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Global Approaches to the Analysis of Cocaine-Induced Gene Expression

Jodi E. Eipper-Mains

University of Connecticut Health Center, jeippermains@student.uchc.edu

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Global Approaches to the Analysis of Cocaine-Induced Gene Expression

Jodi Elene Eipper-Mains, MD, PhD

University of Connecticut, 2013

(Dissertation Completed: June 2011)

ABSTRACT

For over a decade, DNA microarrays have dominated genome-wide gene expression studies, revealing widespread effects of drug exposure on neuronal gene expression. More targeted single gene approaches have added invaluable insights into their behavioral, biochemical, and molecular effects. The more powerful next-generation sequencing technology may soon supplant microarrays for the analysis of complex transcriptomes. RNA-Seq is unparalleled in its ability to identify and quantify low-abundance transcripts without prior sequence knowledge, facilitating detection and investigation of known and novel alternative splicing events, imprinting, and RNA editing, among others. We first used RNA-Seq technology to catalog microRNA (miRNA) expression in total tissue and at postsynaptic densities (PSDs) from control and cocaine-treated mice. Neurons modulate gene expression with subcellular precision through excitation-coupled local protein synthesis, a process that may be regulated in part through the involvement of miRNAs. We identified cocaine-responsive miRNAs, synaptically-enriched and depleted miRNA families, and confirmed cocaine-induced changes in protein expression for several predicted target genes. The *miR-8* family was found to be highly enriched and cocaine-regulated at the PSD, where its members

may modulate expression of cell adhesion molecules. We then used next-generation sequencing of mRNAs purified from the same brain regions to gain a more complete picture of the molecular response to chronic cocaine exposure and withdrawal. Bioinformatic analysis revealed paradigm-specific regulation of multiple components of a limited number of signaling pathways. We identified signaling pathways that utilize distinctly different sets of receptors and signaling molecules under control conditions, after chronic exposure to cocaine, and during withdrawal. The Rho-GEFs expressed in the nucleus accumbens and localized to the postsynaptic density were reviewed because they are strategically positioned to effect rapid alterations in dendritic spine structure. Structural changes mediated by synaptic Rho guanine nucleotide exchange factors (GEFs) control actin cytoskeletal rearrangements and contribute to the long-lasting effects of cocaine. This review is then followed by a brief look at several ongoing and completed collaborative bioinformatics projects. Future studies will employ integrative approaches that combine mRNA, miRNA, and protein expression profiling with focused single gene studies and innovative behavioral paradigms and will facilitate development of integrated approaches to treat addiction.

Global Approaches to the Analysis of Cocaine-Induced Gene Expression

Jodi Elene Eipper-Mains, MD, PhD

ScB, Brown University, 2004

A Dissertation

Submitted in Partial Fulfillment of the

Requirements for the Degree of

Doctor of Philosophy

at the

University of Connecticut

2013

(Dissertation Completed: June 2011)

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Jodi Elene Eipper-Mains

2013

APPROVAL PAGE

Doctor of Philosophy Dissertation

Global Approaches to the Analysis of Cocaine-Induced Gene Expression

Presented by

Jodi Elene Eipper-Mains, ScB

Major Advisor

Brenton R. Graveley

Associate Advisor

Gordon G. Carmichael

Associate Advisor

Marc E. Lalande

Associate Advisor

Blanka Rogina

University of Connecticut

2013

(Dissertation Completed: June 2011)

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CHAPTER 1

Global approaches in the analysis of drug-induced gene expression

System-wide analysis of gene expression

Microarray versus RNA-Seq: The advantages of sequencing

DNA microarrays have dominated genome-wide studies of gene expression for more than a decade. However, the use of next-generation sequencing methods for the analysis of complex transcriptomes may soon supersede the use of microarrays¹. Microarrays have been instrumental in the interrogation and profiling of DNA-protein interactions, identification of single-nucleotide polymorphisms, and comparative analyses of mRNA expression². Any approach that attempts to deliver a global snapshot of gene expression has limitations, however microarrays are limited in a number of ways that next-generation sequencing is not. For example, microarrays rely on base complementarity between the probe and the mRNA and therefore researchers must consider artifacts of cross-hybridization when dealing with highly homologous genes or isoforms³. Microarrays contain a pre-defined set of oligonucleotide probes designed using existing gene annotations for organisms with known genome sequences¹, whereas sequencing facilitates the identification and absolute quantification of low-abundance transcripts without prior knowledge of the sequence³⁻⁶. The sequencing approach, unlike microarray, has low background signal because of the unambiguous nature of sequence mapping and has increased sensitivity for low-abundance transcripts^{1,6}. Additionally, sequencing enables the detection and investigation of alternative splicing (both known and novel)^{4,5,7}, imprinting and allele specific expression⁸, sequence

variation⁶, and RNA editing⁹. And finally, microarrays provide far less data than current next-generation sequencing technologies, which can produce gigabases (10^9 nucleotides) of sequence from a single experiment, enabling detection of very minor transcripts¹.

Explosion of the sequencing field

In 1975, Sanger and Coulson published their breakthrough paper on rapid determination of sequences in bacteriophage ϕ X174 DNA using synthetic oligonucleotide primers and *Escherichia coli* DNA polymerase I¹⁰. This was followed in 1977 by two landmark Sanger *et al.* publications detailing the use of radioactively labeled, chain-terminating 2',3'-dideoxynucleotide phosphates (ddNTPs) for rapid DNA sequencing with DNA polymerase¹¹, revolutionizing modern molecular biology and characterizing the full genome of ϕ X174¹². Also in 1977, Maxam and Gilbert presented an innovative method of sequencing by partial cleavage of terminally labeled DNA molecules at each of the four bases followed by gel electrophoresis¹³.

Since its introduction in the mid-1970s, rapid sequencing research has exploded, as evidenced by the exponential increase in publications in the past few years (**Fig. 1.1**). The first truly high-throughput sequencing device was introduced by Lynx Therapeutics in the 1990s. Massively parallel signature sequencing (MPSS) employed microbead arrays for the analysis of mRNA transcript levels. This approach involved *in vitro* cloning of cDNA templates on a monolayer of slide-fixed microscopic beads followed by ligation of tagged adaptor sequences, and produced 'signature' sequences ranging from 16 to 20 nucleotides in length^{14,15}.

In 2005, two new methods of next-generation sequencing were introduced: (1) pyrosequencing, or 'sequencing-by-synthesis', which detects pyrophosphate release on nucleotide incorporation, was developed by 454 Life Sciences¹⁶, and (2) multiplex 'sequencing-by-ligation' of mate-paired polymerase colonies ('colonies')¹⁷. Also in 2005, Lynx

Figure 1.1 PubMed references for high-throughput sequencing by year.

The number of publications in Entrez PubMed (<http://www.ncbi.nlm.nih.gov/pubmed>) by indicated year for the search term “RNA-seq” or “next-generation sequencing” or “massively parallel sequencing” or “high-throughput sequencing” or “deep sequencing”. Hashed bar depicts the 2011 predicted total. Symbols: ‡, publication total obtained May 11, 2011; \$, projected total for December 31, 2011.

Therapeutics merged with another sequencing company, Solexa. In 2006, Solexa introduced the Genome Analyzer, which, using sequencing by synthesis with fluorescent reversible terminator technology, provided up to 1 gigabase (Gb) of sequence in a single sequencing run. In 2007, Illumina acquired Solexa, and as of 2011, Illumina sequencers generate up to 600 Gb of sequence per run¹⁸. An additional technology, SOLiD (Sequencing by Oligonucleotide Ligation and Detection) from Applied Biosystems, gained renown in 2008 with the sequencing of the stem cell transcriptome⁴. Next-next-generation (3rd generation) sequencing, in which single molecules are sequenced without prior amplification, are considered the future of the sequencing field¹⁹.

Limitations in current RNA-Seq methods

High-throughput sequencing is an extremely powerful tool for biological analysis, but it would be shortsighted not to touch on some of its limitations. The density of sequence reads varies along the length of a transcript, indicating the existence of sequence bias at some point during the library preparation or sequencing process (**Fig. 1.2**)^{20,21}. There are data showing an underrepresentation of AT-rich and GC-rich regions in Illumina RNA-Seq data, which is likely attributable to sequence bias during the reverse transcription and amplification steps of library preparation^{3,22,23}. Strand-specific sequencing, in which the sequencing adapters are ligated to the RNA fragment prior to amplification, may show a more even distribution of aligning reads along the length of the gene; this method, however, requires additional manipulation of highly labile RNA prior to its conversion to more stable cDNA¹⁹. Other difficulties of traditional RNA-Seq include issues of quantifying very low abundance transcripts, problems with profiling repetitive regions of the genome, and trouble in quantifying small RNAs and RNA with a short half-life¹⁹. Next-next-generation sequencing provides an alternative that avoids many of the weaknesses of currently used methods.

Figure 1.2 Exon coverage profile with RNA-Seq using different sequencing platforms.

Expression profile of poly(A)⁺ mRNAs from NAc tissue lysates and striatal PSDs. Data from NAc samples were obtained from 37-cycles of sequencing on the Genome Analyzer IIx. Data from PSD samples were generated using the HiSeq 2000 platform and sequencing was carried out for 100-cycles. Numbers to the left of each track indicate maximal read number for that sample.

Challenges accompany the rapidly improving technology and accumulation of datasets containing vast amounts of information, however. Many biologists struggle to make scientific sense of the data at hand, while many computer scientists are not equipped with the biology training to tackle the relevant questions on their own. A growing number of tools are available to assist in the analysis of high-throughput datasets, including packages for sequence alignment²⁴, *de novo* genome and transcriptome assembly²⁵, analysis of alternative splicing²⁶, functional category assessment, and identification of signaling pathway enrichment²⁷⁻³⁰. The field is in its infancy, and, at present, few 'user friendly' and fully customizable packages exist for the comprehensive analysis of sequencing data. It will be exciting to follow new and improving developments as they become available.

Applying high-throughput techniques to the analysis of gene expression in the brain

mRNA studies

In one of the first global analyses of mRNA expression in the mammalian brain, Eberwine *et al.* amplified transcripts from single dissociated rat hippocampal neurons³¹. Through this analysis they found differing expression profiles in morphologically indistinguishable neurons, highlighting the extraordinary complexity that dictates neuronal gene regulation³¹.

In 2003, Ule and colleagues presented a novel method to analyze direct interactions between the RNA binding protein and splicing cofactor, Nova, and its target mRNAs³². The technique, cross-linking and immunoprecipitation (CLIP), involves direct ultraviolet irradiation of brain tissue, causing the formation of covalent bonds between directly juxtaposed proteins and RNAs. They then used coimmunoprecipitation (co-IP) of Nova, followed by partial RNA digestion and proteinase K digestion to obtain RNA sequences of 60-100 nt. They sequenced

340 Nova CLIP tags, identifying many novel Nova alternative splicing targets³².

Expanding on the role of Nova proteins in the control of neuronal alternative splicing, Ule *et al.* used a specially-designed Affymetrix splicing array to assess alternative splicing in the brains of two knockout mice, Nova1^{-/-} and Nova 2^{-/-}, identifying nearly 50 differentially spliced alternative exons³³. Shortly thereafter, Ule *et al.* used bioinformatic techniques to characterize an RNA map of Nova-dependent alternative splicing in the brain, identifying splicing enhancer and silencer motifs in and around alternatively spliced exons³⁴.

With the advent of next-generation sequencing techniques, the same group developed HITS-CLIP, high-throughput sequencing of RNA isolated by crosslinking immunoprecipitation³⁵. This technique used the previously described³² CLIP method paired with RNA-Seq of isolated mRNAs. They performed a genome-wide characterization of Nova-RNA interactions, identifying an added role for Nova in the regulation of polyadenylation in the mammalian brain³⁵. These examples, and others not included, underscore the utility and power of genome-wide approaches in the study of gene expression in the mammalian brain.

microRNA studies

microRNAs (miRNAs) are a class of endogenous 21-25 nucleotide small RNAs that modulate gene expression through binding complementary sequences in the 3'-untranslated regions (3'-UTRs) of target mRNAs³⁶. Hundreds of miRNAs are expressed in the mature mammalian brain³⁷⁻⁴⁰, where they are involved in the control of synapse development and neuronal plasticity⁴¹. In the biogenesis of mature miRNAs, long primary miRNA (pri-miRNA) transcripts are successively cleaved into shorter preliminary miRNA (pre-miRNA) hairpins, exported from the nucleus, and cleaved to make ~22 nt duplex RNAs prior to loading into RNA-induced silencing complexes (RISCs)³⁶. Each fully assembled RISC contains an Argonaute protein (AGO1-4 in human and mouse), and a single mature miRNA⁴². The RISC interacts with

target mRNAs via complementary base pairing interactions specified by the miRNA sequence, and either represses translation or induces mRNA degradation. AGO2 is the only catalytically active AGO family member in humans and mice; it has endonuclease ('slicer') activity and the ability to cleave target mRNAs³⁶.

Neurons can modulate gene expression with subcellular precision; polyribosomes and mRNAs are found at the base of dendritic spines and are involved in localized protein synthesis^{43,44}. Two recent publications present evidence that individual synapses may act independently in the local control of excitation-coupled protein translation^{41,45}. A subset of miRNAs copurify with polyribosomes isolated from E18 rat cortices, suggesting a role for miRNAs in fine-tuning localized translation of mRNAs in dendrites⁴⁶. Localization of the miRNA processing enzyme DICER and the critical RISC component AGO2 in mouse cortical and hippocampal slices by electron microscopy lent credence to this hypothesis⁴⁷. Further studies identified the RISC component and RNA helicase Armitage (also known as MOV10) at the synapse and established a role for RNA-induced silencing in the control of synaptic gene expression, learning, and memory^{48,49}. The demonstration that *miR-134* was a synapto-dendritically localized miRNA with a role in activity-dependent regulation of hippocampal dendritic spine size through its effects on the kinase LIMK1⁵⁰ was followed by studies demonstrating roles for *miR-132* and *miR-138* in dendritic remodeling and synaptogenesis^{51,52}.

Applying the HITS-CLIP technique³⁵ of protein crosslinking and high-throughput sequencing, Chi *et al.* simultaneously generated two distinct RNA-Seq databases by immunoprecipitating AGO2 from mouse brain⁵³. One database provided a genome-wide map of AGO2-mRNA binding sites, and the other detailed the association of AGO2 with specific miRNAs. Through bioinformatic means, they were able to characterize a combined global AGO2-miRNA-mRNA map, providing further evidence for the importance of miRNA-mediated control of gene expression in the brain⁵³.

miRNAs are implicated in the pathophysiology of a variety of neuropsychiatric disorders and mental retardation syndromes, including Alzheimer's disease, Parkinson's disease⁵⁴, Huntington's disease⁵⁵, schizophrenia^{56,57}, bipolar disorder⁵⁸, Fragile X mental retardation⁵⁹, and Rett syndrome^{60,61}. Studies suggest that miRNAs play an important role in the local control of dendritic morphology through fine-tuning translation of synaptically-localized mRNAs⁴¹. miRNAs likely participate in long-lasting forms of synaptic plasticity through the modulation of regulatory pathways that involve controlled dendritic mRNA trafficking, excitation-coupled modulation of synaptic mRNA translation, alterations to the actin cytoskeleton, neurotransmitter metabolism and peptide hormone processing⁶².

Proteomic studies

Characterization of the complete complement of proteins expressed in the brain has been ongoing for years. Current proteomic approaches lack the same level of sensitivity seen in current high-throughput sequencing techniques, limited by our inability to amplify proteins as we amplify nucleic acids⁶³.

Expression neuroproteomic studies focus on quantitative and qualitative profiling of the neural proteome using gel electrophoresis, 2-dimensional difference in gel electrophoresis (2D-DIGE), and liquid chromatography-tandem mass spectrometry (LC-MS/MS)⁶⁴. Functional neuroproteomic studies tackle the characterization of regulatory networks, signaling pathways, subcellular localization, and complex formation⁶⁴. The Human Brain Proteome Project is an organization that oversees large-scale integration of brain proteome studies⁶⁵.

Over 1,000 proteins have been identified in the mammalian postsynaptic proteome⁶⁶. The postsynaptic proteins are organized into signaling complexes and networks, including the NMDA receptor complex⁶⁷, dozens of actin cytoskeletal and scaffolding proteins⁶⁸, and approximately 60 kinases⁶⁴.

One aspect of proteomics addresses post-translational modifications to proteins, including phosphorylation, glycosylation, nitrosylation, carbonylation, palmitoylation, etc. Phosphoproteomic studies have identified more than 1,500 phosphorylation sites in isolated postsynaptic densities (PSDs) from different brain regions⁶⁴. Studies have revealed widespread activity-dependent phosphorylation of specific target proteins in the PSD in response to neurotransmitter stimulation⁶⁹. For example, a recent study of the postsynaptically-localized Rho GDP/ GTP exchange factor (GEF) Kalirin-7 found 39 different phosphorylation sites using LC-MS/MS⁷⁰.

Cocaine abuse

History of cocaine

In 1977, the National Institute on Drug Abuse (NIDA) released its first report on recreational cocaine use in the United States⁷¹. The report detailed the present state of cocaine-related research, including the history of cocaine use and abuse, the chemical structure and metabolism of the drug, behavioral studies, and a survey of the human impact of cocaine use. The coca plant, *Erythroxylon coca*, is indigenous to Western South America, where for millennia people have chewed the coca leaf for its stimulant and perceived medicinal qualities. Cocaine extract was first purified in 1880, and by the turn of the 20th Century it was widely used in a variety of commercially available products, such as soft drinks, wines, and many over-the-counter medicines. The Harrison Narcotics Act of 1914 ushered in a new era of Federal oversight, restricting the availability of cocaine and coca extracts, and imposing significant fines for non-medical use⁷¹.

By the late 1960s, researchers had developed many of the animal models of substance abuse still in use today, including the paradigm of self-administration. The molecular and biochemical underpinnings of substance abuse and addiction were nearly entirely unknown,

and the impact on the general population of the United States was quite minor, accounting for just 111 documented deaths from 1971 to 1976⁷¹. In the subsequent 35 years, our knowledge of cocaine and its biological activity has increased immensely, as has the burden on the general population of cocaine-related morbidity and mortality.

Societal impact of substance abuse and addiction

Repeated cocaine use may lead to addiction and a number of other serious and adverse health consequences, and cocaine accounts for nearly one third of all drug-related emergency room visits⁷². In 2007, there were 1.6 million cocaine users in the United States who met the Diagnostic and Statistical Manual of Mental Disorders criteria for dependence or abuse of cocaine in the past year^{73,74}. Approximately 90% of addicted individuals eventually resume drug taking, many after prolonged periods of abstinence⁷⁵. Our understanding of the molecular underpinnings of cocaine abuse and addiction is far from complete, and there are currently no FDA-approved medications to treat cocaine addiction⁷⁶. Studies such as those detailed herein aim to elucidate the genetic changes underlying the response of the brain to cocaine and may ultimately help identify new targets for treating cocaine addiction.

Drug abuse research primarily aims to uncover the molecular and cell biological mechanisms causing addiction in humans. Addiction is currently a clinical diagnosis, defined as the compulsive need for and use of a habit-forming substance despite its harmful consequences⁷³. Until recently, evidence in support of an appropriate animal model of addiction was limited. In 2004, Piazza and colleagues put forth convincing evidence in support of addiction-like behavior in the rat⁷⁷. The authors described rats trained to self-administer cocaine that, when cocaine was paired with an aversive foot shock, persisted seeking drug⁷⁷; this behavior is present in a minority of rats and human drug addicts and is a strong predictor of relapse potential after withdrawal. The endogenous reward system of the brain, on which

drugs of abuse act, is similar in many different species. For decades, the use of a variety of animal models, to study the behavioral and neurochemical response to drug exposure, has shed light on the neuroadaptations occurring inside the brains of human drug abusers.

The reward circuitry of the brain

Drugs of abuse induce persistent biochemical and morphological changes in a number of regions throughout the brain via distinct acute mechanisms of action. Abundant evidence, from both animals and humans, identifies the limbic system as the convergence point for all drugs of abuse⁷⁸⁻⁸³. The limbic regions of the brain control emotions, motivations, and behavior. The mesocorticolimbic dopamine pathway, which involves a subset of all limbic structures, is activated by normal physiologic rewards such as food, drink, social interaction, and sex⁸⁴⁻⁸⁶. The mesocorticolimbic system includes dopaminergic projections from the ventral tegmental area (VTA) to the nucleus accumbens (NAc) and prefrontal cortex (PFC) as well as glutamatergic projections from the PFC to the NAc (**Fig. 1.3**)⁸⁷. Although drugs of abuse originate from a diverse array of chemical classes and have a variety of different primary targets, their effects all ultimately converge on the mesocorticolimbic dopamine neurons of the VTA⁸⁸. Other neurotransmitters, including γ -aminobutyric acid (GABA), opioid peptides, serotonin, acetylcholine, and endogenous cannabinoids, are also involved in the signaling of addiction⁸⁹⁻⁹¹. The convergence of these myriad signaling systems has led many to hypothesize that the NAc is the key integration point in the rewarding effects seen with drugs of abuse^{83,92-95}.

The neuronal circuitry of drug craving is complex and incompletely understood; however, data from animal studies and functional imaging of the brains of human addicts have uncovered a number of important details (**Fig. 1.3**). A variety of drug-related cues may spur a reformed addict to resume drug-seeking behavior; these cues include stress, exposure

Figure 1.3 The reward circuitry of the mesocorticolimbic dopamine system.

The VTA is comprised of both GABAergic and dopaminergic neurons that project to the NAc, dorsal striatum, and PFC. The neurons of the NAc receive glutamatergic input from the PFC, amygdala, and hippocampus and send GABAergic efferents to the VP, back to the VTA, and to motor regions. Glutamatergic afferents carry motivational signals to the NAc. The NAc signals function to direct the execution of goal-oriented behavior, including sending signals to motor control regions (not shown). The mixed cholinergic, glutamatergic, and GABAergic efferents from the LDT and PPT modulate signaling in the VTA. Coloring of structures reflects neurotransmitter type, with multi-colored structures (eg. VTA) using more than one neurotransmitter to signal.

Abbreviations: LDT/PPT, laterodorsal tegmental nucleus/pedunculopontine tegmental nucleus; NAc, nucleus accumbens; PFC, prefrontal cortex; VP, ventral pallidum; VTA, ventral tegmental area.

* Figure adapted from Kauer and Malenka, *Nat. Rev. Neurosci.*, (2007)⁸⁷ and Wolf, M.E. in *Basic Neurochemistry: Molecular, Cellular, and Medical Aspects*, (2006)⁸⁸.

to a small dose of drug, and/or exposure to drug paraphernalia or the drug-related environment^{88,90}. These cues activate glutamatergic neurons from the PFC that synapse on the medium spiny neurons of the NAc (shown in blue in **Fig. 1.3**). Aspects of the normal stress response in the brain, mediated by the circuitry of the hypothalamic-pituitary-axis, exhibit profoundly disrupted regulation in the brains of chronic drug users⁸⁸. The PFC controls the cognitive and executive function aspects of drug-taking behavior while the other brain regions involved in the mesocorticolimbic circuitry are thought to control various components of learning, memory, and motivational significance that modulate drug-seeking behavior⁹⁰. It is likely a combination of cognitive dysfunction with increased motivation to take a drug, enhanced conditioned emotional responses, and decreased inhibitory control/judgment that dictate compulsive drug-seeking behavior⁸⁸.

Chronic cocaine exposure elicits morphological changes in multiple areas of the mesolimbic dopaminergic pathway, including the NAc⁸⁸. For example, increased dendritic spine size and density are observed in the MSNs of mice following both experimenter-administered^{68,96-98} and self-administered cocaine⁹⁹. The biology underlying the behavioral and motivational responses experienced by drug abusers are generally described by two phenomena: sensitization and synaptic plasticity. Sensitization is the progressive and persistent amplification of behavioral and motivational responses to a fixed dose of drug^{93,95}. It persists for weeks, months, and years after cessation of drug taking and is thought to play an important role in the risk of a reformed addict for relapse to drug taking behavior¹⁰⁰⁻¹⁰². Synaptic plasticity is the phenomenon whereby the strength of the connection between two neurons changes strength. This change could be due to alterations in presynaptic neurotransmitter release, postsynaptic ion channel composition or conductivity, the morphology of the synapse, and/or second messenger signaling, all of which may be controlled by changes in gene transcription⁸⁶. Synaptic plasticity is strongly implicated in all

types of learning, and current research suggests that drugs of abuse hijack normal synaptic plasticity mechanisms in the mesolimbic system with higher potency than natural rewards, leading to the development of sensitization⁸⁴; addicts effectively “learn” to like the drug.

Overview of signaling pathways

Medium spiny neurons (MSNs) are the inhibitory GABAergic neurons that comprise 90-95% of the neurons in the nucleus accumbens⁸⁷. Dendritic spines of medium spiny neurons receive dopaminergic inputs on their spine necks and glutamatergic inputs on their spine heads, positioning them well to integrate the complex signaling of the mesolimbic system^{95,103}. When cocaine is present at the synapse, it blocks presynaptic dopamine re-uptake by the dopamine transporter (DAT), enhancing postsynaptic dopamine signaling by prolonging the time dopamine is present in the synapse and can bind postsynaptically. Dopamine signaling is central to the mechanism by which rewarding stimuli, both natural and synthetic, modulate behaviors and promote learning of rewarding behaviors⁸⁸. Drugs of abuse activate dopamine neurons of the VTA through direct or indirect mechanisms, eliciting increased dopamine release and causing widespread changes – in gene expression, neurotransmitter release, signal transduction, neuronal excitability, and morphology – in dopamine-targeted neurons^{83,88}.

