

Neuroproteomics of the Synapse and Drug Addiction

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ABSTRACT

Proteomic analyses of brain tissues are becoming an integral component of neuroscientific research. In particular, the essential role of the synapse in neurotransmission and plasticity has brought about extensive efforts to identify its protein constituents. Recent studies have used a combination of subcellular fractionation and proteomic techniques to identify proteins associated with different components of the synapse. Thus, a coherent map of the synapse proteome is rapidly emerging, and a timely review of these data is warranted. In the first part of this review, neuroproteomic techniques that have been used to analyze the synapse proteome are described. We then summarize the results from several recent proteomic analyses of

mammalian synapses and discuss the similarities and differences in their profiling of synaptic proteins. Important advances in this field of research include the use of proteomics to analyze synaptic function and drug effects on synaptic proteins. This article presents an overview of proteomic analyses of the phosphorylation states of synaptic proteins and recent applications of neuroproteomic techniques to the study of drug addiction. Finally, we discuss the challenges in comparing proteomic studies of drug addiction and the future directions of this field in furthering our understanding of the molecular mechanisms underlying synaptic function and drug addiction.

Proteomic approaches are being extensively used to study global protein expression profiles in various tissues and to compare these profiles between physiological and perturbed states. The use of proteomics allows the investigator to study perturbed states, such as disease states or drug effects, without the need for a prior hypotheses. Diseases of the central nervous system, such as neuropsychiatric and neurodegenerative diseases, tend to involve multiple interacting proteins and are thus well suited for proteomic analysis (see Kim et al., 2004). In addition to allowing the unbiased identification of molecular markers for disease states, neuroproteomic approaches permit a greater understanding of the processes underlying them. Thus, although there is still a paucity of information regarding the proteome of the brain, the interest

in proteomics for the purpose of neuroscientific research is rapidly increasing.

In the central nervous system, synapses are known to be the key structure involved in neurotransmission and neuroplasticity. At the synapse, neurotransmitters are released from the axon terminal of the presynaptic neuron to bind to receptors on the postsynaptic target neuron. Using subcellular proteomics, several research groups have proceeded to identify the proteins associated with different components of the synapse, including synaptosomes (Schimpf et al., 2005; Witzmann et al., 2005), synaptic membranes (Stevens et al., 2003), postsynaptic densities (PSDs) (Walikonis et al., 2000; Satoh et al., 2002; Jordan et al., 2004; Li et al., 2004; Peng et al., 2004; Yoshimura et al., 2004; Phillips et al., 2005; Cheng et al., 2006; Dosemeci et al., 2006), synaptic vesicles (Coughenour et al., 2004; Morciano et al., 2005), and the presynapse (Phillips et al., 2005). Thus, a coherent map of the synapse proteome is rapidly emerging. These analyses are important for future studies examining the role of synaptic proteins in disease states, such as drug addiction. In this article, we

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ABBREVIATIONS: PSD, postsynaptic density; 16-BAC, benzyldimethyl-*n*-hexadecylammonium chloride; 2-DE, two-dimensional gel electrophoresis; ICAT, isotope-coded affinity tag; IEF, isoelectric focusing; IMAC, immobilized metal affinity chromatography; IPG, immobilized pH gradient; AMPA, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; MudPIT, multidimensional protein identification technology; PAZ, presynaptic active zone; PAGE, polyacrylamide gel electrophoresis.

review advances in neuroproteomic techniques, the findings from proteomic analyses of mammalian synapses, and recent efforts to apply proteomic techniques to the study of drug addiction.

Techniques in Neuroproteomics

Sample Preparation

To study neuropsychiatric or neurodegenerative diseases, the brain can be divided anatomically to target specific brain regions known to be affected or involved in the disease state. Proteomic analysis can then be performed on homogenized preparations of the whole brain region, or different strategies can be employed to simplify the experimental sample to examine only a fraction of interest. This latter approach is highly advantageous, because it provides increased information on protein localization. One technique that can be used to increase the precision of neuroproteomic analyses is laser capture microdissection (Emmert-Buck et al., 1996). With this dissection technique, it is possible to target specific brain nuclei, subpopulations of neurons, and even individual neuron types from fixed or frozen tissue, with a minimal effect on gene and protein expression profiles (Mouledous et al., 2003). Another strategy is to establish subcellular proteomes, taking advantage of the compartmentalization of cells and the assembly of functional units, such as protein complexes. This approach has been applied to characterize AMPA-receptor complexes and *N*-methyl-D-aspartate-receptor complexes in the brain (Collins et al., 2006). Generally, to characterize signaling complexes, the protein of interest is immunoprecipitated, and all coprecipitated proteins are separated by electrophoresis and analyzed by mass spectrometry (MS). The identified proteins are assumed to take part in the signaling complex. Specific classes of proteins can also be isolated using affinity-based methods. For example, phosphoproteins are commonly enriched using immobilized metal affinity chromatography (IMAC) (Andersson and Porath, 1986). Finally, subcellular fractionation methodologies allow

the enrichment of a subset of proteins associated with a specific subcompartment of the nervous system. This is especially useful in neuroproteomic research, as many of the proteins expressed in the nervous system are low in abundance and escape proteomic characterization from whole tissue. Thus, a typical neuroproteomic experiment often begins with the isolation, using cell-biological techniques, of an enriched fraction containing the subcellular structure of interest.

