

# Proteomic Approaches and Identification of Novel Therapeutic Targets for Alcoholism

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Recent studies have shown that gene regulation is far more complex than previously believed and does not completely explain changes at the protein level. Therefore, the direct study of the proteome, considerably different in both complexity and dynamicity to the genome/transcriptome, has provided unique insights to an increasing number of researchers. During the past decade, extraordinary advances in proteomic techniques have changed the way we can analyze the composition, regulation, and function of protein complexes and pathways underlying altered neurobiological conditions. When combined with complementary approaches, these advances provide the contextual information for decoding large data sets into meaningful biologically adaptive processes. Neuroproteomics offers potential breakthroughs in the field of alcohol research by leading to a deeper understanding of how alcohol globally affects protein structure, function, interactions, and networks. The wealth of information gained from these advances can help pinpoint relevant biomarkers for early diagnosis and improved prognosis of alcoholism and identify future pharmacological targets for the treatment of this addiction.

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## INTRODUCTION

The harmful use of alcohol is a worldwide problem that jeopardizes individual and social development and causes disability, loss of health, and millions of deaths (World Health Organization, 2010). Of all the other substances of abuse, alcohol is by far the most harmful despite it being a legal drug (Nutt *et al*, 2010). Genetic, neurobiological, environmental, and psychosocial risk factors influence some individuals to develop compulsive and uncontrolled consumption of alcoholic beverages despite serious negative consequences (Koob and Le Moal, 2001; Koob, 2006; Mayfield *et al*, 2008; Phillips and Belknap, 2002; Potenza *et al*, 2011; Tabakoff *et al*, 2009). The molecular mechanisms underlying alcohol dependence involve different neurochemical systems and brain regions. Indeed, long-term alcohol abuse can permanently modify brain structure and function while inducing tolerance, physical dependence, craving, and other behavioral changes. Specifically, researchers have shown that long-term exposure to alcohol induces changes in microRNA (miRNA), gene, and protein expression levels in specific brain regions (Crabbe *et al*, 2010; Gorini *et al*, 2011; Nunez and Mayfield, 2012; Renthal

and Nestler, 2009; Wong *et al*, 2011). These modifications in turn mediate altered function of individual neurons and related neural circuits, which ultimately underlie behavioral abnormalities characteristic of addiction (Hyman *et al*, 2006; Nestler, 2000). Therefore, the identification of genes and proteins associated with the predisposition to, or development and maintenance of alcohol dependence, is crucial to shed light on molecular mechanisms underpinning addiction and to ultimately identify novel therapeutic targets for alcoholism. In the past decade, comparison studies of subcellular amounts of transcripts and proteins showed with increasing accuracy that altered mRNA expression does not completely explain changes in protein levels (Baek *et al*, 2008; Chen *et al*, 2002; Greenbaum *et al*, 2003; Guo *et al*, 2010b; Hendrickson *et al*, 2009; Schwanhäusser *et al*, 2011). As a result, the use of proteomic techniques has grown in addiction-related research because of their ability to precisely measure global dynamic modifications in proteomes (Abul-Husn and Devi, 2006; Drabik *et al*, 2007; Hemby and Tannu, 2009; Li and Wang, 2007; Lull *et al*, 2010; Marcotte *et al*, 2003; Plazas-Mayorca and Vrana, 2011; Wang *et al*, 2011). Similar to other neuropsychiatric addictive disorders, alcoholism engages a considerable collection of protein complexes and is thus suitable for proteomic investigations. In addition, the application of proteomics allows researchers to study alcohol-perturbed protein and gene regulatory networks without the need of *a priori* assumptions. A number of proteins potentially related to alcohol exposure and

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dependence have been reported by proteomic studies from cell cultures, animal models, and human alcoholic brain (Anni and Israel, 2002; Gorini *et al*, 2011; Kasinathan *et al*, 2004; Lovinger, 2006; Matsumoto, 2009; Neuhold *et al*, 2004; Torrente *et al*, 2012; Witzmann and Strother, 2004); such studies offer invaluable information on neurobiological mechanisms underlying the basis of addiction, on identification of proteins as diagnostic biomarkers, and on their exploitation as putative therapeutic targets for drug development. The purpose of this commentary is to provide a comprehensive summary of the alcohol-sensitive proteins identified from proteomic studies with an eye on their potential biological, diagnostic, prognostic, and therapeutic significance.

## ALCOHOL ABUSE AND ADDICTION

Alcoholism is a complex-trait disorder, and molecular mechanisms and genetic influences underpinning excessive alcohol consumption are not entirely understood (Gunzerath *et al*, 2011). Crucial risk factors include genetic predisposition, social environment, stress, mental health, age, and gender (Foroud and Phillips, 2012; Koob and Le Moal, 2001; Mayfield *et al*, 2008; Phillips and Belknap, 2002, 2002; Procopio *et al*, 2012; Schuckit, 2009; Volkow and Li, 2005). In particular, genetic risk factors have a key role in the etiology of alcohol abuse. The amount of risk related to heritability is estimated to be between 40 and 60%, as determined from family and twin studies (Schuckit, 2009).

Long-term alcohol abuse causes persistent modifications in brain function contributing to the development and expression of tolerance, symptoms of withdrawal upon removal of alcohol, and compulsive behavior focused on obtaining more alcohol. From a clinical perspective, this condition is diagnosed when such behavioral changes are evident and lead an individual to give up occupational, familial, social, and other important responsibilities. Although the treatment of alcoholism represents a central aim in public health care, only a few medications are currently approved for this disease, whereas others are still in clinical trials (see Box 1). Another limitation to treatment is the fact that pharmacogenetic studies show that the efficacy of these drugs varies across the heterogeneous clinical population of alcoholics (Addolorato *et al*, 2012; Anton *et al*, 2006; Garbutt, 2009; Heilig and Egli, 2006; Leggio and Addolorato, 2010; Volkow and Li, 2005). Therefore, an increase in effective medications for the treatment of alcohol abuse is of critical importance; drug development depends initially on the identification of novel therapeutic targets determined from preclinical research studies. Because the effects of alcohol involve complex system interactions, numerous neuropharmacological targets associated with these systems are currently under investigation, including the following: neurotransmission systems ( $\alpha$ -adrenergic, dopaminergic, endocannabinoid, GABAergic, glutamatergic, nicotinic cholinergic, neuropep-

### Box 1 Current Available Treatments for Alcohol-Use Disorders

Although the development of effective treatments for alcohol-use disorders (AUDs) represents an important public health concern, only a few medications have been approved by the US Food and Drug Administration (FDA) for the specific treatment of AUDs. However, several drugs, approved for the treatment of different diseases and addictive disorders, are under clinical trial for the treatment of AUDs.

FDA-approved medications for the treatment AUDs:

- Disulfiram is an acetaldehyde dehydrogenase inhibitor that causes an unpleasant reaction when alcohol is consumed.
- Acamprosate is a functional glutamatergic antagonist with neuroprotective effects.
- Naltrexone is a pan-opioid antagonist that reduces relapse rates after abstinence.

FDA-approved medications for the treatment of other diseases, under clinical trial for the treatment of AUDs:

- Aripiprazole is a partial dopamine agonist with antidepressant properties.
- Baclofen is an agonist for GABA<sub>B</sub> receptors that inhibits alcohol withdrawal symptoms and craving.
- Gabapentin is a non-benzodiazepine anticonvulsant GABA analog used in the treatment of partial seizures.
- Lamotrigine is an anticonvulsant used in the treatment of epilepsy and bipolar disorder.
- Ondansetron is a serotonin 5-HT<sub>3</sub> receptor antagonist used mainly as an antiemetic.
- Topiramate is an anticonvulsant, most recently also approved for weight loss.
- Varenicline is a nicotinic receptor partial agonist which reduces cravings for tobacco products.

tide Y, serotonergic, and substance P), pathways associated with acetaldehyde-related enzymes, corticotropin-releasing factor, feeding-related peptides, neuroinflammation, and nociception, among others (Addolorato *et al*, 2012; Leggio and Addolorato, 2010; Spanagel, 2009). Advances in high-throughput technologies promise to accelerate the process of pinpointing therapeutic targets and offer invaluable diagnostic and screening potential for a multifaceted disease like alcoholism.

### Relevance of Proteomics to Alcohol Research

The relevance of 'omic' strategies to the study of alcoholism can be better appreciated by considering the influence of similar approaches to other psychiatric, drug abuse, neurodegenerative disorders (Geschwind, 2003; Marcotte *et al*, 2003; Mirnics *et al*, 2000; Tkachev *et al*, 2003) and even cancer, where improved pharmacotherapies (Okutsu *et al*, 2002; Taxman *et al*, 2003; Zembutsu *et al*, 2002) have led to improved molecular classification of diseases (Davis and Hanash, 2006; Kemming *et al*, 2006; Konkimalla *et al*, 2007; Lea and Ling, 2008). The advances in proteomics offer distinct advantages over conventional molecular approaches when studying genes and encoded proteins perturbed by alcohol or that mediate its effects. Such a high-throughput strategy allows investigators to simultaneously survey large numbers of potential target molecules in an unbiased manner without prior knowledge of which molecules might be involved. However, proteomic efforts have so far not completely succeeded in providing exclusive, unanimous, and definitive insights, because of procedural

drawbacks and potential interpretational pitfalls. When applied to addiction research, proteomic methods do present some technical limitations and require robust animal model selection with adequate sample size, accurate sample quality control, sample preparation and/or fractionation, rigorous statistical analysis of results, and validation of changes in protein expression using alternative methods (Abul-Husn and Devi, 2006; Li and Wang, 2007; Lull *et al*, 2010). Moreover, many proteomic studies analyzed whole brain tissue or relatively large brain regions because of the need of greater amounts of starting material compared with microarray experiments, which can potentially yield extensive genome coverage from single cells. As a result, proteomic findings are often less specific in terms of providing molecular snapshots of small neurocircuits with a key role in addiction. Despite the challenges intrinsic to these still emerging techniques, these methods have nonetheless changed the way genes and proteins are studied.