A variety of drugs of abuse induce a sensitized state, akin to the increased synaptic efficacy of long-term potentiation (LTP), in the dopamine neurons of the VTA; dopamine transmission in response to the drug or drug-related cues is greatly increased over the drug-naïve baseline⁸³. Increased responsiveness of ionotropic glutamate receptors of the α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) subtype govern this sensitization and may reflect altered AMPA receptor trafficking or subunit composition⁸⁷. Furthermore, altered glutamatergic signaling has been implicated in behavioral responses to drugs of abuse⁸³.

Decreased GABA transmission in the VTA, mediated by exposure to opiates, ethanol, and cannabinoids, disinhibits efferent dopamine neurons, increasing dopamine release and signaling in the NAc, PFC, and other target areas (**Figs. 1.3**)⁸⁸. Additionally, opiate drugs activate signaling in the NAc independent of their effects on VTA dopamine transmission through the binding of opiate peptides to presynaptic κ -opioid receptors and postsynaptic μ -opioid receptors in the NAc^{88,90}.

Chronic exposure to cocaine markedly increases expression of μ - and κ -opioid receptors in the NAc. Activation of μ -type receptors on dopamine neurons of the VTA stimulates synaptic dopamine release while presynaptic κ -type activation in the NAc is homeostatic, diminishing dopamine release^{104,105}. In the NAc, μ -opioid receptor expression is primarily postsynaptic and κ -opioid receptors are both pre- and postsynaptic, although the postsynaptic subset predominates¹⁰⁶. Cocaine exposure induces striatal expression of the opiate peptide dynorphin (and its precursors), which primarily binds the κ -opioid receptor, and treatment with κ -opioid agonists significantly reduces the behavioral effects of cocaine¹⁰⁷.

Drugs of abuse directly and indirectly activate of $G\alpha_s$ and $G\alpha_{i/o}$ G-protein coupled receptors (GPCRs). $G\alpha_s$ GPCRs, such as the D1-like dopamine receptors, produce the second messenger cyclic adenosine monophosphate (cAMP) through activation of adenylyl cyclase. The resultant increase in intracellular cAMP levels increases the activity of the Protein Kinase A (PKA) family of Ser/Thr kinases. Activated PKA translocates to the nucleus where it phosphorylates the transcription factor CREB (cAMP response element binding), inducing transcription of a variety of target genes. Alternatively, $G\alpha_{i/o}$ GPCRs, such as the D2-like dopamine receptors, inhibit adenylyl cyclase, decreasing the amount of cAMP and decreasing downstream PKA signaling events¹⁰⁸. Both D1 and D2 receptors act through additional signaling mechanisms to exert their full effects⁸⁸.

Importantly, dopamine signaling in mesolimbic MSNs performs a neuromodulatory role. The D1 receptor-PKA pathway influences neuronal excitability through PKA-mediated phosphorylation of voltage- and ligand-gated ion channels. Examples of regulated channels include glutamate receptors of both the AMPA and NMDA type, Na⁺ channels, and Ca²⁺ channels⁸⁸. Increased intracellular Ca²⁺ levels mainly activate calcium/calmodulin-dependent protein kinases (CaM-kinases), ultimately turning on CREB and affecting gene expression¹⁰⁸. Repeated stimulation produces persistent changes in gene expression that cause changes in pre- and postsynaptic neuronal morphology and alter the underlying neuronal circuitry⁸⁸. Drugs of abuse exert their influence on the reward and motivation pathways in the brain by hijacking the normal signaling of activity-dependent plasticity and LTP.

Global analyses of cocaine-mediated gene expression

mRNA expression in postmortem human overdose victims and rodent models of addiction

The number of studies examining the effects of cocaine on one or a small number of genes dwarf the number of genome-wide expression studies. A number of comprehensive analyses, primarily using microarray, of cocaine-induced gene expression have been published over the years, in both animal models and human postmortem brain. These studies highlight the importance of components of the extracellular matrix, including cadherins, integrins, and matrix metalloproteinases¹⁰⁹⁻¹¹². In addition to the extracellular changes that occur in synapse formation, a significant body of work has focused on the role of actin cytoskeletal rearrangements, mediated in large part by the Rho-family of proteins, in the neural plasticity of drug abuse^{68,98,109,113-116}. A variety of signaling molecules and receptors have also been implicated in the molecular responses of the brain to cocaine. Examples include the synaptic vesicle associated proteins α -synuclein and synaptotagmin, clathrin, several dopamine

receptors and the dopamine transporter, a number of ionotropic and metabotropic glutamate receptors, opiate receptors and opiate peptides, other neuropeptides, second messenger molecules such as the mitogen-activated protein kinases (MAPKs), calmodulin proteins, Ca^{2+} /calmodulin-dependent protein kinases, other kinases, phosphatases, and a number of different transcription factors^{30,117-122}. Despite decades of dedicated work, a complete picture of the gene expression changes that accompany cocaine exposure and withdrawal remains elusive.

microRNAs and their emerging role in cocaine research

Several recent studies have investigated the role of AGO2 and miRNAs in biochemical and behavioral responses to cocaine exposure¹²³⁻¹²⁷. For example, qPCR and *in situ* analysis of specific bioinformatically-determined miRNAs identified *let-7d*, *miR-124*, and *miR-181a* as cocaine-regulated in rats^{123,127}. Two studies by the Kenny group identified induction of *miR-132* and *miR-212* in dorsal striatum after 7 days of cocaine self-administration in rats and implicate *miR-212* in the behavioral and motivational response to cocaine through CREB, MeCP2, and brain derived neurotrophic factor (BDNF) signaling^{124,125}. In addition, Schaefer *et al.* identified an overlapping subset of cocaine-induced and AGO2-knockdown-depleted miRNAs in D2 dopamine receptor expressing neurons of the NAc, showing reduced cocaine self-administration when AGO2 is depleted from D2 receptor expressing neurons¹²⁶.

Together, these studies provide evidence that miRNA-mediated gene regulation plays an important role in the cocaine response. However, all of these studies were targeted to a small number of candidate miRNAs or used microarrays, which suffer from issues that plague microarray studies; these include cross-hybridization, high background signal, low dynamic range, and the inability to identify unknown miRNAs³, and thus these studies may not provide a complete picture of cocaine-induced changes in miRNA levels.

Proteomics and drug abuse research

Very few proteomic studies have looked at quantitative changes in postsynaptic proteins following exposure to drugs of abuse¹²⁸. In one of the only drug-response proteomic studies to date, Devi and colleagues examined changes in the hippocampal postsynaptic proteome of mice after morphine injection, identifying just over 100 differentially regulated proteins¹²⁹. They uncovered changes to a variety of proteins involved in mRNA and protein trafficking, intracellular signaling, rearrangement of the actin cytoskeleton, and other aspects of synaptic plasticity. They found significant upregulation of clathrin in the postsynaptic compartment after morphine, suggesting a role for the endocytic pathway in the response to drugs of abuse¹²⁹.

Another recent study in rhesus monkeys used a combined proteomic approach to identify cocaine-regulated changes in cytosolic proteins from the NAc¹³⁰. They uncovered cocaine-altered expression of proteins with known roles in the control of cell morphology, signal transduction, and synaptic plasticity, as well as components of the AMPA and NMDA receptor signaling complexes. They also looked for differentially regulated phosphorylation after cocaine, finding 15 proteins with increased phosphorylation following cocaine, including structural, signaling, and metabolic proteins¹³⁰.

The future of drug abuse research

Knockout and transgenic mouse models have been invaluable for investigations into the behavioral, biochemical, and molecular effects mediated by single genes. It is becoming increasingly clear, however, that the changes that occur as a result of drug abuse are incredibly widespread, affecting multiple brain regions and cell-types in a system-wide manner. These global studies emphasize the broad effects of drug exposure on neuronal gene expression, underscoring the need for an integrative approach that combines mRNA, miRNA,

and protein expression profiling in combination with focused single gene studies and innovative behavioral paradigms in the quest to uncover what drives addiction and develop integrated therapeutic approaches.

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CHAPTER 2

Cocaine-regulated expression of striatal microRNAs

The following is a duplicate version of a published manuscript:

Eipper-Mains, J.E.* , Kiraly, D.D., Palakodeti D., Mains, R.E., Eipper, B.A., Graveley B.R. *RNA* **17**, 1529-1543 (2011).

Abstract

MicroRNAs (miRNAs) are small RNAs that modulate gene expression by binding target mRNAs. The hundreds of miRNAs expressed in the brain are critical for synaptic development and plasticity. Drugs of abuse cause lasting changes in the limbic regions of the brain that process reward, and addiction is viewed as a form of aberrant neuroplasticity. Using next-generation sequencing, we cataloged miRNA expression in the nucleus accumbens and at striatal synapses in control and chronically cocaine-treated mice. We identified cocaine-responsive miRNAs, synaptically-enriched and depleted miRNA families, and confirmed cocaine-induced changes in protein expression for several predicted synaptic target genes. The *miR-8* family, known for its roles in cancer, is highly enriched and cocaine-regulated at striatal synapses, where its members may affect expression of cell adhesion molecules. Synaptically-enriched cocaine-regulated miRNAs may contribute to long-lasting drug-induced plasticity through fine-tuning regulatory pathways that modulate the actin cytoskeleton, neurotransmitter metabolism, and peptide hormone processing.

Introduction

Hundreds of microRNAs (miRNAs), a class of endogenous 21-25 nucleotide small RNAs that modulate gene expression by binding complementary sequences in the 3'-untranslated regions (3'-UTRs) of their target mRNA transcripts³⁶, are expressed in the mature mammalian

brain³⁷⁻⁴⁰ and are thought to be involved in synapse development and plasticity⁴¹. The biogenesis of mature miRNAs is characterized by successive RNA cleavage events in which long primary miRNA (pri-miRNA) transcripts are processed into shorter preliminary miRNA (pre-miRNA) hairpins, exported from the nucleus, and cleaved into ~22 nt duplex RNAs prior to being loaded into RNA-induced silencing complexes (RISCs)³⁶. Each RISC contains an Argonaute protein (AGO1-4 in human and mouse), and a miRNA⁴². The RISC then interacts with the target mRNAs through base pairing interactions specified by the miRNA, and either represses translation or induces mRNA degradation. AGO2, the only catalytically active AGO family member in humans and mice, has endonuclease ('slicer') activity and the ability to cleave target mRNAs³⁶.

Exposure to drugs of abuse such as cocaine triggers persistent cellular and molecular changes in the limbic regions of the brain that process reward. The limbic system, in particular the striatum, is considered the epicenter of the long-term molecular and morphological changes that underlie addiction⁷⁹. The ventral striatum (nucleus accumbens) is considered the primary striatal subregion with limbic functions. Neurons can modulate gene expression with subcellular precision; polyribosomes and mRNAs are found at the base of dendritic spines and are involved in localized protein synthesis^{43,44}. Both AGO2 and DICER, an RNase III enzyme required for miRNA biogenesis, have been localized to dendritic spines⁴⁷. Emerging evidence supports an autonomous role for individual synapses in the local control of protein translation coupled to neuronal excitation^{41,45}.

Data from broad expression studies using microarray technology and from focused single-gene approaches indicate that cocaine exposure elicits widespread changes in the transcriptional landscape⁸⁷. Several recent studies have begun to investigate the role of AGO2 and miRNAs in the biochemical, molecular, and behavioral response to cocaine¹²³⁻¹²⁶. For example, qPCR and *in situ* analysis of specific bioinformatically-determined miRNAs identified

let-7d, *miR-124*, and *miR-181a* as cocaine-regulated in rats¹²³. Two studies by the Kenny group identified induction of *miR-132* and *miR-212* in dorsal striatum after 7 days of cocaine self-administration in rats and implicate *miR-212* in the behavioral and motivational response to cocaine through CREB, MeCP2, and BDNF signaling^{124,125}. In addition, Schaefer *et al.* identified an overlapping subset of cocaine-induced and AGO2-knockdown-depleted miRNAs in D2 dopamine receptor (*Drd2*) expressing neurons of the NAc, and showed a reduction in cocaine self-administration when AGO2 is depleted from *Drd2* neurons¹²⁶. Consistent with these findings, we observed cocaine-induced increases in *Ago2* mRNA levels upon performing RNA-Seq (JEE-M *et al.* in preparation). Together, these studies provide evidence that miRNA-mediated gene regulation plays an important role in cocaine-related changes in neurotransmission and behavior. However, all of these studies were targeted to a small number of candidate miRNAs or used microarrays, which suffer from issues of cross-hybridization, higher background signal, lower dynamic range, and lack the ability to identify unknown miRNAs³, and therefore do not provide a complete picture of cocaine-induced changes in miRNA levels.

Here, we have more closely examined the role of AGO2 and miRNAs in response to chronic cocaine exposure. First, we confirmed synaptic *Ago2* mRNA and protein expression in the striatum and increased expression upon chronic cocaine exposure. We also determined the repertoire of miRNAs expressed in total nucleus accumbens (NAc) tissue lysates and purified striatal postsynaptic densities (PSDs) harvested from saline- and cocaine-treated mice. This led to the identification of a subset of cocaine-regulated miRNAs in whole tissue and at the synapse, and the characterization of miRNAs by degrees of synaptic enrichment and depletion. We also identified mRNAs that are potentially targeted by the differentially expressed miRNAs, and validated cocaine-induced changes in protein levels for several of the identified targets. These data provide an overview of the global regulation and synaptic

enrichment of miRNAs in response to cocaine.

Results

Cocaine regulates striatal AGO2

To assess whether AGO2 protein was present at striatal synapses, we subjected adult mouse striatum to subcellular fractionation (**Fig. 2.1A**). Strong enrichment of NR2B, an NMDA receptor subunit, in the PSD fraction, and depletion of Synaptophysin, a presynaptic protein, verified the success of the preparation¹³¹⁻¹³³. The ER luminal protein BiP showed strong enrichment in the ER and Golgi fraction. β III-tubulin was most abundant in the cytoplasmic fractions. Likewise, the striatally-enriched cytoplasmic protein DARPP32¹³⁴ was highly enriched in soluble fractions and was absent from synaptic fractions. As expected, AGO2 was enriched in the ER/Golgi fraction and present in the cytoplasmic fractions. Consistent with prior studies demonstrating synaptic localization of AGO2 in hippocampus and frontal cortex^{47,135}, our data indicate that AGO2 was present in striatal PSDs (**Fig. 2.1A**).

We next used quantitative-PCR to examine whether chronic cocaine administration affected *Ago2* mRNA expression in the ventral striatum and found a significant increase in the level of *Ago2* mRNA following cocaine exposure (**Fig. 2.1B**). We then determined the impact of chronic cocaine on AGO2 protein levels at the PSD. Striata (dorsal and ventral) and medial prefrontal cortices (mPFCs) from 12 cocaine-treated and 12 saline-treated wild-type male C57BL/6 mice were pooled and subjected to subcellular fractionation. Levels of AGO2 and β III-tubulin in PSDs purified from striata and mPFCs were assessed by quantitative Western blotting. We observed a 24% increase in synaptic AGO2 protein after cocaine treatment in the striatum, but not the mPFC (**Fig. 2.1C,D**). Together these results indicate that AGO2 is localized to the striatal PSD and increases after cocaine treatment. Because up-regulation of AGO2 was specific to the striatum, we chose to focus our subsequent analyses on this brain region.

Figure 2.1 Subcellular fractionation

(A) Wildtype mouse striata were subjected to subcellular fractionation. Aliquots containing equal amounts of protein (10 μ g; 5 μ g, PSD) were fractionated by SDS-PAGE, transferred, and analyzed by Western blot. NR2B, AGO2, and Synaptophysin were visualized from one gel; BiP, β III-tubulin, and DARPP32 were visualized from an identical gel. PSD, postsynaptic density. (B) Total NAC RNA from adult male mice treated with saline or cocaine (4 per group) was analyzed by qPCR; *Ago2* mRNA expression was normalized to *GAPDH*. (C,D) PSDs purified from the striata (C) and PFCs (D) of saline (S)- and cocaine (C)-treated mice were fractionated by SDS-PAGE. AGO2 levels were normalized to β III-tubulin by densitometry for the varying amounts of protein loaded; plot shows average C/S ratio for AGO2. Significance was tested by the 2-tailed Student's *t*-test with unequal (B) or equal (C,D) variance. **P* < 0.05; ***P* < 0.01.

The nucleus accumbens and purified striatal PSDs share abundant microRNAs

Given the synaptic localization and cocaine responsiveness of AGO2 protein, we hypothesized a role for AGO2 and miRNAs in regulating mRNA stability or translation at the striatal PSD in response to cocaine. To explore this idea, we used next-generation sequencing to analyze the global miRNA expression profiles in both NAc total lysates and purified striatal PSDs. For the NAc lysates, we sequenced 2.53 and 4.46 million reads from the saline and cocaine samples, respectively (**Table 2.S1**). We also generated 3.67 and 4.60 million reads from the striatal PSD saline and cocaine samples, respectively (**Table 2.S1**). After removing the 3' linker sequence, reads ranging from 18-30 nt were aligned to the miRBase database (version 16)^{136,137} using Bowtie (version 0.12.7)²⁴ to identify and assess the abundance of known miRNAs.

We generated pairwise scatter plots to compare expression of each mature miRNA (as a percentage of total reads) across all four samples (**Fig. 2.2A**). The two NAc total lysate samples correlated more closely with each other ($R^2 = 0.97$) than with either striatal PSD sample ($R^2 = 0.90-0.93$). The converse was also true, with striatal PSD miRNAs exhibiting a higher correlation to one another ($R^2 = 0.97$) than to the NAc samples. For both the total tissue lysates and the PSDs, a handful of individual miRNAs appeared to be cocaine-regulated; cocaine treatment did not cause widespread alterations in the global miRNA profile.

While the total tissue lysate and purified PSD expression profiles differed, the most highly expressed miRNAs were largely identical; 11 of the 15 most abundantly sequenced miRNAs in both groups were the same (**Fig. 2.2B,C**). Of note, a number of miRNAs known to be brain-specific or neurally-enriched, such as *miR-9*, *miR-30*, *miR-127*, *miR-138*, *miR-181*, and *let-7*³⁷⁻⁴⁰, were very abundant in these samples.

We detected one or more reads mapping to a total of 500 (74%) of the 672 mouse pre-

Figure 2.2 High-throughput sequence analysis of NAc and PSD miRNA

(A) Pairwise scatter plots of miRNA expression data for NAc lysate and striatal PSD libraries (Saline and Cocaine) prepared from adult mouse; shown as $\log_{10}$ (normalized miRNA frequency) per sample. R^2 values were calculated by least squares best fit. Expression data for the 15 most abundant miRNAs in NAc lysates (B) and striatal PSDs (C) are shown as reads (adjusted for equal total reads per sample). The miRNAs in black italics are highly expressed in both NAc and PSD; miRNAs in red italics are highly expressed only in that sample.

Classification of miRNAs detected by RNA-Seq

	miRBase v16	All data	NAc lysates	Striatal PSDs
pre-miRNAs	672	500 (280)	475 (242)	476 (264)
mature miRNAs	1055	771 (342)	718 (283)	732 (312)
miRNA families	253	222 (159)	214 (137)	216 (147)
miRNAs in families	502	410 (266)	392 (230)	394 (253)

Table 2.1 Classification of miRNAs detected by RNA-Seq

Number of pre-miRNAs, mature miRNAs, miRNA families, and miRNAs in families detected by RNA-Seq at one or more reads and 100 or more reads (in parentheses).

miRNAs annotated in miRBase, of which 475 and 476 miRNAs were detected in the NAc and striatal PSD libraries, respectively (**Table 2.1**). We set a lower limit of 100 reads per miRNA for further analysis since we found that reliable qPCR validation of changes necessitated this level of expression. Although this read requirement decreased the diversity of miRNAs considered for further analysis, the miRNAs meeting the expression cut-off account for 99.7% and 99.8% of all reads in the NAc and striatal PSD libraries, respectively. A summary of the individual miRNAs and miRNA families identified in these experiments is presented in **Table 2.1**.

Cocaine modulates expression of ventral striatum microRNAs

There were 283 mature miRNAs detected with at least 100 reads in both NAc lysate libraries (**Table 2.1**); 10% of these miRNAs exhibited at least a 1.5-fold change in expression, with 16 miRNAs up-regulated and 13 down-regulated after cocaine treatment. The NAc total lysate miRNAs that exhibit the largest changes in expression following cocaine are shown in **Fig. 2.3A**. The regulated miRNAs were generally of low- to moderate-abundance, detected at a level between 100 and 1000 reads. One notable exception, *miR-29b*, was among the 15 most abundantly expressed miRNAs in the NAc (**Fig. 2.2B**).

We used qPCR to validate cocaine-induced changes in several of the most highly regulated miRNAs and to validate RNA-Seq data for miRNAs known to be brain-enriched or synaptically-relevant. Commercially available probes were used for qPCR; these probes measure only one mature miRNA product, which did not always correspond to the most abundantly sequenced product due to 3'-end heterogeneity (**Fig. 2.4**). Nevertheless, strong agreement across the two methods ($r = 0.68$) was observed when the cocaine/saline ratios obtained by qPCR and RNA-Seq were plotted (**Fig. 2.3B**). Importantly, inter-platform agreement was consistent over a broad range of overall expression levels.

A) NAc total lysates, RNA-Seq cocaine-regulated miRNAs

B) qPCR vs. RNA-Seq

Figure 2.3 Cocaine-regulated miRNAs in NAc total lysates

(A) NAc lysate libraries (Saline and Cocaine) analyzed by high-throughput sequencing of miRNAs. The eight most cocaine down- and up-regulated miRNAs detected at >100 reads are shown as adjusted reads (equal total reads per sample) on $\log_{10}$ scale. The number above the bars indicates fold change. (B) Pairwise scatter plot comparing $\log_{10}$ ratio of Coc/Sal expression for selected miRNAs in NAc tissue lysate as computed from RNA-Seq data (vertical axis) and qPCR data (horizontal axis). Size of circle is proportional to average RNA-Seq expression value. Pearson's Coefficient represents the linear correlation coefficient, r .

Figure 2.4 3'-end heterogeneity and varying RNA-Seq read abundance

Expression data across all samples are shown as reads (adjusted for equal total reads per sample) for sequences mapping to *miR-132* and *miR-143*. Arrowhead (►) marks annotated mature miRNA sequence from miRBase version 16. Commercially available qPCR primers amplify sequence marked by arrowhead.

Cocaine regulates microRNAs in striatal PSDs

A specific subset of mRNAs is transported along dendrites and local protein synthesis occurs in stimulated dendritic spines, altering the components of the synapse^{43,44}. Since AGO2 protein is localized to the PSD and its levels at the PSD increased following chronic cocaine exposure, we determined whether any PSD localized miRNAs were responsive to cocaine. There were 312 mature miRNAs detected at 100 reads or more in both striatal PSD libraries (**Table 2.1**); 11% of these miRNAs exhibited at least a 1.67-fold change in expression, with 4-times as many miRNAs down-regulated (28) as up-regulated (7) after cocaine treatment. **Fig. 2.5A** shows those striatal PSD miRNAs with at least 100 reads that demonstrated the largest change in expression following cocaine. Similar to what was observed for the NAc miRNAs, the cocaine-regulated PSD miRNAs were generally of low- to medium-abundance, detected with 100 to 10,000 reads.

We again turned to qPCR to confirm responses observed with RNA-Seq. A comparison of the ratios of cocaine/saline measured by RNA-Seq and qPCR showed a high degree of concordance between the two techniques ($r = 0.76$) (**Fig. 2.5B**). Cross-platform agreement spanned a very broad range of expression levels, as seen in NAc lysates.

Synaptically-enriched microRNA families

To determine the subset of miRNAs that are enriched or depleted at the synapse, we calculated the relative abundance of each miRNA in all four samples. We then computed the average abundance of each miRNA in the two NAc lysate and striatal PSD samples, and calculated the $\log_{10}$ ratio of the PSD average over the NAc average. The miRNAs with higher PSD-to-NAc lysate ratios are synaptically-enriched (**Fig. 2.6**), while those with lower ratios are synaptically-depleted (**Fig. 2.7**). Z-scores of relative expression were calculated for each sample and the data displayed as heat maps (**Fig. 2.6A,B**).

A) Striatal PSDs, cocaine-regulated miRNAs

B) qPCR vs. RNA-Seq

Figure 2.5 Cocaine-regulated miRNAs in striatal PSDs

(A) Striatal PSD libraries (Saline and Cocaine) analyzed by high-throughput sequencing of miRNAs. The eight most cocaine down- and up-regulated miRNAs detected at >100 reads are shown as adjusted reads as described in Fig. 3. (B) Pairwise scatter plot comparing log₁₀ ratio of Coc/Sal expression for selected miRNAs in striatal PSDs as computed from RNA-Seq data (vertical axis) and qPCR data (horizontal axis) as described in Fig. 2.3.

Figure 2.6 Identification of miRNA families enriched at the PSD

(A) Normalized miRNA frequency data for saline- and cocaine-treated NAc lysate and striatal PSD libraries of wildtype mice were calculated; heat map is sorted by decreasing average PSD frequency/NAc frequency ratio. Z-score was computed on normalized miRNA frequency across all samples. Blue indicates low expression and yellow indicates high expression. (B) Expansion of heat map showing top PSD-enriched miRNAs. Text color indicates miRNA families with 2 or more family members appearing in the list.