Proteome Profiling

A wide range of proteomic techniques can be used to identify the proteins associated with the sample of interest or to quantitatively compare the protein profile of a sample under different conditions. Neuroproteomic techniques have been recently reviewed elsewhere (Kim et al., 2004a; Williams et al., 2004); those techniques that have been used to analyze the synapse proteome and to study drug addiction are described here and summarized in Table 1.

Protein profiling generally involves the separation of proteins or tryptic fragments of proteins followed by MS analysis. Proteins can be separated using one-dimensional or, more commonly, two-dimensional gel electrophoresis (2-DE) (Rabilloud, 2002). In 2-DE, the first dimension consists of separating proteins by their isoelectric point (pI) or isoelectric focusing (IEF), using an immobilized pH gradient (IPG) strip. The second dimension consists of separating proteins by their molecular weight using SDS-polyacrylamide gel electrophoresis (PAGE). Separation by 2-DE allows the characterization of post-translational modifications and isoforms of proteins. For example, phosphorylation alters the pI value (and mobility) of proteins and is seen as a horizontal series of spots on the gel. However, it can be difficult to compare the protein profiles of control and experimental samples using 2-DE, because this approach is limited by the degree of gel-to-gel variability. A more quantitative technique, differential in-gel electrophoresis, uses fluorescent dyes to label two different protein samples before their separation by 2-DE (Unlu

TABLE 1
Overview of neuroproteomic techniques

Technique	Uses	Limitations	References
Proteome profiling			
2-DE (IEF/SDS-PAGE)	Separation of soluble proteins	Poor detection of proteins with extreme pI or molecular weight, of low abundance, or hydrophobic	Rabilloud, 2002
	Detection of post-translational modifications and isoforms of proteins	Quantification requires consistency and multiple replicates because of gel-to-gel variability	
DIGE	More quantitative than 2-DE	Only detects proteins that are amenable to 2-DE	Unlu et al., 1997
16-BAC/SDS-PAGE	Superior separation of membrane proteins compared to 2-DE	Quantification requires consistency and multiple replicates, because of gel-to-gel variability	Hartinger et al., 1996
MudPIT	Identification of membrane and soluble proteins	Not quantitative, limited information on post-translational modifications	Wolters et al., 2001
ICAT	Quantitative analysis of membrane and soluble proteins	Only detects cysteine-containing tryptic peptides; limited information on post-translational modifications	Gygi et al., 1999
Protein identification			
MALDI-TOF MS	Rapid "peptide mass fingerprint" obtained	Difficult to analyze complex mixtures of proteins	Henzel et al., 1993
Tandem MS	Unambiguous protein identification using "peptide sequence tag", superior analysis of complex mixtures of proteins	Requires longer time to run sample, interpret data, and identify proteins	Mann and Wilm, 1994

DIGE, differential in-gel electrophoresis.

et al., 1997). This allows the control and experimental samples to be run in the same gel, which greatly reduces variability.

2-DE is most successfully used for the separation of soluble proteins; however, it is generally not useful for the separation of proteins that are extreme in size (e.g., over 100,000 Da) or in pI (e.g., greater than 9), low in abundance, or hydrophobic (e.g., membrane proteins). An alternative two-dimensional approach that improves the separation of membrane proteins uses a cationic detergent benzyldimethyl-*n*-hexadecylammonium chloride (16-BAC) instead of IEF in the first dimension, followed by anionic SDS in the second dimension (Hartinger et al., 1996). In this technique, proteins are separated by their molecular weight in both dimensions, but because 16-BAC and SDS bind proteins differently, they produce different migration patterns (Coughenour et al., 2004). This method has been used to successfully resolve and identify integral proteins with one or more transmembrane domains from synaptic vesicles (Coughenour et al., 2004; Morciano et al., 2005).

Recent advances have also allowed the comprehensive profiling of proteins without the need to first carry out protein separation by electrophoresis. For example, an alternative approach to protein separation and in-gel digestion is to perform “shot-gun proteomics” by multidimensional protein identification technology (MudPIT) (Wolters et al., 2001). For this, the protein sample is first trypsin-digested, and the resulting peptides are fractionated by multidimensional liquid chromatography, usually strong cation exchange, and reverse-phase high-performance liquid chromatography (HPLC). MudPIT is useful for the identification of both membrane and soluble proteins in the same sample.

Isotope-coded affinity tag (ICAT) labeling is a quantitative proteomic technique that obviates the need for protein separation by electrophoresis (Gygi et al., 1999). ICAT technology uses a chemical reagent that reacts with the sulfhydryl group of cysteine residues. This reagent contains either a light (^{12}C) or heavy (^{13}C) isotope and a cleavable biotin moiety. The relative levels of protein in two different samples can be examined by labeling one sample with the light isotope and the other sample with the heavy isotope. The two samples are then pooled together and trypsin-digested. This is followed by cation-exchange and avidin chromatography to purify only the labeled cysteine-containing tryptic peptides. Reverse-phase-HPLC and liquid chromatography-tandem MS (LC-MS/MS) are then used to identify peptide pairs and to quantify the relative heavy/light ratios, which indicate the relative levels of expression of the parent proteins in the two samples. It is important to note that this technique is limited to cysteine-containing proteins and does not provide information regarding the absolute protein concentrations in each sample.