Proteomic techniques have evolved rapidly over the past few years, and the integration and interpretation of the huge amounts of data obtained from such studies are complicated by many factors. It has been shown that the magnitude of changes in brain gene and protein expression induced by alcohol exposure is less pronounced than in other diseases (Bull *et al*, 2001; Liu *et al*, 2006; Smith, 2002). Therefore, to avoid the possibility that individual diversity could overshadow the discrete differences underlying alcohol-related disorders, expression data for a large number of proteins should be combined and interpreted with rigorous appropriate statistical tests and bioinformatics. Advances in bioinformatics will undoubtedly simplify the interpretation of large amounts of data and pinpoint common underlying areas of biological significance. Although the methodologies are continuously evolving, proteomic strategies have already become essential tools for elucidating the molecular effects of addictive substances. In the past decade, the number of laboratories using protein expression profiling to define changes in global proteomes in cultured neurons or different brain regions of animals or humans in response to substances of abuse has been steadily growing (Bierczynska-Krzysik *et al*, 2006; Hemby and Tannu, 2009; Hemby, 2010; Li and Wang, 2007; Romanova *et al*, 2012; Song *et al*, 2012; Wang *et al*, 2011; Witzmann and Strother, 2004).

## PROTEOMIC STRATEGIES FOR DISCOVERY OF THERAPEUTIC TARGETS OF ALCOHOL

Chronic alcohol exposure can induce a wide range of adaptations in brain function. Alcohol has been shown to damage neurons in multiple brain regions, leading to cognitive impairment and other abnormalities of brain function (Harper, 2009; Pfefferbaum, 2004; Sullivan and Pfefferbaum, 2005). The neuronal and behavioral adaptation of the brain to the constant presence of alcohol can lead to severe withdrawal symptoms when alcohol levels

## Box 2 Post-Translational Modifications

Post-translational modifications (PTMs) of proteins are controlled and reversible changes that can amplify the functional dynamic spectrum of the proteome by adding biochemical functional groups to the amino acids included in the polypeptide chain, altering their structure, and/or their chemical nature. As an example, hydrophobic groups can be added for membrane localization and cofactors for enhanced enzymatic activity. PTM is a crucial regulatory step involved in a number of different cellular activities and processes and serves as a key mechanism to increase proteomic diversity. PTMs influence proteins' activity, localization, and targeting, including their interactions with ligands, cofactors, nucleic acids, lipids, and other proteins to assemble macromolecular complexes. Thus, protein properties are not fully determined by the primary protein structure or by its levels of expression. The PTMs produced by specific enzymes have a major role in determining protein functionalities. A variety of enzymes are involved in these processes, including kinases, ligases, phosphatases, proteases, and transferases. After translation, polypeptide chains are usually subject to PTMs before processing into a final protein product. PTMs define protein folding and stability, and/or direct proteins to distinct cellular compartments; however, PTMs can occur at any step of the protein cycle. Additional PTMs can take place once protein folding and localization are completed to switch on/off their catalytic activity or to modify their biological activity. In addition, when proteins need to be degraded, they are covalently linked to tags, which identify them as targets for proteolysis. Hundreds of different PTMs have been characterized, and novel examples are still being discovered.

### Classification of post-translational modifications

1. Enzymatic addition of biochemical functional groups:
  - cofactors (eg, lipopates, flavins, heme C groups, etc)
  - hydrophobic groups (eg, myristates, palmitates, etc)
  - small chemical groups (acetylation, alkylation, methylation, glycosylation, phosphorylation, adenylation, etc)
  - unique modifications of translation factors
2. Non-enzymatic addition of biochemical functional groups (eg, glycation)
3. Addition of other proteins or peptides (eg, SUMOylation, ubiquitination, etc)
4. Changes in the chemical nature of amino acids (eg, carbamylation, deamidation, etc)
5. Changes in the structure (eg, cleavages, addition of disulfide bridges, etc)

suddenly drop. The damaging effects of alcohol on the brain as well as on other peripheral adaptive responses are mediated, at least in part, by altered gene and protein expression (Nestler, 2000, 2005).

Proteomic methods have been extensively utilized in several fields, and over the past decade investigators have gradually begun to use proteomic analyses to advance our understanding of the effects of alcohol on the brain. Compared with the genomic studies, proteomic approaches entail distinctive challenges. For example, the proteome is much larger than the genome. A large diversity in proteins results from differential gene splicing, trans-splicing, post-translational modifications (PTMs) (Box 2), and other mechanisms (Moreira *et al*, 2012). Although the human genome is estimated to contain 20–25 thousand protein-coding genes, the human proteome is estimated to range in the low millions (Jensen, 2004; Uhlen and Ponten, 2005). Protein expression levels exhibit different variability compared with gene expression levels (Anni and Israel, 2002), the same amount of protein is not translated from each gene, and some proteins can be expressed in distinct cell types only. In addition, many proteins are expressed only in incredibly small amounts, especially those with regulatory functions. Therefore, their identification is challenging, relative to background noise from other proteins

simultaneously expressed in much larger amounts in the same cells or tissues.

### Correspondence Between Gene and Protein Expression

Molecular modifications in gene expression levels following alcohol exposure have been reported in many alcohol-related studies (Gorini *et al*, 2011). These studies are limited by the fact that genes and their encoded proteins do not necessarily follow parallel trends in their expression. It has been shown that alcohol administration produces changes in protein translation without affecting corresponding mRNA levels (Dodds and Lewohl, 1998), and fluctuations in mRNA levels do not always reflect altered protein expression (Chen *et al*, 2002; Greenbaum *et al*, 2003; Gygi *et al*, 1999). miRNAs have been used as tools to compare mRNA abundance and translational rates. Related studies showed that mRNA degradation accounts for about 75% of the changes observed in protein synthesis (Hendrickson *et al*, 2009) and that miRNAs cause a decrease in mRNA levels that can explain most of the reduction (84%) in protein production (Guo *et al*, 2010b). Advances in mass spectrometry (MS) and bioinformatics have been used to estimate the absolute copy number of cell proteins to compare turnover and expression levels of mRNA and proteins. The results showed that mRNA levels account for only about 40% of the variability in protein levels (Schwanhäusser *et al*, 2011). As protein abundance is predominantly controlled at the level of translation, proteomic methods provide invaluable tools to detect brain modifications underlying alcohol and drug addiction.

### Introduction to the Technologies Currently Used in Neuroproteomic Alcohol Research

Proteomes are encoded from thousands of genes, with a high degree of complexity and a broad dynamic range due to alternative splicing variants and PTMs. The measurement of important effector proteins is an essential component of alcohol research. The simultaneous quantitative analysis of thousands of proteins has become possible with recent advances in high-resolution MS and bioinformatics. These methodologies can provide the quantitative analysis of hundreds to thousands of proteins in complex mixtures, thus representing tools of choice for the elucidation of molecular mechanisms that underlie the neurobiology of addiction.

In the recent past, many proteomic platforms have been developed for the qualitative and quantitative analysis of protein mixtures and PTMs. Selecting a proteomic method depends on the nature of the biological question, researchers' expertise, availability of efficient MS core facilities with up-to-date instrumentation, time frame available for the experiment, and budget limits. The major limitation in proteomic technologies is the incomplete proteome coverage when analyzing complex protein samples in a single

experiment. For efficient analysis, many protein fractionation methods must be implemented to simplify sample complexity and focus on subproteomes of interest. Another strategy involves the combination of complementary proteomic methods; a number of studies have shown that several proteomic methods can detect different sets of proteins and can therefore be used in parallel to achieve higher resolution of the proteome. A full review of proteomic methods is beyond the scope of this article; in Box 3, we briefly describe proteomic techniques in the past decade that have been applied to study changes in brain protein expression underlying genetic predisposition to, or induced by excessive, alcohol consumption/exposure.

### Proteomic Profiling Studies in Cell Cultures

The effects of alcohol exposure on cell cultures have been studied with proteomic techniques, for example, two-dimensional (2D) electrophoresis. Although studies with the intact nervous system are more relevant for elucidating mechanisms of alcohol abuse-related behaviors, the use of proteomic profiling with *in vitro* neuronal culture models has many advantages. The integration of protein expression studies with *in vitro* models of alcohol exposure can provide key insights on mechanisms of cellular signaling and toxicity.

