Neuropharmacology 56 (2009) 196–204

Contents lists available at ScienceDirect

Neuropharmacology

journal homepage: www.elsevier.com/locate/neuropharm

Review

Characterizing intercellular signaling peptides in drug addiction

Elena V. Romanova, Nathan G. Hatcher, Stanislav S. Rubakhin, Jonathan V. Sweedler*

Department of Chemistry and Beckman Institute, University of Illinois, 600 South Mathews Avenue 63-5, Urbana, IL 61801, USA

ARTICLE INFO

Article history:
Received 5 May 2008
Received in revised form 23 July 2008
Accepted 28 July 2008

Keywords:
Mass spectrometry
Neuropeptides
Bioinformatics
Peptidomics

ABSTRACT

Intercellular signaling peptides (SPs) coordinate the activity of cells and influence organism behavior. SPs, a chemically and structurally diverse group of compounds responsible for transferring information between neurons, are broadly involved in neural plasticity, learning and memory, as well as in drug addiction phenomena. Historically, SP discovery and characterization has tracked advances in measurement capabilities. Today, a suite of analytical technologies is available to investigate individual SPs, as well as entire intercellular signaling complements, in samples ranging from individual cells to entire organisms. Immunochemistry and *in situ* hybridization are commonly used for following preselected SPs. Discovery-type investigations targeting the transcriptome and proteome are accomplished using high-throughput characterization technologies such as microarrays and mass spectrometry. By integrating directed approaches with discovery approaches, multiplatform studies fill critical gaps in our knowledge of drug-induced alterations in intercellular signaling. Throughout the past 35 years, the National Institute on Drug Abuse has made significant resources available to scientists that study the mechanisms of drug addiction. The roles of SPs in the addiction process are highlighted, as are the analytical approaches used to detect and characterize them.

1. Introduction: NIDA's role in the investigation of intercellular signaling mechanisms in the nervous system

Drug addiction involves complex physiological and behavioral phenomena, characterized by compulsive drug use and the concomitant physical and mental degradation of the drug user (Goodman, 2008; Kalivas and Volkow, 2005; Koob and Kreek, 2007; Koob and Le Moal, 2008; Nestler, 2001). Drugs act on endogenous neurochemical pathways in the brain; accordingly, understanding these pathways is important for revealing the mechanisms of drug addiction. Established in 1974, the National Institute on Drug Abuse (NIDA) recognized drug abuse and addiction not only as a devastating social problem, but as a significant neurological disease. NIDA has led the scientific effort to address issues of drug addiction and dependence, including understanding how illicit drugs function in the brain and body. As a result, extraordinary progress has been achieved in understanding chemical signaling in the brain.

Because of their importance to brain function and behavior, the characterization of novel neurotransmitter and receptor systems under the influence of drugs had enormous impact in addiction research (for reviews, see Gintzler and Chakrabarti, 2000; Jaworski et al., 2003; Przewlocki, 2004). The discovery of a family of opioid peptides in 1970s (Goldstein et al., 1979; Guillemin et al., 1977; Hughes et al., 1975) stimulated interest in examining the roles of peptide transmitters and modulators in addiction. NIDA eagerly supported both fundamental research on SPs (Fig. 1) and the development of technical innovations capable of advancing this field. Analyses of literature and records in the Computer Retrieval of Information on Scientific Projects database (CRISP: http://crisp.cit.nih.gov/crisp/crisp_query.generate_screen) indicate that peptide research sponsored by NIDA initially focused on pharmacological properties and the potency of opioid peptides for producing physical dependence. Likewise, interactions between endogenous opiates and exogenous narcotic analgesics in the development of tolerance and dependence have been well studied. Significant attention was devoted to analysis of the cellular origin, distribution and functional properties of the endogenous opiates and on comparing the structure–activity relationships of opiate peptide agonists and antagonists with narcotic analgesics. Later, attention shifted to biochemical studies of the mechanism of opioid peptide interactions with receptors and with other known neurotransmitter systems of the brain. In 1980s, the involvement of other non-opiate peptides in the neurochemical response to drugs of abuse was discovered. For example, as a result of behavioral and neurochemical studies, neurotensin was implicated in the regulation of the mesolimbic dopamine system, and thus was named as one of the first non-opiate 'reward peptides' (Glimcher et al., 1984).

According to NIDA records in CRISP, peptide research rapidly grew in 1980s and 1990s (Fig. 1). Progress included the identification of brain-reward regions, the development of increasingly

* Corresponding author. Tel.: +1 217 244 7359; fax: +1 217 265 6290.
E-mail address: jsweedle@uiuc.edu (J.V. Sweedler).

0028-3908/$ – see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuropharm.2008.07.036

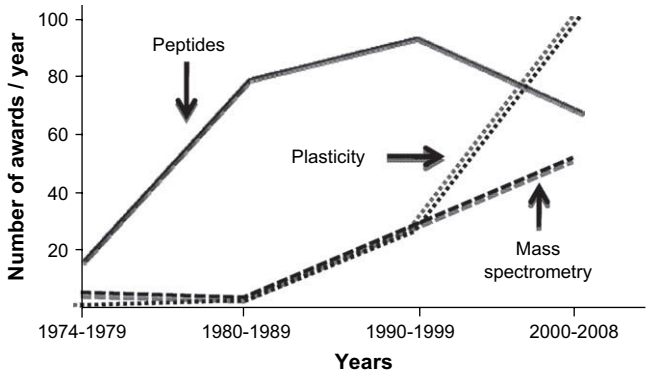

Fig. 1. Trends in NIDA funding by selected key words—peptides, plasticity and mass spectrometry. Average number of awards per year in each period is shown. Source: CRISP database (http://crisp.cit.nih.gov/).

sophisticated animal models reflecting human substance abuse behaviors with remarkable fidelity, and the invention of differential display techniques (Liang and Pardee, 1992). Together, these advances made it possible to study the drug-induced changes affecting neuropeptide signaling in the reward circuitry, accounting for the complex behavioral abnormalities of the addicted state. Ultimately, scanning brain regions for specific mRNAs that are transcriptionally regulated by administration of psychostimulants led to discovery of cocaine- and amphetamine-regulated transcript (CART) mRNA in the shell of the nucleus accumbens and other areas associated with reinforcement (Douglass et al., 1995), even though the existence of a CART peptide fragment had been reported 14 years earlier (Spiess et al., 1981). Characterization of the molecular and cellular pathways by which drugs of abuse produce prolonged changes in brain circuits shed light on mechanisms of motivation-reward and stimulus–response learning during addiction phenomena. Over the past decade, the focus of drug addiction research supported by NIDA has shifted to investigating complex events such as neuronal adaptations and synaptic plasticity (Fig. 1), which convert drug-induced neurochemical signals into long-term alterations in neural function, ultimately remodeling neuronal circuits.

Commemorating the significant contribution of NIDA to the advancement of our fundamental knowledge of brain function, here we present a review of the methods and discoveries that yielded important insights into SP signaling and their importance in drug addiction phenomena.

2. Intercellular signaling peptides in mechanisms of drug abuse

Intercellular signaling peptides play an important role in normal cell–cell signaling in the brain and are critical elements in the mechanisms of addiction. There are several major categories of SPs—cytokines, neurotrophins, neuronal guidance cues, hormones, and neuropeptides. Cytokines, neurotrophins, and neuronal guidance cues are synthesized and widely distributed in the brain (Loughlin and Fallon, 1993; Singh, 2003), and are involved in regulating cell division, maintenance, survival and growth. Other functions such as neuromodulation (Juttler et al., 2002; Lu and Chow, 1999; Pan et al., 1997) are also common. Endogenous neuropeptides and hormones are produced through the intracellular synthesis and processing of a wide variety of precursor proteins. Each prohormone precursor may yield multiple, distinct, and highly potent neuropeptides (Aebischer and Hefti, 1999; Balkwill, 2000a,b; Boulton et al., 1993; Fitzgerald, 2001; Singh, 2003; Thomson and Lotze, 2003). Neuropeptides are involved in a broad range of physiological functions including neurotransmission, neuromodulation, and hormonal signaling.

Evidence has accumulated on the important roles of intercellular signaling molecules in addiction mechanisms (Akbarian et al., 2001; Berhow et al., 1995; Hall et al., 1991; Morley et al., 1980; Narita et al., 2003; Peterson et al., 1998; Pierce and Bari, 2001; Pierce et al., 1999; Smith et al., 2003; Solbrig et al., 2000; Torres and Horowitz, 1999). In particular, SPs have been implicated in a variety of drug dependence phenomena including tolerance (Smith et al., 2003), withdrawal (Akbarian et al., 2001; Grimm et al., 2003), and sensitization (Pierce and Bari, 2001; Pierce et al., 1999). Exposure to substances of abuse can change SP expression, localization, and action. Acute exposure to drugs such as cocaine and morphine influences the expression of a number of genes, resulting in alteration of SP synthesis as well as complex downstream changes in related systems (Gan et al., 1999; Grimm et al., 2003; Peterson et al., 1998). As several examples, CART peptide mRNA expression is upregulated in the presence of psychostimulants (Douglass et al., 1995) and can promote feeding (Kristensen et al., 1998) and locomotion behaviors (Kimmel et al., 2000, 2002) with acute exposure. Chronic cocaine administration is correlated with marked, long-lasting changes in the distribution of neuroactive peptide mRNAs within the central nervous system where they have been suggested to contribute to the reinforcing properties associated with the development and persistence of cocaine addiction. Several reports show a direct link between cocaine exposure and the expression of prohormones such as preproenkephalin in the hypothalamus, nucleus accumbens, amygdala, and prefrontal cortex, thus altering the opioid signaling within these regions (LaForge et al., 2003). Methamphetamine modulates levels of tumor necrosis factor-alpha and interleukin-1β mRNA in striatum (Flora et al., 2003).