Mass Spectrometric Identification of Proteins

Mass spectrometry is a sensitive, high-throughput method of identifying proteins that has revolutionized the proteomics field. There are two main ionization techniques used in mass spectrometric analysis: 1) matrix-assisted laser desorption/ionization (MALDI) and 2) electrospray ionization (Fenn et al., 1989). The ion source is coupled with a mass analyzer, such as time of flight (TOF), quadrupole ion trap, quadrupole mass filter, or Fourier transform-ion cyclotron resonance,

which can be used alone or in tandem. MALDI, which is commonly paired with a TOF mass analyzer (MALDI-TOF), provides a “peptide mass fingerprint” of each protein (Papoin, 2003). This requires a sufficient number of peptides to match with the protein in a theoretical database for unambiguous identification. MALDI-TOF MS is often used to identify proteins separated by SDS-PAGE or 2-DE.

In tandem MS or MS/MS, a first run is used to select peptides, and a second run is used to fragment those peptides. In brief, individual peptides are first resolved from a mixture by LC-MS/MS or nano-electrospray, and then each peptide is dissociated into fragments by collision-induced dissociation. The fragmentation pattern of each peptide provides information on the identity and location of amino acids within the peptide or a “peptide sequence tag,” which is used for specific protein identification (Mann and Wilm, 1994). There are several advantages to using MS/MS for protein identification. This method provides a high level of certainty in protein identification, because it relies on specific sequence information rather than peptide masses. Fragmentation data from tandem MS/MS can also be used to search expressed sequence tag and genomic databases, in addition to protein sequence databases. Finally, MS/MS allows a superior analysis of complex mixtures of proteins.

The Synapse Proteome

The application of novel proteomic techniques to neuroscientific research is starting to provide us with a greater understanding of nervous system structure and function. Given the large degree of heterogeneity within the nervous system, subcellular fractionation techniques have emerged as the key to successful neuroproteomic analyses. In particular, several research groups have begun to dissect the protein composition of the synapse, with a particular emphasis on the PSD. Fewer studies have tackled the analysis of other compartments of mammalian synapses, including synaptosomes, synaptic membranes, synaptic vesicles, and the presynapse. The various neuroproteomic analyses of the synapse are described in this section and are outlined in Table 2. Finally, attempts to characterize the synaptic phosphoproteome are well underway and are discussed below.

Synaptosomes and Synaptic Membranes

Subcellular proteomics have recently been used to isolate and identify the components of synaptosomes and synaptic membranes. Synaptosomes are subcellular structures containing the presynaptic nerve terminal (with its synaptic vesicles and mitochondria), the PSD, and parts of the postsynaptic membrane. Synaptosomes are commonly isolated from brain homogenates using discontinuous sucrose gradients (Hajos, 1975). The protein profile of synaptosomes from the rat forebrain has been studied using 2-DE as well as LC-MS/MS (Witzmann et al., 2005). Over 900 protein spots were detected by 2-DE, from which 91 unique proteins were identified by MALDI-TOF MS or LC-MS/MS. One hundred fifty-five additional proteins were identified by in-solution digestion followed by LC-MS/MS. Of the total 246 proteins, 61 were proteins with known functions at the synapse. A separate study characterized the proteome of synaptosomes from mouse brains using ICAT followed by LC-MS/MS (Schrimpf et al., 2005). This study identified a total of 1131

TABLE 2
Neuroproteomic analyses of the synapse

Synaptic Fraction	Proteomic Method and MS Platform	# of Proteins Identified	References
Synaptosomes and synaptic membranes			
Rat forebrain synaptosomes	2-DE, MALDI-TOF and LC-MS/MS; LC-MS/MS	246	Witzmann et al., 2005
Mouse brain synaptosomes	ICAT and LC-MS/MS	1131	Schrimpf et al., 2005
Rat forebrain synaptic membranes	SDS-PAGE, RP-HPLC, SCX-LC, MALDI-TOF, and LC-MS/MS	107	Stevens et al., 2003
Mouse forebrain plasma membranes	LC-MS/MS	862	Nielsen et al., 2005
Mouse hippocampus plasma membranes	LC-MS/MS	1685	Nielsen et al., 2005
PSD			
Rat forebrain	SDS-PAGE and MALDI-TOF	31	Walikonis et al., 2000
Mouse forebrain	2-DE and LC-MS/MS	47	Satoh et al., 2002
Rat forebrain	2-DE and MALDI-TOF;	233	Li et al., 2004
	ICAT and LC-MS/MS	61	
Rat forebrain	MudPIT	492	Yoshimura et al., 2004
Rat and mouse brain	SDS-PAGE and LC-MS/MS	452	Jordan et al., 2004
Rat forebrain	SDS-PAGE and LC-MS/MS	374	Peng et al., 2004
Rat forebrain	MudPIT	231	Phillips et al., 2005
Rat hippocampal slice	MudPIT	139	Dosemeci et al., 2006
Presynapse			
Rat brain synaptic vesicles	2-DE and LC-MS/MS, 16-BAC/SDS-PAGE and LC-MS/MS	36	Coughenour et al., 2004
Rat brain free synaptic vesicles	16-BAC/SDS-PAGE and MALDI-TOF	72	Morciano et al., 2005
Rat brain synaptic vesicles associated with presynaptic plasma membrane	16-BAC/SDS-PAGE and MALDI-TOF	81	Morciano et al., 2005
Rat forebrain presynaptic fraction	MudPIT	50	Phillips et al., 2005

proteins, including representatives of most synaptic structures and functions.

Synaptic membranes can be obtained by lysis of synaptosomes via osmotic shock (Kanner and Sharon, 1978). Using several complementary proteomic techniques for protein separation and MS, over 100 proteins from rat forebrain synaptic membranes have been identified (Stevens et al., 2003). These included several presynaptic vesicle fusion proteins, as well as postsynaptic receptors. Another study using a novel technique of plasma membrane fractionation identified 862 proteins from mouse cortical membranes and 1685 proteins from hippocampal membranes (Nielsen et al., 2005). This fractionation technique involved the high-speed shearing of tissues (to remove soluble proteins) followed by density gradient centrifugation to enrich plasma membrane proteins. Of the proteins identified (by LC-MS/MS), over 60% were membrane proteins, including several classes of ion channels and receptors.