There is increasing evidence that alcohol, as a psychoactive drug, can affect immune responses. For example, reactive oxygen species derived from the alcohol oxidation metabolic pathway induce an unfolded protein response (UPR) as a protective mechanism from the toxic effects of misfolded proteins causing endoplasmic reticulum (ER) stress. UPR in turn can alter the expression of genes involved in antioxidant defenses, inflammation, energy metabolism, protein synthesis, apoptosis, and cell cycle regulation. In a proteomic study (Boukli *et al*, 2010), the alterations in protein expression of human dendritic cells, treated with 0.1% alcohol for 24 h, were investigated by 2D gel electrophoresis followed by liquid chromatography (LC)-MS and confirmed at the gene expression level by qRT-PCR. Alcohol exposure significantly changed the expression of 32 proteins compared with the control cultures. Changes in the proteome affected several components of the UPR stress-induced pathways that include chaperones, ER stress, antioxidant enzymes, proteases, alcohol dehydrogenase, cytoskeletal, and apoptosis regulating proteins. The expression of relevant UPR and antioxidant genes was also increased as evaluated by qRT-PCR analysis. The results suggest that alcohol exposure induces UPR in dendritic cells, and the authors speculate that although activation of UPR by alcohol may protect the cells from oxidation injury, it may also lead to the development of alcohol-related diseases.

As breast cancer, compared with other cancers affecting women, is associated with alcohol consumption, a related study (Lee *et al*, 2013) investigated the effect of alcohol exposure (100 mM for 48 h) on the growth regulation in T47D breast cancer cells. The researchers used 2D electrophoresis and matrix assisted laser desorption, time of flight

### Box 3 Proteomic Approaches Used to Study the Neurobiology of Alcoholism

Proteomic approaches are sometimes described as top-down or bottom-up. Top-down approaches are used for profiling proteomes at the intact protein level to preserve biochemical characteristics and modifications of proteins. Conversely, bottom-up strategies involve analysis of peptide fragments resulting from enzymatic cleavage of proteins. These fragments are still sufficiently distinctive for the identification of the parent protein but are rarely used to provide information on the entire sequence; peptide fragments are even more effective for protein identification than intact protein level analyses. Bottom-up approaches need to be combined with modern chromatographic or electrophoretic technologies to simplify highly complex peptide mixtures. Methods for protein identification include mass measurements for a set of peptide fragments from the parent protein (peptide mass fingerprinting) or analysis of information derived from the fragmentation process of these peptides.

Two-dimensional electrophoresis (2-DE) is a consolidated technique, which separates mixtures of proteins in two dimensions (electric charge and mass). It can resolve isoforms due to PTMs and splice variants. When combined with MS and advanced image analysis software, 2-DE can deliver important information on protein expression. However, this technology is limited by its relatively small dynamic range (extremely acidic, basic, or hydrophobic proteins are difficult to detect, as well as those with high or small molecular weights and those present in low abundance). For these reasons, 2D differential in-gel electrophoresis (2D-DIGE) represents a superior technique, given its more precise, reproducible, and sensitive approach. The use of fluorescent dye labeling allows loading of multiple samples on the same gel, eliminating gel-to-gel variation. The major disadvantages of 2D-DIGE are the high cost for reagents, instruments, and software. Also, individual spots can contain two or more proteins that differ only minimally in their isoelectric point and mass, which can lead to complications in the subsequent analyses. Furthermore, to minimize the influence of variable labeling efficiency, the dyes should be appropriately swapped to exclude any related bias. Overall, 2D-DIGE will remain a valuable complementary method for proteome characterization in the near future (Rogowska-Wrzesinska *et al*, 2013). Given that current MS-based techniques typically sample only a fraction of all the proteins present in a sample, only a fraction of all identified proteins can be reliably quantified (Bantscheff *et al*, 2007).

The basic concept of non-gel-based methods, termed shotgun proteomic analyses, is the identification of proteins in complex mixtures derived from tissues or cells by combining various fractionation techniques, like liquid chromatography (LC), directly online with MS analysis. As a sample extract can easily contain several thousand proteins at variable concentrations, this approach has become possible only through the development of automated processes. Multidimensional protein identification technology (MudPit or LC LC MS/MS) (Wu *et al*, 2003) refers to the use of two LC columns to separate complex mixtures of proteins before applying MS.

The two main MS-combined strategies currently used for protein quantitation are generally referred to as labeling and label-free methods. Labeling techniques use isotopic labels as a reference for relative or absolute quantitation. These labels can be introduced into samples *in vivo* by metabolic labeling (Wu *et al*, 2004) or SILAC (Ong *et al*, 2002), and *in vitro* by iTRAQ (Ross *et al*, 2004) or TMT tags (Dayon *et al*, 2008). In particular, iTRAQ is a comprehensive and efficient method for proteomic quantification that provides multiplex capability and can identify low-expressed proteins with high confidence in complex samples. Nevertheless, iTRAQ requires sample fractionation, high sample abundance, high costs for reagents, instruments, and software, and involves variable labeling efficiency, complex sample preparation procedures, and inconsistent ratios from complex samples. Thus, investigators are progressively switching to label-free quantitative proteomic methods given their lower costs, higher dynamic range of quantification, and faster results (Mann, 2009; Patel *et al*, 2009). MS-based, label-free quantitative proteomics embraces two general categories (Zhu *et al*, 2010): ion abundance-based measurements (peptide peak areas or peak heights) and spectral counting of identified proteins after MS/MS analysis. Compared with labeling approaches, these methods have to be carefully controlled given the possible errors caused by run-to-run variations when performing LC and MS. However, rapid advances in label-free methods have improved the reliability and accuracy of protein expression measurement in complex biological samples (Coombs, 2011). Finally, quantitative methods for targeted analysis of protein-specific peptides in complex protein samples include selected reaction monitoring (SRM) (Shi *et al*, 2012) and multiple reaction monitoring (Sherwood *et al*, 2009). SRM can identify and quantify specific peptides with very high sensitivity and reproducibility.

(MALDI-ToF) MS to evaluate changes in protein expression in addition to various other techniques. Results showed 14 downregulated and 8 upregulated proteins. The increased

expression of c-ros receptor tyrosine kinase (ROS1) was confirmed by western blotting. This study demonstrated the relationship between alcohol and cell proliferation associated with activation of the MAPK pathway by ROS1 protein.

### Proteomic Studies in Zebrafish

Zebrafish exhibit strain differences in terms of sensitivity and tolerance to alcohol and are sensitive to pharmacological concentrations of alcohol (Dlugos and Rabin, 2003). A proteomic study (Damodaran *et al*, 2006) investigated the effects of 4 weeks of chronic alcohol treatment (0.5% (v/v)) on the brain of *Danio rerio* using a 2D-differential in-gel electrophoresis (2D-DIGE) system combined with MALDI-ToF MS. The results reported 13 differentially regulated proteins in the supernatant fraction and 18 in the pellet fraction. Voltage-dependent anion channels, heat-shock protein 70 (HSP70), and  $\alpha$ -subunit of Go (GNAO1) were upregulated following alcohol exposure. This study reported some novel protein targets as well as substantiated some previously identified alcohol targets.

### Proteomic Studies in Mice

Several mouse models for increased alcohol consumption have been investigated using different proteomic techniques. Many researchers have used proteomic profiling to identify line- and/or strain-specific changes in protein expression patterns after alcohol administration. In several mouse studies, protein expression levels were compared between B6 and D2 strains (see Box 4), and transgenic and knockout mice have also been utilized. Different brain regions and different methods of alcohol administration were studied.

In a 2-DE proteomic-based study (Park *et al*, 2004), whole brain tissue obtained from two inbred mouse strains, C57BL/6J (B6, alcohol-preferring) and DBA/2J (D2, alcohol-avoiding), were analyzed to identify alcohol-responsive proteins. Both strains were subjected to oral administration of a single dose of alcohol (1.5 g/kg). Over 50 proteins showed differential expression patterns between the two strains. The authors first classified those proteins according to their frequency of repetition and rate of changed expression profiles over time, then combined the proteomic data with predicted protein interactions to distinguish proteins with respect to their potential function and shared pathways. Finally, they concluded that the different responses to alcohol between B6 and D2 strains may be due to differences in response rates and interactions of different variants of the alcohol-responsive protein family.

Some researchers used proteomics to investigate the anxiolytic properties of alcohol (Sikela *et al*, 2006). For example, they used a transgenic mouse model for anxiety (adenylyl cyclase 7, ADCY7) based on the observation that a predisposition to anxiety may promote alcohol intake as a form of negative reinforcement, and on the evidence that a polymorphism in the ADCY7 gene is associated with risk for

## Box 4 Animal Models Used in Alcohol-Related Proteomic Studies

The identification of molecular determinants of excessive alcohol consumption remains a major challenge. Proteomic studies have provided valuable new insights into protein regulation in genetically complex diseases such as alcoholism. Over the past decade, protein expression profiling has been used to identify alcohol-responsive proteins and associated pathways in different animal models of alcoholism. Rodents are the prime organisms of choice for modeling human diseases, and the genetically based propensity of some rats and mice to prefer and others to avoid alcohol has provided the basis for alcohol behavioral neuroscience preclinical research (Bell *et al*, 2012; Crabbe *et al*, 2010; Reilly *et al*, 2012).