Perhaps the best studied neuropeptide signaling system linked to mechanisms of drug abuse is the opioid system, which includes neuropeptides such as the endorphins, enkephalins and dynorphins, as well as specialized membrane receptors. The endogenous opioids mediate a wide array of physiological effects, including analgesia, through three categories of opiate receptors: μ, κ and δ. Opioid peptides share the same structural motif (Y-G-G-F) at the N-terminus. Structural variability occurs in the rest of the sequence and follows from differences in prohormone sequence, processing and posttranslational modifications (PTMs). As an example, endorphins are derived from pro-opiomelanocortin (POMC). POMC undergoes tissue-specific processing via endogenous enzymatic cleavage and can yield different ratios of α-, β-, and γ-endorphins as well as Arg- and Met-enkephalins, depending on the neuroanatomical location of POMC processing.

Specific modifications of SPs often vary by brain region as well. For example, β-endorphin is present across multiple brain regions in its native form, and yet exhibits N-terminal acetylation specifically within the intermediate lobe of the pituitary. Morphine shares sequence similarity with the N-terminal amino acids of a number of opioid peptides, perhaps explaining this alkaloid's high affinity for opioid receptors (with the highest affinity for the μ-receptor subtype). In addition to analgesia, opiates such as morphine produce widespread effects across the nervous system, notably within the hypothalamic/pituitary axis, midbrain, brain stem and spinal cord. The sedation and reward effects of morphine reflect the diverse interplay of multiple brain systems incorporating endogenous opioid peptide signaling. Moreover, the behavioral and physical aspects of reward seeking, withdrawal, and relapse are outward manifestations of the broad neurochemical perturbations that opiates induce within these systems.

Peptides endogenous to the nervous system can function as regulators of opioid signaling by either enhancing or decreasing response efficacy. Such is the case with peptides derived from the RFamide family, neuropeptides FF and SF. Neuropeptide FF (SF in

rodents) is present in the spinal cord where it is thought to contribute to analgesia (Yang et al., 1985). When intrathecally injected, neuropeptide FF exhibits strong neuromodulation, enhancing morphine-induced analgesia (Gouarderes et al., 1993). Thus, the coincident presence of opiates and RFamides may result in enhanced effects of endogenous opioid responses to pain. These neuropeptides are also found in the brain and cerebral spinal fluid, but the function of these peptides in these "higher" centers is not known.

In contrast, other endogenous peptides have antagonistic effects on opiates. Glycyl-glutamine, a dipeptide endogenously derived from β-endorphin 1–31, reduces both establishment and reinstatement of morphine- and nicotine-induced place preference in animal models (Cavun et al., 2005; Goktalay et al., 2006). Cleavage of β-endorphin 1–31 to form glycyl-glutamine concomitantly results in the co-formation of a second opiate antagonist, β-endorphin 1–27. Thus, β-endorphin 1–27 and glycyl-glutamine likely are endogenous attenuators of the opiate systems and hold promise for developing pharmacological agents capable of decreasing morphine and nicotine tolerance, dependence, and withdrawal.

Drugs of abuse affect multiple, often interconnected intercellular signaling systems utilizing both classical neurotransmitters and SPs. Cocaine and morphine induce persistent alterations within peptidergic elements of the mesolimbic dopamine circuits involved not only in the formation and maintenance of addiction but also relapse. Orexigenic/hypocretinergic neurons within the lateral hypothalamus mediate arousal behaviors that can reinforce previously extinguished drug-seeking and food-seeking behaviors (Georgescu et al., 2003; Harris et al., 2005). Orexin A causes long-term potentiation in the ventral tegmental area (VTA) through the recruitment of *N*-methyl-D-aspartic acid (NMDA) receptors (Borgland et al., 2006). This NMDA-mediated enhancement of glutamatergic transmission in the VTA profoundly enhances behavioral sensitization to cocaine and provides a strong link between corticotrophin-releasing factor mediated-stress responses and hyperarousal associated with increased drug craving (de Lecea et al., 2006).

3. Analytical technologies for discovery and characterization of signaling peptides

SPs typically exert their effects at the nanomolar to picomolar concentration range, are present in multiple isoforms, often contain multiple posttranslational modifications, and frequently exhibit a variety of actions. Many SP-mediated signals converge on a single cell or small group of functionally interconnected cells. Elucidating the inherent chemical and functional complexity within highly peptidergic, biochemically interconnected systems is critical to understanding the neurobiological underpinnings of drug addiction, and presents significant analytical challenges.

Investigating the role SPs play in the mechanisms of drug addiction requires the detection and identification of these substances in complex chemical matrices as well as quantitative analysis of their dynamic changes with spatial and temporal resolution. Approaches currently used to examine SPs may be categorized as preselected approaches (often presented as hypothesis-driven) and high-throughput (often presented as discovery-oriented). Approaches that incorporate knowledge of the signaling pathways and specific molecular targets are the workhorses of neuroscience and neurochemistry. As an alternative, high-throughput platforms interrogate, ideally comprehensively, the genome, transcriptome, proteome, peptidome, and even secretome to uncover even unsuspected pathways affected by abused drugs and discover potential new pharmacological targets. Both approaches are outlined below.

3.1. Directed strategies for SP characterization

In these approaches, a specific signaling pathway or compound(s) of interest is measured using a molecular probe. Such methods have allowed the analysis of peptides at many stages, including gene expression, peptide synthesis, cellular localization, intracellular transport, secretion, binding to receptors, and catabolism (Hook et al., 2008; Nillni, 2007). A variety of analytical approaches have enabled labeling, purification and sorting of selected SPs in models ranging from single organelles to whole animals, with the sensitivity of some of these approaches reaching the single molecule level (Yeung, 2004).

Most often, experimental designs relying on directed characterization use immunoaffinity interactions of compounds of interest with selective molecular probes such as antibodies. The power of immunohistochemistry and other affinity methods is in the specificity and sensitivity of the immune reaction ($\sim 10^{-15}$ g: Coons, 1956), as well as the sensitivity and spatial resolution of compatible detection readouts. The methodology is indispensable in detection and characterization of signaling pathways. For example, initial knowledge was attained on the cellular and subcellular localization of enkephalin (Elde et al., 1976; Simantov et al., 1977), substance P (Pickel et al., 1979), CREB (Walters et al., 2003), and many other intracellular and intercellular signaling molecules currently implicated in the long-term neuronal plasticity associated with drug addiction via immunostaining followed by optical or electron microscopy. Using affinity purification, automated Edman sequencing and mass spectrometry (MS), Thim et al. (1999) demonstrated differential proteolytic processing of splice variants of the CART prohormone in various brain regions and the pituitary. Radioimmunoassay (RIA) and enzyme-linked immunosorbent assay are routinely used to quantitatively measure the levels of SPs. Elucidation of opioid peptide structures allowed RIA applications in drug addiction research (Simantov and Snyder, 1976; Yang et al., 1977a,b). However, immunoaffinity methods are limited in that the probe can be created only if the sequence of the SP of interest is known.

Besides examining larger samples, the mechanisms of SP synthesis, intracellular packaging and transport, as well as their release into the extracellular space, can be studied at the subcellular level. Detecting SPs in cellular organelles such as dense core vesicles is important but presents challenges because of the low amounts of compounds available and small volume of many organelles. Affinity detection with immunogold staining and electron microscopy is, perhaps, the oldest and most often used technology (for review see Larsson, 1996) and enables the analysis of SPs at the subcellular level with low nanometer lateral resolution (Bergersen et al., 2008). Using the immunogold approach, prodynorphin storage in axons and dendrites (Yakovleva et al., 2006), localization of substance P and calcitonin gene-related peptide in large granular vesicles (Aimar et al., 1998), and the presence of corticotropin-releasing hormone in secretory granules (Schmidt et al., 1995) have been investigated. Confocal microscopy also allows study of fluorescently labeled SPs with organelle resolution (Rutter et al., 2006).

Alternatively to imaging approaches, organelles in nervous tissue can be sorted using flow cytometry (Rajotte et al., 2003) or via microfluidic platforms (Lu et al., 2004). Isolation and consecutive analysis of functionally and biochemically similar organelles has been successfully used to decipher mechanisms generating active neuropeptides in secretory vesicles (Hook et al., 2008).

What happens when a specific SP is not known and a small-volume sample needs to be assayed? Examples of peptide analysis with samples as small as a secretory vesicle have appeared in literature using capillary electrophoresis (Chiu et al., 1998) and MS (Ciobanu et al., 2004; Rubakhin et al., 2000). Despite progress in

sample characterization, peptide analysis at the organelle level remains challenging. In contrast, cellular-level studies are more accessible and yield exceptionally rich chemical information linked to morphological, electrophysiological, and molecular biology data. Because many analytical figures-of-merit scale with concentration rather than mass, sample preparation using low volume samples such as single cells can be studied at native high local concentrations. A "typical" mammalian cell is 10 μm in diameter, has a volume of 500 fL, and contains approximately 50 pg or 2 fmol of protein (Hu et al., 2000). Thus, if only a few compounds are present, these are within the detection range for a number of measurement methods including affinity recognition with fluorescence, enzymatic, and radioactivity detection (Baggerman et al., 2004; Popii and Baumann, 2004). Single cell MS is a powerful tool for the investigation of the intercellular SP complement in cells and organelles (for reviews see Hummon et al., 2006a, 2003b; Li et al., 2000). In many cases, new prohormones have been reported, and the cells containing them identified, the prohormone processing revealed, and the physiology induced by the resulting peptides presented in the same study (Floyd et al., 1999; Hoek et al., 2005; Li et al., 2001; Santama and Benjamin, 2000; Smit et al., 2003; Vilim et al., 2001). As shown in Fig. 2(A), SPs in single pituitary cells have been profiled by matrix-assisted laser desorption/ionization (MALDI) MS (Rubakhin and Sweedler, 2007). Another methodology—capillary electrophoresis–laser induced florescence—allowed Dovichi et al. (Hu et al., 2002, 2001a,b; Zhang et al., 2000) to study proteins in a single cell with higher sensitivity than offered by MS. We have demonstrated a broad range of single cell detection approaches, including *de novo* sequencing of a peptide from a single neuron (Li et al., 1999).

