56	96.868	0.3911	1.2484	Ring finger protein 20, RNF20
337	10434221	Unnamed protein product	63177.7	8.73	9	57	97.144	0.1442	−1.4787	Hypothetical protein FLJ10498, FLJ10498

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439	11374664	Isocitrate dehydrogenase (NADP) (EC 1.1.1.42), cytosolic	46596.5	6.19	6	67	99.757	0.7066	−1.1504	
242	12804225	CCT5, chaperonin-containing TCP1, subunit 5 (epsilon)	59886.9	5.45	11	159	100	0.1577	−1.611	
680	12860410	Unnamed protein product	15612.5	10.08	5	51	88.887	0.6782	1.1754	AU RNA binding protein/ enoyl-coenzyme A hydratase
516	13279173	Similar to COP9	46524.8	5.5	15	346	100	0.1907	1.1899	COP9 constitutive photomorphogenic homolog subunit 4, COPS4
728	13435960	Similar to hypothetical protein FLJ23571	41024.6	9.4	10	60	98.664	0.3842	−1.1525	Hypothetical protein DKFZp434B227
534	13435960	Similar to hypothetical protein FLJ23571	41024.6	9.4	7	50	87.531	0.6963	−1.0929	Hypothetical protein DKFZp434B227
317	13623415	Fascin 1	55151.3	6.84	18	311	100	0.1424	−1.3277	FSCN1
149	13676857	HSP70 protein 2	69977.9	5.56	20	467	100	0.885	1.0331	HSPA2
332	13938355	Unknown	55708.4	5.4	15	345	100	0.07185	−1.6655	ATPase, H+ transporting, lysosomal 56/58 kDa, V1 subunit B, isoform 2, ATP6V1B2
359	15099973	Thrombospondin immunoglobulin heavy chain variable region	12778.3	8.67	4	54	94.557	0.3817	1.2396	
888	15680064	Similar to stathmin 1/ oncoprotein 18	17325.9	5.76	4	58	98.069	0.3664	1.0782	STMN1
880	15824412	Neuronal protein 22	22629.2	6.84	6	143	100	0.3978	1.2247	NP22, NP25
669	15930083	Calbindin 2	31663.6	5.06	7	83	99.993		1.1627	Calretinin, calbindin 29kDa
760	16198390	Unknown (protein for MGC:27286)	33535.7	5.4	6	238	100	0.1244	1.2993	CGI-150 protein
659	16198390	Unknown (protein for MGC:27286)	33535.7	5.4	4	78	99.98	0.1916	1.1935	CGI-150 protein
581	16307182	Similar to transaldolase 1	35534.5	9.07	9	124	100	0.8523	−1.0085	TALDO1
473	16924319	Unknown (protein for IMAGE:3538275)	40819.4	5.78	16	644	100	0.913	−1.0861	Actin
643	17389815	Triosephosphate isomerase 1	26909.8	6.45	4	74	99.944	0.09687	1.7767	TPI
348	18202063	Endothelial-monocyte-activating polypeptide II (EMAP-II)	39975.2	9.37	7	60	98.809	0.1527	−1.3207	Small inducible cytokine subfamily E, memeber 1
950	18202063	Endothelial-monocyte-activating polypeptide II (EMAP-II)	39975.2	9.37	5	53	93.893	0.499	1.1811	Small inducible cytokine subfamily E, memeber 1
117	18256043	Glycyl-tRNA synthetase	81798.7	6.24	8	98	100	0.1313	−1.7071	Gars
911	18307562	Unnamed protein product	69825.2	9.55	7	62	99.249	0.1397	7.2612	
295	18307562	Unnamed protein product	69825.2	9.55	5	49	85.009	0.3296	−1.1903	
224	18307562	Unnamed protein product	69825.2	9.55	8	58	97.678	0.4037	−1.1735	
301	19705447	CDC-ike kinase 3	59262.5	9.53	8	61	99.01	0.3224	−1.1692	Clk3