A common approach in alcohol research involves the identification of differences in gene/protein expression between strains of animals selectively bred for divergent alcohol-related phenotypes. These studies are particularly challenging in terms of statistical power to identify small but reliable differences in brain gene/protein expression. Indeed, the magnitude of these alcohol-induced changes is small if compared with widespread alterations characteristic of other substance abuse disorders. Access to large databases of expression data and meta-analytic approaches can mitigate these obstacles (Bhave *et al*, 2007; Mulligan *et al*, 2006 (meta-analysis of several microarray datasets to study alcohol preference phenotypes)). The studies reported in this review aim to identify constitutive differences in protein expression among diverse inbred strains or selectively bred alcohol-naïve animals or to identify the effects of different alcohol intake/administration paradigms on protein expression.

anxiety in humans. First, synaptosomal preparations from whole brains of naive wild-type and ADCY7 transgenic mice were analyzed using Multidimensional Protein Identification Technology (MudPIT) (Wu *et al*, 2003), as part of a larger set of experiments combining *in silico*, genomic, and proteomic strategies. Five of the identified proteins mapped to a quantitative trait locus (QTL) for anxiety previously reported by the same group. To estimate the quantitative differences in protein levels between the same mice in an unbiased manner, the researchers used metabolic labeling with <sup>15</sup>N to generate (as an internal standard) labeled brain tissue, which was then cofractionated as synaptosomes using either unlabeled tissue from wild-type or transgenic mice. Mixed synaptosomal samples resulting from each fractionation were analyzed using MudPIT, and the relative abundance ratios of wild-type/standard and transgenic/standard were measured and compared (Wu *et al*, 2004). With their multifaceted approach, the researchers quantified about 150 proteins, with 26 proteins showing significant differences between the two mouse strains. The results demonstrated predominant protein upregulation in transgenic mice compared with wild type, and specifically identified proteins related to calcium transport and signaling and proteins involved in anxiety-related behavior. Overall, this study showed the effective use of global, unbiased strategies to identify alcohol candidate genes and proteins associated with anxiety-related behaviors, pathways, and QTLs.

Another group (Sari *et al*, 2010) used a label-free quantitative proteomic analysis to investigate the effect of ethanol on central nervous system development in fetal brains of prenatally alcohol-treated C57BL/6 mice. Moderate prenatal alcohol exposure results in brain defects at different stages of development, interferes with the growth of the central nervous system, and induces neurodegeneration through cellular apoptosis. Pregnant mice were exposed from the 7th to the 13th embryonic day with either a 25% ethanol derived calorie diet or pair-fed liquid diet.

Fetal brains were subjected to chromatography combined with an LC-MS/MS system. Prenatal alcohol exposure induced significant downregulation of several important mitochondrial enzymes, cytoskeletal, nuclear, and cytosolic proteins such as Bcl-2, 14-3-3, calmodulin, prohibitin, and doublecortin. These findings highlighted impairments in energy generation, growth and development, cellular signaling, and neuronal migration as possible mechanisms underlying the effects of prenatal alcohol exposure during early embryonic stages.

Mice lacking type 1 equilibrative nucleoside transporter (ENT1<sup>-/-</sup>) represent a model for increased alcohol consumption. The increased alcohol preference in these mice involves increased glutamate neurotransmission in the nucleus accumbens (NAcc). This model has been studied using isobaric tags for relative and absolute quantitation (iTRAQ) proteomics, combined with other techniques, to elucidate the compensatory mechanisms following deletion of ENT1 and their effect on glutamate signaling in the NAcc (Nam *et al*, 2011). The results showed that out of 533 identified proteins, only five signaling proteins were significantly changed in the NAcc of ENT1<sup>-/-</sup> mice compared with ENT1<sup>+/+</sup> controls: neurogranin, calmodulin, and phosphatidylethanolamine-binding protein 1 were upregulated, whereas cAMP-dependent protein kinase catalytic  $\beta$ -subunit and sodium-dependent glutamate/aspartate transporter 2 were downregulated. As neurogranin and calmodulin are essential components of N-methyl-D-aspartate receptor (NMDAR)-mediated signaling, the authors propose that the deletion of ENT1 results in altered NMDAR signaling, which provides a molecular basis for the ethanol-preferring behavior of ENT1<sup>-/-</sup> mice.

A recent study (Gorini *et al*, 2013a) used 2D-DIGE to analyze cerebral cortices and midbrains from C57BL/6J mice subjected to a chronic intermittent ethanol (CIE), two-bottle choice (2BC) paradigm, which induces heavy drinking and represents one of the best available animal models for alcohol dependence (Lopez and Becker, 2005). The CIE-2BC protocol included ethanol vapor-exposed mice (dependent) and air-exposed mice (non-dependent), which both had access to alcohol, plus a third group of alcohol-naïve mice. CIE-2BC induced significant protein expression changes in ethanol vapor-exposed mice compared with their air-exposed matched controls and with alcohol-naïve mice. Multiple protein isoforms showed region-specific differential regulation as a result of PTMs. This study reported molecular changes in endocytic- and energy-related pathways. The results were supported by a coexpression analysis of the proteomic data, with specific proteins involved, such as dynamin-1 and its isoforms, mostly upregulated in alcohol-dependent mice. The protein changes identified in this comprehensive analysis could have a key role in the escalation of ethanol consumption associated with dependence and may in part relate to relapse vulnerability since the paradigm used included two 3-day 'respite' periods, 2 weeks of withdrawal, and brains were collected 3 days after the last drinking episode.

## Proteomic Studies in Rats

Proteomic approaches involving rat models of alcohol consumption have examined direct strain-dependent differences in protein expression in response to alcohol (see Box 4). For example, the proteomes of relevant brain regions from inbred alcohol-preferring (iP) and -non-preferring (iNP) rats have been compared in three studies using a variety of techniques.

The first study (Witzmann *et al*, 2003) analyzed protein expression differences in two brain regions, amygdala (Amy) and NAcc of alcohol-naïve iP and iNP rats, using 2D electrophoresis and MALDI-ToF MS. Among the significant differentially expressed proteins, the levels of expression were generally lower in the P rats compared with the NP rats in both regions, and the largest differences were detected for cellular retinoic acid-binding protein 1 and a calmodulin-dependent protein kinase, both involved in cellular signaling pathways. Additional changes were identified in the NAcc, where proteins involved in the following functions were reported: metabolism, cytoskeletal and intracellular protein transport, molecular chaperoning, cellular signaling pathways, synaptic function, reduction of oxidative stress, and growth/differentiation. This study provided the first proteomic evidence showing that selective breeding for disparate alcohol drinking behaviors can induce innate modifications in protein levels with the potential to impact neuronal function.

Another study (Bell *et al*, 2006) investigated the effect of continuous *vs* binge-like alcohol drinking on protein expression levels in the same regions (NAcc and Amy) with the same techniques, but using only the iP rats. Three alcohol groups were compared: ethanol-naïve (EN), continuous 24/7 free-choice access to ethanol (CE), and multiple scheduled access (MSA). The paradigm lasted over 6 weeks, and rats were killed 10 h after ethanol removal. Analysis of drinking patterns revealed a significant alcohol deprivation effect in MSA group, which represents a transient increase in alcohol intake occurring in laboratory animals after a period of alcohol deprivation (Martin-Fardon and Weiss, 2013; Spanagel, 2005). The results showed region-specific changes induced by alcohol that were dependent on the type of alcohol paradigm. Notably, changes specifically associated with MSA (*Tpm1*, *Tpm3*, *Enog2*, *Ywhaz*, *Prkar2b*, *Vdac1* in the NAcc and chaperonin containing TCP1 subunit  $\gamma$ , heat-shock protein 70 kDa, serum albumin precursor, and ubiquitin-conjugating enzyme E2N in the Amy) may be related to withdrawal and relapse liability. MSA induced greater changes in protein levels in the NAcc, whereas CE had greater effects on the Amy. In general, alcohol consumption had modulatory effects on proteins associated with the cytoskeleton, cellular stress response, membrane transport, metabolism and energy production, and neurotransmission. As only two of the differentially expressed proteins were common between the NAcc and Amy, the authors concluded that the diverse pattern of protein expression changes observed between the

two regions might reflect correspondent differences in neuroanatomical and functional characteristics associated with ethanol self-administration and possibly withdrawal.

In a third proteomic study, the same group (McBride *et al*, 2009) used a label-free, LC-MS-based protein quantification method (Higgs *et al*, 2005) to determine the effects of repeated systemic administration of a moderate dose (1 g/kg) of ethanol on protein levels in the NAcc shell of inbred alcohol-preferring (P), alcohol-non-preferring (NP), and non-inbred Wistar (W) rats. The shell portion of the NAcc was selected due to its involvement in mediating reinforcement processes, and using such a selective area also increases the resolution of the analysis. Rats received five daily injections with either saline or ethanol (Smith and Weiss, 1999) and experienced 24 h of withdrawal after the last injection. Ethanol administration modified the expression patterns of a larger number of proteins in NP compared to both P and W rats. Few of the changes observed with ethanol treatment for NP rats were observed for P and W rats. Many of the changes occurred in calcium-calmodulin signaling systems, G-protein signaling systems, synaptic structure, and histones. Approximately half of the changes observed in the NAcc shell of P rats were also observed for W rats. Overall, this study showed a unique response to ethanol within the NAcc shell of NP rats compared to P and W rats; this unique response may reflect changes in neuronal function in this brain area that could contribute to the low alcohol drinking behavior and/or higher sensitivity to alcohol exhibited by NP rats.