SP localization in single cells and tissues is often achieved by *in situ* hybridization, which visualizes the expression of peptide coding mRNA. One advantage of this approach is its selectivity and sensitivity; as an example, the presence of low levels of POMC mRNA in regions of the mesocorticolimbic system, an important part of the reward circuit, was recently demonstrated using *in situ* hybridization techniques (Leriche et al., 2007). However, often, mRNA levels and amounts of SPs encoded by them do not correlate, perhaps because of the vastly different lifetimes of the diverse peptides and proteins in the brain and the variety of mechanisms used to regulate protein synthesis from RNA. Such differences between RNA and protein levels have been reported for prodynorphin mRNA and alpha-neoendorphin levels in the nucleus accumbens and striatum following an acute cocaine dose (Turchan et al., 1998), as well as for preprotachykinin-A mRNA and substance P peptide levels in the caudate putamen and substantia nigra (Kraft et al., 2001).

Importantly, it is possible to follow SPs *in vivo* in animal models of drug addiction, thus providing an opportunity to link changes in brain neurochemistry to behavioral output. One method of choice for *in vivo* sampling of SPs is microdialysis sampling (Davies et al., 2000) in combination with RIA (Torregrossa and Kalivas, 2008) or liquid chromatography and MS (Lanckmans et al., 2008). The advantage of the microdialysis procedure is simplification of the collected sample to a particular molecular size range. In addition, the stability of collected peptides is expected to improve with the exclusion of non-specific proteolytic enzymes commonly present in the brain. However, low amounts of SPs in dilute microdialysis samples from the brain require sensitive methods of detection and measurement. Coupling microdialysis to MS has allowed detection of released enkephalin in pallidum at low fmol levels, and neurotensin in hypothalamus at amol levels (Andrén and Caprioli, 1999; Emmett et al., 1995). Changes in concentration of corticotropin-releasing factor measured with RIA observed in elegant microdialysis studies have been suggested to play a role in mediating behavioral sensitization to cocaine (Richter et al., 1995) and in the drug withdrawal process (Richter and Weiss, 1999).

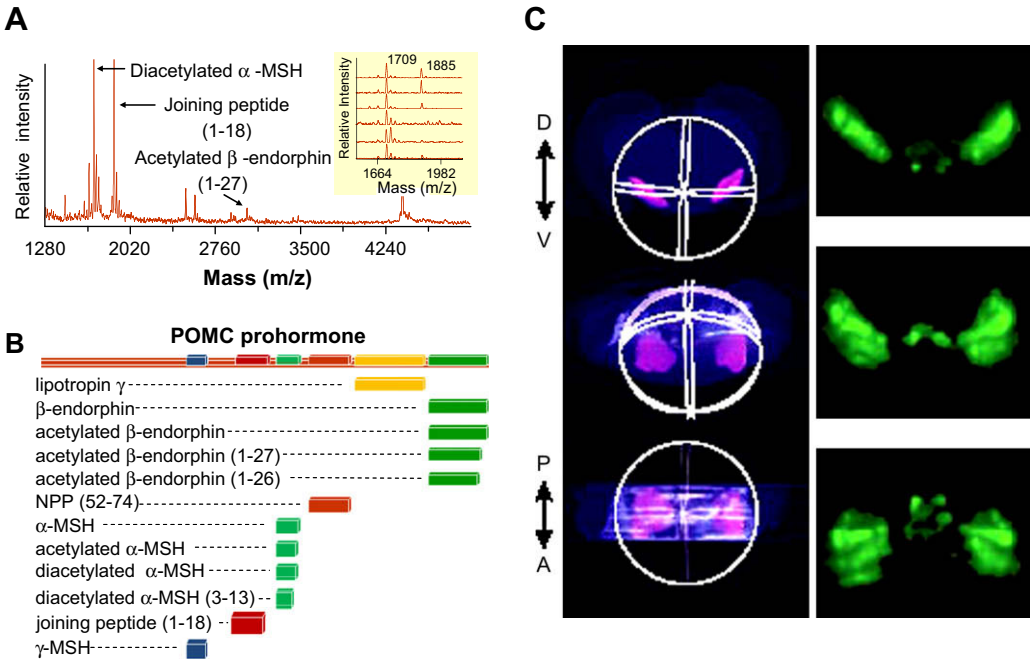

Fig. 2. Mass spectrometric technologies allow detection and characterization of SPs in a vide variety of biological specimens. (A) Representative mass spectrum of an individual pituitary cell obtained using single cell MALDI MS. Not all identified peptides are labeled. Inset: mass spectra obtained from six different isolated pituitary cells. The 1709 *m*/*z* and 1885 *m*/*z* peaks correspond to diacetylated α-MSH and joining peptide (1–18). (Reprinted with some modifications from Rubakhin et al., 2008, Nat. Protoc., 2, 1987–1997, with permission.) (B) Summary of POMC prohormone processing based on a single pituitary cell spectra showing the excellent prohormone coverage. (C) 3D visualization of substance P distribution in the rat brain created with MALDI MSI and a bundle of software programs allowing processing of mass spectra, creation of 2D images, coregistration of information obtained from adjacent brain slices, and finally, formation of the 3D image. Volume reconstruction of substance P localization shown from frontal (top rows of images) and dorsal (bottom row) views. Brain slice positioning: A – anterior, P – posterior, V – ventral, D – dorsal. (Reprinted with some modifications from Andersson et al., 2008, Nat. Methods, 5, 101–108, with permission.)

Moving up from individual cells and tissues to organs, and even entire organisms, makes more material available for analysis. However, the increased chemical complexity of these larger samples requires additional separation and purification steps in order to isolate compounds of interest. Consequently, for these more complex investigations, more global methods of peptide analysis have become increasingly common.

3.2. High-throughput, wholesale characterization strategies

A key advantage of high-throughput exploratory analysis, relative to many other molecular screening methods, is that the approach does not require *a priori* information on the identity of the compound(s) of interest. Moreover, instead of measuring changes in just a few components such as a preselected mRNA or a protein, entire classes of compounds are measured. Such strategies, as discussed below, offer fast and sensitive means to uncover critical genes and signaling pathways affected by abused dugs, and can lead to the discovery of novel pharmacological targets.

3.2.1. Gene expression analysis

With the advent of techniques for analysis of global gene expression it became possible to assay drug-related changes in the entire transcriptome. Microarray technology (Fodor et al., 1993; Schena et al., 1995) enables high-throughput, simultaneous assay of thousands of genes in a single experiment and has revolutionized how scientists approach investigating cellular processes (Lockhart and Winzeler, 2000). Expression profiling using microarrays has revealed psychostimulant-induced alterations in genes underlying neuronal growth, synaptogenesis, signal transduction, apoptosis and cell metabolism (Pollock, 2002; Yuferov et al., 2005), as well as multiple structural protein genes (Bannon et al., 2005). Microarray analysis used in analyzing gene expression in genetic models of alcoholism revealed elevated hippocampal neuropeptide Y expression in alcohol-avoiding animals (Arlinde et al., 2004). Genomics research in psychiatry and neuroscience has provided important insights into gene expression in perturbed states, including drug induced, without the need for a prior hypothesis. Contrary to some expectations, genomics has not yet markedly improved the therapeutic options in addiction, but it has identified candidate genes involved with signaling pathways that are potentially associated with addiction phenomena (Kreek, 2008; Uhl et al., 2008).

The complexity of relating genetic information to the actual functions of encoded proteins partly arises from alternative splicing of gene transcripts and PTMs, which yield a multitude of products from a single gene. According to the ENCODE pilot project (Tress et al., 2007), which annotates alternatively spliced gene products, the number of protein forms for each coding region ranges from 1 to 17, with 2.5 being the average in humans. In addition, a direct correlation between levels of gene transcripts and their encoded proteins often is not observed (Gygi et al., 1999). Investigation of peptide roles in drug dependence presents additional challenges that stem from the processing of the SP prohormone by tissue-specific enzymes, generating overlapping but not identical sets of bioactive peptides. Proteomics approaches based on robust mass spectrometric profiling and identification can reveal actual gene products and their PTMs, at times, with spatial resolution (Fig. 2(B)). Comparison of global protein/peptide expression profiles in the brain-reward circuitry aids understanding of the molecular mechanisms governing the synaptic transmission and plasticity underlying the complex process of drug addiction.

3.2.2. Bioinformatics

High-throughput experiments generate large amounts of complex data. In fact, the biological sciences are experiencing a globalization phase in their development, characterized by an exponential growth in the amount of information on biochemical structures and interactions in living systems. This information is becoming heavily cross-referenced with the help of bioinformatics, which in turn creates important tools for prediction, discovery, characterization, and confirmation. The availability of genome, transcriptome, and proteome information for a growing number of species allows *in silico* discovery of new SPs in terms of structure and possible function. Examination of the strategies used in SP research suggests that bioinformatics serves two important roles: as a discovery/prediction tool, helping us analyze massive genome, transcriptome and proteome databases, and as a tool for confirmation/verification.