Of the 672 annotated murine miRNAs, 75% (502) can be grouped into 253 families based on sequence homology in the 'seed region' (nts 2-8 at the 5'-end of the miRNAs) and the observation that these miRNAs are often functionally redundant³⁶. We detected expression of at least one member of 222 (88%) of the 253 annotated miRNA families, with 159 families expressed at 100 reads or more (**Table 2.1**). Strikingly, several of the synaptically-enriched miRNAs we identified were members of the same family, as indicated by text color in **Fig. 2.6B**. All 5 members of the *miR-8* family (*miR-141*, *miR-200a*, *miR-200b*, *miR-200c*, and *miR-429*) were synaptically-enriched. The same was true for the *miR-7* and *miR-142* families. The mouse genome contains 12 loci encoding members of the *let-7* family, three of which were synaptically-enriched (**Fig. 2.6B**). In contrast, we did not observe family enrichment in the subset of PSD-depleted miRNAs (**Fig. 2.7**).

PSD-localized, cocaine-regulated microRNAs target a functionally-related subset of mRNAs

To provide insight into the role of the cocaine-regulated miRNAs, we performed a computational search for potential target mRNAs. First, we compiled a list of 1,041 dendritically- and synaptically-localized mRNAs reported in the literature¹³⁸⁻¹⁴³ (**Table 2.S4**). 424 of those mRNAs were PSD-enriched in rat forebrain by microarray¹⁴³, and this subset of synaptically-localized genes (**Table 2.S4**) was cross-referenced with the putative miRNA target genes identified by analysis using the miRanda microRNA target prediction algorithm^{144,145}. Briefly, each predicted miRNA binding site in the 3'-UTR of a given gene has a pre-computed mirSVR score; this value directly correlates with the extent of mRNA down-regulation predicted by miRNA binding, and the composite score for each gene in **Fig. 2.8** represents the sum of all cataloged mirSVR scores across the queried miRNAs¹⁴⁵.

We performed target prediction on the 16 PSD-localized cocaine-regulated miRNAs

Figure 2.7 miRNAs depleted at the PSD

(A) High-throughput sequencing data for saline- and cocaine-treated NAC lysates and striatal PSDs of wildtype mice; normalized miRNA frequency was calculated and heat map is sorted by decreasing average PSD frequency/NAC frequency ratio (as in Fig. 2.6). Z-score was computed on normalized miRNA frequency across all samples. Blue indicates low expression and yellow indicates high expression. (B) Expansion of heat map showing top PSD-depleted miRNAs. Text color indicates miRNA families with more than two miRNAs appearing in the list.

Figure 2.8 Target prediction for cocaine-regulated miRNAs

The 16 most cocaine-regulated striatal PSD miRNAs from Fig. 4.6 were subjected to bioinformatic target prediction using miRanda. The list of potential target mRNAs was cross-referenced with the 424 PSD-enriched mRNAs identified by Suzuki *et al.* 2007 (**Table S4**) and sorted by ascending mirSVR score. + symbols show presence of predicted miRNA binding site in 3'-UTR of target gene; number of + signs denotes number of predicted miRNA binding sites. The 36 target genes shown were run through gene ontology (GO) analysis using DAVID; colored boxes indicate enriched GO functions with $P < 0.05$.

depicted in **Fig. 2.5A**. A subset of the list of synaptically-localized predicted target genes for the cocaine-regulated striatal-PSD miRNAs sorted by mirSVR score is shown in **Fig. 2.8**. A number of the identified target genes were predicted to have multiple binding sites for a given miRNA in their 3'-UTR (depicted by the number of + symbols) and the majority of the predicted target genes had putative binding sites for both up- and down-regulated miRNAs.

We carried out gene ontological analysis using DAVID^{28,146,147} to investigate the functional properties of the identified target genes. The colored boxes next to the genes in **Fig. 2.8** show the enriched gene ontological categories ($P < 0.05$). Most of the enriched categories (cell junction, regulation of synaptic transmission, calcium ion binding, dendrite/neuron projection, cell-cell signaling, cytoskeleton, phosphate metabolic process, transmembrane, growth factor binding) are processes generally linked to synaptic plasticity, learning, and memory.

Identified target genes exhibit differential expression at striatal synapses after cocaine

Schematic drawings for *Ntrk2*, *E-cadherin*, *Mtdh* and *Pcsk2*, potential target genes of cocaine-regulated synaptic miRNAs (**Fig. 2.9**), depict the sites within their annotated 3'-UTRs that are predicted to bind up- and down-regulated miRNAs. Since these synaptically-enriched transcripts may interact with multiple regulated miRNAs, the direction of cocaine-mediated changes in synaptic protein levels are difficult to predict.

We used quantitative Western blotting to investigate the effect of cocaine administration on the protein level of several potential target genes of cocaine-regulated synaptic miRNAs (**Fig. 2.9**). One identified target gene is *Ntrk2* (also known as *TrkB*), a member of the neurotrophin receptor tyrosine kinase family, which binds brain-derived neurotrophic factor (BDNF) and is critically involved in the process of activity-dependent synaptic plasticity

and long-term potentiation (LTP)¹⁴⁸. The *Ntrk2* 3'-UTR is targeted by four *miR-8* family members (*miR-200a*, *miR-200b*, *miR-200c*, and *miR-429*), which are down-regulated by cocaine, and by *miR-33* and *miR-130b*, which are up-regulated by cocaine (**Fig. 2.8**). The level of synaptic TRK protein increased 18% in the striatum after chronic cocaine administration (**Fig. 2.9A**).

Similarly, synaptic expression of Metadherin (MTDH), encoded by *Mtdh*, the top synaptically-regulated miRNA target gene (**Fig. 2.8**), increased to 142% of the saline level after chronic cocaine treatment (**Fig. 2.9B**). *Mtdh* is targeted by *miR-183* and four *miR-8* family members, which are down-regulated by cocaine, and by *miR-16*, *miR-32*, *miR-33*, *miR-130b*, and *miR-153*, which are up-regulated by cocaine (**Fig. 2.8**). While not a direct target of the identified cocaine-regulated synaptically-localized miRNAs, E-cadherin (also known as *Cdh1* or *ECAD*) expression is controlled by transcriptional repressors (ZEB1 and ZEB2) which are directly targeted by members of the *miR-8* family of miRNAs¹⁴⁹, and by several other miRNAs which are down-regulated by cocaine and by a number of miRNAs which are up-regulated by cocaine (**Fig. 2.9B**). Two forms of ECAD were detected at the synapse by Western blot (**Fig. 2.9B**). The 105 kDa major band is mature ECAD (black arrow) and the higher molecular weight band represents proECAD^{150,151}. The level of mature ECAD decreased to 68% of the saline level following chronic cocaine.

The prohormone convertase PC2 (also known as *Pcsk2*) was also identified in our bioinformatic analysis as a potential target of the cocaine-regulated miRNAs. *Pcsk2* is targeted by three *miR-8* family members and *miR-370*, which are down-regulated by cocaine, and by *miR-32* and *miR-218*, which are up-regulated by cocaine (**Fig. 2.8**). Several forms of PC2 were detected at striatal PSDs by Western blot (**Fig. 2.9C**). The smaller molecular weight band represents mature PC2, the middle band represents proPC2 and the upper band is the size of preproPC2¹⁵², indicative of dendritic synthesis and processing. When normalized to β III-

Figure 2.9 Validation of target predictions

(A,B) PSDs purified from the striata of saline (S)- and cocaine (C)-treated mice were fractionated by SDS-PAGE; three different amounts of protein were analyzed to establish response linearity. Levels for pan-TRK (a), ECAD (◀), and MTDH (b), and PC2 (◀), proPC2 (◀), and preproPC2 (◀) (c) were normalized to βIII-tubulin by densitometry for the varying amounts of protein loaded; plot shows average C/S ratio. Based on published reports, the higher molecular weight band (~120 kDa) above the major 105 kDa ECAD band corresponds to the precursor, proECAD (◀)^{150,151}. For PC2, the smaller molecular weight band represents mature PC2 (◀), the middle band represents proPC2 (◀) and the upper band is the size of preproPC2 (◀)¹⁵². Schematic drawings represent mRNAs of indicated genes; open reading frame (ORF) is gray, 3'-UTR is striped, arrowheads show miRanda predicted miRNA binding sites for miRNAs up-regulated (▲) or down-regulated (▼) after cocaine at PSD. Significance was tested by the 2-tailed Student's *t*-test with equal variance. **P* < 0.05; ***P* < 0.01.

tubulin, mature PC2 and proPC2/preproPC2 increased significantly, 67% and 61%, respectively, after cocaine treatment.

Discussion

The role of miRNAs in response to drugs of abuse is largely unknown. Here, we used deep sequencing to identify miRNAs that are differentially regulated in response to cocaine exposure, both in purified NAc tissue and at striatal synapses. This catalog of synaptically enriched and cocaine-regulated miRNAs provides new insight into the molecular mechanisms of the cocaine-response and serves as a resource for future studies.

Intriguingly, many (18) of the synaptically-enriched miRNAs we identified are encoded in miRNA clusters – precursor transcripts that contain multiple miRNAs – or are members of the same miRNA families. Moreover, the expression patterns of the clustered miRNAs are similar to one another. Particularly strong examples include the two clusters encoding *miR-8* family members (*miR-429/200a/b* and *miR-141/200c*), the cluster encoding *let-7* family members (*let-7f/miR-98*), and clusters *miR-1/133a*, *miR-182/96/183*, and *miR-216a/217* (**Fig. 2.10**). This indicates that many of the miRNAs we identified are coordinately regulated by virtue of being encoded in clusters and potentially control the same set of mRNA targets as they share the same seed sequences.

It is well established that the growth and remodeling that occur in dendritic spines following synaptic activity require *de novo* protein synthesis, and that blocking protein synthesis attenuates formation of long-term memory⁴¹. The ability to modulate the synthesis of a subset of synaptic proteins affords neurons the capacity to integrate and fine-tune extracellular cues with subcellular resolution, in individual dendrites or at specific synapses¹⁵³. MicroRNAs represent ideal candidates for regulating local translation of specific mRNAs at the synapse. It is therefore intriguing that we observed both increased levels of synaptically

Figure 2.10 Analysis of miRNA clusters and families

Heat map shows expression of striatal PSD-enriched miRNAs and accompanying members of their genomic cluster; "chr" denotes chromosomal location of each miRNA cluster. Normalized miRNA frequency was calculated from high-throughput sequencing data; Z-score was computed on normalized miRNA frequency across all samples. Blue indicates low expression and yellow indicates high expression. Text color identifies miRNA families with two or more miRNAs; bold text indicates presence in PSD-enrichment list from Fig. 5; gray italic text indicates miRNAs not enriched at striatal PSD and/or not belonging to listed miR families.

Figure 2.11 Comparison of data for striatum and cortex/hippocampus

(A) Pairwise scatter plot comparing miRNA synaptic enrichment ratios from Lugli *et al.*, 2008 (adult mouse cortex and hippocampus; measured by microarray) with RNA-Seq data (adult mouse striatum) from these experiments. Horizontal axis, data from Lugli *et al.*, 2008 computed as ratio of PSD (Syn)/total tissue (Tot) expression; vertical axis, data from RNA-Seq computed as PSD/NAc lysate ratio of average of saline and cocaine expression levels for adult mouse striatal PSD and NAc lysate. Blue circles were identified as cocaine-regulated and/or were assayed by qPCR, green circles indicate miRNAs enriched at striatal PSDs by RNA-Seq, red circles indicate miRNAs depleted at striatal PSDs by RNA-Seq, and gray circles represent all other miRNAs. Size of circle is proportional to average RNA-Seq expression value. (B) Synaptosomal enrichment and depletion categories for miRNAs from Siegel *et al.*, 2009 as determined by microarray analysis of rat forebrain samples. Heat map shows high-throughput sequencing data for saline- and cocaine-treated NAc lysates and striatal PSDs of wildtype mice; normalized miRNA frequency was calculated and Z-score was computed on normalized miRNA frequency across all samples. Blue indicates low expression and yellow indicates high expression.

localized AGO2 protein and altered levels of specific miRNAs upon cocaine exposure. These observations indicate that modulations in miRNA-mediated gene regulation play an important role in the response to cocaine. Programmed changes in miRNA levels may result in specific changes in the stability and/or the translation rate of target mRNAs at the synapse.

We identified several miRNAs that were synaptically enriched or depleted (**Fig. 2.6B, 2.7B**). A comparison of our data to earlier microarray experiments performed in adult (2-month old) mouse cortex and hippocampus¹³⁵ (**Fig. 2.11A**) revealed significant overlap. Notably, 4 of the 5 *miR-8* family members (*miR-200a/b/c/429*) are highly enriched in both datasets. Additionally, *miR-182* and *miR-183* are highly PSD-enriched in both datasets while *miR-126*, *miR-143*, *miR-145*, *miR-150*, and *miR-451* are synaptically depleted in both datasets (**Fig. 2.11A**). We also observed strong agreement between our data and a second microarray study that identified synaptosomally-enriched and depleted miRNAs from rat P15 total forebrain samples⁵². In both datasets, *miR-219-5p*, *miR-21*, *miR-377*, *miR-98*, *miR-376b*, *miR-218*, *miR-7a/b*, and *miR-29a* are synaptosomally- or PSD-enriched, while *miR-143*, *miR-145*, and *miR-150* are depleted (**Fig. 2.11B**). The high degree of concordance between these three datasets, which used different experimental platforms and strikingly different samples, may reflect the prevalence of glutamatergic synapses and other features common to these brain regions.

The increased levels of AGO2 and altered levels of specific miRNAs at the synapse in response to cocaine strongly suggest that these factors play a role in controlling translation of localized mRNAs. To explore this, we identified potential mRNA targets from a set of PSD-enriched mRNAs¹⁴³ (**Fig. 2.8**). Due to the fact that most mRNAs we identified are predicted to be targeted by many of the regulated miRNAs, it was difficult to predict whether translation of the targets would increase or decrease in response to cocaine. Nonetheless, we confirmed changes in protein levels for three predicted targets (TRKB, MTDH, and PC2) and one indirect target (ECAD) upon exposure to cocaine. One of the validated targets, TRKB (NTRK2), has also

been shown to increase in the NAc of rats after chronic cocaine self-administration¹⁵⁴, and TRKB-BDNF signaling in the NAc may modulate behavioral responses to cocaine¹⁵⁵.

Importantly, several other predicted targets have been implicated in various aspects of the cocaine response¹⁵⁶⁻¹⁵⁸.

Recent studies have demonstrated the importance of miRNAs in the pathophysiology of several neuropsychiatric disorders and mental retardation syndromes^{157,158}, including schizophrenia^{56-58,159}, bipolar disorder, Fragile X mental retardation¹⁶⁰⁻¹⁶², Rett syndrome⁶¹, Alzheimer's disease, Parkinson's disease¹⁶³, and Huntington's disease⁵⁵. A number of the cocaine-responsive miRNAs identified in this study are important in controlling dendritic morphology; these miRNAs, and many others, may contribute to long-lasting forms of drug-mediated synaptic plasticity through control of regulatory pathways that modulate changes to the actin cytoskeleton, neurotransmitter metabolism and peptide hormone processing. It is likely that the miRNAs we identified are but one aspect of an exquisitely complex regulatory system which includes dendritic mRNA trafficking and excitation-coupled modulation of translation and protein phosphorylation to fine-tune translation of synaptically-localized miRNAs. As the breadth of information about the molecular changes that occur in the brain in response to cocaine and other drugs of abuse increases, the importance of integrating these data in a meaningful and biologically informative way becomes increasingly clear.

Methods

Cocaine treatment of mice

Adult wild-type C57BL/6 mice (Jackson Laboratories, Bar Harbor, Maine; 2-5 months old) were used for these experiments. All mice were allowed to acclimate to the colony for at least one week before handling or injections. Mice were kept in the University of Connecticut Health Center animal facility on a 12 h light/dark cycle (lights on 7:00 am to 7:00 pm) and

handled in accordance with University of Connecticut Health Center Institutional Animal Care and Use Committee guidelines. To reduce stress from handling during the injections, animals in all groups were handled for 1 min/animal/day for 2-3 days prior to the start of injections. During injections, animals were brought into the behavior room to acclimate to the new environment for 45-60 minutes each day before the beginning of experiments.

For these studies, we used two slightly different cocaine injection paradigms, both of which have been shown to produce robust locomotor sensitization. For all experiments, animals were given a saline or cocaine injection and had their locomotor activity monitored for the subsequent 45 minutes. In the experiments in which RNA from NAc lysates was used for miRNA-Seq library preparation, animals were given injections of 20 mg/kg cocaine or saline (10 ml/kg) intraperitoneally for 7 days. Our group and others have demonstrated the efficacy of this method at eliciting robust locomotor sensitization^{98,164}. For all other experiments (both RNA and protein), animals received a lower dose of cocaine (10 mg/kg) on the first and last (7th) day, with the standard 20 mg/kg cocaine dose on days 2-6^{165,166}. The extent of locomotor sensitization was taken as the ratio of activity on day 7/day 1¹⁶⁶. We chose to switch to this method because it provided a clearer distinction between those animals that had sensitized and those that did not. Protein biochemistry from our laboratory has shown no biochemical differences between sensitized animals from the two paradigms (DDK, unpublished observations). Neither tissue nor data from these different treatment paradigms were pooled. For all experiments, animals were sacrificed 24 hours after the final injection.

Whole tissue lysate preparation and subcellular fractionation

Adult male mouse ventral striatum (nucleus accumbens, NAc) punches were harvested. For whole tissue protein studies, samples were sonicated and boiled in 1% SDS, 50mM TrisHCl [pH 7.4], 5mM EDTA, 50mM NaF, 2mM sodium orthovanadate, 1mM PMSF, and

protease inhibitor cocktail as described¹³³. Total RNA from tissue lysates for individual mice was prepared from freshly dissected tissue with TRIzol (Invitrogen) following the manufacturer's instructions, except that isopropanol precipitation was carried out overnight at -20°C with 2 ethanol washes instead of one.

For subcellular fractionation, whole striata (40 mg wet weight/mouse) or medial prefrontal cortices (mPFC) (30 mg/mouse) from 12 identically-treated male mice were pooled, homogenized and fractionated by differential centrifugation followed by sucrose gradient centrifugation. After extraction of the PSD-enriched fraction with 0.5% Triton X-100, PSDs were pelleted and subjected to Western blot analysis as described^{98,133}. The protocol is similar to that of Lugli *et al.*¹³⁵ and was modified to protect against RNA degradation as follows: all rotors, homogenizers, pestles, and ultracentrifuge tubes were rinsed with RNaseZAP (Applied Biosystems) and DEPC water and air dried prior to use; fractionation buffers were prepared using DEPC water and were treated with RNASecure (1:25 dilution from stock; Applied Biosystems) at 60°C for 10 min; aliquots of different subcellular fractions were diluted into TRIzol for preparation of total RNA as described above.

Small RNA cloning and sequencing

Small RNA sequencing libraries were prepared following the manufacturer's instructions for the Small RNA Sample Prep Kit (Illumina). Total RNA used for NAc tissue lysate library preparation was pooled in equal parts from RNA isolated from 8 male mice from each treatment group. One saline and one cocaine NAc lysate library were prepared from the pooled RNA and these are referred to as NAc lysate saline and cocaine libraries. Total RNA used for striatal postsynaptic density (PSD) library preparation was isolated as outlined above from the pooled striata of 11 mice (7 male, 4 female) from each treatment group; these libraries are referred to as striatal PSD saline and cocaine libraries. Small RNAs of 18-35 nt were gel-purified

from 1 µg of pooled total RNA. Eluted RNAs were ligated to 5' and then to 3' adaptors with RNA ligase, with gel purification following each ligation. RNAs were reverse transcribed and cDNAs were PCR-amplified for 16 cycles. PCR products were purified on a non-denaturing acrylamide gel and sequenced on the Illumina Genome Analyzer II_x for 50 (NAc lysate libraries) or 40 (striatal PSD libraries) cycles.

Small RNA genomic analysis

Sequences were extracted from image files using Firecrest and Bustard. The 3' adaptor sequences were trimmed from the reads, and reads 18-30 nt in length were aligned to all annotated mouse microRNAs from miRBase (www.mirbase.org; version 16) with Bowtie²⁴ (bowtie-bio.sourceforge.net; version 0.12.7); only perfectly aligning reads were considered for miRNA expression level analysis. A custom Perl script was used to calculate the number of reads aligning to each specific miRNA gene and data were normalized by the total number of reads obtained in that sequencing run. A cut-off of 100 reads or more for an individual miRNA was required for it to be included in further expression analyses.

To identify reads mapping to a variety of non-coding genes and repetitive elements, the remaining non-miRNA sequences were aligned to the Rfam database (rfam.sanger.ac.uk; version 10.0), the Repbase database (www.girinst.org/rebase/index.html; version 16.01), and NONCODE (noncode.org; version 2.0). We subsequently mapped the remaining reads to the mouse transcriptome, including exons, introns, and exon-exon junctions annotated on the UCSC genome browser (genome.ucsc.edu; mm9; NCBI Build 37) as the following tracks: UCSC Genes, RefSeq Genes, Vega Protein Coding Annotations, and Ensembl Gene Predictions. Finally, the remaining reads were aligned to the mouse genomic sequence (genome.ucsc.edu; mm9; NCBI Build 37). All of these alignments were performed using Bowtie and allowing 1-mismatch. Alignment data are shown in **Fig. 2.12**.

Figure 2.12 Identification of mapped sequences

After trimming 5' and 3' adaptors from extracted sequences for all four libraries, those between 18 and 30 nt in length were aligned to miRBase annotated mouse microRNAs using Bowtie²⁴ (version 0.12.7); perfect alignment was required. Remaining non-miRNA sequences were aligned to non-coding and repetitive elements annotated in the Rfam, Repbase, and NONCODE databases, and then to the mouse transcriptome (exons, introns, exon-exon junctions), and mouse genome. These non-miRNA alignments were performed permitting 1-mismatch. The percentage of the total reads mapped to each category is shown graphically in the pie-chart and numerically in tabular form.

Importantly, the trimmed reads were also aligned to miRBase allowing 1- or 2-mismatches. Neither the overall expression pattern nor the list of the most cocaine-responsive miRNAs changed significantly when allowing for more mismatches (JEE-M, unpublished observations). Read numbers presented in this paper were calculated from the 0-mismatch alignment.

Generation of heat maps

Normalized miRNA frequency across all 4 sequenced libraries was calculated; expression in one sample of 100 reads or more was required. Z-scores were computed from frequency data using the following equation: $(x - \mu)/\sigma$; x , sample frequency; μ , mean across all samples; σ , standard deviation across all samples. Blue indicates low expression and yellow indicates high expression; heat maps were generated using Java Treeview software (sourceforge.net/projects/jtreeview/files).

Target gene prediction

MicroRNA target genes were identified using miRanda^{144,145} (www.microrna.org; August 2010 release). Prediction scores were computed using the miRanda database of highly conserved target sites with good mirSVR scores¹⁴⁵. We chose this database because programs requiring target conservation across a broad range of species are more stringent than those with more relaxed definitions of conservation. For the miRanda-generated target gene list for PSD-localized cocaine-regulated miRNAs, we filtered the list to include only those target genes with supporting experimental data indicating dendritic or synaptic localization; data compiled from six studies¹³⁸⁻¹⁴³ yielded a list of 1,041 target genes (**Table 2.S2**).

Functional analysis of target genes

We used the Database for Annotation, Visualization, and Integrated Discovery (DAVID;

david.abcc.ncifcrf.gov) to interrogate multiple databases in parallel with a list of target genes²⁸.

We used the GoCharts module to analyze a subset of the target mRNAs identified from miRanda analysis. GoCharts uses the classifications of the Gene Ontology Consortium (GO; www.geneontology.org), which assigns functional terms to annotated genes and proteins. Only those GO terms identified by DAVID with enrichment $P < 0.05$ were included in **Fig. 2.8**.

Quantitative polymerase chain reaction (qPCR)

The cDNAs from tissue lysate total RNA were prepared using iScript (BioRad) with random primers; a 5 min 65°C step was added to the iScript protocol before chilling and adding the enzyme. Pairwise statistical comparisons used the t-test for two samples assuming unequal variance and the two-tailed value are reported. Real-time PCR for mRNAs was performed using an Eppendorf Realplex2 machine and Sybr-Green (BioRad), with the following parameters: 95°C 2 min; 95°C 15 sec, 55°C 15 sec, 68°C 40 sec; repeat 40x. Maximal rates of amplification per cycle were calculated for all primer pairs and all samples in all assays and averaged 2.03 ± 0.06 (SD) in 6 runs of 96-well plates. Data are calculated with respect to GAPDH for each sample within an assay, and data across assays were then averaged because the values with respect to GAPDH for each transcript were very consistent. Primer pairs were chosen to keep the melt temperature (T_m) between 60 and 62°C (calculated using www.basic.northwestern.edu/biotools/oligocalc.html) and products in the 120 ± 5 nt size range. Differences between saline and cocaine samples were evaluated using the 2-tailed Student's *t*-test with unequal variances.