Alcohol abuse during adolescence represents a major health concern. During adolescence the brain continues to undergo critical developmental changes, and alcohol abuse can induce particularly adverse neural and cognitive changes. Adolescents are typically more susceptible to alcohol-induced memory impairment, and hippocampal damage has a crucial role in these memory dysfunctions (Pitel *et al*, 2007). Accordingly, adolescent rats have been shown to display greater impairment in spatial memory acquisition and longer memory performance deficits than adult rats under the influence of alcohol (Sircar and Sircar, 2005; White *et al*, 2000). Thus, several research groups are focusing their studies on age-specific effects of chronic alcohol abuse. For example, a 2D-electrophoresis based proteomic study (Hargreaves *et al*, 2009) has investigated the effects of chronic alcohol intake on the protein levels in the hippocampus of adolescent compared with adult wistar rats. After 4 weeks of access to alcohol (beer), rats were subjected to an additional 2-week period of alcohol withdrawal. Beer consumption resulted in modest changes in the adult group, with only two proteins upregulated compared with controls. Conversely, alcohol exposure induced widespread changes in the hippocampus of adolescent rats relative to their controls, involving proteins related to glycolysis, glutamate metabolism, neurodegeneration, synaptic function, and cytoskeletal structure. These results highlighted the vulnerability of adolescent brain to lasting effects induced by alcohol exposure and suggested putative

mechanisms by which alcohol abuse and binge drinking can induce damage in the developing hippocampus, leading to lasting cognitive and emotional disturbances.

More recently, another group (Maldonado-Devincci *et al*, 2012) proposed a different methodology for assessing the effects of chronic ethanol exposure during adolescence in rats. This approach incorporates either a label-free or a chemical tagging method using iTRAQ, and can complement other proteomics-based studies such as 2D gel electrophoresis described earlier, providing an unbiased global assessment of age-specific expression of neuroproteins induced by alcohol.

The studies mentioned above utilized relatively short alcohol paradigms. Rat models using only short periods of alcohol exposure might not adequately represent the specific neuroadaptations in motivation-related brain pathways that contribute to the long-term process of addiction, where alcohol consumption becomes an established compulsive routine that is resistant to reprogramming. This discrepancy was assessed by a recent study (Kashem *et al*, 2012) in which proteome changes were examined after 8 months of moderate daily alcohol consumption obtained with the 'beer model'. The whole striatum was chosen for this study, because of its contribution to decision making, emotional and motivational aspects of behavior, and major role in addiction-related neuroadaptations. Proteomic 2-DE analysis showed that a total of 28 striatal proteins were significantly altered in the beer-drinking rats relative to controls. The most salient changes were in proteins associated with dopamine biosynthesis and related intracellular signaling, energy metabolism, and oxidative stress. These results showed how daily alcohol consumption at relatively modest levels causes major biochemical adaptations in dopaminergic signaling pathways in the rat striatal proteome.

### Proteomic Studies in Human Post-Mortem Brain

Global proteomic approaches on human post-mortem brain have been used to study several different alcohol-sensitive brain regions from both uncomplicated and hepatic cirrhosis-complicated human alcoholics.

Several considerations need to be made when applying proteomic profiling to human post-mortem brain. The post-mortem interval, agonal state, and tissue pH are important parameters that can influence the quality of the analysis. In addition, comparisons across proteomic studies performed from different laboratories are complicated by differences in tissue harvesting, proteomic platforms, sample processing, use of labeling, and data analysis procedures. Brain regional differences may be related to differences in case selection criteria such as age, gender, alcohol drinking history, etc. In addition, the effects of comorbidities (polydrug use, poor diet, smoking, etc) can diminish and potentially compromise the reliability of results. Availability of accurate clinical information is therefore critical for experimental design and for the evaluation of individual differences across cases.

The most studied region of human brain is the prefrontal cortex (PFC), an area important for judgment, decision making and other cognitive functions (Godefroy and Rousseaux, 1997; Rahman *et al*, 1999; Ratti *et al*, 2002). This region is also associated with neurocircuitry involved with mediating reward, which influences the development and expression of alcohol tolerance and dependence (Vetulani, 2001).

Analyses of post-mortem brain of long-term alcohol abusers have reported neuronal loss in gray matter and loss of white matter volume (Harper, 2009; Kril and Harper, 1989; Pfefferbaum, 2004; Sullivan and Pfefferbaum, 2005). These effects are less pronounced in motor, temporal, or cingulate cortices, suggesting that alcohol-induced brain damage is likely brain region selective. The impact of concomitant liver cirrhosis on the brain proteome has also been tested in different brain regions of cirrhotic vs non-cirrhotic alcoholics. Cirrhosis, the widespread disruption of liver structure with fibrosis and regenerative nodules, represents a common comorbid condition resulting from long-term alcohol abuse.

The first application of proteomics in human alcoholic brain compared pooled autopsy samples from four long-term, well-characterized chronic alcoholics to four healthy control subjects using two dimensional electrophoresis (Lewohl *et al*, 2004). This study reported about 180 differentially expressed proteins in the superior frontal cortex, with predominant downregulation (139 down- vs 35 upregulated proteins) observed in alcoholics. Using both MALDI-MS and MS-MS, the authors were able to identify 63 proteins, including enzymes, ion channels, and signaling-related proteins.

Proteomics was also used to investigate the effects of alcohol abuse in the dorsolateral PFC, which includes Brodmann area 9, with white (wBA9) (Alexander-Kaufman *et al*, 2006, 2007a) and gray matter (gBA9) (Alexander-Kaufman *et al*, 2007a). The dorsolateral PFC is connected to many brain regions involved in cognitive functions by reciprocal projections and is subject to significant shrinkage in alcoholics, particularly the white matter. Autopsy samples obtained from alcoholics with and without cirrhosis and non-alcoholics were compared using 2D electrophoresis combined with MALDI-ToF MS analyses. In both wBA9 and gBA9 regions, transketolase and pyruvate dehydrogenase E1  $\beta$ -subunit were differentially expressed in both groups of alcoholics, suggesting alterations in the thiamine-dependent cascade. Furthermore, the levels of many metabolism-related enzymes were differentially regulated, in particular those related to energy transduction (glycolysis, Krebs cycle), suggesting energy deficit, loss of viability, and cell death in the dorsolateral PFC of alcoholics.

The cerebellar vermis (CV) of uncomplicated and cirrhotic alcoholics was also analyzed with the same techniques (Alexander-Kaufman *et al*, 2007b). The CV receives somatic sensory input and is associated with bodily posture and locomotion. Owing to its connections with PFC, the cerebellum is indirectly involved in cognitive functions,

and the CV is susceptible to general shrinkage or atrophy in alcoholics, with loss of Purkinje cells and reduced dendritic arborization. As found in the dorsolateral PFC, transketolase and pyruvate dehydrogenase E1  $\beta$ -subunit were also differentially regulated in both groups of alcoholics, indicating that changes in vitamin B1-dependent and energy metabolism-dependent enzyme levels could contribute to CV damage in alcoholic brain. In addition, the proteomic analysis revealed some unique changes in the CV of cirrhotic alcoholics, suggesting specific effects on CV protein expression resulting from liver dysfunction.

A concomitant study from the same group focused on the proteomic analysis of the hippocampus (Matsuda-Matsumoto *et al*, 2007), which has crucial roles in cognitive processes, memory, and spatial navigation. Autopsy samples from uncomplicated alcoholics were compared with normal, non-alcoholic controls using the same 2D gel approach combined with MS identification. This study reports 18 out of 20 identified proteins as downregulated in alcoholics, and while some of them were identified in other brain regions mentioned above, hippocampal proteomic analysis showed unique, significant changes in the level of glutamine synthetase expression, an enzyme predominantly located in astrocytes that can detoxify hepatic products like ammonia.

Further studies investigated the corpus callosum (CC) in detail by distinct analyses of its posterior part (splenium), its anterior part (genu), and the truncus (body) among complicated *vs* uncomplicated alcoholics and controls (Kashem *et al*, 2007, 2008, 2009). The CC connects the left and right cerebral hemispheres, is the largest white matter structure in the brain, and regulates various cognitive functions. This region is also subject to volume reduction following excessive alcohol consumption, resulting in cognitive dysfunctions. The comparative analysis of CC subregions showed region- and group-specific chronic alcohol-induced alterations of various biochemical pathways. Several proteins involved in different metabolic pathways such as lipid peroxidation, oxidative stress, and apoptosis showed specific differential expression in the CC body. Thiamine/energy biosynthesis enzymes were differentially regulated in the genu, whereas glutamate carboxypeptidase (GCP1) was identified in both splenium and genu of complicated alcoholics only.