Bioinformatic analyses become essential steps at multiple stages of SP investigation, including SP discovery, detection, identification in complex samples and prediction of function (Amare et al., 2006; Hummon et al., 2003a; Southey et al., 2008). For example, prediction of bioactive peptides can be aided by homology searches that exploit similarity between prohormone sequences in different species (Altschul et al., 1990). Because neuropeptides are fairly short and the conserved sequences even shorter, similarity searches alone may not always be effective. Codon scanning methods that search for the nucleotide codes of conserved motifs within the database have been found effective as many prohormones contain repeating amino acid sequences separated by dibasic sequences such as KK, KR, and RR. Hence, searching for the repeated codons within a 100 kb region of the genome can reveal potential neuropeptide precursors. Codon scanning can also be applied using sequences obtained from measurements such as tandem MS (Hummon et al., 2006b). In many cases, codon scanning and Blast searches help identify a portion of the peptide or prohormone, but regions surrounding the active peptide, including signal sequence, must be reconstructed from the genomic sequence. To reconstruct the complete precursor, the nucleotides surrounding the active sequence are examined for the presence of various gene features such as translation start-sites, exons and introns, as well as termination signals. These gene features can be determined using a variety of bioinformatics programs and web applications and eventually lead to complete reconstruction of the gene.

Once the prohormone is known, the most likely set of bioactive peptides can be predicted from the new prohormone using several binary logistic and expert systems using the NeuroPred discovery tool (Southey et al., 2006). A list of predicted peptides provides information for follow-up MS analyses. Application of bioinformatics to novel genomes allows the annotation of many novel prohormones for some models (Amare and Sweedler, 2007; Clynen et al., 2006; Hewes and Taghert, 2001; Li et al., 2008; Riehle et al., 2002). Bioinformatics approaches are also capable of predicting new SPs; Mirabeau (Mirabeau et al., 2007) designed and applied the hidden Markov model to alignment of proteins constructed from the Ensemble database or known protein sequences from the SWISS-PROT/TrEMBL database. As proof of concept, in both cases, known SPs were observed, however, with less efficiency in the case of the SWISS-PROT/TrEMBL database. From the 300 top candidates picked by this analysis, 61% represented known SPs and 20% were hypothetical or poorly characterized proteins. Application of additional criteria to the last group helped to determine two potential novel SP precursors. As a result of this search, followed by molecular biology and electrophysiological experiments, two new SP candidates have been described: spexin and augurin.

Despite the success of bioinformatics in SP discovery, this approach is only partially successful when applied to species with unannotated genomes. Fortunately, technologies such as MS, when combined with bioinformatics, enable discovery of unknown SPs and detection of previously characterized SPs in complex biological samples.

3.2.3. Bioanalytical mass spectrometry

Mass spectrometry has evolved into a central technique for SP discovery and characterization. High sensitivity, structural characterization capabilities, and new bioinformatics tools for processing data have empowered modern MS-based strategies. Initially used for measurements of abused drugs as early as 1965 (Audier et al., 1965), MS is now a favored method for measurement, identification and quantification of peptides and proteins in many drug addiction studies (Abul-Husn and Devi, 2006; Che et al., 2006; Prokai et al., 2005; Yang et al., 2007). Pioneering applications of MS in drug addiction-related research has led to pivotal discoveries such as the natural ligands for opiate receptors, Met-enkephalin and Leu-enkephalin (Hughes et al., 1975). Partial amino acid sequences of these small peptides were initially obtained by the dansyl–Edman procedure. Uncertainties about peptide length and amino acid composition were resolved by measuring masses of derivatized peptide ions and their fragments using electron impact ionization MS. The utility of MS greatly increased in the late 1980s following breakthrough developments in soft ionization methods for larger biomolecules by Tanaka (Tanaka et al., 1988), Fenn (Fenn et al., 1989), and Hillenkamp and Karas (Karas et al., 1987).

Currently, a suite of analytical techniques is available to undertake the discovery of the biochemical organization and the molecular identity of the constituents in cells. When combined with gene expression data and physiological activity measurements in the brain, MS reveals a great deal about complex signaling processes between and within the cells that affect the activity of an organism as a whole. The ability of MS to structurally characterize SPs, complements single cell differential expression analyses by providing information on final products of gene expression.

Notwithstanding these advances in measurement technologies, analysis of SPs in the brain remains challenging because of its inherent structural and chemical heterogeneity. Furthermore, detection of native SPs in the mammalian brain may be obscured by post-mortem protein degradation products. Typically, SPs present at low levels relative to the ubiquitous proteins and they may easily be overwhelmed if even a small fraction of the abundant cellular proteins break down (Che et al., 2005; Svensson et al., 2003). Robust fractionation techniques, such as two-dimensional gel electrophoresis, liquid chromatography, and affinity purifications, are commonly used in conjunction with MS to enhance investigation at the protein level. Separated analytes can be detected using UV absorbance, fluorescence, electrochemical, nuclear magnetic resonance (NMR), and MS methods (Sandberg and Weber, 2003). The first three approaches typically demand analyte standards for SP identification, while NMR and MS are capable of identifying molecules directly.

The combination of liquid chromatography and mass spectrometry (LC–MS) creates a major discovery platform for high-throughput analysis of SPs. Early attempts to combine separation and MS for identification of peptides via fragmentation perhaps date back to 1975 (Jayasimhulu and Day, 1975). One of the first successful interfaces using a continuous sample flow was reported in the following decade (Caprioli et al., 1987), which coincided with the invention of softer methods of ionization. These instrumental advances expanded the analytical capability for fast and robust analysis of biological molecules and made LC–MS technology indispensable for the analysis of SPs.

Matrix-assisted laser desorption/ionization time-of-flight MS is a common technique for identification of differentially regulated proteins due to its robustness and sensitivity to low amounts of peptides and proteins in chemically heterogeneous samples. Structural identification of proteins is often achieved by peptide mass fingerprinting (Henzel et al., 1993) of protein mixtures fractionated by gel electrophoresis and digested into smaller peptides with proteolytic enzymes such as trypsin. Masses of resulting peptide ions are measured by MS and searched against databases for statistically the most probable mass match to amino acid sequences from known proteins deposited in the database. Alternatively, peptide sequences deduced *in silico* on platforms with tandem MS capability can be statistically matched to a protein in the database.

Bioinformatics plays a vital role in processing the MS-generated information. One of the most commonly used applications, Mascot (Perkins et al., 1999), accurately models the actions of exogenous proteolytic enzymes and searches experimentally observed mass values (whole peptide or tandem MS fragment ions) against predicted ones, yielding a probability score relating whether the observed match may be due to a random match.

MS-based platforms are well-suited for the analysis and identification of signaling proteins and protein signatures associated with exposure to abused drugs in animal models and human addicts. In a non-candidate study using tandem MS of purified cytosolic fractions of NAc, Tannu (Tannu et al., 2007) determined differences in the abundance levels of membrane-associated proteins and proteins involved in cytoskeletal transformation, 15 proteins total, in human cocaine overdose victims. Mass spectrometric examination of the protein constituents of the NAc in rodent models of morphine abuse revealed changes in structural proteins (Li et al., 2006) characteristic of opioid addiction and neurodegenerative diseases in humans (Ferrer-Alcón et al., 2000; Lariviere and Julien, 2004). Likewise, Li et al. (2006) pinpointed morphine-induced changes in synapsin and Lin-7 (also known as veils), known to be involved in synaptic transmission. Long-lasting molecular neuroadaptations in the medial prefrontal cortex have been found in animal models of a natural reward, such as sucrose self-administration. Differentially regulated proteins unambiguously identified by peptide mass fingerprint analyses were involved in cytoskeleton organization, energy metabolism, neurotransmission, oxidative stress, and differentiation and outgrowth (VandenOever et al., 2006). Geng et al. (2006) applied a surface-enhanced laser desorption/ionization method to study protein expression in the brain of rats withdrawn from repeated cocaine exposure. Two small proteins were found to be significantly increased in the VTA of the brain in these animals.

Often, determining the SP complement in biological samples requires several proteomics approaches, especially when the SP genes are not annotated in advance. As one example, the honey bee, *Apis mellifera*, has become an exciting sociogenomic model that allows one to relate specific gene levels to the social roles of an individual in the hive (The Honey Bee Consortium, 2006). Hummon et al. (2006b) integrated bioinformatics, small-volume MS, and *de novo* peptide sequencing to discover SPs during the genome annotation process; 36 genes encoding more than 200 putative and known SPs were reported, of which 33 had not been known previously.

A newer capability, mass spectrometric imaging (MSI) is becoming increasingly practical for mapping the spatial distribution of peptides and proteins and their structural variances directly in tissue such as the brain and spine (Andersson et al., 2008; Cornett et al., 2007; Monroe et al., in press; Rubakhin et al., 2005), allowing both the chemical identity and localizations to be explored in a single experiment. Whole body imaging of SP expression can be investigated by several unique approaches including three-dimensional mass spectrometric imaging (3D MSI) (Andersson et al., 2008), positron emission tomography (Sprenger et al., 2005), and fluorescence whole body imaging (Fujiki et al., 2008). In principal, 3D MSI (see Fig. 2(B)) is an exceptionally powerful technology, allowing simultaneous determination and visualization of numerous SPs contained in the sample at detectable levels (Fig. 2).