Primers used for mRNA qPCR

Primer name	Sequence				T _m (°C)	Product length (nt)
<i>Gapdh</i> -for	5'	TTGTCAGCAATGCATCCTGCACCACC	3'		61	119
<i>Gapdh</i> -rev	5'	CTGAGTGGCAGTGATGGCATGGAC	3'		61	
<i>Ago2</i> -for	5'	GACTATCAGCCAGGAATCACGTTTCATCG	3'		61	121
<i>Ago2</i> -rev	5'	CCACGGTTGTGCCTGCGGGAATG	3'		62	

Table 2.2 Primers used for mRNA qPCR

MicroRNA qPCR

Reverse transcription using mature miRNA-specific primers was performed on 1-10 ng total RNA from tissue lysates per the manufacturer's instructions for the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) using the suggested parameters (ice 5 min; 16°C 30 min; 42°C 30 min; 85°C 5 min). Quantification was performed using the TaqMan MicroRNA Assay (Applied Biosystems) with the manufacturer's recommended amplification parameters (95°C 10 min; 95°C 15 sec, 60°C 60 sec; repeat 40x). NAc lysate qPCR was performed on RNA isolated from individual animals while striatal-PSD qPCR was carried out on the pooled preparations. Data are calculated with respect to an empirically determined endogenous control miRNA; total NAc lysate and striatal-PSD miRNA levels were calculated with respect to *miR-9* and *miR-101a*, respectively. Normalization data are shown in **Fig. 2.13**, and qPCR data for **Fig. 2.3B** and **2.5B** are in **Fig. 2.14**.

Figure 2.13 Use of *miR-101b* for normalization and assessment of brain-enriched and synaptically-important miRNAs

(A) Expression of *miR-101b* from NAc tissue lysates from wildtype adult male mice treated with saline and cocaine was evaluated by RNA-Seq (left) and by qPCR (right). RNA-Seq data for *miR-101b* are shown as reads (adjusted, equal total reads/sample) on log₁₀ scale; qPCR data for *miR-101b* are normalized to *miR-9* on linear scale. (B) Expression of *miR-101a* and *miR-101b* in striatal PSDs from saline- and cocaine-treated wildtype mice was also evaluated by RNA-Seq (left) and qPCR (right). RNA-Seq data are shown as reads (adjusted, equal total reads/sample) on log₁₀ scale; qPCR data are shown as the ratio of *miR-101b*/*miR-101a* expression. Expression of brain-enriched and/or synaptically important miRNAs in NAc lysates and striatal PSDs by RNA-Seq (C,E) or qPCR (D,F).

A) NAc total lysates, qPCR

B) Striatal PSDs, RNA-Seq C) Striatal PSDs, qPCR

Figure 2.14 Validation of RNA-Seq cocaine-induced expression changes by qPCR

qPCR analysis of cocaine-regulated miRNAs identified in Figures 3 and 4 from NAc lysates (A) and striatal PSDs (C). (B) Expression of select PSD-expressed cocaine-regulated miRNAs not appearing in Fig. 4A by RNA-Seq. Significance was evaluated using the 2-tailed Student's *t*-test with unequal (A) or equal (C) variance. **P* < 0.05; ***P* < 0.005.

Western blot analysis

Gels (4-15% Mini-PROTEAN TGX precast gels, BioRad) of subcellular fractions were loaded with 1 to 10 μ g protein, which was measured using the Bicinchoninic Acid Assay with bovine serum albumin as the standard (Pierce, Rockford, IL). Commercially available mouse monoclonal antibodies were used: β III-tubulin (TUJ1; Covance; 1:1000), NR2B (clone N59/20; NeuroMab; 1:1000), Ago2 (2E12-1C9; Novus Biologicals; 1:1000), Synaptophysin (SVP-38; Sigma; 1:10,000). Commercially available rabbit polyclonal antibodies were also used: BiP/GRP78 (Affinity BioReagents; 1:500), DARPP32 (2303; Cell Signaling Technology; 1:1000), pan-Trk (MCTrks, SC-414; Santa Cruz Biotechnology; 1:250), E-cadherin (ab53033; Abcam; 1:1000) and Metadherin (Ab2989; Millipore; 1:500). Rabbit polyclonal antibody JH1159 (1:250)¹⁶⁷ to the C-terminal 13 residues (amino acids 626-638) of rat PC2 was affinity-purified using the antigenic peptide linked to AffiGel 15 beads (Bio-Rad Laboratories).

For quantitative analysis of purified PSDs from Saline and Cocaine treated mice, three different amounts of sample were analyzed to verify the linearity of the response. After transfer to polyvinylidene difluoride membranes (Millipore), blots were stained with Coomassie Brilliant Blue R-250 and cut to separate the proteins of interest. Blots were incubated in primary antibody overnight at 4°C, washed and incubated for 1h at room temperature in the appropriate horseradish peroxidase-conjugated secondary antibody (Pierce). Washed blots were incubated in Super Signal West Pico chemiluminescence substrate (Pierce) for 5min and multiple exposures were collected using a Syngene imaging system (Syngene, Frederick, MD). Signals were quantified using GeneTools software; for each dilution of each PSD sample, the signal for each protein was normalized to β III-tubulin and the cocaine-to-saline ratio was determined.

Statistical analyses

Data across qPCR assays were averaged because the values with respect to the endogenous control for each miRNA were very consistent. Outliers were determined with the GraphPad Grubbs' test outlier calculator (graphpad.com/quickcalcs/Grubbs1.cfm). Differences between saline and cocaine samples were evaluated using the 2-tailed Student's *t*-test with unequal variances (NAc lysates) or equal variance (striatal-PSDs). For PSD Western blot quantification, cocaine-to-saline ratios for the three amounts of each sample were averaged and differences were evaluated using the 2-tailed Student's *t*-test with equal variance.

Pearson's Coefficient represents the linear correlation coefficient between 2 samples, *X* and *Y*.

Pearson's $r = \text{covariance}(X, Y) / \sigma_X \sigma_Y$; σ , standard deviation.

Online Supporting Material

Supplementary tables are available online at:

http://intron.ccam.uchc.edu/data/pipeline/jodi/THESIS_supplementary_files/CHAPTER_2

These materials are also available at the journal of publication:

<http://rnajournal.cshlp.org/content/17/8/1529/suppl/DC1>

CHAPTER 3

Effects of Cocaine and Withdrawal on the Mouse Nucleus Accumbens Transcriptome

The following is a duplicate version of a published manuscript:

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Abstract

Genetic association studies, pharmacological investigations, and analysis of mice lacking individual genes have made it clear that cocaine administration and withdrawal have a profound impact on multiple neurotransmitter systems. The GABAergic medium spiny neurons of the nucleus accumbens (NAc) exhibit changes in the expression of genes encoding receptors for glutamate and in the signaling pathways triggered by dopamine binding to G-protein coupled dopamine receptors. Deep sequence analysis provides a sensitive, quantitative and global analysis of the effects of cocaine on the NAc transcriptome. RNA prepared from the NAc of adult male mice receiving daily injections of saline or cocaine, or cocaine followed by a period of withdrawal, was used for high-throughput sequence analysis. Changes were validated by qPCR or Western blot. Based on pathway analysis, a preponderance of the genes affected by cocaine and withdrawal were involved in the cadherin, heterotrimeric G-protein, and Wnt signaling pathways. Distinct subsets of cadherins and protocadherins exhibited a sustained increase or decrease in expression. Sustained down-regulation of several heterotrimeric G-protein β - and γ -subunits was observed. In addition to altered expression of receptors for small molecule neurotransmitters, neuropeptides and endocannabinoids, changes in the expression of plasma membrane transporters and vesicular

neurotransmitter transporters were also observed. The effects of chronic cocaine and withdrawal on the expression of genes essential to cholinergic, glutamatergic, GABAergic, peptidergic, and endocannabinoid signaling are as profound as their effects on dopaminergic transmission. Simultaneous targeting of multiple withdrawal-specific changes in gene expression may facilitate development of new therapeutic approaches that are better able to prevent relapse.

Introduction

Chronic drug exposure induces persistent changes in the brain that underlie the addiction-associated behavioral abnormalities seen in human addicts and rodent models of addiction. Data from broad expression studies using microarray technology and from single-gene approaches suggest that cocaine exposure elicits widespread modifications to the transcriptional landscape, including drug-induced changes in epigenetics, RNA processing, microRNAs, and gene transcription^{168,169}. These alterations are believed to be integral to the neural plasticity seen in addiction.

Dopaminergic, glutamatergic, and peptidergic signaling in the mesolimbic system are altered in response to cocaine. Repeated exposure to cocaine leads to sensitization, the progressive and persistent amplification of behavioral and motivational responses to a fixed dose of the same drug⁹⁵. Sensitization may persist for weeks to years after cessation of drug taking, presumably contributing to a reformed addict's risk for relapse¹⁷⁰, which occurs in up to ninety percent of addicted individuals^{171,172}.

A majority of current research focuses on the effects of cocaine on the mesolimbic dopaminergic system, identified in prior studies as the reward center of the brain⁸³. Dopaminergic projections from the ventral tegmental area as well as glutamatergic projections from the prefrontal cortex synapse on inhibitory GABAergic medium spiny

neurons in the ventral striatum (nucleus accumbens; NAc)⁸⁷. This convergence of dopaminergic and glutamatergic projections, as well as accompanying biochemical and morphological changes, identified the NAc as a key integration point in the rewarding and addictive effects of drugs of abuse^{86,95,170,171}.

High-throughput sequencing has a low background signal, facilitates identification and absolute quantification of low-abundance and novel transcripts^{3,5,6,8,9,169,173}. We used next-generation sequencing of mRNAs purified from the NAc to obtain a more complete picture of the molecular response to chronic cocaine exposure and withdrawal. Bioinformatic analysis of cocaine-regulated transcripts revealed substantial involvement of the Wnt/cadherin and heterotrimeric G-protein signaling pathways. Evaluation of the synthetic enzymes, transporters, and receptors involved in catecholaminergic, glutamatergic, GABAergic, cholinergic, and lipid signaling identified complex responses unique to cocaine and withdrawal.

Results

Genome coverage

To characterize the effects of chronic cocaine and withdrawal on mRNA expression in the NAc, we prepared mRNA-Seq libraries from RNA pooled from mice subjected to daily saline injections (Saline), daily cocaine injections (Cocaine) or daily cocaine injections followed by a week with no injections (Withdrawal). Paired-end sequencing yielded approximately 20 million uniquely mapped reads for each library (**Table 3.1A**). The current annotation of the *Mus musculus* genome (2007, mm9) spans ~1.6 billion bases; 3.2% of the genome encodes mature mRNAs and 40.6% encodes primary transcripts. We detected 5-6% of the genome in the poly(A)⁺ RNA-Seq data from each NAc library. Approximately 80% of the reads in each library mapped to annotated exons; 15-16% of reads mapped to introns and 5% of reads

aligned to intergenic regions (**Table 3.1B**). Mitochondrial reads accounted for roughly 2% of all mapped sequences in these libraries (**Fig. 3.1**). Scatter plots of mitochondrial (**Fig. 3.2**) and ribosomal (**Fig. 3.3**) gene expression demonstrated no evidence for cocaine-regulation, so subsequent analyses were performed on a filtered gene list that excluded both ribosomal and mitochondrial genes.

A. High-throughput sequencing alignment information

Sample	Treatment	Number aligning reads	Total genome coverage
NAc lysate	Saline	26,563,428	3.8%
	Cocaine	24,696,981	3.8%
	Withdrawal	19,767,382	3.1%

B. Read mapping

Sample	Treatment	Exonic	Intronic	Intergenic
NAc lysate	Saline	79.4%	13.5%	3.1%
	Cocaine	79.0%	16.1%	4.9%
	Withdrawal	79.0%	16.2%	4.8%

Table 3.1 Read mapping and base coverage for RNA-Seq data

Sequences from NAc libraries were aligned to the *Mus musculus* reference genome (2007, mm9; NCBI Build 37) using Bowtie²⁴ and allowing up to 2-mismatches. (A) Aligning read numbers and percent coverage of the genome. (B) Reads were characterized as aligning to exonic, intronic, or intergenic regions based on the existing annotation.

Figure 3.1 Contribution of reads mapping to mitochondrial chromosome

The proportion of mapped sequence reads from NAc lysate libraries aligning to the mitochondrial chromosome was determined. Abbreviations: Sal, Saline; Coc, Cocaine; W/D, Withdrawal; chrM, mitochondrial chromosome.

Mitochondrial Gene Expression

Figure 3.2 High-throughput sequence analysis of mitochondrial NAc mRNAs

Pairwise scatter plots of mRNA expression data for NAc lysate (A-C) libraries (Saline, Cocaine, and Withdrawal) prepared from adult mouse; shown as $\log_{10}$ (RPKM) per sample. Scatter plots include only mitochondrial genes (from GO:0005840). R^2 values were calculated by least squares best fit.

Abbreviation: RPKM, reads per kilobase gene model per million mapped reads⁵.

Ribosomal Gene Expression

Figure 3.3 High-throughput sequence analysis of ribosomal NAc mRNAs

Pairwise scatter plots of mRNA expression data for NAc lysate (A-C) libraries (Saline, Cocaine, and Withdrawal) prepared from adult mouse; shown as $\log_{10}$ (RPKM) per sample. Scatter plots only include ribosomal genes (from GO:0005739). R^2 values were calculated by least squares best fit. Abbreviation: RPKM, reads per kilobase gene model per million mapped reads⁵.

Genome-wide assessment of the effect of cocaine on gene expression

Gene expression over a broad (10^6) dynamic range was evaluated in each library. Expression was quantified in reads per kilobase of exon per million mapped sequence reads (RPKM), a normalized measure of exonic read density⁵. For the three NAc libraries, pairwise scatter plots revealed similarly varied expression between the Saline/Cocaine, Saline/Withdrawal and Cocaine/Withdrawal libraries (**Fig. 3.4A-C**). The variability of gene expression between samples was highest for genes expressed at lower levels. For this reason, a lower limit of expression was applied before examining regulation. Since biogenic amine transporter (*Slc6a2*, NET; *Slc6a3*, DAT; *Slc6a4*, SERT) transcripts are not expressed in the NAc (<http://mouse.brain-map.org/>), we used their RPKM values to set this lower limit; for inclusion in subsequent analyses, a gene had to have an RPKM of > 1.0 in at least one of the three treatment groups. We used qPCR to validate the RNA-Seq data (**Fig. 3.4D,E**). The RNA-Seq and qPCR expression levels of 47 transcripts from five neurotransmitter systems from the Saline sample showed strong agreement across the two methods (Pearson's $r = 0.75$) over a greater than 1000-fold range of expression (**Fig. 3.4D**). A comparison of the ratios of Cocaine/Saline measured by RNA-Seq and qPCR was also carried out (**Fig. 3.4E**). Cocaine-mediated changes in gene expression estimated from the RNA-Seq data were consistently larger than the changes observed with qPCR; nevertheless, the ratios show good qualitative agreement (Pearson's $r = 0.60$). Differences presumably reflect the much larger dynamic range of RNA-Seq and quantification across entire transcripts rather than between single primer pairs.

Regulation of NAc transcript levels by cocaine and withdrawal

To evaluate enrichment and depletion of transcripts in NAc lysates prepared from mice in the Saline, Cocaine, and Withdrawal groups, we calculated the relative abundance of

Figure 3.4 High-throughput sequence analysis of NAc mRNA

(A-C) Pairwise scatter plots of mRNA expression data for NAc libraries; shown as $\log_{10}(\text{RPKM})$ per sample. Ribosomal and mitochondrial genes are not included in the scatter plots. R^2 values were calculated by least squares best fit. Abbreviations: Sal, Saline; Coc, Cocaine; W/D, Withdrawal; RPKM, reads per kilobase gene model per million mapped reads⁵. (D) RPKM data for 47 transcripts in the Saline library are compared to qPCR data (normalized to Glyceraldehyde 3-phosphate dehydrogenase, GAPDH) for the same transcripts; Pearson's Coefficient represents the linear correlation coefficient, r . (E) Cocaine/Saline ratios for the same 47 transcripts were calculated using RPKM data and qPCR data. Pearson's Coefficient represents the linear correlation coefficient, r , between two samples, X and Y . Pearson's $r = \text{covariance}(X, Y) / \sigma_X \sigma_Y$, where σ is the standard deviation.

each mRNA in the three treatment groups, computed Z-scores across samples, and hierarchically clustered genes for visualization in a heat map. Of the 19,890 detected genes, 13,184 were expressed in at least one library at an RPKM > 1.0 and were used in downstream analyses. Several patterns of expression emerged from hierarchical clustering, including transcripts showing (i) sustained increases in both Cocaine and Withdrawal, (ii) increases only in Withdrawal, (iii) transient increases only in Cocaine, (iv) transient decreases only in Cocaine, and (v) sustained decreases in Cocaine and Withdrawal (**Fig. 3.5A**). The full gene list for each cluster is available in **Table 3.S2**.

Since transcripts exhibiting a sustained change in response to cocaine are included in Clusters i (sustained increase), ii (increase, Withdrawal), and v (sustained decrease), we used Panther Pathway (**Table 3.2A** and **Table 3.S3**) analysis to identify common pathways. Three pathways were enriched ($P < 0.005$) in these three Clusters: cadherin signaling, heterotrimeric G-protein signaling ($G\alpha_i$ and $G\alpha_s$ -mediated), and Wnt signaling; since there was a great deal of overlap in the cadherin and Wnt signaling pathways, they are discussed together. Other pathways were unique to specific clusters (**Table 3.S3**). For example, components of the ionotropic glutamate receptor, metabotropic glutamate receptor group III, and nicotinic acetylcholine receptor pathways were enriched only in Cluster i (sustained increase) while components of the muscarinic acetylcholine receptor 1 and 3 pathway were enriched in Cluster ii (increase, Withdrawal).

The pathways exhibiting sustained changes were examined in more detail. Unique sets of cadherins and protocadherins appeared in each Cluster (**Table 3.3**). While many cadherins and protocadherins showed a sustained increase in expression (Cluster i), others increased only in Withdrawal (Cluster ii) or showed a transient response to Cocaine (Clusters iii and iv). The clustered families of protocadherins, *Pcdh α* , *- β* , and *- γ* , are important in neuronal

Figure 3.5 Differential regulation of clusters of genes in the NAc following cocaine and withdrawal

(A) Normalized mRNA frequency as a function of total reads for NAc lysate libraries (Saline, Cocaine, Withdrawal) was calculated for all genes in which one sample had an RPKM ≥ 1.0 ($n = 13,184$). Hierarchical gene expression clustering using Gene Cluster 3.0 and Java TreeView generated the dendrogram and heat map. Z-score was computed on normalized mRNA frequency across all samples. Blue, low expression; yellow, high expression; grey, no expression. Gene lists from the indicated Clusters (i-v) were used in subsequent protein class and pathway analyses. (B) The effects of Cocaine on one of the protocadherins identified as a target were verified. Subcellular fractionation of striatal tissue revealed enrichment of PCDH10 at the PSD. PSDs purified from the striata of Saline and Cocaine treated mice were analyzed for PCDH10; after normalization to bIII tubulin, the Coc/Sal ratio for PCDH10 rose 24% ($P < 0.01$; t -test).

PANTHER Pathway	<i>Mus musculus</i> genes (26,185)	(i) Sustained Increase, Coc & W/D (1,387)	(i) <i>P</i>-value	(ii) Increase, W/D only (1,001)	(ii) <i>P</i>-value	(v) Sustained Decrease, Coc & W/D (2,670)	(v) <i>P</i>-value
Cadherin signaling	167	19	1.94E-03	16	9.25E-04	30	2.70E-03
Heterotrimeric G-protein signaling – G α_i & G α_s	169	19	2.21E-03	18	1.32E-04	32	8.89E-04
Wnt signaling	348	46	3.67E-08	27	5.77E-04	64	9.02E-06

Table 3.2 Panther Pathways from Cocaine- and Withdrawal-regulated genes in the NAc

Gene lists from Clusters *i-v* (Fig. 3.5A) were used in Panther Pathway and Protein Class analysis (<http://www.pantherdb.org>); pathways enriched ($P < 0.005$) in all three lists relative to the *Mus musculus* reference genome are indicated. Complete pathway lists are provided in Table 3.S3. Abbreviations: Coc, Cocaine; W/D, Withdrawal.

development and are synaptically enriched in mature myelinated neurons¹⁷⁴. The *Pcdhb* gene cluster includes 22 genes; three showed a sustained increase (Cluster i), five were increased only after Withdrawal (Cluster ii), five exhibited an increase only after Cocaine (Cluster iii), and one showed a sustained decrease in expression (Cluster v). In contrast, the *Pcdhg* cluster of 23 genes exhibited a more homogeneous expression pattern in which expression of all 21 detected isoforms showed a sustained decrease (Cluster v).

We used an antibody to protocadherin 10 (PCDH10), which is known to play a role in the development of striatal axons and thalamocortical projections¹⁷⁵, to evaluate its subcellular localization and quantify its levels in postsynaptic densities (PSDs) purified from the striata of saline- and cocaine-treated mice. PCDH10 was enriched in PSD preparations (**Fig. 3.5B**); furthermore, PSDs purified from striatal extracts of cocaine-treated mice contained more PCDH10 than striatal PSDs from saline controls. A role for extracellular matrix proteins, including cadherins and protocadherins, is consistent with previous studies of cocaine-regulated gene expression in mice¹⁶⁹ and postmortem human brain^{112,176}.

Consistent with the literature, heterotrimeric G-protein signaling ($G\alpha_i$ and $G\alpha_s$ -mediated) was identified as a major pathway targeted by cocaine. In addition to G-protein coupled receptors (GPCRs) for the major neurotransmitters (discussed below), neuropeptide (opioid, galanin, somatostatin), lipid, and cadherin-type GPCRs showed cocaine-related changes in expression (**Table 3.3**).

Panther Pathway analysis identified cocaine-regulated changes in the repertoire of intracellular signaling proteins downstream of the regulated $G\alpha_i$ and $G\alpha_s$ -coupled GPCRs (**Table 3.3** and **Fig. 3.6**). The diagram in **Fig. 3.6** depicts the complex interplay of intracellular signaling proteins. For example, only two members of the *Mus* arrestin gene family are expressed outside of the retina¹⁷⁷; *Arrb1* showed a sustained increase in expression (Cluster i)

Figure 3.6 $G\alpha_i$ and $G\alpha_s$ heterotrimeric G-protein signaling schematic.

Model based on Panther Pathway diagram (<http://www.pantherdb.org>). Venn diagram depicts color key for signaling components. Genes labeled yellow showed a sustained increase in Coc and W/D (cluster i), red genes increased in W/D only (cluster ii), and blue genes had decreased expression in both Coc and W/D (cluster v); overlap colors indicate contribution of > 1 cluster to signaling group. For example, GPCRs, which appear in all three clusters, are shown in magenta. Genes from Clusters i, ii, and v were used for this analysis. Abbreviations: Coc, Cocaine; W/D, Withdrawal.

	(i) Sustained Increase, Coc & W/D	(ii) Increase, W/D only	(iii) Transient Increase, Coc only	(iv) Transient Decrease, Coc only	(v) Sustained Decrease, Coc & W/D
Cadherin / Wnt Signaling					
Cadherins	<i>Cdh4, Cdh5, Cdh6, Cdh8, Cdh11</i>	<i>Cdh7, Cdh19, Cdh22</i>	<i>Cdh24</i>	<i>Cdh13</i>	
Protocadherins	<i>Pcdh1, Pcdh10, Pcdh18, Pcdhb13, Pcdhb14, Pcdhb16</i>	<i>Pcdhb2, Pcdhb6, Pcdhb7, Pcdhb8, Pcdhb9</i>	<i>Pcdh20, Pcdhb5, Pcdhb10, Pcdhb11, Pcdhb19, Pcdhb20</i>		<i>Pcdha4, Pcdhb12, Pcdhga1, Pcdhga2, Pcdhga3, Pcdhga4, Pcdhga5, Pcdhga6, Pcdhga7, Pcdhga8, Pcdhga9, Pcdhga10, Pcdhga11, Pcdhga12, Pcdhgb1, Pcdhgb2, Pcdhgb4, Pcdhgb5, Pcdhgb6, Pcdhgb7, Pcdhgb8, Pcdhgc3, Pcdhgc4, Pcdhgc5</i>
Heterotrimeric G-Protein Signaling (Gα_i & Gα_s)					
Type of GPCR:					
Neuropeptide binding	<i>Fzd5, Fzd8, Hcrtr2, Npffr1, Oprk1, Oprm1, Prlhr, Tacr1</i>	<i>Fzd6, Fzd7, Mc3r, Oprd1, Pth1r, Smo, Trhr2</i>	<i>Hcrtr1, Npbwr1, Npy2r, Oxtr, Sstr3, Tacr3</i>	<i>Fzd9, Gipr, Nmbr, Npy5r</i>	<i>Mc4r, Mchr1, Mtnr1a, Sstr2</i>
Lipid binding		<i>S1pr3</i>		<i>S1pr4</i>	<i>Lpar1</i>
Cadherin type	<i>Celsr2, Celsr3</i>	<i>Celsr1</i>			
Intracellular signaling proteins:					
GPCR kinases				<i>Grk6</i>	<i>Grk1</i>
Arrestins	<i>Arrb1</i>				<i>Arrb2</i>
G β subunits		<i>Gnb4</i>			<i>Gnb1, Gnb11, Gnb2, Gnb211, Gnb5</i>
G γ subunits		<i>Gng7</i>	<i>Gng2</i>		<i>Gng3, Gng8, Gng10, Gng11, Gng13</i>
RGSs	<i>Rgs6</i>	<i>Rgs8, Rgs11</i>	<i>Rgs17</i>	<i>Rgs9, Rgs19</i>	<i>Rgs2, Rgs4, Rgs10, Rgs16, Rgs20</i>
Adenylate cyclases	<i>Adcy1, Adcy4, Adcy6, Adcy9</i>	<i>Adcy2, Adcy5</i>	<i>Adcy8</i>		
Phosphorylase b kinases	<i>Phka2</i>				<i>Phkg1, Phkg2</i>
CREBs	<i>Creb3l2, Crebbp</i>				<i>Creb3</i>

Table 3.3 Clustered genes from the Cadherin/Wnt and Heterotrimeric G-Protein Signaling Pathways

The cadherin and protocadherin genes appearing in Clusters *i-v* are identified. Genes in the heterotrimeric G-protein signaling pathway (G α_i and G α_s) that fell into Clusters *i-v* are grouped by function; GPCRs for dopamine, GABA, glutamate and acetylcholine are discussed separately and GPCRs without known ligands are not listed. Intracellular signaling proteins are grouped by function. Abbreviations: Coc, Cocaine; W/D, Withdrawal.

while *Arrb2* exhibited a sustained decrease (Cluster v). Transcripts encoding the β - and γ -subunits of heterotrimeric G-proteins were primarily identified in Cluster v (sustained decrease). In contrast, Regulators of G-protein signaling (RGS genes) appeared in all clusters. Transcripts encoding four adenylate cyclase genes showed a sustained increase in expression (Cluster i) while the transcripts encoding two additional adenylate cyclase genes demonstrated increased expression only in the Withdrawal group (Cluster ii).