Two proteomic studies on alcoholic human brain analyzed synaptosomal preparations from superior frontal gyrus (SFG). This area is part of the PFC, which controls executive functions; it is generally considered severely affected by alcoholism, and is susceptible to alcoholism-induced neuronal loss. In an effort to unravel the mechanism underpinning synaptic disorders in alcoholism, the first study (Etheridge *et al*, 2009) compared SFG with occipital cortex (OC), a brain region that does not lose gray matter in response to alcoholism and does not exhibit obvious functional changes; OC was therefore considered by the authors as an alcohol-spared brain region in comparison with the alcohol-susceptible SFG. Synaptosomes from

chronic alcoholics and non-alcoholic controls were compared using 2D electrophoresis and MS, and the results showed that alcoholism induced changes in the synaptic proteome in both the SFG and the OC compared with controls. This study confirmed a number of proteins previously linked to alcoholism as well as pinpointing novel alcoholism-affected proteins, owing to the specific fractionation used. Unexpectedly, the majority of proteins altered were in the OC rather than in the SFG. The identified proteins are involved in vesicle transport, metabolism, folding and trafficking, and signal transduction. The changes relative to vesicle transport and cytoskeleton proteins suggested alterations in synaptic transmission pathways and may contribute to the neurodegenerative effects of alcohol and to its general disruption of cognitive function.

In their second study (Etheridge *et al*, 2011), the group above used the same techniques to analyze synaptosomes from the SFG of controls and from uncomplicated and cirrhotic alcoholics. Cirrhotic alcoholics showed changes in protein expression levels compared with both controls and non-comorbid alcoholics, likely caused by differences in disease severity between the subgroups of alcoholics. This disease-state specific regulation was accompanied with significant differences between post-translationally modified protein isoforms that are known to be involved in basal energy metabolism, synaptic vesicle recycling, and chaperoning.

All of the studies above indicate that high-throughput neuroproteomic approaches can potentially dissect the mechanisms of complex brain disorders and suggest that chronic alcohol consumption can directly alter the levels of several important brain proteins. Nevertheless, some major observations should be pointed out. First, even though there is a partial correspondence in the differentially expressed proteins across multiple alcohol-sensitive brain regions and distinct fractionation methods, from the studies described above it appears evident that every region shows specific patterns of protein expression. Although these corresponding changes confirm the relevance of pathways previously described as general reaction mechanisms to prolonged alcohol abuse, distinct region-specific alterations may be induced as exclusive mechanisms of neurobiological adaptation. In addition, the human studies are complicated by gaps in the knowledge of the subjects' history, and the observed protein changes could result from other unknown concomitant conditions. Studies comparing complicated cirrhotic alcoholics with non-comorbid alcoholics show how hepatic-induced factors affect protein expression profiles. This suggests that improving liver function in alcoholics could potentially prevent further brain damage (Matsumoto, 2009). Finally, the investigation of specific changes in isoform regulation due to diverse PTMs in different disease states can provide novel insight into the mechanisms involved in cerebral damage in alcoholics. Understanding the exact role that these widespread changes have in cellular regulation during alcohol dependence continues to be a challenge for addiction biologists.

## Proteins and Pathways Associated with Alcohol Exposure

Proteomic techniques applied to alcohol research can shed light on multiple molecular targets and pathways, improving our understanding of the complex consequences of alcohol on brain function. From the 22 published alcohol-related proteomic studies reviewed above, we collected 460 unique proteins reported to be associated with alcohol exposure. Twenty-eight proteins have been identified in at least four of these studies and are listed in Table 1. Although this might appear as little concordance, it is noteworthy that the studies described above differ in methodologies, statistical analysis of results (and number of proteins reported), species, brain region, sample preparation, and alcohol protocol used. On a different viewpoint, 26 of 348 proteins (7.5%) were reported by at least 4 out of 17 studies (~25%). Creatine kinase B-type, which has a central role in energy transduction in the brain, was identified by nine independent proteomic investigations and therefore represents one of the most important alcohol targets. Similarly, changes in dihydropyrimidinase-like 2, enolase, heat-shock 70 kDa protein 8, and lactate dehydrogenase B protein levels were described by eight different alcohol-related proteomic publications. Fifty-four well-consolidated, alcohol-sensitive proteins, reported by at least three of these studies, were analyzed for functional category enrichment. Owing to the large diversity in the functions of the proteins annotated, we used Ingenuity Pathway Analysis (IPA) (Ingenuity Systems, [www.ingenuity.com](http://www.ingenuity.com)) to more clearly highlight the major enriched biological pathways underlying alcohol's effects. Interestingly, the top networks associated with the collected proteomic signatures include neurological disease, immunological-inflammatory disease, carbohydrate metabolism, and cell morphology. In addition cell death, cellular assembly, organization, function, and maintenance, as well as nucleic acid metabolism, are the main molecular/cellular functions affected. Some of the representative biochemical pathways enriched among these proteins are glycolysis/gluconeogenesis, mitochondrial dysfunction, and protein ubiquitination. Signal-transduction pathways related to neuronal functions include Parkinson's signaling, clathrin-mediated endocytosis signaling, 14-3-3-mediated signaling, p70S6K signaling, and Huntington's disease signaling (Table 2). These pathways are involved in important neuronal functions and have also been associated with general molecular mechanisms of drug addiction.

Proteomic approaches represent powerful tools for the investigation and identification of biological processes underlying alcohol dependence. Nevertheless, owing to the limitations of the methods utilized to date, not all the proteins associated with alcohol exposure have been identified by proteomic technologies. Similar to what has been found for other proteomic studies of addictive disorders (Wang *et al*, 2011), the majority of identified proteins related to alcohol abuse belong to a relatively small number of functional categories (Figure 1). This is

consistent with the observation that some genetic influences may not be specific for alcohol-use disorders, but likely reflect a general vulnerability for polysubstance abuse (Gorini *et al*, 2011; Mayfield *et al*, 2008; Nestler, 2005). Other proteins such as neurotransmitter receptors, well known to have critical roles in the development of alcohol dependence, are under-represented in proteomic studies despite the fact that their genes have been associated with alcohol exposure at both mRNA and protein levels. This can be explained by the small dynamic range of the proteomic techniques used in the above studies. Advances in sample preparation and separation techniques will improve the sensitivity and power of proteomic analyses.

## Interaction Proteomics Approaches

Protein-protein interactions (PPIs) have a central role in virtually every process of a living cell. Proteins interact with other proteins by assembling into macromolecular complexes that are part of dynamic PPI networks of high complexity; these networks coordinate the variety of processes that impact cellular function, and their topology, with the association and dissociation of protein complexes, has broad biological importance. Biologists rely on interaction data in several ways: layering PPIs onto other networks, exploring how sets of proteins are interconnected, classifying proteins with the most interacting partners, and uncovering new biological functions by the superposition of biophysical and functional data. For example, we used an online PPI database (STRING, <http://string-db.org>) to explore the interaction network of alcohol-sensitive proteins described by proteomic studies and listed in Table 1. Network analysis revealed that the most connected interacting partner protein is polyubiquitin-C (Figure 2), thus confirming the involvement of ubiquitination pathways suggested by IPA functional analysis. PPIs participate in various physiological processes and are essential for neurotransmission. The release of neurotransmitter molecules responsible for signaling among neurons involves regulated PPIs (Brodin *et al*, 2000), and ion channels or neurotransmitter transporters in synaptic membranes are regulated by complex PPIs (Deken *et al*, 2000; Garner *et al*, 2000; Maiya *et al*, 2007; Muth *et al*, 1998; Staub and Rotin, 1997; Sung *et al*, 2005). Thus, identification and characterization of PPIs can not only advance our understanding of the processes that occur during normal neurotransmission but also highlight cell function adaptations because of the presence of alcohol. Importantly, this could provide the basis for new therapeutic approaches since newly identified PPIs may represent novel targets for drug development. This strategy has emerged as an important subfield in medication development (Arkin and Wells, 2004; Dev, 2004; Reilly *et al*, 2009), although PPIs do not always have detectable effects. Three-dimensional structural information can be used to predict PPIs (Zhang *et al*, 2012). There is also growing evidence that select clusters of functionally related gene expression profiles