Above and beyond the identification of new molecular players, quantitation of peptide levels is important and is becoming

common in MS applications, with stable isotope labeling being the mainstream approach. As one example, Che et al. (2006) used tandem MS to identify and stable isotopic labeling to quantify changes in levels of 10 peptides induced in the rodent brain by cocaine administration. Intriguingly, the measured peptides were not previously reported to change under cocaine treatment. Using analogous approaches with stable isotopic tags and MS, Decaillot et al. (2006) identified 27 endogenous peptides and revealed small, consistent changes in the levels of several of them in the hypothalamus and striatum of Cpefat/fat mice chronically treated with morphine.

4. Conclusions

Deciphering the overall mechanisms governing drug addiction requires an understanding of how dynamic and interconnected intercellular signaling systems integrate. Anatomically and functionally distinct neural networks mediate, not only adaptive responses to environmental stimuli, but also maladaptive addiction behaviors brought about by a hijacked reward mechanism. The study of these systems and the pathophysiologies associated with addiction have generated far-reaching insights into brain circuitry. As a result, drug abuse research has made major contributions to the generalized mechanisms of neuronal plasticity and the elucidation of neural substrates of behavior, a fundamental goal of neuroscience research. Our understanding of reward, tolerance, withdrawal, and even relapse, extends from behavior to network, from cells to receptors, from prohormone processing to SP release. A current challenge is to provide a comprehensive view of the chemical makeup of defined brain regions in normal states and to reveal the changes that occur with specific aspects of addiction. Mass spectrometry-based proteomics is the newest of these technologies, and is in the midst of rapid methodological advancement. With improvements in instrumentation, sampling, and bioinformatics, large-scale measures of drug of abuse-induced changes in SPs can now be made. These studies fill critical gaps in our knowledge about drug-induced alterations in cell–cell signaling and point to an exciting new range of possible pharmacological targets.

Acknowledgements

This material is based upon work supported by the National Institute of Drug Abuse under Award No. DA017940 and No. P30DA018310 to the UIUC Neuroproteomics Center on Cell–Cell Signaling.

References

Abul-Husn, N.S., Devi, L.A., 2006. Neuroproteomics of the synapse and drug addiction. J. Pharmacol. Exp. Ther. 318, 461–468.
Aebischer, P., Hefti, F., 1999. Neurotrophic Factors. Handbook of Experimental Pharmacology. Springer, Berlin, New York.
Aimar, P., Pasti, L., Carmignoto, G., Merighi, A., 1998. Nitric oxide-producing islet cells modulate the release of sensory neuropeptides in the rat substantia gelatinosa. J. Neurosci. 18, 10375–10388.
Akbarian, S., Bates, B., Liu, R.J., Skirboll, S.L., Pejchal, T., Coppola, V., Sun, L.D., Fan, G., Kucera, J., Wilson, M.A., Tessarollo, L., Kosofsky, B.E., Taylor, J.R., Bothwell, M., Nestler, E.J., Aghajanian, G.K., Jaenisch, R., 2001. Neurotrophin-3 modulates noradrenergic neuron function and opiate withdrawal. Mol. Psychiatry 6, 593–604.
Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410.
Amare, A., Hummon, A.B., Southey, B.R., Zimmerman, T.A., Rodriguez-Zas, S.L., Sweedler, J.V., 2006. Bridging neuropeptidomics and genomics with bioinformatics: prediction of mammalian neuropeptide prohormone processing. J. Proteome Res. 5, 1162–1167.
Amare, A., Sweedler, J.V., 2007. Neuropeptide precursors in *Tribolium castaneum*. Peptides 28, 1282–1291.
Andersson, M., Groseclose, M.R., Deutch, A.Y., Caprioli, R.M., 2008. Imaging mass spectrometry of proteins and peptides: 3D volume reconstruction. Nat. Methods 5, 101–108.
Andrén, P.E., Caprioli, R.M., 1999. Determination of extracellular release of neurotensin in discrete rat brain regions utilizing in vivo microdialysis/electrospray mass spectrometry. Brain Res. 845, 123–129.
Arlinde, C., Sommer, W., Bjo?rk, K., Reimers, M., Hyytia, P., Kiianmaa, K., Heilig, M., 2004. A cluster of differentially expressed signal transduction genes identified by microarray analysis in a rat genetic model of alcoholism. Pharmacogenomics J. 4, 208–218.
Audier, H., Fetizon, M., Ginsburg, D., Mandelbaum, A., Ruell, T., 1965. Mass spectrometry of the morphine alkaloids. Tetrahedron Lett. 57, 13–22.
Baggerman, G., Verleyen, P., Clynen, E., Huybrechts, J., De Loof, A., Schoofs, L., 2004. Peptidomics. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 803, 3–16.
Balkwill, F.R. (Ed.), 2000a. Cytokine Cell Biology: a Practical Approach. Oxford University Press, New York.
Balkwill, F.R., 2000b. The Cytokine Network. Oxford University Press, Oxford.
Bannon, M., Kapatos, G., Albertson, D., 2005. Gene expression profiling in the brains of human cocaine abusers. Addict. Biol. 10, 119–126.
Bergersen, L.H., Storm-Mathisen, J., Gundersen, V., 2008. Immunogold quantification of amino acids and proteins in complex subcellular compartments. Nat. Protoc. 3, 144–152.
Berhow, M.T., Russell, D.S., Terwilliger, R.Z., Beitner-Johnson, D., Self, D.W., Lindsay, R.M., Nestler, E.J., 1995. Influence of neurotrophic factors on morphine- and cocaine-induced biochemical changes in the mesolimbic dopamine system. Neuroscience 68, 969–979.
Borgland, S.L., Taha, S.A., Sarti, F., Fields, H.L., Bonci, A., 2006. Orexin A in the VTA is critical for the induction of synaptic plasticity and behavioral sensitization to cocaine. Neuron 49, 589–601.
Boulton, A.A., Baker, G.B., Hefti, F., 1993. Neurotrophic Factors. Humana Press, Totowa, NJ.
Caprioli, R.M., DaGue, B., Fan, T., Moore, W.T., 1987. Microbore HPLC/mass spectrometry for the analysis of peptide mixtures using a continuous flow interface. Biochem. Biophys. Res. Commun. 146, 291–299.
Cavun, S., Goktalay, G., Millington, W.R., 2005. Glycyl-glutamine, an endogenous beta-endorphin-derived peptide, inhibits morphine-induced conditioned place preference, tolerance, dependence, and withdrawal. J. Pharmacol. Exp. Ther. 315, 949–958.
Che, F.Y., Lim, J., Pan, H., Biswas, R., Fricker, L.D., 2005. Quantitative neuropeptidomics of microwave-irradiated mouse brain and pituitary. Mol. Cell. Proteomics 4, 1391–1405.
Che, F.Y., Vathy, I., Fricker, L.D., 2006. Quantitative peptidomics in mice: effect of cocaine treatment. J. Mol. Neurosci. 28, 265–275.
Chiu, D.T., Lillard, S.J., Scheller, R.H., Zare, R.N., Rodriguez-Cruz, S.E., Williams, E.R., Orwar, O., Sandberg, M., Lundqvist, J.A., 1998. Probing single secretory vesicles with capillary electrophoresis. Science 279, 1190–1193.
Ciobanu, L., Rubakhin, S.S., Stuart, J.N., Fuller, R.R., Webb, A.G., Sweedler, J.V., 2004. Characterization of the physicochemical parameters of dense core atrial gland and lucent red hemiduct vesicles in *Aplysia californica*. Anal. Chem. 76, 2331–2335.
Clynen, E., Huybrechts, J., Verleyen, P., De Loof, A., Schoofs, L., 2006. Annotation of novel neuropeptide precursors in the migratory locust based on transcript screening of a public EST database and mass spectrometry. BMC Genomics 7, 201.
Coons, A.H., 1956. Histochemistry with labeled antibody. Int. Rev. Cytol. 5, 1–23.
Cornett, D.S., Reyzer, M.L., Chaurand, P., Caprioli, R.M., 2007. MALDI imaging mass spectrometry: molecular snapshots of biochemical systems. Nat. Methods 4, 828–833.
Davies, M.I., Cooper, J.D., Desmond, S.S., Lunte, C.E., Lunte, S.M., 2000. Analytical considerations for microdialysis sampling. Adv. Drug Deliv. Rev. 45, 169–188.
Decaillot, F.M., Che, F.Y., Fricker, L.D., Devi, L.A., 2006. Peptidomics of Cpefat/fat mouse hypothalamus and striatum: effect of chronic morphine administration. J. Mol. Neurosci. 28, 277–284.
Douglass, J., McKinzie, A.A., Couceyro, P., 1995. PCR differential display identifies a rat brain mRNA that is transcriptionally regulated by cocaine and amphetamine. J. Neurosci. 15, 2471–2481.
Elde, R., Hökfelt, T., Johansson, O., Terenius, L., 1976. Immunohistochemical studies using antibodies to leucine-enkephalin: initial observations on the nervous system of the rat. Neuroscience 1, 349–351. IN311–IN314.
Emmett, M.R., Andrén, P.E., Caprioli, R.M., 1995. Specific molecular mass detection of endogenously released neuropeptides using in vivo microdialysis/mass spectrometry. J. Neurosci. Methods 62, 141–147.
Fenn, J.B., Mann, M., Meng, C.K., Wong, S.F., Whitehouse, C.M., 1989. Electrospray ionization for mass spectrometry of large biomolecules. Science 246, 64–71.
Ferrer-Alcón, M., García-Sevilla, J.A., Jaquet, P.E., Harpe, R.L., Riederer, B.M., Walzer, C., Guimón, J., 2000. Regulation of nonphosphorylated and phosphorylated forms of neurofilament proteins in the prefrontal cortex of human opioid addicts. J. Neuroscience Res. 61, 338–349.
Fitzgerald, K.A., 2001. The Cytokine Factsbook. Academic Press, San Diego, London.
Flora, G., Lee, Y.W., Nath, A., Hennig, B., Maragos, W., Toborek, M., 2003. Methamphetamine potentiates HIV-1 Tat protein-mediated activation of redox-sensitive pathways in discrete regions of the brain. Exp. Neurol. 179, 60–70.
Floyd, P.D., Li, L., Rubakhin, S.S., Sweedler, J.V., Horn, C.C., Kupfermann, I., Alexeeva, V.Y., Ellis, T.A., Dembrow, N.C., Weiss, K.R., Vilim, F.S., 1999. Insulin prohormone processing, distribution, and relation to metabolism in *Aplysia californica*. J. Neurosci. 19, 7732–7741.
Fodor, S.P.A., Rava, R.P., Huang, X.C., Pease, A.C., Holmes, C.P., Adams, C.L., 1993. Multiplexed biochemical assays with biological chips. Nature 364, 555–556.
Fujiki, Y., Tao, K., Bianchi, D.W., Giel-Moloney, M., Leiter, A.B., Johnson, K.L., 2008. Quantification of green fluorescent protein by in vivo imaging, PCR, and flow