Panther Protein Class analysis (**Table 3.S4**) identified Cytoskeletal Proteins, Transcription Factors, and Transferases as the three Protein Classes enriched in genes exhibiting a sustained response (Clusters i, ii, and v; $P < 0.0001$). Sustained up-regulation of G-protein modulators and guanyl-nucleotide exchange factors (Cluster i) was also observed. Several Protein Classes involved in interactions with Ca^{2+} (calcium-binding proteins, calmodulin and intracellular calcium-sensing proteins) were enriched only in Cluster v (sustained down-regulation). GPCRs, ligand-gated ion channels, membrane trafficking proteins and neuropeptides were also enriched in Cluster v.

Widespread effects of cocaine and withdrawal on catecholaminergic, GABAergic, cholinergic, glutamatergic, and endocannabinoid pathways

Dopamine, norepinephrine, and serotonin receptors expressed in the NAc play a critical role in addiction. Transcripts encoding the dopamine receptors, *Drd1a*, *Drd2*, *Drd3*, and *Drd5*, exhibited different patterns of response to cocaine and withdrawal (**Fig. 3.7**). *Drd1a* was known to decrease in response to cocaine treatment, while *Drd2* was known to increase¹⁷⁸. *Adra1a*, *Adra2a* and *Adrb1* adrenergic receptor expression rose in response to cocaine treatment, while expression of *Adra1b*, *Adra2b*, and *Adra2c* fell. Six of the twelve serotonin receptors (*Htr1b*, *Htr2a*, *Htr2c*, *Htr4*, *Htr5a*, *Htr6*) exhibited increased expression in response to cocaine and/or withdrawal. The long-term changes during withdrawal were verified by qPCR

Figure 3.7 Regulation of Receptors for Biogenic Amines

RPKM data are shown for dopamine, adrenergic and serotonin receptor transcripts (RPKM > 1) in the Saline library. Z-scores were calculated as described in **Methods**. Transcripts included in this group but expressed at an RPKM < 1 are listed to the side. *Ppp1r1b* (*DARPP32*), a multiply phosphorylated phosphatase inhibitor highly expressed in medium spiny neurons, is included for comparison.

for *Drd1a*, *Drd2*, *Adra1a*, *Adra2b*, *Htr1d*, *Htr2a*, *Htr2c*, and *Ppp1r1b* (DARPP32).

Over 90% of the neurons in the NAc are GABAergic medium spiny interneurons¹⁷⁹; the genes involved in synthesizing, storing and retrieving GABA (γ -aminobutyric acid) are expressed in the NAc, along with ionotropic and metabotropic GABA receptors (**Fig. 3.8**). Expression of the GABA-synthesizing enzymes (*Gad65*, *Gad67*), the vesicular inhibitory amino acid transmitter transporter (*Viatt*) and the plasma membrane transporters (*Gat1*, *Gat3* and *Gat4*) rose in response to cocaine and withdrawal. The three Na⁺/Cl⁻-dependent GABA transporters are expressed by astrocytes and retrieve GABA after secretion¹⁸⁰⁻¹⁸²; neurons also express *Gat1*¹⁸³. Levels of *Gat4* were highest after Cocaine while levels of *Gat1* were highest in Withdrawal. Multiple GABA_A receptor subunits are expressed at similar levels; while some showed a sustained increase in expression (*Gabrb2*, *Gabrb3*, *Gabrg3*, *Gabrq*), *Gabrb1* expression decreased in Withdrawal and *Gabrd* expression decreased after Cocaine but recovered in Withdrawal. Expression of GABA_B receptor subunits (*Gabbr1*, *Gabbr2*) increased after Cocaine and increased further during Withdrawal.

Glutamatergic signaling plays an essential role in drug abuse, with major glutamatergic inputs onto NAc medium spiny neurons (MSNs) coming from amygdala, hippocampus, and prefrontal cortex^{184,185}. The effects of cocaine on expression of ionotropic and metabotropic glutamate receptors have been studied in detail^{171,184,186} and were largely confirmed in our analysis (**Fig. 3.9**). Glutamatergic inputs acutely excite NAc MSNs primarily via activation of AMPA receptors, a step considered necessary for initiation of drug seeking behavior¹⁸⁷. AMPA receptor expression showed only small changes during Cocaine administration; *Gria1* increased substantially while *Gria3* decreased during Withdrawal (confirmed by qPCR). Expression of several NMDA receptor subunits increased during Cocaine administration (*Grin1*, *Grin2a*, *Grin2b*, *Grin2d*, *Grin3a*) (several confirmed by qPCR). Expression of kainate receptors *Grik1*, *Grik2*, and *Grik3* showed a sustained increase while expression of

Figure 3.8 Regulation of Genes Affecting GABAergic Transmission

Data were analyzed as described for Fig. 3.7. The transcripts encoding glutamic acid decarboxylase 65 and 67 (*Gad1* and *Gad2*) are expressed at similar levels. GABA transport into synaptic vesicles requires *Slc36a1*; retrieval of GABA from extracellular space requires *Slc6a1*, *Slc6a13*, or *Slc11*. Multiple ionotropic GABA_A receptor subunits are expressed in the NAc; expression of metabotropic GABA_B receptor *Gabbr1* greatly exceeds expression of *Gabbr2*.

Figure 3.9 Regulation of Genes Affecting Glutamatergic Transmission

Data were analyzed as described for Fig. 3.7. Expression of all three vesicular glutamate transporters (*Vglut*) and four of the plasma membrane glutamate transporters (*Slc1a1*, *Slc1a2*, *Slc1a3*, *Slc1a6*) was above the cutoff level. Multiple subunits of the ionotropic glutamate receptors (AMPA, NMDA, and Kainate) and multiple metabotropic glutamate receptors are expressed at similar levels.

Grik4 and *Grik5* showed a sustained decrease. Expression of the five most-highly expressed metabotropic receptors for glutamate (*Grm1*, *Grm3*, *Grm4*, *Grm5*, and *Grm7*) showed a sustained increase. Vesicular glutamate transporters (*Vglut*) can be expressed in GABAergic and cholinergic neurons as well as in glial cells¹⁸⁸. Transcripts encoding all three *Vgluts* were detectable in the NAc, which contains no primarily glutamatergic neurons; expression of *Vglut1* mRNA exceeded that of *Vglut2*. Na⁺-dependent plasma membrane glutamate transporters (*Eaat1* through *Eaat5*), which remove glutamate from the synaptic cleft and perisynaptic area, also play a critical role in glutamatergic transmission. Three of the four *Eaats* expressed in the NAc (*Eaat1*, *Eaat2*, and *Eaat3*) exhibited a sustained increase in expression while *Eaat4* exhibited reduced expression in Withdrawal. *Eaat1* and *Eaat2* are primarily expressed in glia, while *Eaat3*, *Eaat4* and *Eaat5* show widespread neuronal expression throughout the brain¹⁸⁹.

A small population of giant aspiny cholinergic interneurons is found in the NAc¹⁹⁰; expression of both the plasma membrane choline transporter (*Cht1*), which provides the rate-limiting substrate, and choline acetyltransferase (*ChAT*) was increased by Cocaine and further increased by Withdrawal (**Fig. 3.10**). Expression of each of the major nicotinic ACh receptor subunits found in the NAc (*Chrna4*, *Chrna7*, and *Chrn2*) and three muscarinic ACh receptors (*Chrm2*, *Chrm3*, and *Chrm5*) showed sustained increases, many verified by qPCR.

The endocannabinoid system is heavily involved in many reward-seeking responses to drugs of abuse¹⁹¹. Levels of transcripts encoding *Cnr1* (the CB1 endocannabinoid receptor) and the cannabinoid synthetic enzymes *Dagla* and *Napepld* showed sustained increases in Cocaine and Withdrawal, while *Daglb* showed a sustained decrease (**Fig. 3.11**). The effect of Cocaine expression on each of these genes was confirmed by qPCR analysis of two separate sets of animals.

Figure 3.10 Regulation of Genes Affecting Cholinergic Transmission

Data were analyzed as described for Fig. 3.7. Although only a small percentage of the neurons in the NAc are cholinergic, choline acetyltransferase (*ChAT*), high affinity plasma membrane choline transporter (*Slc5a7*) and vesicular acetylcholine transporter (*Slc18a3*) transcript levels all fall above the 1 RPKM cutoff. Only three of the many nicotinic acetylcholine receptor subunits are expressed in the NAc while all five muscarinic receptors are expressed. Expression of each of the genes involved in cholinergic transmission is elevated in withdrawal.

Figure 3.11 Regulation of Genes Affecting Endocannabinoid Signaling

RPKM data were analyzed as described for Fig. 3.7. While transcripts encoding the two enzymes that synthesize the endocannabinoid 2-arachidonoylglycerol (diacylglycerol lipase α and β ; *Dagla* and *Daglb*) are expressed at similar levels in the NAc, they respond to cocaine in an opposite manner. Expression of transcripts encoding cannabinoid receptor 1 (*Cnr1*) greatly exceeds that of cannabinoid receptor 2 (*Cnr2*) and is responsive to cocaine.

Discussion

Single-gene, proteomic, and microarray analyses demonstrated that cocaine produces widespread, multi-faceted responses in the NAc^{168,169}. Next-generation sequencing identified pathways not previously thought to play any role in addiction and facilitated systematic cataloging of changes in all components of known signaling pathways. With greater sequencing depth, changes in editing, splicing, imprinting and allele-specific expression can be quantified^{3,5,6,8,9,169,173}. Components of the Wnt, cadherin, and heterotrimeric G-protein signaling pathways were over-represented among cocaine-responsive transcripts; cross-talk between these signaling cascades is extensive¹⁹².

Global comparisons of gene expression identify signaling pathways targeted by cocaine

Cadherin/Wnt pathways

By sequencing PSD-localized, cocaine-regulated striatal microRNAs, we previously identified *miR-8* family members as key players in the response to cocaine¹⁶⁹. Although the *miR-8* family has primarily been studied for its role in cancer progression and metastasis, we verified cocaine regulation of two bioinformatically-predicted cadherin family targets¹⁶⁹. We identified over forty cocaine-regulated cadherin and protocadherin transcripts; genes from the *Pcdhβ* cluster were primarily up-regulated following cocaine in both a transient and sustained fashion (Clusters i-iii), whereas genes from the *Pcdhγ* cluster were exclusively found to undergo a sustained down-regulation following cocaine exposure (Cluster v).

A few studies have suggested a role for cell adhesion and extracellular matrix proteins in the response to cocaine. Disturbances in cortical cytoarchitecture in animal models of prenatal cocaine exposure resembled changes observed when Wnt/cadherin pathway function was altered; this lead to the prediction that these pathways play a role in the

response to cocaine¹⁹³. As observed here (Cluster i), *Cdh6* and *Cdh11* expression increased in the frontal cortex of E18 pups taken from dams treated with cocaine for 10 days¹⁹³. Also contributing to alterations in the extracellular milieu is cocaine-mediated down-regulation of glycosyltransferase expression; these enzymes are crucial for the synthesis of cell surface glycoproteins. In a compilation documenting changes in gene expression in postmortem tissue from cocaine addicts, cell adhesion molecules were identified as major targets^{109,112}.

Heterotrimeric G-protein pathways

Transcripts encoding GPCRs, heterotrimeric G-protein subunits, regulators of G-protein signaling (RGS), GPCR kinases (GRK) and adenylate cyclases (Adcy) responded differently to cocaine and withdrawal. Genes encoding GPCRs and RGSs were identified in all five clusters. Particularly striking was the prevalence of GPCRs for neuropeptides, with different receptor subtypes in different clusters (e.g., Wnt, galanin, somatostatin, and opioid receptors). The μ -opioid (*Oprm1*) receptor, associated with increased sensations of reward and pleasure, showed a sustained increase in expression in response to cocaine, as expected^{104,172}, as did the endocannabinoid (*Cnr1*) receptor^{194,195}. The δ -opioid receptor (*Oprd1*), also involved in positive reinforcement of drugs of abuse¹⁹⁶, increased expression only in Withdrawal. The κ -opioid receptor (*Oprk1*), crucial to the negative emotional state of withdrawal¹⁷², showed a sustained increase, as also observed in postmortem tissue from cocaine overdose victims¹⁹⁷. Transcripts encoding several neuropeptides were substantially down-regulated by cocaine: *Pomc* (proopiomelanocortin), *Tac1* (tachykinin), *Oxt* (oxytocin) and *Cartpt* (CART; cocaine and amphetamine regulated peptide).

Consistent with a major role for cadherins in the response to cocaine, seven transmembrane cadherins (*Celsr*) were identified in clusters i and ii. Genes encoding adenylate cyclases appeared in Clusters i, ii, and iii. On the other hand, the majority of genes encoding

the β - and γ -subunits of the heterotrimeric G-proteins fell into cluster v (sustained decrease). The few genes whose expression is uniquely regulated in Withdrawal are of special interest; these transcripts may provide insight into the biochemical and cell biological alterations underlying reinstatement of drug-seeking behavior, ultimately helping identify potential therapeutic approaches to prevent relapse¹⁸⁵.

Neurotransmitter-specific effects

Expression of the D3 dopamine receptor (*Drd3*) increased in response to Cocaine and remained elevated after Withdrawal (cluster i); in contrast, D2 dopamine receptor (*Drd2*) expression increased only after Withdrawal. An increase in D3 receptor expression was observed in the NAc of cocaine overdose victims¹⁹⁷ and in rats 45 days after cocaine self-administration ceased¹⁵⁷. Recent work suggests that dopamine receptor dysregulation, mediated by decreased D2 and increased D3 receptor expression, contributes to increased cocaine-seeking behavior after prolonged withdrawal¹⁵⁷. Alterations in serotonin signaling are also recognized as important in drug addiction¹⁹⁸, and we observed cocaine-responsive changes in gene expression across many of the serotonin receptors.

Expression of many of the genes responsible for GABA synthesis, transport into synaptic vesicles, and retrieval from the synaptic cleft rose following cocaine treatment and increased further during withdrawal. Daily cocaine injections decreased GABA_A receptor-mediated maximal evoked currents and miniature inhibitory postsynaptic currents on dopamine neurons in the VTA¹⁹⁹; furthermore, GABA_A receptor function has been implicated in dopamine-mediated alcohol reward²⁰⁰. GABA_B receptor agonists attenuated cocaine reinforcement and reduced cocaine craving in a study of human cocaine users²⁰¹; while the precise mechanism of these behavioral effects is unknown, activation of GABA_B receptors in the NAc reduces dopamine release from dopaminergic projections from the VTA²⁰². Changes

in GABA_A receptor subunit expression, which fell during withdrawal, and in GABA_B receptor expression, which rose, could contribute significantly to altered synaptic transmission.

A growing body of literature has focused on the role of AMPA, NMDA and kainate receptor-mediated glutamatergic transmission into the NAc in the development of addiction and addictive-like behaviors¹⁸⁶. Increased glutamate receptor mRNA expression parallels increases in cell surface expression of GluR1-containing AMPA receptors following cocaine withdrawal²⁰³, along with a specific population of GluR2-lacking calcium-permeable AMPA receptors¹⁸⁵. We saw sustained up-regulation of *Gria1* (GluR1) transcripts, a transient increase in *Gria4* (GluR4) transcripts, and no change in *Gria2* (GluR2) transcripts. While expression of kainate receptor 3 (*Grik3*) rose substantially, expression of *Grik4* and *Grik5* fell. NMDA receptors are critical for the behavioral response to drugs of abuse^{204,205}; new spines appearing in the NAc following cocaine are enriched in NMDA receptors and are not formed if NMDA receptor currents are blocked^{206,207}. Correspondingly, transcripts encoding several NMDA receptor subunits rose in response to cocaine. Expression of the more prevalent metabotropic glutamate receptors also increased. Perhaps the most significant finding is that expression of *Slc17a7* and *Slc17a6* (Vglut1 and Vglut2) exhibited sustained down-regulation after cocaine treatment and withdrawal.

Cholinergic transmission in the NAc contributes to addiction through the modulation of dopamine signaling and reward processing²⁰⁸. Increased expression of both choline acetyltransferase and the plasma membrane choline transporter in the giant aspiny cholinergic interneurons could increase cholinergic signaling at both nicotinic and muscarinic ACh receptors expressed on the GABAergic output neurons.

Conclusion

We used next-generation sequencing to generate a comprehensive catalog of transcriptional changes that occur in response to cocaine treatment and withdrawal in the primary reward center of the brain. Pathway analysis enabled the complete categorization of transcriptional changes in signaling pathways known to be important in addiction along with the identification of pathways not previously considered important. These data should serve as a resource for development of new targets for cocaine therapy and facilitate selection of protein interaction and pathway targets for future study. With more information about their normal physiological roles, the extracellular domains of the many cadherins and protocadherins that respond to cocaine may be useful therapeutic targets. Pharmaceutical cocktails targeted to a subset of the specific GPCRs whose expression is altered during withdrawal may prove to be more effective than treatments targeting a single pathway.

Methods and Materials

Cocaine Treatment of Mice

Adult male C57BL/6 mice (Jackson Laboratories, Bar Harbor, Maine; 2-5 months old) were group-housed in the animal facility at the University of Connecticut Health Center on a 12 h light/dark cycle (lights, 7:00am-7:00pm) and handled in accordance with Institutional Animal Care and Use Committee guidelines. After acclimation for one week, animals were handled for 1 min/day for 2-3 days prior to injections. On each treatment day, animals were allowed to acclimate to the behavior room for 45-60 minutes before beginning injections.

Cocaine treatment was performed as described¹⁶⁹. Animals were given saline (10 ml/kg/day) or cocaine (10 or 20 mg/kg/day) intraperitoneally for 7 days and locomotor activity was monitored daily using a 15" x 15" Plexiglas chamber (PAS Open Field System, San Diego Instruments); after the 7th injection, locomotor activity was three-fold higher in mice receiving

cocaine than in mice receiving saline (**Fig. 3.12**)^{98,209}. Administering 10 mg/kg cocaine on the first and last days produces more reliable locomotor sensitization (locomotor ambulations on day 7 ÷ day 1), as originally demonstrated in rats¹⁶⁶, and was used in later experimental sets in this work. Animals were sacrificed by decapitation 24 hours after the final injection with the exception of mice in the withdrawal groups, which were kept in the home cage for 8 or 28 days after the final cocaine dose prior to tissue harvest; elevated locomotor responding was maintained for the 28 day withdrawal period (**Fig. 3.12**). Quantitative polymerase chain reaction (qPCR) validation was performed on RNA samples used for library preparation (8 animals analyzed separately in each treatment group) and on two biological replicate groups of mice sensitized in a similar manner⁹⁸: 8 each saline and cocaine; 4 each saline, cocaine, and 28 days withdrawal. Pairwise comparisons were performed and subjected to t-test ($p < 0.05$ taken as significant).

RNA and Library Preparation and Sequencing

NAc punches were taken from individual mice (at least 8 animals/treatment group). Total RNA was prepared using TRIzol (Invitrogen)¹⁶⁹. Average yield of total RNA per NAc punch was 6.3 ± 0.6 mg. Total RNA used for NAc library preparation consisted of equal amounts of RNA from 8 individual mice for each treatment. One Saline, one Cocaine, and one 7-day Withdrawal mRNA-Seq library was prepared from each pooled RNA sample according to manufacturer's specifications (Illumina). Briefly, total RNA was treated with DNase I; poly(A)⁺ RNA purified using Dynal magnetic beads (Invitrogen) was fragmented by partial alkaline hydrolysis (Ambion) and reverse-transcribed using random primers and SuperScript II (Invitrogen). cDNA was size-selected (~200 bp insert) on 2% agarose gels. Libraries were prepared for sequencing using the Paired-End DNA Sample Prep Kit (Illumina).

Figure 3.12 Cocaine causes long-lasting locomotor sensitization

(A) Two sets of 8 male mice were subjected to saline or cocaine injection as indicated, and locomotor activity for the subsequent 45 min was recorded. RNA from these mice was used for Q-PCR validation. (B) Two sets of 8 male mice were subjected to saline or 20 mg/kg cocaine injection for 8 days followed by 30 min of locomotor monitoring. A third set of 8 male mice received 20 mg/kg cocaine for 8 days, was subjected to withdrawal for 8 days, and then locomotion was monitored in 3 of the 8 mice following a test injection of 20 mg/kg cocaine. RNA from these mice was used for RNA-Seq. (C) An additional two sets of 4 male mice each were subjected to saline or 20 mg/kg cocaine injection daily for 7 days, followed by 15 min of locomotor monitoring; a third set of 4 mice received cocaine for 7 days, was subjected to withdrawal for 28 days, and then locomotion was monitored after a test injection of 20 mg/kg cocaine. RNA from these mice was used for Q-PCR validation. Abbreviations: Sal, Saline; Coc, Cocaine; W/D, Withdrawal. The number following the abbreviation indicated number of days in a given treatment paradigm.

NAC libraries were sequenced in nine lanes (3 technical replicates per sample) on an Illumina GAll_x using a 37-cycle paired-end sequencing protocol. Replicates were analyzed for intra-sample disparity and read data from all three lanes were then merged into one composite data file per sample; intra-sample coefficient of determination, $R^2 \geq 0.98$. The composite file was used for subsequent analyses.

mRNA-Seq Data Analysis

Sequences and quality scores were extracted from image files using Firecrest, Bustard, and GERALD software. Sequences were aligned to the *Mus musculus* genome (2007, mm9; NCBI Build 37) using Bowtie (<http://bowtie-bio.sourceforge.net>; version 0.12.7)²⁴ permitting multi-alignment and allowing up to 2-mismatches. Ambiguous alignments were resolved using the Spliced Paired-end Aligner Perl script²¹⁰. Robust assembly of novel transcripts and a complete analysis of cocaine-induced alternative splicing requires greater transcript coverage and read depth than we obtained²⁵.

Normalized mRNA expression was calculated as RPKM (reads per kilobase gene model per million mapped reads)⁵ from aligned sequence reads; Z-scores were computed from frequency data using the following equation: $(x - \mu)/\sigma$; x , sample frequency; μ , mean across all samples; σ , standard deviation across all samples. Gene expression data (Z-scores) were hierarchically clustered using Gene Cluster 3.0 (<http://rana.lbl.gov/EisenSoftware.htm>); heat maps were generated using Java Treeview (<http://www.sourceforge.net/projects/jtreeview/files>). Gene lists were submitted to the Panther website (<http://www.pantherdb.org>) for analysis of Pathway and Protein Class enrichment compared to the *Mus musculus* reference gene list. Western and qPCR analyses were performed as described¹⁶⁹; primer sequences are in **Table 3.5**.