**TABLE 1** Proteins Reported as Differentially Expressed by At Least Four Alcohol-Related Proteomic Studies

Hits	Gene symbol	Protein name	Location	Type	Related references
9	<i>Ckb</i>	Creatine kinase, brain	Cyt	Kinase	Alexander-Kaufman et al, 2007b; Etheridge et al, 2009; Gorini et al, 2013a; Kashem et al, 2007, 2008, 2009; Lewohl et al, 2004; Matsuda-Matsumoto et al, 2007; Witzmann et al, 2003
8	<i>Dpysl2</i>	Dihydropyrimidinase-like 2	Cyt	Enzyme	Alexander-Kaufman et al, 2007a, 2007b; Etheridge et al, 2009; Gorini et al, 2013a; Kashem et al, 2007, 2008, 2009; Matsuda-Matsumoto et al, 2007
8	<i>Eno1</i>	Enolase 1, $\alpha$	Cyt	Transcription Regulator	Alexander-Kaufman et al, 2007a, 2007b; Boukli et al, 2010; Gorini et al, 2013a; Hargreaves et al, 2009; Kashem et al, 2009, 2012; Lewohl et al, 2004
8	<i>Hspa8</i>	Heat-shock 70 kDa protein 8	Cyt	Enzyme	Alexander-Kaufman et al, 2007a; Bell et al, 2006; Boukli et al, 2010; Etheridge et al, 2009, 2011; Kashem et al, 2008; Lewohl et al, 2004; Witzmann et al, 2003
8	<i>Ldhb</i>	Lactate dehydrogenase B	Unk	Enzyme	Alexander-Kaufman et al, 2007b; Etheridge et al, 2009, 2011; Gorini et al, 2013a; Kashem et al, 2008, 2009, 2012; Witzmann et al, 2003
7	<i>Anxa5</i>	Annexin A5	PM	Other	Bell et al, 2006; Boukli et al, 2010; Etheridge et al, 2009; Gorini et al, 2013a; Kashem et al, 2007, 2008, 2009
7	<i>Pebp1</i>	Phosphatidylethanolamine-binding protein 1	Cyt	Other	Alexander-Kaufman et al, 2007a, 2007b; Kashem et al, 2012; Matsuda-Matsumoto et al, 2007; McBride et al, 2009; Nam et al, 2011; Witzmann et al, 2003
6	<i>Actb</i>	Actin, $\beta$	Cyt	Other	(Alexander-Kaufman et al, 2007b; Bell et al, 2006; Kashem et al, 2007; Lewohl et al, 2004; Sari et al, 2010; Witzmann et al, 2003)
6	<i>Gfap</i>	Glial fibrillary acidic protein	Cyt	Other	Alexander-Kaufman et al, 2007a, 2007b; Kashem et al, 2007, 2008, 2009; Witzmann et al, 2003
6	<i>Tpi1</i>	Triosephosphate isomerase 1	Cyt	Enzyme	Alexander-Kaufman et al, 2007b; Bell et al, 2006; Etheridge et al, 2009; Hargreaves et al, 2009; Kashem et al, 2009; Matsuda-Matsumoto et al, 2007
5	<i>Actg1</i>	Actin, $\gamma$ 1	Cyt	Other	Alexander-Kaufman et al, 2007b; Boukli et al, 2010; Etheridge et al, 2009; Gorini et al, 2013a; Kashem et al, 2007
5	<i>Aldoc</i>	Aldolase C, fructose-bisphosphate	Cyt	Enzyme	Alexander-Kaufman et al, 2007b; Etheridge et al, 2009; Gorini et al, 2013a; Kashem et al, 2007; Matsuda-Matsumoto et al, 2007
5	<i>Gapdh</i>	Glyceraldehyde-3-phosphate dehydrogenase	Cyt	Enzyme	Alexander-Kaufman et al, 2006; Boukli et al, 2010; Etheridge et al, 2011; Gorini et al, 2013a; Hargreaves et al, 2009
5	<i>Ina</i>	Intermedin neuronal intermediate filament protein, $\alpha$	Cyt	Other	Alexander-Kaufman et al, 2006, 2007a; Kashem et al, 2007, 2008; Witzmann et al, 2003
5	<i>Pdhb</i>	Pyruvate dehydrogenase (lipoamide) $\beta$	Cyt	Enzyme	Alexander-Kaufman et al, 2007a, 2007b; Etheridge et al, 2009; Lewohl et al, 2004; McBride et al, 2009
5	<i>Pgam1</i>	Phosphoglycerate mutase 1 (brain)	Cyt	Phosphatase	Alexander-Kaufman et al, 2007a; Etheridge et al, 2009; Gorini et al, 2013a; Kashem et al, 2009; Witzmann et al, 2003
5	<i>Pkm</i>	Pyruvate kinase, muscle	Unk	Kinase	Alexander-Kaufman et al, 2007a, 2007b; Boukli et al, 2010; Gorini et al, 2013a; Lewohl et al, 2004
5	<i>Sod1</i>	Superoxide dismutase 1, soluble	Cyt	Enzyme	Gorini et al, 2013a; Lewohl et al, 2004; Matsuda-Matsumoto et al, 2007; McBride et al, 2009; Witzmann et al, 2003
4	<i>Atp6v1b2</i>	ATPase, H <sup>+</sup> transporting, lysosomal 56/58 kDa, V1 subunit B2	Cyt	Transporter	Alexander-Kaufman et al, 2006, 2007b; Damodaran et al, 2006; Etheridge et al, 2009
4	<i>Dnm1</i>	Dynamin 1	Cyt	Enzyme	Alexander-Kaufman et al, 2007a; Etheridge et al, 2009, 2011; Gorini et al, 2013a
4	<i>Hspd1</i>	Heat-shock 60 kDa protein 1 (chaperonin)	Cyt	Enzyme	Boukli et al, 2010; Gorini et al, 2013a; Kashem et al, 2009; Witzmann et al, 2003
4	<i>Idh3a</i>	Isocitrate dehydrogenase 3 (NAD <sup>+</sup> ) $\alpha$	Cyt	Enzyme	Etheridge et al, 2009; Kashem et al, 2007, 2008, 2009
4	<i>Nefl</i>	Neurofilament, light polypeptide	Cyt	Other	Alexander-Kaufman et al, 2007a; Gorini et al, 2013a; Kashem et al, 2007, 2008
4	<i>Park7</i>	Parkinson protein 7	Nu	Enzyme	Alexander-Kaufman et al, 2007b; Etheridge et al, 2009; Kashem et al, 2007; Matsuda-Matsumoto et al, 2007
4	<i>Prdx6</i>	Peroxiredoxin 6	Cyt	Enzyme	Alexander-Kaufman et al, 2007b; Kashem et al, 2007, 2009; Lewohl et al, 2004
4	<i>Snca</i>	Synuclein, $\alpha$ (non-A4 component of amyloid precursor)	Cyt	Other	Gorini et al, 2013a; Kashem et al, 2007; Lewohl et al, 2004; McBride et al, 2009
4	<i>Stxbp1</i>	Syntaxin-binding protein 1	Cyt	Transporter	Etheridge et al, 2009; Gorini et al, 2013a; McBride et al, 2009; Park et al, 2004
4	<i>Tpm3</i>	Tropomyosin 3	Cyt	Other	Bell et al, 2006; Boukli et al, 2010; Kashem et al, 2007, 2008

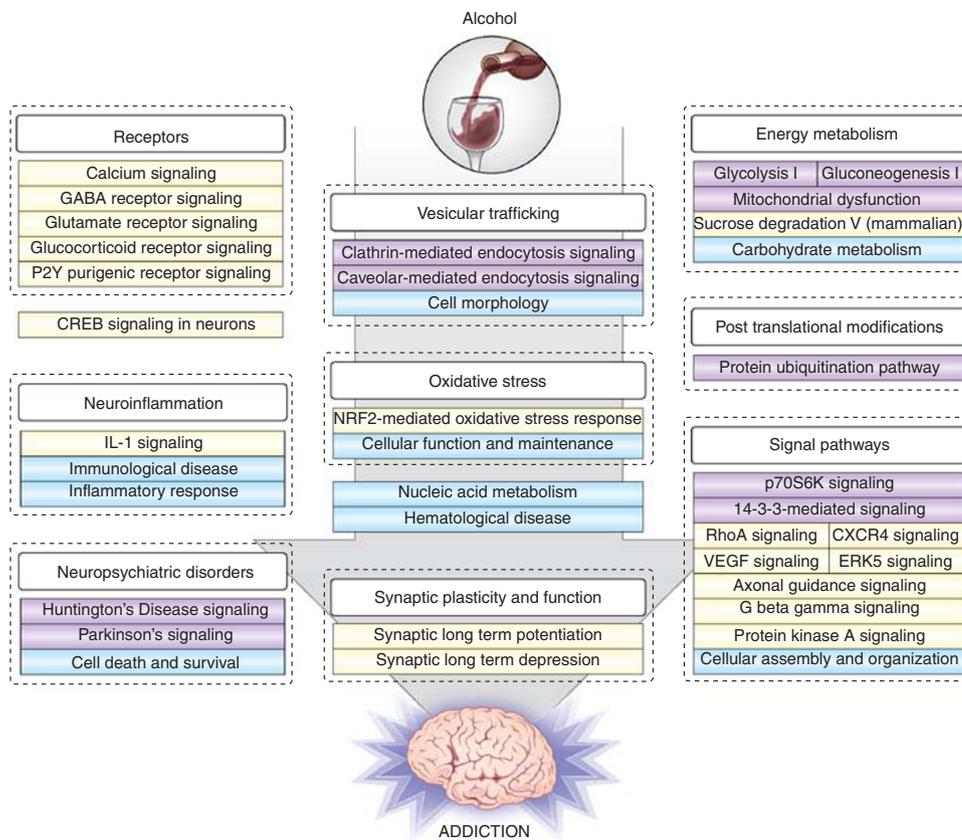
Abbreviations: Cyt, cytoplasm; Nu, nucleus; PM, plasma membrane; Unk, unknown.

Consolidated alcohol-sensitive proteins, described as differentially expressed by at least four alcohol-related proteomic studies. The number of related references referring to each protein is shown under the column Hits. Gene symbol, full name, location, and function are also listed for each protein.