The PSD

The PSD is a highly electron-dense structure located directly beneath the postsynaptic membrane, which contains receptors and their associated signaling and scaffolding molecules that organize signal transduction pathways. Isolation of a PSD fraction was first described in the 1970s (Cotman et al., 1974), and since then, numerous studies have helped to elucidate its protein composition using a variety of biochemical and yeast two-hybrid approaches. More recently, several research groups have applied large-scale proteomic techniques to comprehensively identify proteins associated with the PSD. The first proteomic studies to characterize the PSD used gel electrophoresis: 31 proteins were identified by SDS-PAGE followed by MALDI-TOF (Walikonis et al., 2000), and 47 proteins were identified by 2-DE followed by LC-MS/MS (Satoh et al., 2002). Since then, approximately 500 PSD-associated proteins have been identified (Yoshimura et al., 2004), using a

number of proteomic techniques, including SDS-PAGE (Jordan et al., 2004; Peng et al., 2004), 2-DE and ICAT (Li et al., 2004), and MudPIT (Yoshimura et al., 2004; Phillips et al., 2005). The lists of proteins generated from these studies show considerable overlap but are not identical. This is probably due to differences in species/strains, brain regions, sample preparation, and method of analysis. Nevertheless, these studies have confirmed the essential functions of the PSD as a scaffolding and signaling component of the synapse. A common finding of these studies is that the PSD contains proteins belonging to highly diverse functional classes, including receptors and channels, kinases and phosphatases, G proteins, enzymes, cytoskeletal, trafficking, and metabolic proteins. In addition, a number of novel PSD-associated proteins have been uncovered, the characterization of which will certainly lead to a greater understanding of PSD organization and function.

As most proteomic studies of the PSD have focused on the whole brain or forebrain, an interesting next step is to characterize and compare the PSD subproteome in specific brain regions (Cheng et al., 2006) and even in specific neuronal populations, for example, using laser capture microdissection. A recent study described a protocol for the preparation of a "micro-PSD fraction" from hippocampal slices (Dosemeci et al., 2006). Starting with five to six hippocampal slices (400 μm), a yield of 4 μg of PSD fraction per slice was obtained. Using LC-MS/MS, 139 PSD proteins were identified (from just 10 μg of the micro-PSD fraction), most of which have also been identified in the conventional PSD preparations described above. Because the hippocampal slice preparation allows for the precise manipulation of synaptic activity by both electrophysiological and pharmacological means, this technique will undoubtedly prove useful in characterizing changes in PSD proteins related to hippocampal synaptic function and plasticity.

The Presynapse

Whereas extensive efforts have been made to characterize proteins at the PSD, relatively less is known about proteins of the presynapse. In recent years, a few proteomic studies have described the separation and characterization of presynaptic fractions containing synaptic vesicle proteins, as well as presynaptic membrane proteins, which have shed some light into the composition of the presynapse.

Synaptic vesicles are required for the rapid release of neurotransmitters from the active zone, a specialized membrane compartment of the presynaptic nerve terminal. The characterization of synaptic vesicle proteins and their interacting proteins at the active zone is therefore central to understanding the molecular mechanisms of synaptic vesicle trafficking and neurotransmitter release. Identification of the synaptic vesicle proteome has only recently been made possible, because of the development and optimization of subcellular fractionation methods. In one study, synaptic vesicles were purified from the rat brain using a glycerol velocity gradient (Coughenour et al., 2004). Synaptic vesicle proteins were then separated using traditional 2-DE (IEF/SDS-PAGE), as well as 16-BAC/SDS-PAGE, to resolve both soluble and membrane proteins, respectively. In total, 36 synaptic vesicle proteins were identified by MS/MS, including seven integral membrane proteins. A separate study used subcellular fractionation and immunoaffinity purification to isolate two synaptic vesicle-containing fractions from the rat brain (Moricano et al., 2005). In brief, synaptosomes were disrupted by hypo-osmotic shock, and the released components were further fractionated by continuous sucrose density gradient centrifugation. This resulted in the sedimentation of a low-density fraction containing free synaptic vesicles and a higher-density fraction containing synaptic vesicles associated with the presynaptic plasma membrane. Each fraction was further purified by immunoisolation (using an antibody against the integral synaptic vesicle protein SV2) and analyzed using 16-BAC/SDS-PAGE followed by MALDI-TOF MS. In this study, 72 proteins were identified in the free synaptic vesicle fraction and 81 in the plasma membrane-containing denser fraction, which included proteins involved in vesicle fusion and retrieval.

In addition to synaptic vesicle proteins, further efforts have been made to identify the protein composition of the presynapse. The separation of a fraction enriched in presynaptic proteins from synaptic junctions has been described using Triton X-100 at pH 8 to extract presynaptic components, which are mostly soluble, from the PSD fraction, which is relatively insoluble (Phillips et al., 2001). Proteins in the presynaptic fraction (and its corresponding PSD fraction) were recently identified using MudPIT (Phillips et al., 2005). The presynaptic fraction contained 50 unique proteins, including a number of proteins involved in synaptic vesicle trafficking and neurotransmitter release.