Table 3.4 Primers used for quantitative PCR

Gene Name	Sequence	T _m (°C)	Length (nt)
GAPDH (G3PDH) NM_008084.2	TTGTCAGCAATGCATCCTGCACCACC	61	119
	CTGAGTGGCAGTGATGGCATGGAC	61	
glutamate receptor, ionotropic, AMPA4	CCCAGTATTCTAGAGGGTGTGTTGCC	61	123
	CTTCAGTGGGAAGCTTGGTGTGATG	61	
glutamate receptor, ionotropic, AMPA3 (alpha 3)	CACACGACGCAATACTGGTCATAGCAG	61	120
	GACTCCAGGGCACAGCAGGATTTG	61	
glutamate receptor, ionotropic, NMDA2B	CTGAGACTGAAGAACAGGAAGATGACCATC	62	119
	CGGGACTGTATTCCGCATGCAGG	61	
dopamine receptor D1A	GCTGGCTTTTGGCCCTTTGGGTC	61	123
	GCTGGAGATAGCCCAATACCTGTCC	61	
dopamine receptor 2	CCATCTCTTGCCCACTGCTCTTTGG	61	122
	GGTGACGATGAAGGGACGTAGAAC	61	
glutamate receptor, ionotropic, AMPA1	GTCAGCGACGGCAAATACGGAGC	61	120
	GACCAAGGTTATGGTCAAGGGGGC	61	
	CCTCGCTTGTACGCCCCC	60	
mNR2A	CTCTCGTAGCATAAGCCTCAAGGACAG	61	123
	CAGACCTTGGGGGAAAAGGGAGCTTTTG	61	
mDARPP32	AGACCCACAGGGCAGGTCCTAAG	61	126
	GTTGAAGTCTATGAGAGGGTTGGGGAC	61	
GAD65	GAGGGTTACTGATGTCCCGAAACAC	61	120
	CCAGGAGAGCTGAACACTGCAAGG	61	
GAD67	TCTCCTATGACACCGGGGACAAGG	61	129
	GGCATTGTGTGATCTGGTTTTCAAATCCCAC	60	
GABA-a1 Gabar1	CCAGTTTCGGACCACTTCAGACCAC	61	120
	TGTTTAGCCGGAGCACTGTCATGGG	61	
DGL-alpha	CTCACGCACAGAGCTGCTGGCA	60	120
	ATGGTTGCACTTGGTGGTCGCTCAG	61	
DGL-beta	CTGCATGGCTGTTGGTACGGACTG	61	120
	GGTTAGAAGTGCTGCTCTCAAGAAGA	60	
NAPE-PLD	GGATGAGTATGAGGACAGCCAGTCTC	61	120
	GGAGAACCTAGAAGACACACTTCCTCC	61	
Choline acetyltransferase	GTTATAACCCCCAGCCTGAGGCC	61	120
	GGTCTCTCATGTCAACAAGGCTCGC	61	
M1 muscarinic R	AGGAACAACCTGGCCAAGAGAAAGACCTT	60	120
	GCACCATGATGTTATATGGTGTCCAGGT	60	
B2 Nicotinic R	TCCCTCGACGTACCGCTGGTG	60	115
	GCGTGGTAGGCGAACGGTGG	60	
GABA-B1	CCGTGGCTTGACTCGCGACC	60	117
	AGGCACTTGCGCACCTTGGGC	60	
EAAT1 glial	ACCATTAAACATGGATGGGACCGCCC	61	121
	CGGCCGTGGCTGTGATGCTTATTG	61	
EAAT2 glial	CATTAAACATGGATGGCACAGCCCTTAC	60	120
	TGCCAGGTTTCGGTGTCTTGGC	61	
EAAT3 neuronal	ACTGTCCTGAGCGGGCTTGCAATC	61	117
	TGTCAGGAGAGCCTGCGCCATAC	61	
EAAT4 neuronal	GCCTGCAGACCATGACCCGAG	60	125
	GAGCTGGTACGGGCGCAAGG	60	
GABA-B1	CCGTGGCTTGACTCGCGACC	60	117
	AGGCACTTGCGCACCTTGGGC	60	
GABA transporter	CAGGGTGGCATTATGTCTTCAAACCTGTTTG	60	123
	ATAGAACCGGTTGACACCATAAAACCAGG	60	

Gene Name	Sequence	T _m (°C)	Length (nt)
Kainate 5	TCCTGGTTGTCGTTCTCGGGC	60	117
	AGCCGGCATCTTCTCCCTC	60	
NR1	AACAAGCGCGGACCCAAGGCAG	60	121
	CGCTTGAGAAAGGATGATGACCCG	61	
mGluR5	TGGCTCTGGCCCCGGTGCA	60	121
	GAAGCGCTCCTCTGCCTCTGC	60	
5HT-1d	CGGTGAACGAGCGGTGACCC	60	117
	TGGTTCTGCTCTGCTGACGGCTTTG	61	
5HT-2c	CGTCGGCGTCGTGGAGATCG	60	120
	TCCAGCATCTCCGCGCCAATCG	60	
vAChT	TCCTTCGCGCCCTTAGTGGTCTC	61	120
	GCCATAGACTGATACGTGGCGCAC	60	
vGlut1	AGAAGCGGCAGGAAGGCGCG	60	120
	TGTAGCGACGAGGGAGGCCAAAG	61	
vGlut2	TTGGTGCTTGCAGTAGGATTCAGTGGATT	60	120
	CAGCGTGCCAACGCCATTTGAAATGC	61	
VIATT	CGCTGAGGCTACGCGAGGTG	60	118
	CCGAAGCCTCTGTGTGGACATGG	61	
Cnr1; CB1-Receptor	AAGGCTCACAGCCACGAGTTCTG	61	120
	TAATGTCCATGCGGGCTTGATCAGG	61	
Adra1a	TGCATCAACCCATCATATACCCATGCTC	60	120
	AGTGTAGCCAGGGCATGCTTGG	61	
Adra2b	CCACCTTGACGACGCTCAG	60	120
	GCTCAGCTGTGTCCGACGGC	60	
Htr1b	GAACCAAGTCAAAGTGCAGTCTCAGAC	61	120
	GCCAACACACAATAAATGCTCCTAAATGATCC	61	
Htr2a	GGACGATGCAGTCCATCAGCAACG	61	120
	TGCAGATGACGGCCATGATATTGGTGATG	61	
Gabbr2	GAAGATTGGAAGACCTCCACCTCAGTC	61	120
	GTCTTTGTCCAGCTCTGTGATCTTCATTC	60	
Gabrd	CAGGAAGAAACGGAAAGCCAAGGTCAAG	61	120
	GAGATAGCCAACTCCTGACTGACC	61	
Grik3	CATCGACTCCAAGGGCTATGGCATC	61	120
	CACTTCTCCTTCATGATGTGCAGTTTGTC	60	
Chrm5	TGTTCTTCCCAGTGTCCAAAGACCC	61	120
	CAAGGTCTGCGCGCTTCTCTCT	61	
Chrna7	CGCTGCAGCCTGGCCAGTGTGGAGC	60	120
	AGTCTGGAGTTGGGGCACAGTGC	61	
Sesn3	CGAATGGTCTACAATCTCACCTACAACAC	60	120
	CATAGTCATCATACCTGATTCCAAACATACAGTG	61	
Hipk2	CAGCAGCGCACAGGGCACAAAC	60	120
	TTCAGTGGCTGGGTCTTCAGTGG	61	
Add2	GACCACGTGGATGAAGGCTGATGAAG	61	120
	ATGTCCAGAACTTCTGGGGGTCAG	61	
Kcnb1	AGACTCATGGAGACCAACCCCATCC	61	120
	GAAGTTAACCTTGAGCGCTCGCAGC	61	
Shc3	ACGGAAGGACCTCTTTGACATGAAGCC	61	120
	GGGGCTCTGGGTGTGACCG	60	

Online Supporting Material

Supplementary tables are available online at:

http://intron.ccam.uchc.edu/data/pipeline/jodi/THESIS_supplementary_files/CHAPTER_3

These materials are also available at the journal of publication:

<http://onlinelibrary.wiley.com/doi/10.1111/j.1601-183X.2012.00873.x/supinfo>

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CHAPTER 4

Synaptic plasticity, a symphony in GEF

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^{*} These two authors contributed equally to this work

Abstract

Dendritic spines are the postsynaptic sites for the majority of excitatory synapses in the mammalian forebrain. While many spines display great stability, others change shape in a matter of seconds to minutes. These rapid alterations in dendritic spine number and size require tight control of the actin cytoskeleton, the main structural component of dendritic spines. The ability of neurons to alter spine number and size is essential for the expression of neuronal plasticity. Within spines, guanine nucleotide exchange factors (GEFs) act as critical regulators of the actin cytoskeleton by controlling the activity of Rho-GTPases. In this review we focus on the Rho-GEFs expressed in the nucleus accumbens and localized to the postsynaptic density, and thus positioned to effect rapid alterations in the structure of dendritic spines. We review literature that ties these GEFs to different receptor systems and intracellular signaling cascades and discuss the effects these interactions are likely to have on synaptic plasticity.

Introduction

In the mammalian forebrain, the vast majority of excitatory synapses (75-90%) terminate on dendritic spines^{211,212}. While many of these spines are stable, lasting for months even *in vivo*, one of the key hallmarks of spines is their lability. Dendritic spine size changes in

response to a variety of extracellular stimuli. Of particular importance, stimuli that induce long-term potentiation, believed to be the cellular correlate of learning, result in increases in both spine size and spine density in *ex vivo* slice preparations²¹³⁻²¹⁷. Additionally, animals that have undergone training in a fear conditioning paradigm have altered dendritic spine content and morphology in the amygdala, the brain region responsible for this type of learning^{218,219}.

While alterations in dendritic spine plasticity are important for learning and memory, it is likely that they play a role in pathological plasticity as well. Chronic treatment with cocaine or amphetamine increases spine density in the nucleus accumbens (NAc), while chronic morphine treatment decreases spine density in this crucial region^{96,99,220-222}. In addition to addiction, various forms of mental retardation and psychiatric conditions such as schizophrenia have been linked to changes in spine morphology²²³⁻²²⁸. For example, patients with fragile X syndrome demonstrate a profound increase in spine density²²⁹⁻²³², while a number of non-syndromic mental retardations show decreases in spine density^{233,234}. Down's syndrome patients have significantly decreased dendritic spine density, branching and length in various cortical subregions^{223,235}. Clearly, tight regulation of spine formation is crucial for normal neuronal function.

Key to the regulation of dendritic spine formation and function is the actin cytoskeleton, which makes up the structural core of the spine^{216,236-240}. Actin dynamics are crucial to the plasticity involved in forming and reshaping new spines. Rho-GTPases, a sub-family of the small GTP-binding proteins, are critical for regulation of the actin cytoskeleton and myriad other cellular functions^{115,216,240-243}. We focus here on the signaling pathways used to control these molecular switches at the postsynaptic density.

Control of the Actin Cytoskeleton: Rho Proteins

Rho-GTPases function as tightly regulated molecular switches. In their basal state,

these proteins are GDP-bound and inactive. Activation is controlled by guanine nucleotide exchange factors (GEFs), which catalyze the exchange of GDP for GTP. GTP-bound Rho-GTPases are active and can form high-affinity interactions with their downstream targets, regulating target protein activity and localization²⁴⁴. GTPase activating proteins (GAPs), which accelerate the GTPase activity of their target Rho proteins, return the Rho proteins to their inactive, GDP-bound state. While there are 61 Rho-GEFs and 68 Rho-GAPs encoded by the human genome, there are only 20 Rho-family GTPases²⁴⁵ (<http://www.uniprot.org/>). The gene tallies differ slightly in the mouse, with 58 Rho-GEFs, 56 Rho-GAPs and 20 Rho-GTPases (<http://www.uniprot.org/>). In both species, the excess of GEFs and GAPs over GTPases presents an interesting regulatory quagmire and puts a premium on sites of expression and subcellular localization.

Since we are interested in understanding how drugs of abuse hijack the mechanisms that control synaptic plasticity, we focus on the nucleus accumbens (NAc), a region of the ventral striatum containing primarily GABAergic medium spiny neurons that receive heavy dopaminergic innervation from the ventral tegmental area and glutamatergic inputs from the prefrontal cortex^{87,184}. Based on extensive behavioral data, the nucleus accumbens is frequently called the “reward center” of the brain^{94,95,121,246-248}. mRNA-Seq technology³ was used to identify 31 Rho-GEF, 26 Rho-GAP and 14 Rho-GTPase genes whose transcripts are expressed at significant levels in the adult mouse NAc (Eipper-Mains, J.E., unpublished observations). Official gene names, synonyms and associated identifiers for all annotated Rho-GEF, Rho-GAP and Rho-GTPase genes in the mouse genome are provided in **Supplementary Table 1**.

The epicenter of the dendritic spine is the postsynaptic density (PSD), an electron dense structure composed of over 500 proteins, including scaffolds, ligand gated ion channels, receptors and signaling molecules²⁴⁹⁻²⁵¹. In order to focus on the Rho-GEFs, GAPs and GTPases that are localized to the PSD, we compared our list of expressed transcripts to the

forebrain PSD proteome^{66,252,253}. Ten of the Rho-GEFs expressed at significant levels in the NAc have been identified in purified PSDs or otherwise localized to the PSD²⁵⁴ (J E-M, unpublished observations). Official gene names, synonyms and SMART structures of the major neuronal isoforms for these Rho-GEFs are shown in **Fig. 4.1**; five terminate with a PDZ-binding motif and five include an SH3 domain. Of the Rho-GTPases expressed in the NAc, only Rac1 and RhoG have been localized to the PSD (J E-M, unpublished observations). Localization to the PSD puts these Rho-GEF/Rho-GTPase pairs in a unique position to respond rapidly to extracellular stimuli and modify the actin cytoskeleton in a manner dependent on synaptic activity. Of the Rho-GAPs expressed in the NAc, only 3 are known to localize to the PSD (AU040829, Bcr, D15Wsu169e) (J E-M, unpublished observations). While Rho-GAPs clearly play an integral part in the regulation of Rho-GTPase activity, their role in synaptic plasticity is outside the scope of this review. We focus our review on how the PSD-localized Rho-GEFs are regulated by extracellular and intracellular signals and the roles they might play in modulating dendritic spine function.

With respect to dendritic spine dynamics, the best characterized Rho GTPases are Rac1, RhoA and Cdc42. Within spines, Rac1 and RhoA seem to play antagonistic roles, with Rac1 promoting spine formation and growth and RhoA promoting spine retraction and decreases in spine density^{241,255,256}. Cdc42 also promotes spine formation and is present in spines^{227,257}, but has not been localized to the PSD. We begin with a review of the Rho-GEFs that preferentially activate Rac1 before moving on to those that activate RhoA and Cdc42.

Rac1-Specific Rho-GEFs

Tiam1

Tiam1 (T-cell lymphoma invasion and metastasis 1) is a Rac1-specific GEF with a single Dbl homology-pleckstrin homology (DH-PH) domain near its C-terminus, a PDZ-binding motif

Figure 4.1 Rho-GEFs and Rho-GTPases expressed in the NAc and localized to the PSD

Gene diagrams are drawn to scale and represent SMART structures (<http://smart.embl-heidelberg.de/>) with several modifications (see **Supplementary Table 2**). Abbreviations: C1, Protein kinase C conserved region 1 (C1); C2, Protein kinase C conserved region 2 (CalB); EH, Eps15 homology domain; IQ, Short calmodulin-binding motif containing conserved Ile and Gln residues; PDZ, Domain present in PSD-95, Dlg, and ZO-1/2; PH, Pleckstrin homology domain; RasGEF, Guanine nucleotide exchange factor for Ras-like small GTPases; RasGEFN, Guanine nucleotide exchange factor for Ras-like GTPases, N-terminal motif; RBD, Raf-like Ras-binding domain; RhoGAP, GTPase-activator protein for Rho-like GTPases; RhoGEF, Guanine nucleotide exchange factor for Rho/Rac/Cdc42-like GTPases; SEC14, Domain in homologues of a *S. cerevisiae* phosphatidylinositol transfer protein (Sec14p); SH3, Src homology 3 domains; SPEC, Spectrin-like repeats.

at its C-terminus, and an additional PH domain closer to its N-terminus (**Fig. 4.1**)²⁵⁸.

Additionally, Tiam1 has an internal PDZ domain that binds to the C-terminal peptide sequence Tyr-Tyr-(Phe/Ala), although neuronal interaction partners have not yet been identified²⁵⁸.

When expressed in fibroblasts, Tiam1 induces membrane ruffling and a morphology resembling that of constitutively activated Rac1²⁵⁹. Interestingly, it is the more N-terminal PH domain of Tiam1 that is essential for its membrane localization, and deletion of this domain leads to cytosolic dispersal and a lack of induced membrane changes²⁶⁰. Studies in neuroblastoma lines demonstrated that over-expression of Tiam1 increases Rac1-dependent neurite outgrowth and insensitivity to RhoA-induced neurite retraction²⁶¹. While Tiam1 was not identified in purified rat forebrain PSDs^{66,252}, immunogold electron microscopy demonstrates that Tiam1 is intercalated into the PSD²⁵⁴. This study, which was the first to examine Tiam1 in primary neurons, demonstrated that Tiam1 is necessary for the elaboration of a full dendritic arbor. Neurons transfected with RNAi targeted against Tiam1 showed a dramatic reduction in spine density, and the remaining spines were longer and more filopodial than spines in control cultures²⁵⁴.

Neurons expressing Tiam1 RNAi also exhibited a sharp decrease in miniature postsynaptic currents, indicating a functional as well as a morphological change²⁵⁴. The ability of Tiam1 to interact directly with the NR1 NMDA receptor subunit, but not with any of the NR2 subunits, presumably contributes to this deficit (**Fig. 4.2A**). Stimulation of NMDA receptors in control cultures leads to an increase in spine density and size; neurons expressing Tiam1 RNAi showed no such changes in spine morphology²⁵⁴. This result is particularly compelling because it presents a strong functional connection for the interaction of Tiam1 and NR1. Further linking NMDA receptors to Tiam1 signaling, Tiam1 is phosphorylated on unknown Thr residues by CamKII and protein kinase C (PKC), enhancing its GEF activity about 2-fold^{262,263} (**Fig. 4.2A**). Given that CamKII and PKC are indirectly activated by calcium^{264,265} and both represent major

Figure 4.2 Rac-specific GEFs at the PSD

(A) Tiam1 with NR1 subunit of NMDA receptor, EphB and TrkB. (B) Kalirin7 with PSD-95, PI-3-P/PI-3,5-P2, NR2B subunit of NMDA receptor, EphB, Cdk5, and AF-6/N-cadherin. (C) Arhgef7/ β -Pix with CamKK/CamKI/PKA, GluR2/3 subunit of AMPA receptor via GIT1/liprin- α /GRIP, Pak, Shank, and EphB. (D) Rasgrf2 with Cdk5, NMDA and AMPA receptors.

components of the PSD⁶⁶, the anchoring of Tiam1 to NMDA receptors may enhance the response of the synapse to the calcium influx that results from NMDA receptor stimulation²⁶⁶.

In addition to NMDA receptors, Tiam1 also forms a stable interaction with the receptor tyrosine kinase EphB2²⁶⁷ (**Fig. 4.2A**). EphB2 is stimulated by its presynaptic binding partner EphrinB, increasing dendritic spine number and size²⁶⁸. Stimulation of cultured neurons with EphrinB leads to a clustering of Tiam1 at the sites of new synaptic contacts. At these sites, Tiam1 and EphB2 form a stable complex with EphB2 binding to the N-terminal PH domain of Tiam1. As a result, EphB2 phosphorylates Tiam1 at Tyr⁸²⁹²⁶⁷ (**Fig. 4.2A**). In COS7 cells, a phosphomimetic point mutant at this site leads to an increase in Rac1 activation²⁶⁹. Importantly, RNAi knockdown of Tiam1 ablates the ability of activated EphB2 to increase spine formation in cultured hippocampal neurons, indicating that this pathway is Tiam1-dependent²⁶⁷. The interaction of Tiam1 with both NMDA receptors and EphB2 begins to paint an interesting picture of an important molecular complex at the PSD. EphB2 has repeatedly been shown to be important for NMDA receptor clustering and function²⁷⁰⁻²⁷³. Additionally, stimulation of both EphB2^{268,270} and NMDA receptors²⁷⁴ has been implicated in the growth and formation of new spines. As a direct interactor with both proteins, Tiam1 is well-positioned to integrate voltage-sensitive, ligand-gated ion channel signaling and receptor tyrosine kinase signaling to effect activity-dependent changes in the actin cytoskeleton.

Finally, Tiam1 interacts with another receptor tyrosine kinase, TrkB²⁶⁹ (**Fig. 4.2A**). The binding of BDNF to TrkB has long been known to be important for the formation and maturation of synapses²⁷⁵. Tiam1 interacts with TrkB via its N-terminal PH domain and is phosphorylated on Tyr⁸²⁹ (as with EphB2)²⁶⁹. Expression of a non-phosphorylatable Tiam1 mutant (Tyr⁸²⁹Phe) blocked the BDNF-induced increase in dendritic spines, demonstrating an essential role for phosphorylation at this site²⁶⁹. Although a Tiam1 knockout mouse has been created and is viable²⁷⁶, it has primarily been used for cancer research.

Kalirin7

In the adult brain, Kalirin7, with a single GEF domain specific for Rac1 and RhoG, is the most abundant splice variant of the *Kalrn* gene^{277,278} (**Fig. 2.1**); several larger Kalirin isoforms, with a second GEF domain specific for RhoA, are expressed at lower levels, but are not localized to the PSD²⁷⁹⁻²⁸². Kalirin7 terminates with a PDZ-binding motif and it is estimated that the average hippocampal PSD, which contains about 20 NMDA receptors and 15 AMPA receptors²⁴⁹, contains 10 copies of Kalirin7¹³³. At the N-terminus of Kalirin7 is a Sec14p domain that binds phosphatidylinositol-3-phosphate and phosphatidylinositol-3,5-bisphosphate and is essential for membrane localization²⁸³; this is followed by 9 spectrin-like repeats (**Fig. 2.1** and **2.2B**).

A yeast two-hybrid screen with the PDZ-binding motif of Kalirin7 identified 16 PDZ domain-containing interactors²⁸⁴. Given that these PDZ domain-containing proteins interact with a variety of receptor subtypes (e.g. NMDA, AMPA, 5-HT), protein kinases (e.g. PKA, CamKII) and filamentous actin, this places Kalirin7 in a unique position to integrate a wide variety of signals at the PSD²⁸⁵. The interaction of Kalirin7 with PSD-95 decreases its ability to activate Rac1 (**Fig. 2.2B**). Kalirin7 was identified in the NMDA receptor interactome after affinity-purification using a peptide from the C-terminus of the NR2B subunit⁶⁶; this interaction was verified by co-immunoprecipitation of Kalirin7 with NR2B (Kiraly, D.D., unpublished observations). Consistent with this, PSDs purified from mice lacking Kalirin7 have decreased levels of NR2B¹³³. Unlike Tiam1, Kalirin7 does not co-immunoprecipitate with NR1²⁵⁴. Tiam1, through its association with the NR1 subunit²⁵⁴, and Kalirin7, through its association the NR2B subunit, may serve to modulate critical signaling steps downstream of specific NMDA receptor subsets.

The ability of Kalirin7 to increase spine formation *in vitro* has been demonstrated in cultured cortical and hippocampal neurons^{284,286,287}. Perhaps most compelling, Kalirin7

expression in otherwise aspiny interneurons leads to the formation of spine-like structures that acquire apposing presynaptic terminals and appear to function as active synapses²⁸⁸. Recently, these findings were validated *in vivo* with the development of a Kalirin7 knockout mouse. These mice display a decrease in dendritic spine density in the hippocampus as well as impaired hippocampal LTP and focal hippocampus-dependent learning impairments¹³³. Much has been done to clarify the intracellular molecular interactors with Kalirin7. In cultured cortical neurons, Kalirin7 is essential for EphB2-induced spine formation^{287,289} (**Fig. 2.2B**). Stimulation of cell cultures with clustered EphrinB led to a modest tyrosine phosphorylation signal on Kalirin7, although the location of the phosphorylated residue was not established²⁸⁷. Kalirin7 and Tiam1 may work in parallel pathways to propagate signals downstream of this receptor tyrosine kinase.

It has been suggested that the N-terminus (Thr⁹⁵) of Kalirin7 is phosphorylated by CamKII and that phosphorylation at this site is essential for Kalirin7 to display GEF activity²⁹⁰ (**Fig. 2.2B**). However, mutation of Thr⁹⁵, which is not a consensus CamKII site^{264,291,292}, to a non-phosphorylatable Ala (Thr⁹⁵Ala) did not eliminate its GEF activity, and the GEF activity of Kalirin7 and shorter splice variants lacking this region (Δ Kal7) are indistinguishable *in vitro*^{283,293,294}. Co-immunoprecipitation of Kalirin7 and GluR1 led Xie and colleagues to hypothesize that Kalirin7 is necessary for the movement of GluR1 into newly formed dendritic spines upon NMDA receptor stimulation of cortical cultures²⁹⁰. Mice genetically lacking Kalirin7 have normal levels of GluR1 in both tissue homogenates and purified PSDs¹³³, so Kalirin7 is not essential for AMPA receptor localization *in vivo*.

The Kalirin7 PDZ-binding motif interacts with the PDZ domains of AF-6/Afadin, Neurabin and Spinophilin, each of which can bind directly to filamentous actin. AF-6 also mediates the interaction of Kalirin7 with N-cadherin, a trans-synaptic protein that forms homophilic interactions with presynaptic N-cadherin^{284,295} (**Fig. 2.2B**). N-cadherin plays an

important role in the regulation of dendritic spine morphology^{296,297}. Stimulation of neuronal cultures with clustered N-cadherin results in an increase in dendritic spine size and recruits AF-6, Kalirin7 and Rac1 into dendritic spines²⁹⁵. When expression of all isoforms of Kalirin was reduced using RNAi, clustered N-cadherin was no longer able to change dendritic spine morphology²⁹⁵.

Kalirin7 is phosphorylated at a single site (Thr¹⁵⁹⁰) by Cdk5 (**Fig. 2.2B**), a synaptically-localized serine/threonine kinase known to play an important role in synapse development and modulation; Cdk5 is localized to membranes and activated by binding to p35 or p39, not to a cyclin^{114,120,294,298-303}. Phosphorylation of Thr¹⁵⁹⁰ increases the GEF activity of Kalirin, with the phosphomimetic mutant (Thr¹⁵⁹⁰Asp) showing a two-fold increase in activity over the non-phosphorylatable mutant (Thr¹⁵⁹⁰Ala). Mutation of Thr¹⁵⁹⁰ to Asp or Ala did not affect the ability of Kalirin7 to increase spine density. While neurons expressing Thr¹⁵⁹⁰Asp-Kalirin7 exhibited increased dendritic spine size, neurons expressing Thr¹⁵⁹⁰Ala-Kalirin7 did not²⁹⁴. The fact that Kalirin7 knockout mice show a decrease in Cdk5 levels in purified synaptosomes and PSDs¹³³ suggests a role for reciprocal regulation of Kalirin7 and Cdk5 *in vivo* as well as *in vitro*.