cytometry: comparison of transgenic strains and relevance for fetal cell microchimerism. Cytometry A 73, 11–118.
Gan, X.H., Zhang, L., Berger, O., Stins, M.F., Way, D., Taub, D.D., Chang, S.L., Kim, K.S., House, S.D., Weinand, M., Witte, M., Graves, M.C., Fiala, M., 1999. Cocaine enhances brain endothelial adhesion molecules and leukocyte migration. Clin. Immunol. 91, 68–76.
Geng, T., Seitz, P.K., Thomas, M.L., Xu, B., Soman, K.V., Kurosky, A., Luxon, B.A., Cunningham, K.A., 2006. Use of surface enhanced laser desorption/ionization–time of flight mass spectrometry (SELDI-TOF MS) to study protein expression in a rat model of cocaine withdrawal. J. Neurosci. Methods 158, 1–12.
Georgescu, D., Zachariou, V., Barrot, M., Mieda, M., Willie, J.T., Eisch, A.J., Yanagisawa, M., Nestler, E.J., DiLeone, R.J., 2003. Involvement of the lateral hypothalamic peptide orexin in morphine dependence and withdrawal. J. Neurosci. 23, 3106–3111.
Gintzler, A., Chakrabarti, S., 2000. Opioid tolerance and the emergence of new opioid receptor-coupled signaling. Mol. Neurobiol. 21, 21–33.
Glimcher, P.W., Margolin, D.H., Giovino, A.A., Hoebel, B.G., 1984. Neurotensin: a new 'reward peptide'. Brain Res. 291, 119–124.
Goktalay, G., Cavun, S., Levendusky, M.C., Hamilton, J.R., Millington, W.R., 2006. Glycyl-glutamine inhibits nicotine conditioned place preference and withdrawal. Eur. J. Pharmacol. 530, 95–102.
Goldstein, A., Tachibana, S., Lowney, L.I., Hunkapiller, M., Hood, L., 1979. Dynorphin-(1–13), an extraordinarily potent opioid peptide. Proc. Natl. Acad. Sci. U.S.A. 76, 6666–6670.
Goodman, A., 2008. Neurobiology of addiction. An integrative review. Biochem. Pharmacol. 75, 266–322.
Gouarderes, C., Jhamandas, K., Cridland, R., Cros, J., Quirion, R., Zajac, J.M., 1993. Opioid and substance P receptor adaptations in the rat spinal cord following sub-chronic intrathecal treatment with morphine and naloxone. Neuroscience 54, 799–807.
Grimm, J.W., Lu, L., Hayashi, T., Hope, B.T., Su, T.P., Shaham, Y., 2003. Time-dependent increases in brain-derived neurotrophic factor protein levels within the mesolimbic dopamine system after withdrawal from cocaine: implications for incubation of cocaine craving. J. Neurosci. 23, 742–747.
Guillemin, R., Ling, N., Burgus, R., Bloom, F., Segal, D., 1977. Characterization of the endorphins, novel hypothalamic and neurohypophysial peptides with opiate-like activity: evidence that they induce profound behavioral changes. Psychoneuroendocrinology 2, 59–62.
Gygi, S.P., Rochon, Y., Franza, B.R., Aebersold, R., 1999. Correlation between protein and mRNA abundance in yeast. Mol. Cell. Biol. 19, 1720–1730.
Hall, N.R., O'Grady, M.P., Menzies, R.A., 1991. Neuroimmunopharmacologic effects of drugs of abuse. Adv. Exp. Med. Biol. 288, 13–23.
Harris, G.C., Wimmer, M., Aston-Jones, G., 2005. A role for lateral hypothalamic orexin neurons in reward seeking. Nature 437, 556–559.
Henzel, W.J., Billeci, T.M., Stults, J.T., Wong, S.C., Grimley, C., Watanabe, C., 1993. Identifying proteins from two-dimensional gels by molecular mass searching of peptide fragments in protein sequence databases. Proc. Natl. Acad. Sci. U.S.A. 90, 5011–5015.
Hewes, R.S., Taghert, P.H., 2001. Neuropeptides and neuropeptide receptors in the *Drosophila melanogaster* genome. Genome Res. 11, 1126–1142.
Hoek, R.M., Li, K.W., van Minnen, J., Lodder, J.C., de Jong-Brink, M., Smit, A.B., van Kesteren, R.E., 2005. LFRFamides: a novel family of parasitation-induced-RFamide neuropeptides that inhibit the activity of neuroendocrine cells in *Lymnaea stagnalis*. J. Neurochem. 92, 1073–1080.
Hook, V., Funkelstein, L., Lu, D., Bark, S., Wegrzyn, J., Hwang, S.R., 2008. Proteases for processing proneuropeptides into peptide neurotransmitters and hormones. Annu. Rev. Pharmacol. Toxicol. 48, 393–423.
Hu, S., Jiang, J., Cook, L.M., Richards, D.P., Horlick, L., Wong, B., Dovichi, N.J., 2002. Capillary sodium dodecyl sulfate-DALT electrophoresis with laser-induced fluorescence detection for size-based analysis of proteins in human colon cancer cells. Electrophoresis 23, 3136–3142.
Hu, S., Lee, R., Zhang, Z., Krylov, S.N., Dovichi, N.J., 2001a. Protein analysis of an individual *Caenorhabditis elegans* single-cell embryo by capillary electrophoresis. J. Chromatogr. B Biomed. Sci. Appl. 752, 307–310.
Hu, S., Zhang, L., Cook, L.M., Dovichi, N.J., 2001b. Capillary sodium dodecyl sulfate-DALT electrophoresis of proteins in a single human cancer cell. Electrophoresis 22, 3677–3682.
Hu, S., Zhang, Z.R., Cook, L.M., Carpenter, E.J., Dovichi, N.J., 2000. Separation of proteins by sodium dodecyl sulfate capillary electrophoresis in hydroxypropylcellulose sieving matrix with laser-induced fluorescence detection. J. Chromatogr. A 894, 291–296.
Hughes, J., Smith, T.W., Kosterlitz, H.W., Fothergill, L.A., Morgan, B.A., Morris, H.R., 1975. Identification of two related pentapeptides from the brain with potent opiate agonist activity. Nature 258, 577–580.
Hummon, A.B., Amare, A., Sweedler, J.V., 2006a. Discovering new invertebrate neuropeptides using mass spectrometry. Mass Spectrom. Rev. 25, 77–98.
Hummon, A.B., Hummon, N.P., Corbin, R.W., Li, L., Vilim, F.S., Weiss, K.R., Sweedler, J.V., 2003a. From precursor to final peptides: a statistical sequence-based approach to predicting prohormone processing. J. Proteome Res. 2, 650–656.
Hummon, A.B., Richmond, T.A., Verleyen, P., Baggerman, G., Huybrechts, J., Ewing, M.A., Vierstraete, E., Rodriguez-Zas, S.L., Schoofs, L., Robinson, G.E., Sweedler, J.V., 2006b. From the genome to the proteome: uncovering peptides in the *Apis* brain. Science 314, 647–649.