The Synaptic Phosphoproteome

Protein phosphorylation is known to play a key role in synaptic function. Phosphorylation has traditionally been studied at the level of individual proteins; however, with recent advances in proteomic techniques, global characterization of the synaptic phosphoproteome is now possible.

In one study, a phosphoproteomic analysis of synapto-

somes from human cerebral cortices was performed (DeGiorgis et al., 2005). Synaptosomes were prepared from small amounts of cortical tissue obtained during surgical procedures. Phosphopeptides were enriched using IMAC and were identified by LC-MS/MS. A total of 50 phosphopeptides corresponding to 26 proteins were identified. A similar study characterized the phosphorylation of mouse PSD proteins using IMAC and LC-MS/MS (Trinidad et al., 2005). In this study, 83 phosphopeptides were identified, which originated from 42 proteins. In both of these studies, the phosphopeptides identified did not include any phosphotyrosine residues, suggesting that the identification of synaptic phosphoproteins was not exhaustive. It is likely that future proteomic studies will detect larger numbers of *in vivo* phosphorylation sites in the synapse, as phosphoprotein purification approaches and detection sensitivity improve.

Another study has described the large-scale analysis of protein phosphorylation in mouse synaptosomes, using multiple complementary approaches at the level of protein extraction, phosphoprotein and phosphopeptide enrichment, and MS analysis (Collins et al., 2005). This multifactorial analysis resulted in the identification of 289 phosphorylation sites, representing 79 synaptic proteins. Phosphoprotein components of both presynaptic and postsynaptic multiprotein complexes and signaling pathways were identified. This type of study establishing a global phosphorylation map of the synapse provides the basis for subsequent functional studies, which will increase our understanding of synaptic organization and signaling pathways.

Neuroproteomics and Drug Addiction

Although it is important to identify the proteins present at the synapse, a more central issue is to determine the biological function of these identified proteins. A starting point in obtaining information on the function of a protein is to perform quantitative proteomics, because many changes in synaptic activity are likely to be explained by comparing synaptic protein levels and/or activation states between physiological and perturbed states. In the context of drug addiction, a proteomic approach serves to generate an objective global view of the drug-induced changes in a specific proteome. Proteins that are up- or down-regulated following drug administration may be involved in its addictive potential; of course, this type of association needs to be directly tested by subsequent studies. In addition, by applying quantitative proteomic analyses, it becomes possible to thoroughly characterize and compare the effects of drugs of abuse to better understand their mechanisms of action.

It is thought that addictive drugs produce significant and persistent changes at the synapse, which could account for their long-lasting effects (Robinson and Kolb, 2004). To date, however, surprisingly few studies have taken advantage of subcellular proteomic techniques to examine the role of synaptic proteins in drug addiction. One recent study used ICAT to analyze the effect of chronic morphine administration on synaptic membrane proteins (Prokai et al., 2005). Rats were treated by subcutaneous infusion of saline or morphine for 7 days and were sacrificed on the 8th day. Synaptic membrane fractions were isolated and subjected to ICAT labeling followed by LC-MS/MS. A total of 74 proteins were identified, with 10 showing an increase and 17 showing a decrease

(>32%) after exposure to morphine. The synaptic proteins that were altered included proteins involved in cell adhesion (e.g., neural cell adhesion molecule, neuroligin, and neurofascin), synaptic vesicle trafficking (e.g., *N*-ethylmaleimide-sensitive factor), and endocytosis (e.g., clathrin and adaptor protein complex AP-2). This could account for morphine-induced changes in synaptic structure, as well as alterations in neurotransmitter release and/or neurotransmitter receptor trafficking.

We have initiated studies to examine the effect of morphine on presynaptic active zone (PAZ) proteins by 2-DE (Abul-Husn et al., 2005). For this, we used a subcellular fractionation technique described by Phillips et al. (2001) to separate a fraction enriched in presynaptic proteins from the hippocampi of mice treated with saline or escalating doses of morphine for 2 days. The PAZ proteins from saline- and morphine-treated mice were separated by 2-DE and visualized using Coomassie Blue (Fig. 1). We find that subcellular fractionation before separation by 2-DE results in the efficient resolution of several hundred proteins and that morphine alters the levels as well as the post-translational states of proteins in the PAZ fraction. This type of analysis will enable us to determine the effects of morphine on a specific subset of synaptic proteins that is highly relevant, because morphine is known to produce significant effects on presynaptic neurotransmitter release (Guo et al., 2005).

A separate study used 2-DE to analyze protein expression after chronic morphine administration (Li et al., 2006). Although no subcellular fractionation was performed, this study focused on a specific brain region, the nucleus accumbens, which plays an important role in reward and drug addiction. A 14-day paradigm of intermittent morphine administration, which produces behavioral sensitization, was used. Animals were sacrificed 24 h after the last drug injection. The nucleus accumbens from five individual saline- and five morphine-treated rats were separately extracted and separated by 2-DE. Approximately 1500 spots were visualized per gel, among which 22 showed significant differences of at least 1.5-fold between the saline and morphine groups. Fifteen of these were successfully characterized (by MALDI-TOF MS); however, only three were identified as synaptic proteins (synapsin, Lin-7, and β -synuclein). Thus, it is likely

that the majority of morphine-induced changes at the level of the synapse were missed in this study, possibly because of the limitations of using the entire brain region, which dilutes out low-abundance proteins. A similar study examined the effect of repeated nicotine administration for 7 days on striatal proteins using 2-DE and MALDI-TOF MS (Yeom et al., 2005). In this study, the striata from 10 saline- and 10 nicotine-treated rats were dissected and pooled for 2-DE, which was executed three times per group. Three proteins were increased, and four were decreased after nicotine exposure.