Arhgef7/β-Pix

Arhgef7 (β-Pix) was identified based on its ability to activate Rac1, Cdc42 and Pak (p21 activated kinase)³⁰⁴ (**Fig. 2.2C**). Two isoforms of β-Pix, β1 and β2, which differ only at their C-termini, are the major ones expressed in the mammalian brain³⁰⁵. The β1 isoform C-terminus has a coiled-coil domain and a PDZ-binding motif while the β2 isoform C-terminus has a serine-rich region (**Fig. 2.2C**)³⁰⁵. The N-terminal SH3 domain of β-Pix binds to Pak, recruiting Pak to Rac1- and Cdc42-induced adhesion complexes; mutations that inactivate this SH3 domain lead to mislocalization of Pak³⁰⁴. Crystal structure analysis detailed the interactions of the SH3 domain of β-Pix with Pak³⁰⁶. β-Pix is phosphorylated by Pak on Thr⁵²⁶, increasing its

membrane localization and boosting its GEF activity, ultimately eliciting neurite formation in PC12 cells³⁰⁷.

When studied in primary hippocampal cultures, it became clear that β -Pix is recruited to synapses by GIT1 (G-protein-coupled receptor-kinase-interacting protein 1), which is present in the PSD^{66,308}. GIT1 is a ubiquitously expressed ARF-GAP (ADP-ribosylation factor GTPase Activating Protein) that is enriched at the PSD and complexes with AMPA receptor subunits in the rat brain through its association with liprin- α ³⁰⁹. Liprin- α is a multi-functional protein with a number of known interacting proteins including the GRIP/ABP family (glutamate receptor interacting proteins) of multi-PDZ domain containing proteins. Through their PDZ domains, GRIPs at the PSD interact with a number of different proteins including the AMPA receptor subunits GluR2 and GluR3, the receptor tyrosine kinase EphB2 and the neuronal Ras-GEF GRASP-1^{309,310}.

The GIT1-binding domain of β -Pix lies just upstream of the C-terminal coiled-coil and the C-terminal serine-rich regions of β 1- and β 2-Pix, respectively³⁰⁵. The ability of GIT1 to recruit β -Pix to synapses is necessary for the formation of new dendritic spines with apposing presynaptic terminals. When the β -Pix-binding domain of GIT1 was deleted, β -Pix showed a more diffuse localization, leading to a multitude of aberrant cell protrusions that were not associated with presynaptic terminals. The GEF activity of β -Pix is necessary for the formation of new spine-like structures in hippocampal neurons³⁰⁸. GIT1 is not solely responsible for the localization of β -Pix to synapses. β 1-Pix binds to the PDZ domain of Shank via its C-terminal PDZ-binding motif²⁵⁰ or its coiled-coil domain³¹¹ and over-expression of Shank recruits additional β -Pix to dendritic spines³¹¹ (**Fig. 2.2C**), blocking actin bundling³¹². While β -PIX does not interact with the PDZ domains of PSD95, Sap97, S-Scam, GRIP1, Lin7 or Densilin, it directly interacts with the PDZ domain of Scribble, a protein most studied in cancer biology and

epithelial polarity; in PC12 cells stimulated with KCl, Scribble recruits β -Pix to the plasma membrane³¹³.

β -Pix modulates Ca^{2+} -dependent intracellular signaling. β -Pix co-immunoprecipitates with CamKK (CamK-kinase) in transfected cells and rat brain lysates³¹⁴. This interaction is mediated via the SH3 domain of β -Pix, the region also known to interact with Pak. β -Pix is phosphorylated by CamKI, a downstream target of CamKK, on Ser⁵¹⁶, a site known to be phosphorylated by PKA³¹⁵. The GEF activity of β -Pix is stimulated in a Ca^{2+} -dependent manner and mutation of Ser⁵¹⁶ to a non-phosphorylatable mutant (Ser⁵¹⁶Ala) obviated the Ca^{2+} -dependent changes in Rac1 activation³¹⁴. Expression of dominant negative CamKI, Ser⁵¹⁶Ala- β PIX, or siRNA for either CamKI or β -Pix resulted in a marked decrease in dendritic spine density in cultured neurons³¹⁴. This study clearly demonstrates a role for β -Pix in calcium-calmodulin signaling cascades.

While β -Pix has not been directly linked to any cell-surface receptor in mammals, deletion of the corresponding *Drosophila* gene leads to decreased synaptic levels of the glutamate receptor subunit GluRIIA³¹⁶. In mice, β -Pix is linked to GluR2/3 AMPA receptor subunits via the GIT1/liprin- α /GRIP complex described above. Disruption of the GIT1/liprin- α interaction results in decreased AMPA receptor clustering in cultured neurons³⁰⁹. Taken together, the *Drosophila* and mouse data suggest a role for β -Pix in proper postsynaptic AMPA receptor targeting via its interaction with GIT1/liprin- α /GRIP. Additionally, GIT1 is indirectly recruited to synapses by EphrinB stimulation of the EphB2 receptor tyrosine kinase³¹⁷. Three PSD Rho-GEFs, β -Pix, Tiam1, and Kalirin7, mediate morphogenic signals downstream of EphB2^{267,287,318,319}.

Rasgrf2

Rasgrf2 is unique in that it contains both a Rho-GEF DH-PH domain as well as a Ras-

GEF domain (**Fig. 2.1**). Rasgrf2 has a C-terminal type II PDZ-binding motif with no known binding partners²⁴⁵. Ras promotes the creation of larger spines receiving excitatory synapses and is normally held in check by SynGAP³²⁰. Interestingly, Rasgrf2 has been linked to the Cdk5/p35 signaling pathway in neurons. Cdk5 phosphorylates Rasgrf2 on Ser⁷³⁷, decreasing its Rac1-GEF activity, but has no effect on its ability to activate Ras³²¹ (**Fig. 2.2D**). Additionally, while Rasgrf2 is typically found along dendrites in basal conditions, co-expression of p35 and Cdk5 leads to the retraction of Rasgrf2 into the cell soma³²¹. This is another indication of the many ways in which Cdk5 activity affects PSD-GEF function. The Ras-GEF domain of Rasgrf2 has been implicated in the control of both NMDA and AMPA glutamate receptors, but will not be discussed here as we are focusing on Rho-GEFs at the PSD³²²⁻³²⁴.

RhoA/Cdc42-Specific Rho-GEFs

Ngef/Ephexin1

In the NAc, transcripts encoding Ngef (Ephexin1) are more prevalent than those encoding any other Rho-GEF (JE-M, unpublished observations). Ephexin1 has C-terminally located DH, PH and SH3 domains (**Fig. 4.1**). Ephexin1 activates Rac1, Cdc42, and RhoA³²⁵; different signaling pathways shift its specificity towards Rac1/Cdc42 or towards RhoA, altering the downstream response. Ephexin1-mediated activation of Rac1 has been studied in the context of axon outgrowth and neuronal development³²⁵⁻³²⁸. While the events leading to Rac1 activation by the Rho-GEFs discussed above are generally well characterized, the events involved in Ephexin1-mediated Rac1 and Cdc42 activation are not well understood. Pak, which is involved in β -Pix activation of Rac1, demonstrates increased activity in the context of Ephexin1-mediated axonal outgrowth³²⁵. Pak may complex with Ephexin1 and shift its GEF activity towards Rac1/Cdc42 activation and actin polymerization³²⁵ (**Fig. 4.2A**).

A great deal more is known about the role of Ephexin1 in RhoA activation. The

generation of an Ephexin1 knockout mouse facilitated studies into the role of Ephexin1 in axonal outgrowth and growth cone collapse in cultured retinal ganglion cells (RGCs)³²⁶. Ephexin^{-/-} RGCs exhibit the same number of neurites per cell but have significantly shorter axons than their wild-type counterparts³²⁶. Incubation of Ephexin1^{-/-} RGCs with EphrinA1 does not elicit the wild-type phenotype of growth cone collapse, implicating an EphA4/Ephexin1 interaction in the control of EphrinA1-induced growth cone collapse. It should not go unmentioned that Ephexin1 knockout mice show no overt axonal phenotype *in vivo*³²⁶, perhaps due to functional redundancy amongst RhoGEFs.

The DH-PH domain of Ephexin1 binds the kinase domain of EphA4 in cultured fibroblasts³²⁵; a direct interaction between EphA4 and Ephexin1 has not been demonstrated by co-immunoprecipitation in neurons³²⁶. Ephexin1 is phosphorylated following EphrinA1 stimulation of EphA4 receptors, and this phosphorylation event is required for growth cone collapse in cultured RGCs³²⁶. Stimulation of the EphA4 receptor by EphrinA1 in neuronal cultures results in EphA4 autophosphorylation and recruitment of two kinases, Cdk5/p35 and Src, to EphA4 at the PSD^{301,327}. EphrinA1 binding causes a loss of dendritic spines and a corresponding decrease in miniature excitatory postsynaptic current (mEPSC) frequency³⁰¹. The EphA4 receptor co-immunoprecipitates with Cdk5/p35 from rat brain extracts, suggesting a direct interaction *in vivo*³⁰¹.

Activated EphA4 phosphorylates the recruited Cdk5, stimulating its kinase activity and its subsequent phosphorylation of Ephexin1 at three sites, Thr⁴¹, Thr⁴⁷, and Ser^{139/301} (**Fig. 4.3A**). Mutant Ephexin1, in which the Cdk5 target residues are replaced with non-phosphorylatable Ala residues, eliminates the ability of Src to phosphorylate Ephexin1 on Tyr⁸⁷; Ephexin1 with Glu at these same sites supports robust EphrinA1/EphA4-mediated Ephexin1 phosphorylation at Tyr^{87/301}. The kinase domain of EphA4 does not directly phosphorylate Ephexin1 at Tyr^{87/326}. Rather, EphA4 and Src demonstrate ligand-induced association with subsequent stimulation

Figure 4.3 Rho/Cdc42-specific GEFs at the PSD

(A) Ngef/Ephexin1 with Pak, EphA4, Src, and Cdk5. (B) Arhgef2/Lfc/GEF-H1 with a microtubule, Spinophilin, GluR1 subunit of AMPA receptor, ERK1/2, AKAP121/PKA, and 14-3-3. (C) Arhgef9/Collybistin with Gephyrin and Neuroigin-2. (D) Itsn1/Intersectin-L with N-WASP, EphB2, and Arp2/3.

of the tyrosine kinase activity of Src³²⁷. Activated Src phosphorylates Ephexin1 at Tyr⁸⁷, an event that shifts the GEF activity of Ephexin1 towards RhoA and ultimately results in growth cone collapse^{326,329} (**Fig. 4.3A**). Inhibition of Src-dependent phosphorylation of Ephexin1 at Tyr⁸⁷ obviates EphrinA1/EphA4-induced repulsive axon guidance in retinal axons³²⁷. Similarly, analysis of hippocampal cultures from Cdk5^{-/-} mice, revealed an obligatory role for Cdk5 in the EphrinA1-mediated loss of dendritic spines and decrease in mEPSC frequency³⁰¹.

There is evidence in cultured cells that the FGF and EphA receptors form hetero-oligomers and cross-activate one another³³⁰. Activated FGF receptor phosphorylates Ephexin1 at several tyrosine residues, including Tyr⁸⁷, and shifts its GEF activity towards RhoA³²⁹. While the FGF receptor is known to be present at presynaptic terminals and is expressed in astrocytes³³¹, it is not present at the PSD^{66,252} and is thus unlikely to play a major role in dendritic spine actin dynamics.

Arhgef2/Lfc/GEF-H1

Arhgef2 (Lfc; GEF-H1) is a RhoA-specific GEF with a single DH-PH domain and two coiled coil regions near its C-terminus³³² (**Fig. 4.1**). Under basal conditions Lfc is found predominantly in dendritic shafts, where it interacts with microtubules via its PH domain^{332,333}. In cultured neurons, NMDA receptor activation causes the rapid translocation of Lfc into dendritic spines³³³ (**Fig. 4.3B**). A recent study examining Lfc localization in primate cortex at the electron micrograph level also concluded that Lfc was primarily localized to dendritic shafts under basal conditions, translocating rapidly into spines upon synaptic stimulation³³⁴. Once in spines, Lfc interacts with Spinophilin and Neurabin, which bind filamentous actin³³³ (**Fig. 4.3B**). One study, by Ryan and colleagues, demonstrated that over-expression of Lfc in cultured neurons produces a decrease in spine size and an increase in spine density³³³. Consistent with this, a more recent study by Kang and colleagues demonstrated that

expression of dominant negative Lfc or shRNA targeted to Lfc increased spine size³³⁵. However, in the more mature cultures studied by Kang and colleagues, reductions in Lfc action increased spine density, perhaps suggesting developmental alterations the Lfc/RhoA pathway³³⁵. Activation of RhoA has typically been associated with decreased spine density^{241,336}.

Even under basal conditions, Kang and colleagues found Lfc in the PSD, a finding supported by PSD proteomics studies^{66,252}. Based on both proteomic analyses of AMPA receptor complexes and co-immunoprecipitation with the GluR1 and GluR2 AMPA receptor subunits, Lfc interacts with AMPA receptors. Incubation of hippocampal cultures with an AMPA receptor antagonist normally leads to a decrease in dendritic spine density; in neurons expressing Lfc-shRNA, this effect is ablated³³⁵. Suppression of AMPA receptor function normally increases RhoA activity; this response is diminished when Lfc expression is reduced using Lfc-shRNA, and stimulation of AMPA-R inhibits Lfc activity and RhoA signaling³³⁵ (**Fig. 4.3B**). Lfc seems to play a crucial role in RhoA-mediated spine retraction.

The GEF activity of Lfc is also controlled by phosphorylation³³⁷⁻³³⁹. Phosphorylation by kinases Aurora A/B (at Ser⁸⁸⁵) or Cdk1/Cyclin B (at Ser⁹⁵⁹) decreases Lfc-RhoGEF activity³³⁷ while phosphorylation by ERK1/2 (at Thr⁶⁷⁸) enhances Lfc-RhoGEF activity³³⁸. PKA phosphorylates Ser⁸⁸⁵, with concomitant AKAP121 binding to Lfc in HEK293 cells; the phospho-specific scaffolding protein, 14-3-3, then binds to P-Ser⁸⁸⁵-Lfc, decreasing the GEF activity of Lfc³⁴⁰ (**Fig. 4.3B**). Though proteomics studies have localized PKA to the PSD^{66,252}, the role of PKA-dependent phosphorylation in the regulation of Lfc-RhoGEF activity in neurons remains to be studied.

Arhgef9/Collybistin

Arhgef9 (Collybistin) is a brain-specific Rho-GEF with a single DH-PH domain that is

specific for Cdc42³⁴¹ (**Fig. 4.1**). Two isoforms of Collybistin are expressed in brain: Collybistin1 includes an N-terminal SH3 domain and a C-terminal coiled-coil domain; Collybistin2 lacks these two domains³⁴². Although the identities of the specific players regulating Collybistin-RhoGEF activity and localization remain elusive, it is clear that its SH3 domain plays a key role in these interactions³⁴³.

Collybistin is expressed broadly throughout the forebrain and cerebellum from early embryonic life through adulthood^{342,344}. Collybistin was originally discovered as an interactor with Gephyrin, a protein essential for the formation of inhibitory postsynaptic structures in brain and spinal cord^{341,342}. The linker region between the SH3 and DH domains is essential for the Collybistin-Gephyrin interaction³⁴⁵ (**Fig. 4.3C**). Collybistin with an active PH domain and an intact Gephyrin-binding site is essential for the postsynaptic localization of Gephyrin in dendrites of cultured cortical neurons³⁴³. A patient exhibiting hyperekplexia and epilepsy, both potentially caused by a decrease in inhibitory neurotransmission, had a mutation that disrupted the SH3 region of Collybistin³⁴³. Expression of this mutant protein in cultured cortical neurons led to a loss of GABA_A receptor clustering³⁴³. Another patient with a balanced chromosomal translocation leading to disruption of the Collybistin gene displayed disturbed sleep cycles, increased anxiety, epileptic seizures and mental retardation³⁴⁶. Collybistin clearly plays a key role in the regulation of inhibitory transmission in humans as well as rodents.

The Collybistin knockout mouse displays normal locomotor activity, but anxiety-like behavior is increased dramatically and spatial learning is grossly impaired³⁴⁷. In the hippocampus and amygdala of the Collybistin^{-/-} mouse, GABA_A receptor expression is greatly reduced and inhibitory neurotransmission is decreased. Interestingly, these mice display enhanced CA1 LTP in hippocampal slices while LTD plasticity is impaired³⁴⁷. In contrast, induction of LTP in the dentate gyrus of Collybistin^{-/-} mice is greatly decreased³⁴⁸. Both Collybistin and Gephyrin interact with Neuroligin-2, a trans-synaptic scaffolding protein

essential for the formation of inhibitory synapses³⁴⁹ (**Fig. 4.3C**). The interaction of Collybistin and Neuroligin-2 enhances the ability of Collybistin to recruit Gephyrin to synapses, which in turn leads to the formation of more inhibitory synapses in cultured neurons³⁴⁹. Taken together, these studies demonstrate a clear role for Collybistin in the normal formation of inhibitory synapses and the balance of excitatory/inhibitory transmission in the mammalian forebrain, but leave unanswered the role of the GEF domain of Collybistin in the nervous system.

Itsn1/Intersectin-L

The longer neuron-specific splice variant of the *Itsn1* gene, Intersectin-L, includes C-terminal DH, PH and C2 domains as well as several common protein-protein interacting regions it shares with the shorter, ubiquitously expressed splice variant, Intersectin-S³⁵⁰⁻³⁵² (**Fig. 4.1**). The common region includes two Eps15 homology domains, several coiled-coil regions, and five SH3 domains. Intersectin-S affects neuronal endocytic vesicle formation³⁵². The tandem DH-PH domains of Intersectin-L interact specifically with Cdc42 to stimulate its GTPase activity, and over-expression of Intersectin-L induces formation of filopodia in fibroblasts³⁵⁰.

Regulation of the Rho-GEF activity of Intersectin-L occurs at many levels. Activity assays demonstrate more robust GEF activity in C-terminal DH-PH tail constructs than in full-length Intersectin-L, suggesting a role for N-terminal modulation of the GEF activity of Intersectin-L. N-WASP (Neuronal Wiskott-Aldrich Syndrome Protein), which activates the Arp2/3 actin-nucleating complex, interacts directly with the SH3 domains of Intersectin-L, stimulating its GEF activity; the Intersectin-L/N-WASP complex can be immunoprecipitated from transfected cells and from native brain extracts³⁵⁰ (**Fig. 4.3D**). The N-WASP/Intersectin-L interaction relieves Intersectin-L autoinhibition and may stabilize the GEF domain interaction with GDP-bound Cdc42, catalyzing its activation³⁵⁰. Additionally, the kinase domain of the

receptor tyrosine kinase EphB2 interacts with the N-terminus of Intersectin-L, activating Cdc42 in hippocampal neurons; the Intersectin-L/ EphB2 complex can be immunoprecipitated from extracts of transfected cells³⁵³ (**Fig. 4.3D**).

N-WASP links Cdc42 to the actin cytoskeleton via its ability to stimulate the Arp2/3 complex, causing actin-nucleation^{239,350,354}. Co-localization of EphB2 and N-WASP with Intersectin-L and F-actin in dendritic spines suggests a model in which EphB2 and N-WASP act synergistically to activate Intersectin-L GEF activity^{352,353} (**Fig. 4.3D**). Concomitant binding of N-WASP to the Intersectin-L/Cdc42 complex results in relief of N-WASP autoinhibition and a dose-dependent N-WASP-mediated stimulation of Intersectin-L GEF activity^{239,350}. Activated N-WASP stimulates the Arp2/3 complex, causing actin polymerization^{239,350}. N-WASP plays roles both up- and downstream of Intersectin-L, highlighting the importance that multimolecular complexes play in the regulation of the actin cytoskeleton³⁵⁵.

The *Itsn1* gene has been implicated in Down Syndrome (DS), with DS neurons exhibiting abnormal dendritic spine morphology and altered endosomal pathways³⁵¹. The *Itsn1* gene is located on human chromosome 21, the same chromosome present in extra copy number in Down Syndrome, further suggesting a role for *Itsn1* in the pathophysiology of Down Syndrome³⁵².

Bcr

Bcr (Breakpoint cluster region) negatively regulates cell proliferation and oncogenic transformation³⁵⁶. Bcr and the closely related protein Abr are the only two GTPase-activating proteins that specifically inactivate Rac1³⁵⁷. Bcr both turns Rac1 on (via its GEF domain) and inactivates Rac1 (via its RhoGAP domain) (**Fig. 4.1**). Bcr exhibits GEF activity towards Rac1, Cdc42, and RhoA²⁴⁵. In addition to its DH-PH and GAP domains, Bcr has a serine/threonine kinase domain and a C-terminal PDZ-binding motif that interacts with the PDZ domain of AF-

6/Afadin, a protein known to bind Ras and filamentous actin³⁵⁶. The kinase domain of Bcr phosphorylates AF-6 near its PDZ domain, promoting a direct interaction between Bcr and AF-6³⁵⁶. While the impact of the Bcr/AF-6 interaction on the GEF activity of Bcr remains to be determined, the existence of this phosphorylation-mediated interaction raises the possibility that phosphorylation events can regulate interactions between other PDZ-binding motif-containing Rho-GEFs and their PDZ-domain-containing partners²⁴⁵.

Bcr is among a set of genes expressed very early in development³⁵⁸. Full-length Bcr contributes to NGF-independent neurite formation in PC12 cells³⁵⁹, while a fusion construct including the GEF domain but not the RhoGAP domain suppresses neurite outgrowth³⁵⁹. Mice in which both Bcr and Abr are knocked out show a number of glial cell developmental abnormalities which then precipitate neuronal abnormalities in the cerebellum and midbrain³⁶⁰. A genomic translocation resulting in a fusion between Bcr and the Abl kinase is seen in 95% of patients with chronic myelogenous leukemia (CML)³⁶¹.

Conclusions

As primary regulators of the actin cytoskeleton, Rho-GEFs play the role of conductors in the elegant symphony that is synaptic plasticity. A preponderance of human and animal studies demonstrate that a dynamic balance of synapse formation and elimination is necessary for normal cognition and neuronal function. The delicate balance that is struck between GEF-GTPase activity and the actin cytoskeleton includes several GEFs regulated by the same pathways. A number of GEFs have been shown to be downstream of NMDA receptors (Tiam1, Kalirin7, Rasgrf2, Lfc) and/or Ephrin receptors (Tiam1, Kalirin7, β -Pix, Ephexin1, Intersectin-L), two receptor systems critically linked to the plasticity of the postsynaptic neuron^{319,362}. Additionally, while all of these proteins have been linked to a variety of intracellular signaling cascades, it is noteworthy that a number of them (Kalirin7, Rasgrf2,

Ephexin1) have been linked to Cdk5, a protein known to have myriad effects on the structure and function of PSDs and spines^{120,301,303,363}. Additionally, Tiam1, Kalirin7 and β -Pix have all been linked to calcium-calmodulin kinase signaling pathways, which have a plethora of functions at the synapse and play essential roles in synaptic plasticity^{264,314}. Clearly, this puts a premium on localization of the GEF, its target GTPase, and any other signaling molecules that influence their function.

One of the quandaries raised by this review of the literature is the disparity between cell culture studies and *in vivo* models of these Rho-GEFs. While cell culture studies may indicate that a specific Rho-GEF is “essential” for dendritic spine formation^{254,286}, the phenotype observed in the corresponding knockout animal is often much less profound. Taking Kalirin7 as an example, cultures expressing shRNA for Kalirin7 have almost no normal dendritic spines²⁸⁸; however, Kalirin7 knockout mice display only a 20% decrease in hippocampal spine density. While neither the cell culture nor constitutive knockout system is without its drawbacks, the disparity between these types of studies suggests that serious consideration should be made before interpreting any of these findings in a translational or clinical sense. As new and developing technologies (mRNA-Seq, proteomics, phosphoproteomics, etc.) become more affordable and accessible to researchers, they will facilitate the integration of seemingly solitary signaling pathways into the overlapping web of pathways that is likely occurring *in vivo*.

While extensive research has pushed our understanding of dendritic spines forward, there is clearly still much more that needs to be determined. Localizing activated Rho-GEFs and Rho-GTPases and understanding the kinetics of their activation and inactivation will be critical. It will be important to clarify the conditions under which different receptors activate different Rho-GEFs and how they interact with their target Rho-GTPases. It is only through the clarification of these networks that we will be able to determine their precise role in mental

retardation, drug addiction, schizophrenia or the host of other human pathologies that demonstrate altered synaptic connectivity.

Online Supporting Material

Supplementary tables are available online at:

http://intron.ccam.uchc.edu/data/pipeline/jodi/THESIS_supplementary_files/CHAPTER_4

These materials are also available at the journal of publication:

<http://pubs.acs.org/doi/suppl/10.1021/cn100012x>

Supplementary table legends

Supplementary Table 1: Names and accession numbers for all identified *M. musculus* Rho-GEFs, Rho-GAPs, and Rho-GTPases expressed in the PSD.