Hummon, A.B., Sweedler, J.V., Corbin, R.W., 2003b. Discovering new neuropeptides using single-cell mass spectrometry. Trends Analyt. Chem. 22, 515–521.
Jaworski, J.N., Vicentic, A., Hunter, R.G., Kimmel, H.L., Kuhar, M.J., 2003. CART peptides are modulators of mesolimbic dopamine and psychostimulants. Life Sci. 73, 741–747.
Jayasimhulu, K., Day, R.A., 1975. Sequence determination of peptides by mass spectrometry: methods for combining "wet" separation techniques with mass spectrometry. Anal. Biochem. 67, 359–371.
Juttler, E., Tarabin, V., Schwaninger, M., 2002. Interleukin-6 (IL-6): a possible neuromodulator induced by neuronal activity. Neuroscientist 8, 268–275.
Kalivas, P.W., Volkow, N.D., 2005. The neural basis of addiction: a pathology of motivation and choice. Am. J. Psychiatry 162, 1403–1413.
Karas, M., Bachmann, D., Bahr, U., Hillenkamp, F., 1987. Matrix-assisted ultraviolet-laser desorption of nonvolatile compounds. Int. J. Mass Spectrom. Ion Process. 78, 53–68.
Kimmel, H.L., Gong, W., Vechia, S.D., Hunter, R.G., Kuhar, M.J., 2000. Intra-ventral tegmental area injection of rat cocaine and amphetamine-regulated transcript peptide 55–102 induces locomotor activity and promotes conditioned place preference. J. Pharmacol. Exp. Ther. 294, 784–792.
Kimmel, H.L., Thim, L., Kuhar, M.J., 2002. Activity of various CART peptides in changing locomotor activity in the rat. Neuropeptides 36, 9–12.
Koob, G., Kreek, M.J., 2007. Stress, dysregulation of drug reward pathways, and the transition to drug dependence. Am. J. Psychiatry 164, 1149–1159.
Koob, G.F., Le Moal, M., 2008. Addiction and the brain antireward system. In: Fiske, S.T., Schacter, D.L., Sternberg, R. (Eds.). Annu. Rev. Psychol., 29–53.
Kraft, M., Noailles, P., Angulo, J.A., 2001. Substance P modulates cocaine-evoked dopamine overflow in the striatum of the rat brain. Ann. N. Y. Acad. Sci. 937, 121–131.
Kreek, M.J., 2008. Role of a functional human gene polymorphism in stress responsivity and addictions. Clin. Pharmacol. Ther. 83, 615–618.
Kristensen, P., Judge, M.E., Thim, L., Ribel, U., Christjansen, K.N., Wulff, B.S., Clausen, J.T., Jensen, P.B., Madsen, O.D., Vrang, N., Larsen, P.J., Hastrup, S., 1998. Hypothalamic CART is a new anorectic peptide regulated by leptin. Nature 393, 72–76.
LaForge, K.S., Yuferov, V., Zhou, Y., Ho, A., Nyberg, F., Kreek, M.J., 2003. "Binge" cocaine differentially alters preproenkephalin mRNA levels in guinea pig brain. Brain Res. Bull. 59, 353–357.
Lanckmans, K., Sarre, S., Smolders, I., Michotte, Y., 2008. Quantitative liquid chromatography/mass spectrometry for the analysis of microdialysates. Talanta 74, 458–469.
Lariviere, R.C., Julien, J.-P., 2004. Functions of intermediate filaments in neuronal development and disease. J. Neurobiol. 58, 131–148.
Larsson, L.I., 1996. Immunogold labelling of neuroendocrine peptides with special reference to antibody specificity and multiple staining techniques. Histochem. Cell Biol. 106, 93–103.
de Lecea, L., Jones, B.E., Boutrel, B., Borgland, S.L., Nishino, S., Bubser, M., DiLeone, R., 2006. Addiction and arousal: alternative roles of hypothalamic peptides. J. Neurosci. 26, 10372–10375.
Leriche, M., Cote-Velez, A., Mendez, M., 2007. Presence of pro-opiomelanocortin mRNA in the rat medial prefrontal cortex, nucleus accumbens and ventral tegmental area: studies by RT-PCR and in situ hybridization techniques. Neuropeptides 41, 421–431.
Li, B., Predel, R., Neupert, S., Hauser, F., Tanaka, Y., Cazzamali, G., Williamson, M., Arakane, Y., Verleyen, P., Schoofs, L., Schachtner, J., Grimmelikhuijzen, C.J.P., Park, Y., 2008. Genomics, transcriptomics, and peptidomics of neuropeptides and protein hormones in the red flour beetle *Tribolium castaneum*. Genome Res. 18, 113–122.
Li, K.W., Jimenez, C.R., Schors, R.C.v.d., Hornshaw, M.P., Schoffelmeer, A.N.M., Smit, A.B., 2006. Intermittent administration of morphine alters protein expression in rat nucleus accumbens. Proteomics 6, 2003–2008.
Li, L., Floyd, P.D., Rubakhin, S.S., Romanova, E.V., Jing, J., Alexeeva, V.Y., Dembrow, N.C., Weiss, K.R., Vilim, F.S., Sweedler, J.V., 2001. Cerebrin prohormone processing, distribution and action in *Aplysia californica*. J. Neurochem. 77, 1569–1580.
Li, L., Garden, R.W., Romanova, E.V., Sweedler, J.V., 1999. In situ sequencing of peptides from biological tissues and single cells using MALDI-PSD/CID analysis. Anal. Chem. 71, 5451–5458.
Li, L., Garden, R.W., Sweedler, J.V., 2000. Single-cell MALDI: a new tool for direct peptide profiling. Trends Biotechnol. 18, 151–160.
Liang, P., Pardee, A.B., 1992. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science 257, 967–971.
Lockhart, D.J., Winzeler, E.A., 2000. Genomics, gene expression and DNA arrays. Nature 405, 827–836.
Loughlin, S.E., Fallon, J.H., 1993. Neurotrophic Factors. Academic Press, San Diego.
Lu, B., Chow, A., 1999. Neurotrophins and hippocampal synaptic transmission and plasticity. J. Neurosci. Res. 58, 76–87.
Lu, H., Gaudet, S., Schmidt, M.A., Jensen, K.F., 2004. A microfabricated device for subcellular organelle sorting. Anal. Chem. 76, 5705–5712.
Mirabeau, O., Perlas, E., Severini, C., Audero, E., Gascuel, O., Possenti, R., Birney, E., Rosenthal, N., Gross, C., 2007. Identification of novel peptide hormones in the human proteome by hidden Markov model screening. Genome Res. 17, 320–327.
Monroe, E.B., Annangudi, S.P., Hatcher, N.G., Gutstein, H.B., Rubakhin, S.S., Sweedler, J.V. SIMS and MALDI MS imaging of the spinal cord. Proteomics, doi:10.100/pmic.200800127
Morley, J.E., Yamada, T., Walsh, J.H., Lamers, C.B., Wong, H., Shulkes, A., Damassa, D.A., Gordon, J., Carlson, H.E., Hershman, J.M., 1980. Morphine addiction and withdrawal alters brain peptide concentrations. Life Sci. 26, 2239–2244.