The effects of morphine and butorphanol (a mixed opioid agonist-antagonist) on phosphotyrosyl proteins in the rat frontal cortex have been analyzed by 2-DE followed by MALDI-TOF MS (Kim et al., 2004b, 2005). Rats were given intracerebroventricular infusions of morphine or butorphanol for 72 h and sacrificed 6 h later. Proteins from the frontal cortex were resolved by 2-DE using a wide (pH 3–10) and narrow (pH 3–6, 5–8, and 7–10) range IPG strips. Global alterations in the phosphotyrosine proteome were determined by immunoblotting with an anti-phosphotyrosine antibody and by proteomic analysis. Proteins showing significant changes in tyrosine phosphorylation were excised from the gels and identified by MALDI-TOF MS. The levels of phosphotyrosyl proteins were found to be generally higher in morphine- and butorphanol-dependent rat brains compared with saline controls. Several cytoskeletal proteins (such as various isoforms of actin and tubulin) were significantly increased in the dependent rat brains, suggesting alterations in neuronal morphology and/or axonal transport. In addition, several phosphotyrosyl proteins were expressed only in the dependent rat brains, including the two G proteins, G_i and G_o . This could suggest a modulation of opioid receptor signal transduction during dependence.

The studies described above illustrate the potential of proteomics for studying the complex process of drug addiction. Although no consistent patterns have yet emerged from this limited number of studies, this is likely to change as more and more researchers apply proteomics to the study of addiction. It is clear from the few studies described above that chronic drug administration significantly alters the levels and phosphorylation states of synaptic proteins, including signaling, vesicle trafficking, endocytotic, cytoskeletal, and

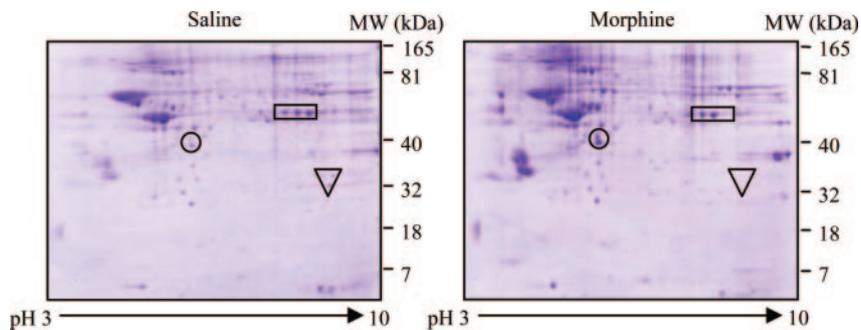


Fig. 1. Comparison of hippocampal PAZ proteins from saline- and morphine-treated mice by 2-DE. Mice ($n = 10$ per group) were treated with saline or escalating doses of morphine (5–15 mg/kg i.p.) every 12 h for 2 days. The hippocampi were dissected 12 h after the last injection and were pooled for subcellular fractionation. In brief, synaptosomes were prepared by sucrose gradient centrifugation of hippocampal homogenate. Synaptic junctions were extracted from the synaptosomal fraction by solubilization with Triton X-100 at pH 6. The PAZ fraction was extracted by further solubilization at pH 8. PAZ fractions (1 mg) from saline- and morphine-treated mice were separated by 2-DE and visualized by Coomassie Blue staining. In the first dimension, IEF was carried out using IPG strips, pH 3 to 10, 18 cm in an Ettan IPGphor II (Amersham Biosciences). In the second dimension, SDS-PAGE was carried out using 12% polyacrylamide gels. The gels were stained with Coomassie Blue. The circles outline examples of proteins that are increased, triangles outline proteins that are decreased, and rectangles outline possible post-translational modifications of proteins upon morphine treatment.

cell adhesion proteins. The underlying mechanisms for these changes are still unknown and will necessitate further exploration.

Challenges and Future Directions

As evidenced by the studies described above, a major challenge in comparing the results of proteomic analyses of drug addiction is the wide range of drug administration paradigms used, which vary in dosage, frequency, method, and duration of drug administration. Even the time at which animals are sacrificed after prolonged drug administration has to be carefully considered, because they can go from a drug-dependent state to a withdrawal state within a short time period, depending on the type and dose of drug administered. Of course, the choice of brain region, subcellular fraction, and/or subset of proteins to analyze are other factors that make such studies difficult to compare.

The choice of proteomic technique used in an experiment can also affect its outcome. For example, the use of two different separation techniques (such as IEF/SDS-PAGE versus 16-BAC/SDS-PAGE) to analyze the same protein sample can produce drastically different results, because the techniques are more or less suitable for specific subsets of proteins (such as soluble versus membrane proteins). In addition, proteomic techniques are often executed in different ways in each laboratory. For example, some groups perform ICAT using forward and reverse labeling (forward: control = light, treated = heavy; reverse: control = heavy, treated = light) to ensure consistency during the labeling procedure. Others perform unidirectional ICAT labeling with an increased number of replicates and use statistical means to determine significant changes in protein levels. In the case of 2-DE, some prefer to compare samples from multiple individual animals, whereas others pool samples within each experimental group (to decrease biological variability) and run multiple replicate gels. Future neuroproteomic studies of drug addiction would certainly benefit from using complementary proteomic approaches as well as some standardization of data representation.