Gene Symbol, Gene Name, MGI ID, and Entrez ID represent the official names and identifiers for each gene as designated by the naming authority. MGI ID and Entrez ID entries are hyperlinked to the specific gene page the Mouse Genome Informatics (MGI) and NCBI (GenBank) websites. Table includes Synonyms, UniGene IDs, UniProt ID, UniProt Accession, Ensemble IDs, and RefSeq IDs.

Supplementary Table 2: Modifications to SMART structures of Rho-GEFs in Fig. 4.1.

Features changed from SMART database (<http://smart.embl-heidelberg.de/>) structures are provided for each Rho-GEF in **Fig. 4.1**. Table includes the accession number used in the SMART query and provides a reference in support of the changes. Accession numbers are from the UniProt (<http://www.uniprot.org/>) and NCBI (<http://www.ncbi.nlm.nih.gov/protein>) databases. SMART designates segments of low compositional complexity, all of which were removed from the gene structures in **Fig. 4.1**.

CHAPTER 5

Collaborative Bioinformatic Projects

Introduction

Collaborative work has been an invaluable part of my graduate education. Through collaborative efforts I have been able to explore a number of aspects of biology that I otherwise might not have. This chapter provides a brief compilation of some of the additional computational work I have done in collaboration with others, much of which has resulted in the generation of published studies.

Alternative exon classification

The first year of my time in graduate school was spent learning the basics of Perl scripting. My first assignment was the characterization of the transcribed elements of the fruit fly (*Drosophila melanogaster*), mouse (*Mus musculus*), and human (*Homo sapiens*) transcriptomes. I generated Perl scripts that processed the annotated genes and created files with the following alternative exon event classifications: alternative 5' and alternative 3' splice sites, alternative initiation and termination exons, retained/unprocessed introns, cassette exons, mutually exclusive exons, and coordinate exons (**Fig. 5.1**).

My classification of alternative and constitutive regions of the *Drosophila* genome was used in the generation of two recent publications^{364,365}:

McManus, C.J., Duff, M.O., Eipper-Mains, J. & Graveley, B.R. Global analysis of trans-splicing in *Drosophila*. *Proc. Natl. Acad. Sci. U.S.A.* **107**, 12975-12979 (2010).

McManus, C.J., *et al.* Regulatory divergence in *Drosophila* revealed by mRNA-seq. *Genome Res.* **20**, 816-825 (2010).

CLASSIFICATION OF ALTERNATIVE SPLICING EVENTS

Figure 5.1 Classification of common types of alternative splicing events

Depiction of the alternative splicing events determined by the Perl script I wrote. Constitutive exons are shown in blue and alternatively spliced exons are shown in yellow.

Two ongoing projects in the lab are making use of the complete classification of *Drosophila* and *Homo* transcribed regions for the analysis of alternative splicing. I wrote additional Perl and R-programming language scripts to analyze splice junction usage and/or exclusion and to provide an output containing a statistical measure of significance (Fisher's Exact Test). These studies are expected to result in publications in the near future.

Analysis of alternate promoter usage in the Kalrn gene

Because of my familiarity with the UCSC Genome Browser, I was asked to assist with analysis of the effects of cocaine on a specific Rho GDP/GTP exchange factor (GEF), *Kalrn*. For this paper, I performed many of the necessary bioinformatic analyses. This included identification and extraction of alternative promoter sequences, characterization of exons, and identification of the corresponding protein domains.

Chronic cocaine exposure induces lasting morphological changes at excitatory glutamatergic synapses on medium spiny neurons of the striatum⁸⁷. Modifications to synaptic morphology are carried out through rearrangements of the actin cytoskeleton and are largely mediated by the activation of the Rho family of proteins⁶⁸. The *Kalrn* gene exhibits complex alternative splicing and multiple distinct isoforms with different functional domains are expressed throughout the body^{280,281}. Full-length *Kalrn* has two GEF domains, through which it activates Rho proteins, as well as additional protein interacting domains²⁷⁷.

Chronic cocaine exposure leads to increased expression of a specific Kalirin isoform, Kalirin 7, in the striatum. Mice specifically lacking this isoform, which is the major *Kalrn* isoform in the adult rodent brain, exhibit behavioral abnormalities and lack the increased dendritic spine density normally observed following chronic cocaine exposure⁹⁸.

I contributed a figure detailing the complex alternative splicing pattern seen in the *Kalrn* gene (**Fig. 5.2**).

Figure 5.2 Structure of the mouse *Kalrn* gene and major transcripts

Genomic and transcript information was compiled from websites (http://www.ensembl.org/Mus_musculus/Info/; <http://genome.ucsc.edu/cgi-bin/>) and previously published work (Johnson *et al.*, 2000, McPherson *et al.*, 2002, and McPherson *et al.*, 2004) to create an accurate map of the mouse *Kalrn* gene and the major brain transcripts^{277,280}. Protein domains (<http://smart.embl-heidelberg.de/>) encoded by groups of exons are indicated.

(Appeared in its original form as **Figure 2** from Mains, R.E., Kiraly, D.D., Eipper-Mains, J.E., Ma, X. & Eipper, B.A. *Kalrn* promoter usage and isoform expression respond to chronic cocaine exposure. *BMC Neurosci.* **12**, 20 (2011)).

This work appeared in a recent publication¹¹⁶:

Mains, R.E., Kiraly, D.D., Eipper-Mains, J.E., Ma, X. & Eipper, B.A. *Kalrn* promoter usage and isoform expression respond to chronic cocaine exposure. *BMC Neurosci* **12**, 20 (2011).

EMBOSS silent restriction sites

I installed, compiled, and executed the EMBOSS silent program (<http://emboss.bioinformatics.nl/>) to identify potential silent restriction site modifications for use in the introduction of specific mutations and features in protein coding genes. These contributions are in use in a number of ongoing projects in another lab and will likely be included in future publications.

CHAPTER 6

Conclusions & Future Directions

Introduction

Global analytical approaches generate enormous amounts of data, creating new opportunities to address important questions in fundamentally different ways. The studies described in the previous chapters provide a framework for many future experiments.

Experimental approaches

The data presented in this thesis were collected over a period of years on a relatively limited number of experimental animals, using slightly different cocaine treatment paradigms and techniques, and two different sequencing machines. Ideally, these experiments could be carried out with larger numbers of mice receiving identical treatments occurring simultaneously for all treatment groups. For mRNA experiments using total tissue, it would be interesting to use multiplex tags for sequencing RNA from individual animals because this would enable us to look for correlations between gene expression changes and differences in cocaine responsiveness between individual mice.

Additionally, as mentioned in the introduction, all drugs of abuse ultimately cause enhanced signaling in the nucleus accumbens (NAc)⁸³. Studies using different drugs of abuse and different drug treatment paradigms would therefore be interesting to compare to the data presented here. Profiling the molecular differences in the brain between experimenter-administered and self-administered cocaine could potentially provide insight into the genes underlying motivation and drug-seeking behavior. Studies to profile gene expression after alternate drugs of abuse, such as morphine, amphetamine, and methamphetamine, would

help establish the overlapping signaling pathways common to all drugs as well as those unique to a particular drug.

The mesolimbic dopamine system is comprised of a variety of cell types. The sequencing data presented here therefore derive from a heterogeneous population of cells. A number of recent experiments suggest that gene expression differs significantly from one cell to the next^{31,366-369}. A recently developed technique allows one to isolate cell-type specific translating RNA using eGFP-tagged ribosomes: the translating ribosome affinity purification (TRAP) method³⁷⁰. In this method, generation of transgenic mice expressing an eGFP/ribosomal fusion protein controlled by a cell-type specific driver enables specific extraction of labeled polysomes using antibodies to eGFP. The characterization of cell-type specific mRNA expression could potentially illuminate important molecules and signaling pathways involved in drug abuse that were obscured by the heterogeneity of mixed tissue lysates.

It would also be interesting to investigate epigenetic modifications to DNA and histones as a result of drug exposure using RNA-Seq technology. Epigenetic changes are known to occur in many neuropsychiatric disorders³⁷¹⁻³⁷³, and recent evidence points to a role for epigenetics in the response of the brain to drugs of abuse³⁷⁴⁻³⁷⁸.

Analytical approaches

The methodology underlying high-throughput sequencing is undergoing constant improvement, which makes comparisons between recent data sets and older data sets, obtained with what was then "state of the art", very difficult. Even using one method and a highly expressed gene such as *Actb* (β -actin), sequence coverage across different exons is far from uniform (**Fig. 1.2**); these differences are even more pronounced when comparisons are

made between data sets generated with 37 bp reads and 100 bp reads. Difficulties of this type will presumably be minimized as the technology matures.

For both editing and alternative splicing, a major impediment to data interpretation is the general lack of knowledge regarding the functional significance of these events. Does a given event occur in a functionally important region of the gene and alter its function? Until more is known about individual events, genome-wide studies of all detected instances of editing or alternative splicing in various treatment paradigms does little more than generate data.

RNA Editing

Adenosine to inosine (A-to-I) RNA editing is a post-transcriptional modification catalyzed by a family of widely expressed double-stranded RNA-specific adenosine deaminases acting on RNA (ADARs)^{9,379-381}. In mammals, RNA editing alters the protein sequence (recoding) at a small proportion of all identified editing sites because the majority of A-to-I events reside in intronic, untranslated, or intergenic regions³⁸²⁻³⁸⁶. Comprehensive analysis of mRNA editing in our existing datasets was complicated by the uneven coverage of aligning reads and the number of genes in which more than one editing event occurs over a short distance. Realignment of sequence reads to allow more than two A/G mismatches could potentially enhance the existing analyses, however deeper sequencing and PCR validation will be required for more complete analysis.

Editing events within the coding regions of a number of neurotransmitter receptors known to respond to cocaine cause changes to their RNA processing, channel subunit maturation/assembly, kinetics, gating properties, and trafficking, altering synaptic transmission³⁸⁷. We identified 15 A-to-I potential editing events occurring in five sequence libraries (NAc Saline, Cocaine, Withdrawal and PSD Saline and Cocaine) and 14 additional

events occurring in four of the five libraries (**Table 6.1**). Editing would lead to a change in protein coding in 12 of the 29 identified sites. The remaining identified sites fell in introns, 5' or 3' UTRs, or were from noncoding RNAs.

We detected many of the functionally significant editing events known to occur in the coding regions of the glutamate, serotonin, GABA, and kainate neurotransmitter receptors (**Fig. 6.1**). Three editing sites were identified in the ionotropic glutamate receptor, *Gria2* (alpha-amino-3-hydroxy-5-methyl-4-isoxazol glutamate receptor, AMPA; also known as *GluR2*). Editing of the Q/R site alters channel calcium permeability and nearly complete Q/R editing is required for viability^{379,389}. While the Q/R site was nearly 100% edited in all samples, partial editing occurred at the R/G site (**Fig. 6.1A**); receptors edited at the R/G site exhibit faster recovery from desensitization³⁷⁹. Under baseline conditions, PSD-localized *Gria2* transcripts show less R/G editing than total tissue *Gria2* transcripts. *Gria2* R/G editing frequency at the PSD increased following cocaine exposure, while total tissue *Gria2* R/G editing was unaltered (**Fig. 6.1A**).

Editing was detected at four sites in the serotonin receptor 5-HT_{2C} (*Htr2c*), one site in *Gabra3* (gamma-aminobutyric acid type A3 receptor), and at several sites in the kainate receptors *Grik1* and *Grik2* (**Fig. 6.1B,C** and **Table 6.1**). The serotonin receptor, 5-HT_{2C} (*Htr2c*), is an extreme example of a gene in which more than one editing event occurs over a short distance; it has 5 A-to-I editing events in the span of 13 nucleotides, and it is known that multiple editing events often occur in a single transcript^{379,388}. Similarly, we observed a K/E recoding editing event of unknown functional significance in *Cyfp2* (cytoplasmic FMR1 interacting protein 2), with lower baseline PSD editing that increased after cocaine (**Fig. 6.1D**). The editing profile for E/G recoding in a region of the calcium-dependent secretion activator 1 (CADPS) protein crucial for its interaction with secretory vesicles³⁹¹ was below 40% in all tissue

Gene(s)	Coordinate	Site	NAc Sal		NAc Coc		NAc Wit		PSD Sal		PSD Coc	
			Read depth (Qual. adj. cov.)	Percent Alt base (% edit ± CI)	Read depth (Qual. adj. cov.)	Percent Alt base (% edit ± CI)	Read depth (Qual. adj. cov.)	Percent Alt base (% edit ± CI)	Read depth (Qual. adj. cov.)	Percent Alt base (% edit ± CI)	Read depth (Qual. adj. cov.)	Percent Alt base (% edit ± CI)
4930578G10Rik	chr4:42804740	Intron	6 (4)	100.0 ± 0.0	7 (6)	100.0 ± 0.0	2 (0)	100.0 ± 0.0	3 (3)	100.0 ± 0.0	14 (13)	100.0 ± 0.0
4930578G10Rik	chr4:42806801	W/R	7 (6)	71.4 ± 17.1	11 (9)	36.4 ± 14.5	11 (6)	63.6 ± 14.5	19 (17)	31.6 ± 10.7	41 (41)	53.7 ± 7.8
Atp2b1	chr10:98378004	5'-UTR	4 (3)	50.0 ± 25.0	5 (4)	60.0 ± 21.9	6 (6)	33.3 ± 19.2	0 (0)		4 (4)	100.0 ± 0.0
Blcap	chr2:157383876	Q/R	48 (23)	31.3 ± 6.7	52 (32)	26.9 ± 6.2	59 (32)	25.4 ± 5.7	16 (6)	37.5 ± 12.1	0 (0)	
C330019G07Rik/ Pisd	chr5:33081060	I/V	60 (45)	65.0 ± 6.2	53 (38)	69.8 ± 6.3	21 (18)	52.4 ± 10.9	76 (73)	35.5 ± 5.5	128 (125)	57.0 ± 4.4
Cadps	chr14:13244096	E/G	34 (22)	35.3 ± 8.2	41 (34)	31.7 ± 7.3	28 (19)	39.3 ± 9.2	76 (71)	26.3 ± 5.1	10 (10)	80.0 ± 12.6
Cyfp2	chr11:46086145	K/E	39 (10)	79.5 ± 6.5	102 (29)	83.3 ± 3.7	80 (34)	77.5 ± 4.7	34 (26)	29.4 ± 7.8	15 (15)	66.7 ± 12.2
Dagla	chr19:10320186	3'-UTR	30 (10)	53.3 ± 9.1	14 (4)	78.6 ± 11.0	14 (3)	78.6 ± 11.0	96 (33)	43.8 ± 5.1	156 (56)	44.2 ± 4.0
Dlgap2	chr8:14306982	Intron	17 (0)	70.6 ± 11.1	18 (9)	61.1 ± 11.5	19 (3)	94.7 ± 5.1	51 (8)	90.2 ± 4.2	123 (34)	60.2 ± 4.4
Gm14391/Gm14399/ Gm2026	chr2:175024814	Intron	11 (7)	100.0 ± 0.0	15 (10)	100.0 ± 0.0	5 (3)	100.0 ± 0.0	7 (7)	100.0 ± 0.0	0 (0)	
Gon4l	chr3:88689333	Intron	3 (1)	100.0 ± 0.0	6 (4)	83.3 ± 15.2	3 (3)	66.7 ± 27.2	0 (0)		8 (8)	100.0 ± 0.0
Gpr137b	chr13:13457408	T/A	16 (12)	37.5 ± 12.1	15 (10)	66.7 ± 12.2	8 (5)	37.5 ± 17.1	22 (21)	81.8 ± 8.2	68 (57)	35.3 ± 5.8
Gria2	chr3:80496208	R/G	125 (91)	63.2 ± 4.3	106 (89)	51.9 ± 4.9	75 (59)	62.7 ± 5.6	37 (34)	32.4 ± 7.7	31 (30)	64.5 ± 8.6
Gria2	chr3:80510834	Q/R	104 (47)	99.0 ± 1.0	114 (51)	100.0 ± 0.0	82 (35)	100.0 ± 0.0	3 (3)	100.0 ± 0.0	37 (21)	100.0 ± 0.0
Gria3	chrX:39007429	R/G	22 (16)	95.5 ± 4.4	28 (17)	92.9 ± 4.9	22 (21)	86.4 ± 7.3	0 (0)		20 (18)	100.0 ± 0.0
Grik1	chr16:87940788	Q/R	7 (4)	71.4 ± 17.1	4 (1)	100.0 ± 0.0	3 (2)	100.0 ± 0.0	12 (12)	100.0 ± 0.0	0 (0)	
Htr2c	chrX:143604241	I/V (D-site)	15 (8)	53.3 ± 12.9	12 (9)	66.7 ± 13.6	0 (0)		14 (14)	100.0 ± 0.0	8 (8)	100.0 ± 0.0
lpw	chr7:66929246	ncRNA	282 (177)	86.5 ± 2.0	601 (373)	93.7 ± 1.0	467 (303)	90.8 ± 1.3	0 (0)		49 (37)	63.3 ± 6.9
lpw	chr7:66931777	ncRNA	19 (16)	26.3 ± 10.1	24 (17)	33.3 ± 9.6	0 (0)		3 (2)	100.0 ± 0.0	9 (7)	100.0 ± 0.0
Myo3a	chr2:22443824	Intron	7237 (556)	99.9 ± 0.0	5124 (452)	99.9 ± 0.0	3466 (328)	99.9 ± 0.0	7888 (125)	99.3 ± 0.1	7953 (162)	99.0 ± 0.1
Myo3a	chr2:22444034	Intron	7353 (547)	99.9 ± 0.0	7299 (784)	100.0 ± 0.0	5066 (545)	99.9 ± 0.0	7949 (172)	98.3 ± 0.1	7940 (167)	98.0 ± 0.2
Myo3a	chr2:22445548	Intron	195 (3)	100.0 ± 0.0	107 (6)	100.0 ± 0.0	82 (4)	100.0 ± 0.0	1518 (8)	99.9 ± 0.1	1435 (16)	99.7 ± 0.1
Myo3a	chr2:22445583	Intron	1209 (909)	99.9 ± 0.1	735 (584)	100.0 ± 0.0	429 (330)	100.0 ± 0.0	7980 (7569)	99.9 ± 0.0	7999 (7707)	100.0 ± 0.0
Myo3a	chr2:22445901	Intron	802 (47)	100.0 ± 0.0	561 (27)	100.0 ± 0.0	357 (17)	100.0 ± 0.0	31 (3)	90.3 ± 5.3	17 (0)	35.3 ± 11.6
Rpa1	chr11:75114517	3'-UTR	16 (12)	81.3 ± 9.8	11 (7)	72.7 ± 13.4	5 (3)	80.0 ± 17.9	135 (111)	97.8 ± 1.3	105 (88)	88.6 ± 3.1
Taf1b	chr12:25194319	N/D or E/G	7 (5)	57.1 ± 18.7	8 (6)	37.5 ± 17.1	4 (4)	75.0 ± 21.7	91 (85)	68.1 ± 4.9	121 (120)	100.0 ± 0.0
Tmem63b	chr17:45799898	Q/R	65 (37)	64.6 ± 5.9	65 (38)	67.7 ± 5.8	31 (22)	61.3 ± 8.7	0 (0)		61 (59)	62.3 ± 6.2
Zfp352	chr4:89892206	3'-UTR	18 (1)	100.0 ± 0.0	8 (0)	100.0 ± 0.0	7 (1)	100.0 ± 0.0	112 (2)	100.0 ± 0.0	118 (6)	100.0 ± 0.0
Zfp804b	chr5:7276845	Intron	1145 (103)	99.8 ± 0.1	573 (47)	99.7 ± 0.2	439 (39)	100.0 ± 0.0	121 (6)	100.0 ± 0.0	190 (6)	100.0 ± 0.0

Table 6.1 Table of A-to-I RNA editing events

Identified potential RNA editing events in RNA-Seq libraries from NAc tissue lysates (Saline, Cocaine, Withdrawal) and striatal PSDs (Saline, Cocaine) . Table details the gene symbol, genomic coordinate, editing site information including recoding event, raw read depth with PHRED-score quality adjusted coverage for reads ≥ 30, and the percent alternate base ± 95% confidence interval (CI). Site D of Htr2c was detailed by Burns *et al.* (1997)³⁸⁸.

Figure 6.1 A-to-I RNA editing in select genes

Mismatch identification and quality filtering was performed using SAMTools pileup and varFilter³⁹⁰. Potential A-to-I editing sites occurring in multiple sequencing libraries were identified. Frequency of A-to-I edits at the indicated genomic coordinate is shown as percent editing for (A) *Gria2*, (B) *Htr2c*, (C) *Gabra3*, (D) *Cyfp2*, (E) *Cadps*, and (F) *Gria3*. Recoding is indicated by the single letter amino acid code before the genomic coordinate. Sites A, B, and D of *Htr2c* were previously described³⁸⁸. Abbreviations: S, Saline; C, Cocaine; W, Withdrawal.

samples and at the PSDs of saline-treated mice but increased to 80% at the PSD after cocaine (Fig. 6.1E).

Editing of the ionotropic glutamate receptor, *Gria3* (also known as *GluR3*) occurs in the ligand interaction domain and causes R/G recoding³⁹². Editing at this site is low in embryonic rat brain and increases markedly as development progresses; 80-90% of *Gria3* transcripts are edited in humans³⁹³. Editing at the R/G site enables rapid recovery from desensitization thereby permitting a sustained response to rapid stimuli. It is believed that editing at this site occurs prior to splicing and can alter splice efficiency³⁸⁷. Consistent with previously published reports, we observed a very high degree of editing (< 80%) at the R/G site in all samples irrespective of treatment paradigm (Fig. 6.1F).

Of note, amongst the genes with editing sites whose usage was responsive to cocaine (4930578G10Rik, *Cadps*, *Cyfp2*, *Gpr137b*, *Gria2*, *Taf1b*), editing at the PSD and in tissue lysates changed in opposite directions or only differed in the PSD (Table 6.1).

Alternative Splicing

Similarly, our analysis of alternative splicing of the described mRNAs was incomplete and merits revisiting. While options exist for alternate isoform analysis^{25,26,394}, the annotation of the mouse genome is far from complete, meaning that the available gene annotations undergo frequent changes. As with editing, the databases of known alternative splicing events are quite imperfect, which can produce misleading analyses.

Between 92 and 97% of multi-exon human genes are alternatively spliced^{7,395,396} and splicing levels are especially high in the brain, where it plays an important role in synaptic plasticity^{33,397,398-402}. For example, inclusion or exclusion of the three alternatively spliced exons of the NR1 subunit of the NMDA receptor is activity-dependent and affects receptor assembly and exit from the endoplasmic reticulum, trafficking, phosphorylation, and pharmacological

properties⁴⁰⁰. Although approximately 10% of aligning reads from the NAc lysates and 20% from striatal PSD libraries map to annotated exon-exon junctions, deeper coverage is needed to evaluate splicing of transcripts like those encoding NR1.

Since Methyl CpG Binding Protein 2 (*Mecp2*) mRNA is known to affect behavioral responses to cocaine and is alternatively spliced^{125,374,377,403}, we carried out a direct analysis of PSD junction reads mapping to it (**Fig. 6.2**). Inclusion of exons 1, 2 and 3 allows translation to start in exon 2, generating MECP2A; when exon 2 is excluded, translation starts in exon 1, generating MECP2B^{404,405}. Preliminary analysis shows that in the Sal PSD library, more reads spanned the junction between exons 1 and 2 or 2 and 3 (26 reads support inclusion of exon 2) than the junction between exons 1 and 3 (3 reads support exclusion of exon 2). In contrast, in the Coc PSD library, no junction reads supported exon 2 inclusion (MECP2A) while 4 junction reads supported exon 2 exclusion (MECP2B) (**Fig. 6.2**). Our data suggest a switch from MECP2A to the more readily translated MECP2B transcript at the PSD following cocaine treatment.

MECP2, a transcriptional regulator, was known to localize to the PSD^{66,406} and transcript levels were high enough to allow a preliminary analysis of its splicing. The longer N-terminus of MECP2B is compositionally similar to extracellular signal-regulated kinase-1 and is hypothesized to play a role in coupling synaptic signaling to regulation of transcription⁴⁰⁴. Exclusion of exon 2 from PSD-localized transcripts following cocaine treatment may facilitate synapse-to-nucleus signaling by shifting expression towards MECP2B. Recent studies implicate MECP2 and other epigenetic modifications in the molecular response to chronic cocaine^{125,374,377,403}. These preliminary observations should be followed up with further studies.

Figure 6.2 Alternative splicing of *Mecp2*

The exon/intron structure of the *Mecp2* gene is shown; exclusion of exon 2 generates MECP2B (NM_001081979, variant 1) while inclusion of exon 2 generates MECP2A (NM_010788, variant 2). Reads observed in the striatal PSD Sal and Coc libraries are shown.

Conceptual and integrative approaches

Knockout and transgenic mouse models have provided invaluable insights for investigations into the behavioral, biochemical, and molecular effects mediated by single genes. The changes that occur as a result of drug abuse are widespread and occur at multiple levels of gene expression: epigenetic, transcriptional, post-transcriptional, translational, and post-translational. These changes most likely involve multiple brain regions and cell-types and act in a system-wide manner. At the present time we lack the data and analytic tools necessary to integrate all of these drug-induced changes into a single cohesive model. Global studies such as those outlined in the previous chapters emphasize the broad effects of drug exposure on neuronal gene expression. They emphasize the need for an integrative approach that combines mRNA, miRNA, and protein expression profiling with focused studies and innovative behavioral paradigms in pursuit of the factors that underlie addiction and develop integrated therapeutic approaches.

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