Narita, M., Aoki, K., Takagi, M., Yajima, Y., Suzuki, T., 2003. Implication of brain-derived neurotrophic factor in the release of dopamine and dopamine-related behaviors induced by methamphetamine. Neuroscience 119, 767–775.
Nestler, E.J., 2001. Molecular basis of long-term plasticity underlying addiction. Nat. Rev. Neurosci. 2, 119–128.
Nillni, E.A., 2007. Regulation of prohormone convertases in hypothalamic neurons: implications for prothyrotropin-releasing hormone and proopiomelanocortin. Endocrinology 148, 4191–4200.
Pan, W., Zadina, J.E., Harlan, R.E., Weber, J.T., Banks, W.A., Kastin, A.J., 1997. Tumor necrosis factor-alpha: a neuromodulator in the CNS. Neurosci. Biobehav. Rev. 21, 603–613.
Perkins, D.N., Pappin, D.J.C., Creasy, D.M., Cottrell, J.S., 1999. Probability-based protein identification by searching sequence databases using mass spectrometry data. Electrophoresis 20, 3551–3567.
Peterson, P.K., Molitor, T.W., Chao, C.C., 1998. The opioid–cytokine connection. J. Neuroimmunol. 83, 63–69.
Pickel, V.M., Joh, T.H., Reis, D.J., Leeman, S.E., Miller, R.J., 1979. Electron microscopic localization of substance P and enkephalin in axon terminals related to dendrites of catecholaminergic neurons. Brain Res. 160, 387–400.
Pierce, R.C., Bari, A.A., 2001. The role of neurotrophic factors in psychostimulant-induced behavioral and neuronal plasticity. Rev. Neurosci. 12, 95–110.
Pierce, R.C., Pierce-Bancroft, A.F., Prasad, B.M., 1999. Neurotrophin-3 contributes to the initiation of behavioral sensitization to cocaine by activating the Ras/Mitogen-activated protein kinase signal transduction cascade. J. Neurosci. 19, 8685–8695.
Pollock, J.D., 2002. Gene expression profiling: methodological challenges, results, and prospects for addiction research. Chem. Phys. Lipids 121, 241–256.
Popii, V., Baumann, G., 2004. Laboratory measurement of growth hormone. Clin. Chim. Acta 350, 1–16.
Prokai, L., Zharikova, A.D., Stevens Jr., S.M., 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.
Przewlocki, R., 2004. Opioid abuse and brain gene expression. Eur. J. Pharmacol. 500, 331–349.
Rajotte, D., Stearns, C.D., Kabcenell, A.K., 2003. Isolation of mast cell secretory lysosomes using flow cytometry. Cytometry A 55, 94–101.
Richter, R.M., Pich, E.M., Koob, G.F., Weiss, F., 1995. Sensitization of cocaine-stimulated increase in extracellular levels of corticotropin-releasing factor from the rat amygdala after repeated administration as determined by intracranial microdialysis. Neurosci. Lett. 187, 169–172.
Richter, R.M., Weiss, F., 1999. In vivo CRF release in rat amygdala is increased during cocaine withdrawal in self-administering rats. Synapse 32, 254–261.
Riehle, M.A., Garczynski, S.F., Crim, J.W., Hill, C.A., Brown, M.R., 2002. Neuropeptides and peptide hormones in *Anopheles gambiae*. Science 298, 172–175.
Rubakhin, S.S., Garden, R.W., Fuller, R.R., Sweedler, J.V., 2000. Measuring the peptides in individual organelles with mass spectrometry. Nat. Biotechnol. 18, 172–175.
Rubakhin, S.S., Jurchen, J.C., Monroe, E.B., Sweedler, J.V., 2005. Imaging mass spectrometry: fundamentals and applications to drug discovery. Drug Discov. Today 10, 823–837.
Rubakhin, S.S., Sweedler, J.V., 2007. Characterizing peptides in individual mammalian cells using mass spectrometry. Nat. Protoc. 2, 1987–1997.
Rutter, G.A., Loder, M.K., Ravier, M.A., 2006. Rapid three-dimensional imaging of individual insulin release events by Nipkow disc confocal microscopy. Biochem. Soc. Trans. 34, 675–678.
Sandberg, M., Weber, S.G., 2003. Techniques for neuropeptide determination. Trends Analyt. Chem. 22, 522–527.
Santama, N., Benjamin, P.R., 2000. Gene expression and function of FMRFamide-related neuropeptides in the snail *Lymnaea*. Microsc. Res. Tech. 49, 547–556.
Schena, M., Shalon, D., Davis, R.W., Brown, P.O., 1995. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science 270, 467–470.
Schmidt, E.D., Janszen, A.W., Wouterlood, F.G., Tilders, F.J., 1995. Interleukin-1-induced long-lasting changes in hypothalamic corticotropin-releasing hormone (CRH) – neurons and hyperresponsiveness of the hypothalamus–pituitary–adrenal axis. J. Neurosci. 15, 7417–7426.
Simantov, R., Kuhar, M.J., Uhl, G.R., Snyder, S.H., 1977. Opioid peptide enkephalin: immunohistochemical mapping in rat central nervous system. Proc. Natl. Acad. Sci. U.S.A. 74, 2167–2171.
Simantov, R., Snyder, S.H., 1976. Morphine like peptides in mammalian brain: isolation, structure elucidation, and interactions with the opiate receptor. Proc. Natl. Acad. Sci. U.S.A. 73, 2515–2519.
Singh, V.K., 2003. Cytokine regulation of autism. In: Kronfol, Z. (Ed.), Cytokines and Mental Health. Kluwer Academic Publishers, Boston, pp. 367–383.
Smit, A.B., van Kesteren, R.E., Spijker, S., van Minnen, J., van Golen, F.A., Jiménez, C.R., Li, K.W., 2003. Peptidergic modulation of male sexual behavior in *Lymnaea stagnalis*: structural and functional characterization of −FVamide neuropeptides. J. Neurochem. 87, 1245–1254.
Smith, D.J., Leil, T.A., Liu, X., 2003. Neurotrophin-4 is required for tolerance to morphine in the mouse. Neurosci. Lett. 340, 103–106.
Solbrig, M.V., Koob, G.F., Parsons, L.H., Kadota, T., Horscroft, N., Briese, T., Lipkin, W.I., 2000. Neurotrophic factor expression after CNS viral injury produces enhanced sensitivity to psychostimulants: potential mechanism for addiction vulnerability. J. Neurosci. 20, RC104.
Southey, B.R., Amare, A., Zimmerman, T.A., Rodriguez-Zas, S.L., Sweedler, J.V., 2006. NeuroPred: a tool to predict cleavage sites in neuropeptide precursors and provide the masses of the resulting peptides. Nucleic Acids Res. 34.
Southey, B.R., Sweedler, J.V., Rodriguez-Zas, S.L., 2008. Prediction of neuropeptide cleavage sites in insects. Bioinformatics 24, 815–825.
Spiess, J., Villarreal, J., Vale, W., 1981. Isolation and sequence analysis of a somatostatin-like polypeptide from ovine hypothalamus. Biochemistry 20, 1982–1988.
Sprenger, T., Berthele, A., Platzer, S., Boecker, H., Tölle, T.R., 2005. What to learn from in vivo opioidergic brain imaging? Eur. J. Pain 9, 117–121.
Svensson, M., Skold, K., Svenningsson, P., Andren, P.E., 2003. Peptidomics-based discovery of novel neuropeptides. J. Proteome Res. 2, 213–219.
The Honey Bee Consortium, 2006. Insights into social insects from the genome of the honeybee *Apis mellifera*. Nature 443, 931–949.
Tanaka, K., Waki, H., Ido, Y., Akita, S., Yoshida, Y., Yoshida, T., 1988. Protein and polymer analysis up to m/z 100 000 by laser ionisation time-of-flight mass spectrometry. Rapid Commun. Mass Spectrom. 2, 151–153.
Tannu, N., Mash, D.C., Hemby, S.E., 2007. Cytosolic proteomic alterations in the nucleus accumbens of cocaine overdose victims. Mol. Psychiatry 12, 55–73.
Thim, L., Kristensen, P., Nielsen, P.F., Wulff, B.S., Clausen, J.T., 1999. Tissue-specific processing of cocaine- and amphetamine-regulated transcript peptides in the rat. Proc. Natl. Acad. Sci. U.S.A. 96, 2722–2727.
Thomson, A.W., Lotze, M.T., 2003. The Cytokine Handbook. Academic Press, Amsterdam; Boston.
Torregrossa, M.M., Kalivas, P.W., 2008. Microdialysis and the neurochemistry of addiction. Pharmacol. Biochem. Behav. 63, 261–272.
Torres, G., Horowitz, J.M., 1999. Drugs of abuse and brain gene expression. Psychosom. Med. 61, 630–650.
Tress, M.L., Martelli, P.L., Frankish, A., Reeves, G.A., Wesselink, J.J., Yeats, C., Olason, P.L., Albrecht, M., Hegyi, H., Giorgetti, A., Raimondo, D., Lagarde, J., Laskowski, R.A., Lopez, G., Sadowski, M.I., Watson, J.D., Fariselli, P., Rossi, I., Nagy, A., Kai, W., Storling, Z., Orsini, M., Assenov, Y., Blankenburg, H., Huthmacher, C., Ramirez, F., Schlicker, A., Denoeud, F., Jones, P., Kerrien, S., Orchard, S., Antonarakis, S.E., Reymond, A., Birney, E., Brunak, S., Casadio, R., Guigo, R., Harrow, J., Hermjakob, H., Jones, D.T., Lengauer, T.A., Orengo, C., Patthy, L., Thornton, J.M., Tramontano, A., Valencia, A., 2007. The implications of alternative splicing in the ENCODE protein complement. Proc. Natl. Acad. Sci. U.S.A. 104, 5495–5500.
Turchan, J., Przewlocka, B., Lason, W., Przewlocki, R., 1998. Effects of repeated psychostimulant administration on the prodynorphin system activity and kappa opioid receptor density in the rat brain. Neuroscience 85, 1051–1059.
Uhl, G.R., Drgon, T., Johnson, C., Fatusin, O.O., Liu, Q.R., Contoreggi, C., Li, C.Y., Buck, K., Crabbe, J., 2008. "Higher order" addiction molecular genetics: convergent data from genome-wide association in humans and mice. Biochem. Pharmacol. 75, 98–111.
VandenOever, M.C., Spijker, S., Li, K.W., Jimenez, C.R., Koya, E., VanderSchors, R.C., Gouwenberg, Y., Binnekade, R., DeVries, T.J., Schoffelmeer, A.N.M., Smit, A.B., 2006. A proteomics approach to identify long-term molecular changes in rat medial prefrontal cortex resulting from sucrose self-administration. J. Proteome Res. 5, 147–154.
Vilim, F.S., Alexeeva, V., Moroz, L.L., Li, L., Moroz, T.P., Sweedler, J.V., Weiss, K.R., 2001. Cloning, expression and processing of the CP2 neuropeptide precursor of *Aplysia*. Peptides 22, 2027–2038.
Walters, C.L., Yuo-Chen Kuo, C.L., Blendy, J.A., 2003. Differential distribution of CREB in the mesolimbic dopamine reward pathway. J. Neurochem. 87, 1237–1244.
Yakovleva, T., Bazov, I., Cebers, G., Marinova, Z., Hara, Y., Ahmed, A., Vlaskovska, M., Johansson, B., Hochgeschwender, U., Singh, I.N., Bruce-Keller, A.J., Hurd, Y.L., Kaneko, T., Terenius, L., Ekstrom, T.J., Hauser, K.F., Pickel, V.M., Bakalkin, G., 2006. Prodynorphin storage and processing in axon terminals and dendrites. FASEB J. 20, 2124–2126.
Yang, H.Y., Fratta, W., Majane, E.A., Costa, E., 1985. Isolation, sequencing, synthesis, and pharmacological characterization of two brain neuropeptides that modulate the action of morphine. Proc. Natl. Acad. Sci. U.S.A. 82, 7757–7761.
Yang, H.Y., Hong, J.S., Costa, E., 1977a. Regional distribution of LEU and MET enkephalin in rat brain. Neuropharmacology 16, 303–307.
Yang, J.T., Bewley, T.A., Chen, G.C., Li, C.H., 1977b. Conformation of beta-endorphin and beta-lipotropin: formation of helical structure in methanol and sodium dodecyl sulfate solutions. Proc. Natl. Acad. Sci. U.S.A. 74, 3235–3238.
Yang, L., Sun, Z.S., Zhu, Y.P., 2007. Proteomic analysis of rat prefrontal cortex in three phases of morphine-induced conditioned place preference. J. Proteome Res. 6, 2239–2247.
Yeung, E.S., 2004. Dynamics of single biomolecules in free solution. Annu. Rev. Phys. Chem. 55, 97–126.
Yuferov, V., Nielsen, D., Butelman, E., Kreek, M.J., 2005. Microarray studies of psychostimulant-induced changes in gene expression. Addict. Biol. 10, 101–118.
Zhang, Z., Krylov, S., Arriaga, E.A., Polakowski, R., Dovichi, N.J., 2000. One-dimensional protein analysis of an HT29 human colon adenocarcinoma cell. Anal. Chem. 72, 318–322.