It is likely that an increasing number of future studies will use subcellular fractionation and quantitative proteomic techniques to examine the global effects of drugs of abuse on synaptic proteins. Such analyses will provide the foundation for further studies to uncover the function of synaptic proteins that are altered during the administration of drugs of abuse and to elucidate the role of these proteins in the development of addictive behavior. Proteomic studies will also facilitate the identification of molecular markers of drug addiction, as well as potential drug targets for the treatment of addiction. It is likely that novel synaptic proteins will continue to be discovered as proteomic techniques improve in the ability to detect low abundance membrane and signaling molecules. This will increase our understanding of synaptic structure, organization, and function and will enable us to construct a map of synaptic proteins, their interactions, and regulations. Thus, neuroproteomic studies will serve to answer many fundamental questions of synaptic function and to increase our understanding of molecular mechanisms of drug addiction.

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References

- Abul-Husn NS, Morón JA, Dolios G, Wang R, and Devi LA (2005) Modulation of presynaptic proteins by morphine. *Soc Neurosci Abstr* **31**:Program 802.7.
- Andersson L and Porath J (1986) Isolation of phosphoproteins by immobilized metal (Fe³⁺) affinity chromatography. *Anal Biochem* **154**:250–254.
- Cheng D, Hoogenraad CC, Rush J, Ramm E, Schlager MA, Duong DM, Xu P, Rukshan S, Hanfelt J, Nakagawa T, et al. (2006) Relative and absolute quantification of postsynaptic density proteome isolated from rat forebrain and cerebellum. *Mol Cell Proteomics*, in press.
- Collins MO, Husi H, Yu L, Brandon JM, Anderson CN, Blackstock WP, Choudhary JS, and Grant SG (2006) Molecular characterization and comparison of the components and multiple protein complexes in the postsynaptic proteome. *J Neurochem* **97** (suppl 1):16–23.
- Collins MO, Yu L, Coba MP, Husi H, Campuzano I, Blackstock WP, Choudhary JS, and Grant SG (2005) Proteomic analysis of in vivo phosphorylated synaptic proteins. *J Biol Chem* **280**:5972–5982.
- Cotman CW, Banker G, Churchill L, and Taylor D (1974) Isolation of postsynaptic densities from rat brain. *J Cell Biol* **63**:441–455.
- Coughenour HD, Spaulding RS, and Thompson CM (2004) The synaptic vesicle proteome: a comparative study in membrane protein identification. *Proteomics* **4**:3141–3155.
- DeGiorgis JA, Jaffe H, Moreira JE, Carlotti CG Jr, Leite JP, Pant HC, and Dosemeci A (2005) Phosphoproteomic analysis of synaptosomes from human cerebral cortex. *J Proteome Res* **4**:306–315.
- Dosemeci A, Tao-Cheng JH, Vinade L, and Jaffe H (2006) Preparation of postsynaptic density fraction from hippocampal slices and proteomic analysis. *Biochem Biophys Res Commun* **339**:687–694.
- Emmert-Buck MR, Bonner RF, Smith PD, Chuaqui RF, Zhuang Z, Goldstein SR, Weiss RA, and Liotta LA (1996) Laser capture microdissection. *Science (Wash DC)* **274**:998–1001.
- Fenn JB, Mann M, Meng CK, Wong SF, and Whitehouse CM (1989) Electrospray ionization for mass spectrometry of large biomolecules. *Science (Wash DC)* **246**:64–71.
- Guo M, Xu NJ, Li YT, Yang JY, Wu CF, and Pei G (2005) Morphine modulates glutamate release in the hippocampal CA1 area in mice. *Neurosci Lett* **381**:12–15.
- Gygi SP, Rist B, Gerber SA, Turecek F, Gelb MH, and Aebersold R (1999) Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat Biotechnol* **17**:994–999.
- Hajos F (1975) An improved method for the preparation of synaptosomal fractions in high purity. *Brain Res* **93**:485–489.
- Hartinger J, Stenius K, Hogemann D, and Jahn R (1996) 16-BAC/SDS-PAGE: a two-dimensional gel electrophoresis system suitable for the separation of integral membrane proteins. *Anal Biochem* **240**:126–133.
- Jordan BA, Fernholz BD, Boussac M, Xu C, Grigorean G, Ziff EB, and Neubert TA (2004) Identification and verification of novel rodent postsynaptic density proteins. *Mol Cell Proteomics* **3**:857–871.
- Kanner BI and Sharon I (1978) Active transport of L-glutamate by membrane vesicles isolated from rat brain. *Biochemistry* **17**:3949–3953.
- Kim SI, Voshol H, van Oostrum J, Hastings TG, Cascio M, and Glucksman MJ (2004a) Neuroproteomics: expression profiling of the brain's proteomes in health and disease. *Neurochem Res* **29**:1317–1331.
- Kim SY, Chudapongse N, Lee SM, Levin MC, Oh JT, Park HJ, and Ho IK (2004b) Proteomic analysis of phosphotyrosyl proteins in the rat brain: effect of butorphanol dependence. *J Neurosci Res* **77**:867–877.
- Kim SY, Chudapongse N, Lee SM, Levin MC, Oh JT, Park HJ, and Ho IK (2005) Proteomic analysis of phosphotyrosyl proteins in morphine-dependent rat brains. *Brain Res Mol Brain Res* **133**:58–70.
- Li KW, Hornshaw MP, Van Der Schors RC, Watson R, Tate S, Casetta B, Jimenez CR, Gouwenberg Y, Gundelfinger ED, Smalla KH, et al. (2004) Proteomics analysis of rat brain postsynaptic density. Implications of the diverse protein functional groups for the integration of synaptic physiology. *J Biol Chem* **279**:987–1002.
- Li KW, Jimenez CR, van der Schors RC, Hornshaw MP, Schoffemeer AN, and Smit AB (2006) Intermittent administration of morphine alters protein expression in rat nucleus accumbens. *Proteomics* **6**:2003–2008.
- Mann M and Wilm M (1994) Error-tolerant identification of peptides in sequence databases by peptide sequence tags. *Anal Chem* **66**:4390–4399.
- Morciano M, Burre J, Corvey C, Karas M, Zimmermann H, and Volkandt W (2005) Immunolocalization of two synaptic vesicle pools from synaptosomes: a proteomics analysis. *J Neurochem* **95**:1732–1745.
- Mouledous L, Hunt S, Harcourt R, Harry J, Williams KL, and Gutstein HB (2003) Navigated laser capture microdissection as an alternative to direct histological staining for proteomic analysis of brain samples. *Proteomics* **3**:610–615.
- Nielsen PA, Olsen JV, Podtelejnikov AV, Andersen JR, Mann M, and Wisniewski JR (2005) Proteomic mapping of brain plasma membrane proteins. *Mol Cell Proteomics* **4**:402–408.
- Pappin DJ (2003) Peptide mass fingerprinting using MALDI-TOF mass spectrometry. *Methods Mol Biol* **211**:211–219.
- Peng J, Kim MJ, Cheng D, Duong DM, Gygi SP, and Sheng M (2004) Semiquantitative proteomic analysis of rat forebrain postsynaptic density fractions by mass spectrometry. *J Biol Chem* **279**:21003–21011.
- Phillips GR, Florens L, Tanaka H, Khaing ZZ, Fidler L, Yates JR 3rd, and Colman DR (2005) Proteomic comparison of two fractions derived from the transsynaptic scaffold. *J Neurosci Res* **81**:762–775.