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896	19716076	Myeloid cell nuclear-differentiation factor	46244.3	9.72	8	64	99.492	0.1551	1.41	
325	19913428	ATPase, H+ transporting, lysosomal 56/58 kD, V1 subunit B, isoform 2	56807	5.57	11	216	100	0.1551	−1.3285	ATP6V1B2
443	19923206	Glutamate–ammonia ligase	42664.5	6.43	9	173	100	0.1807	−1.4274	GLUL
71	20072188	Aconitase 2	86252.3	7.62	21	472	100	0.1309	−1.8516	
934	20385874	Beta-tropomyosin	17808.9	4.6	6	63	99.315	0.4222	1.5841	
541	20563689	Mannose phosphate isomerase isoform	29908.3	5.99	4	80	99.988	0.8909	1.135	MPI
585	20862467	Hypothetical protein XP_164064	14450.5	9.57	5	56	96.566	0.5297	1.0918	
400	20864657	Similar to Retrovirus-related POL polyprotein	21374.9	9.35	6	65	99.547	0.6174	−1.1668	Similar to Cas-Br-M ectropic retroviral-transforming sequence b
712	20865698	Similar to protein phosphatase 1, regulatory (inhibitor) subunit 12A	22293.5	9.19	6	51	90.541	0.07508	1.1249	PPP1R12A
186	20868874	Hypothetical protein XP_160082	25606.8	6.41	6	54	94.43	0.09973	−1.5525	
800	20887601	Hypothetical protein XP_157898	21094.5	8.58	6	58	98.069	0.1626	1.3765	
981	20892463	RIKEN cDNA 1300010H20	13156.8	9.79	4	54	95.036	0.9819	1.0705	Similar to NADH:ubiquinone oxidoreductase B15 subunit
455	20892491	Similar to creatine kinase, brain	19243.9	7.82	4	59	98.279	0.9706	1.0978	
55	20901108	Hypothetical protein XP_157013	13206.1	4.7	4	53	93.893	0.4558	−1.169	
616	20978314	GTP-ase ran	24606.6	6.6	2	52	90.757	0.1235	−1.5667	RAN, member RAS oncogene family
795	20978314	GTP-ase ran	24606.6	6.6	2	52	90.757	0.9104	1.1077	RAN, member RAS oncogene family
48	20984919	Similar to interferon-inducible protein 10 (IP-10) receptor	89422.8	5.14	24	429	100	0.09089	−1.9122	
311	21313234	RIKEN cDNA 1300006M19	57494.2	8.87	7	58	98.024	0.05004	−1.6899	
570	22041696	Similar to ribosomal protein L7a, cytosolic	13035.2	10.46	5	55	95.677	0.1715	−1.1965	
658	22748619	Tropomyosin 3	28262.3	4.72	7	58	97.833	0.2513	−1.1211	TPM3, alpha-tropomyosin 3
615	22748619	Tropomyosin 3	28262.3	4.72	8	69	99.839	0.6958	−1.0386	TPM3, alpha-tropomyosin 3
361	23208520	DNA polymerase kappa	11938	9.04	5	56	97.009	0.5065	−1.0928	
284	23308577	PHGDH, phosphoglycerate dehydrogenase	57355.7	6.29	5	124	100	0.06459	−1.6773	3-Phosphoglycerate dehydrogenase
796	23395758	TPA: aflatoxin B1-aldehyde reductase	40019.9	6.7	7	63	99.403	0.7007	−1.0226	Aldo–keto reductase family 7, member A2 (aflatoxin aldehyde reductase), AKR7A2
255	24987750	Protein phosphatase 3, catalytic subunit, alpha isoform	43482.6	5.9	7	78	99.98	0.07135	−1.3094	Calcineurin A alpha, PPP3CA, PP2BCA

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252	24987750	Protein phosphatase 3, catalytic subunit, alpha isoform	42696	5.26	6	61	98.836	0.1063	−1.4861	Calcineurin A alpha, PPP3CA, PP2BCA
399	24987750	Protein phosphatase 3, catalytic subunit, alpha isoform	3380.3	5.26	8	152	100	0.1853	−1.1264	Calcineurin A alpha, PPP3CA, PP2BCA
723	24987750	Protein phosphatase 3, catalytic subunit, alpha isoform	43380.3	5.26	5	89	99.998	0.8823	1.3475	Calcineurin A alpha, PPP3CA, PP2BCA
106	25020592	Hypothetical protein XP_206488	11818.2	10.44	4	49	84.66	0.7306	1.6247	
375	25777739	Aldehyde dehydrogenase 9A1	54679.3	5.69	13	299	100	0.07027	−1.6495	ALDH9A1
376	25777739	Aldehyde dehydrogenase 9A1	54679.3	5.69	13	299	100	0.453	−1.1089	ALDH9A1
378	26330804	Unnamed protein product	14753.8	10.76	4	52	91.949	0.1878	1.2013	RIKEN cDNA 5730406M06 gene, 5730406M06Rik
687	26336324	Unnamed protein product	47225	8.58	6	56	96.32		3.1461	RIKEN cDNA 1500032A09 gene, 1500032A09Rik
409	27480797	Similar to hypothetical protein DKFZp434D0917.1	26958.3	8.97	4	54	94.43	0.949	1.0125	
453	27503783	Similar to mitochondrial translational release factor 1	52843	8.75	5	54	94.168	0.4426	1.4356	RF1; MTTRF1; MGC47721
964	27574235	Chain B deoxyhemoglobin	16090.3	6.75	3	108	100	0.2269	1.6911	
949	27574235	Chain B deoxyhemoglobin	16090.3	6.75	3	108	100	0.2754	1.3964	
642	27658930	Similar to ATP-dependent chromatin remodeling protein SNF2H	42705.1	9.1	6	55	95.775	0.2464	−1.1293	
789	27658930	Similar to ATP-dependent chromatin remodeling protein SNF2H	42705.1	9.15	6	51	90.095	0.265	−1.1694	
624	27677648	Similar to 60S ribosomal protein L7	17571.1	9.43	5	50	87.241	0.5553	−1.0843	
278	27707686	Similar to ribosomal protein L19	14078	9.87	4	52	91.949	0.2165	−1.3871	
296	27714549	Similar to ribosomal protein L24	12066.3	11.2	4	50	86.943	0.1397	−1.5458	
351	27717139	Similar to 60S ribosomal protein L29	13101.2	10.94	4	52	90.757	0.8939	−1.0125	
411	27960434	Colon cancer autoantigen protein	83725.6	6.11	10	61	98.986	0.5941	1.153	Serologically defined colon cancer antigen 8; Sdccag8
702	28376635	Rab37	24268.2	5.97	7	64	99.417	0.9899	1.0827	
335	28422545	UDP glucose pyrophosphorylase 2	57075.8	8.16	11	101	100	0.1491	−1.3007	UGPP2, UDPG
265	28552838	Hypotheical protein XP_289117	12857.5	9.25	4	57	97.569	0.06322	−1.6146	