- Phillips GR, Huang JK, Wang Y, Tanaka H, Shapiro L, Zhang W, Shan WS, Arndt K, Frank M, Gordon RE, et al. (2001) The presynaptic particle web: ultrastructure, composition, dissolution and reconstitution. *Neuron* **32**:63–77.
- Prokai L, Zharikova AD, and Stevens SM Jr (2005) Effect of chronic morphine exposure on the synaptic plasma-membrane subproteome of rats: a quantitative protein profiling study based on isotope-coded affinity tags and liquid chromatography/mass spectrometry. *J Mass Spectrom* **40**:169–175.
- Rabilloud T (2002) Two-dimensional gel electrophoresis in proteomics: old, old fashioned, but it still climbs up the mountains. *Proteomics* **2**:3–10.
- Robinson TE and Kolb B (2004) Structural plasticity associated with exposure to drugs of abuse. *Neuropharmacology* **47** (Suppl 1):33–46.
- Satoh K, Takeuchi M, Oda Y, Deguchi-Tawarada M, Sakamoto Y, Matsubara K, Nagasu T, and Takai Y (2002) Identification of activity-regulated proteins in the postsynaptic density fraction. *Genes Cells* **7**:187–197.
- Schrimpf SP, Meskenaite V, Brunner E, Rutishauser D, Walther P, Eng J, Aebersold R, and Sonderegger P (2005) Proteomic analysis of synaptosomes using isotope-coded affinity tags and mass spectrometry. *Proteomics* **5**:2531–2541.
- Stevens SM Jr, Zharikova AD, and Prokai L (2003) Proteomic analysis of the synaptic plasma membrane fraction isolated from rat forebrain. *Brain Res Mol Brain Res* **117**:116–128.
- Trinidad JC, Thalhammer A, Specht CG, Schoepfer R, and Burlingame AL (2005) Phosphorylation state of postsynaptic density proteins. *J Neurochem* **92**:1306–1316.
- Unlu M, Morgan ME, and Minden JS (1997) Difference gel electrophoresis: a single gel method for detecting changes in protein extracts. *Electrophoresis* **18**:2071–2077.
- Walikonis RS, Jensen ON, Mann M, Provance DW Jr, Mercer JA, and Kennedy MB (2000) Identification of proteins in the postsynaptic density fraction by mass spectrometry. *J Neurosci* **20**:4069–4080.
- Williams K, Wu T, Colangelo C, and Nairn AC (2004) Recent advances in neuroproteomics and potential application to studies of drug addiction. *Neuropharmacology* **47** (Suppl 1):148–166.
- Witzmann FA, Arnold RJ, Bai F, Hrnčirova P, Kimpel MW, Mechref YS, McBride WJ, Novotny MV, Pedrick NM, Ringham HN, et al. (2005) A proteomic survey of rat cerebral cortical synaptosomes. *Proteomics* **5**:2177–2201.
- Wolters DA, Washburn MP, and Yates JR 3rd (2001) An automated multidimensional protein identification technology for shotgun proteomics. *Anal Chem* **73**:5683–5690.
- Yeom M, Shim I, Lee HJ, and Hahm DH (2005) Proteomic analysis of nicotine-associated protein expression in the striatum of repeated nicotine-treated rats. *Biochem Biophys Res Commun* **326**:321–328.
- Yoshimura Y, Yamauchi Y, Shinkawa T, Taoka M, Donai H, Takahashi N, Isobe T, and Yamauchi T (2004) Molecular constituents of the postsynaptic density fraction revealed by proteomic analysis using multidimensional liquid chromatography-tandem mass spectrometry. *J Neurochem* **88**:759–768.

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