**TABLE 2** Ingenuity Canonical Pathways Enriched in Genes Encoding Proteins Associated with Alcohol Exposure

Ingenuity canonical pathways	P-value	FDR	Molecules
Glycolysis I	6.41E – 13	8.65E – 11	PGK1, TPI1, ENO1, PGAM1, PKM, GAPDH, ENO2, ALDOC
Gluconeogenesis I	6.46E – 09	4.36E – 07	PGK1, ENO1, PGAM1, GAPDH, ENO2, ALDOC
Parkinson's signaling	1.64E – 05	7.37E – 04	UCHL1, PARK7, SNCA
Clathrin-mediated endocytosis signaling	2.77E – 05	9.35E – 04	HSPA8, DNMI1, ALB, APOA1, ACTB, ACTG1
14-3-3-Mediated signaling	3.68E – 05	9.93E – 04	YWHAQ, PDIA3, YWHAZ, GFAP, SNCA
p70S6K signaling	6.58E – 04	1.24E – 02	YWHAQ, PDIA3, EEF2, YWHAZ
Huntington's disease signaling	7.33E – 04	1.24E – 02	GNB1, HSPA8, DNMI1, HSPA2, SNCA
Protein ubiquitination pathway	1.35E – 03	1.65E – 02	UCHL1, HSPA8, HSPD1, HSPA2, HSPA12A
Sucrose degradation V	1.46E – 03	1.65E – 02	TPI1, ALDOC
Mitochondrial dysfunction	1.46E – 03	1.65E – 02	PARK7, ATP5A1, UQCRC1, SNCA
Caveolar-mediated endocytosis signaling	1.80E – 03	1.87E – 02	ALB, ACTB, ACTG1

Consolidated alcohol-sensitive proteins, described as differentially expressed by at least three alcohol-related proteomic studies, were analyzed with ingenuity pathway analysis (IPA). A significantly enriched pathway is defined as one with  $P < 0.05$  and a false discovery ratio (FDR)  $< 0.05$ . The  $P$ -value is calculated by Fisher's exact test right-tailed. The FDR is calculated by the method of Benjamini and Hochberg (1995).



**Figure 1.** Neurobiological effects of alcohol suggested by proteomic studies. To elucidate the complex effects of excessive alcohol consumption on neurons, regulated proteins reported by proteomic studies were analyzed with Ingenuity Pathway Analysis (IPA). Alcohol modulates multiple pathways, affects several biological processes, and likely activates the molecular machinery responsible for preserving cellular homeostasis. Related direct or indirect mechanisms can in turn alter several signaling pathways (including those involved in receptor-mediated neurotransmission), disturb energy metabolism, produce oxidative stress, perturb vesicular trafficking, affect cell fate, and ultimately induce permanent plastic neuronal modifications responsible for addictive behavior. White boxes describe major biological processes; blue boxes indicate enriched Ingenuity molecular and cellular functions; purple boxes display enriched ingenuity canonical pathways resulting from core analysis of 54 proteins differentially expressed in at least three independent alcohol-related proteomic studies; yellow boxes show the pathways resulting from a similar analysis involving 460 proteins collectively reported by 22 proteomic studies. Significantly enriched pathways are defined as the ones with  $P < 0.05$  and a false discovery ratio (FDR)  $< 0.05$ . The  $P$ -value is calculated by Fisher's exact test right-tailed. The FDR is calculated by the method of Benjamini and Hochberg (1995).

identified by array studies can be used to predict meaningful networks of interacting proteins (Bhardwaj and Lu, 2005; Lu *et al*, 2005; Ramani *et al*, 2005). For example, gene

expression studies have shown that alcohol alters the expression pattern of many genes required for normal synaptic function (Gorini *et al*, 2011; Mayfield *et al*, 2008).



be classified as signaling proteins, trafficking proteins, cell adhesion molecules, ion channels, cytoskeletal proteins, metabolic enzymes, and extracellular matrix-associated proteins. Interestingly, DAT was found to specifically interact with the voltage-gated potassium channel Kv2.1 and with the synaptic proteins synapsin-1 and dynamin-1, involved in regulating neurotransmitter release and recycling. An *in silico* analysis was also performed to evaluate the biological significance of these interacting proteins as a group. The correlation between the expression levels of the genes encoding the various interacting proteins was greater than would be predicted by chance alone, suggesting common regulatory mechanisms.

Few studies have shown direct evidence of the effects of alcohol exposure on PPIs. One of these studies (Hong-Brown *et al*, 2010) examined the effect of ethanol on the interaction complex of the mammalian target of rapamycin (mTOR), which is a key regulator of cell growth and proliferation and plays an important role in regulating protein synthesis in response to alcohol. The mTOR interacts with several proteins to form the mTOR complex 1 (mTORC1) consisting of: regulatory-associated protein of mTOR (raptor), TORC subunit LST8 (mLST8/G $\beta$ L), and proline-rich Akt substrate 40 (PRAS40). Incubation of C2C12 myocytes with ethanol (100 mM, 24 h) caused changes in PPIs in the mTORC1 complex. Immunoprecipitations followed by immunoblots showed that ethanol enhanced the binding of raptor and PRAS40 with mTOR and these changes occurred in concert with increased binding of the cytosolic protein 14-3-3 to raptor, whereas the PRAS40 and 14-3-3 interaction was not affected. Taken together with other experimental evidence, the results from this study suggest a possible mechanism for the inhibitory effects of ethanol on mTOR kinase activity and protein synthesis in myocytes.

Prenatal exposure to ethanol could result in fetal alcohol spectrum disorder (FASD), which induces long-term cognitive, behavioral, and developmental defects and is often accompanied by neuronal cell death that in turn reduces brain volume. Clusterin is a glycoprotein with various roles (antiapoptotic, proapoptotic, and chaperone) that is upregulated in some neurodegenerative diseases (Nuutinen *et al*, 2009; Prikrylová Vranová *et al*, 2010). Cell death induced by ethanol in the developing mouse brain requires B-cell lymphoma 2-associated X protein (Bax), a proapoptotic protein (Young *et al*, 2003); clusterin normally interacts with Bax, thus inhibiting apoptosis (Trogakos *et al*, 2009). An interesting study (Kim *et al*, 2012) investigated mechanisms of ethanol-induced cell death in the developing brain of the rat, one of the most commonly used animal models of FASD. This study involved an interaction proteomic approach to test the effect of ethanol on clusterin's PPIs. Seven-day-old rats were subcutaneously injected with 20% ethanol (3 g/kg, administered two times). Following acute ethanol treatment, clusterin upregulation was detected in the cerebral cortex. Co-immunoprecipitation experiments followed by immunoblotting showed the

interaction of nuclear clusterin with Bax and with an antiapoptotic protein of the same family, B-cell lymphoma-extra large (Bcl-XL). Clusterin was co-immunoprecipitated with Bcl-XL and *vice versa* in the cerebral cortices from ethanol-treated rats but not from control rats. Furthermore, the co-immunoprecipitation of Bcl-XL and Bax was reduced by 36% in ethanol-treated rats compared with controls, implying that upregulated clusterin sequestered Bcl-XL, releasing Bax and thus mediating apoptosis. This study concluded that nuclear clusterin has a proapoptotic role in ethanol-induced cell death in the developing brain, providing new insights on the development of FASD.

A comprehensive network of ethanol-related candidate genes has been constructed and analyzed (Guo *et al*, 2010a). The authors collected many large-scale data sets for alcohol dependence and ethanol response in human, rat, mouse, *Drosophila*, and *Caenorhabditis elegans*, and deposited them in a web-based gene resource database (ethanol-related gene resource, ERGR; [bioinfo.mc.vanderbilt.edu/ERGR/](http://bioinfo.mc.vanderbilt.edu/ERGR/)). On the basis of evidence for multiple genes among these data sets, they prioritized 57 ethanol-related candidate genes. Gene ontology term-enrichment tests and pathway analysis revealed that these candidate genes were highly enriched in alcohol dependence and highly expressed in the brain or liver. In addition, their PPI network analysis examined connections of the same candidate genes in the human interactome, from which they extracted a subnetwork of 106 molecules (nodes) and 158 links (edges). Among these nodes, 56 were new genes, not comprised in the original list of candidate genes. Among the new genes, 25 were linked to alcohol according to the ERGR database. In this subnetwork, they ultimately identified six genes that were highly connected to the proteins encoded by ethanol-related genes based on evidence provided by the ERGR database; these genes were tyrosine-protein kinase Fyn, vinculin, Von Hippel-Lindau disease tumor suppressor, calnexin, promelanin-concentrating hormone, and sintaxin-1A. This study is a prime example of how publically available PPI databases can be used to integrate experimental data from different sources with consolidated interactome maps to study the molecular mechanisms of alcohol dependence. Similar approaches could be applied to study the features of other putative, candidate genes from alternative phenotypes.

In summary, examining complex diseases in terms of PPIs, rather than individual genes and proteins, could help elucidate the underlying neurobiology of alcoholism and other addictive disorders. Select clusters of genes can predict meaningful networks of interacting proteins that are sensitive to the effects of alcohol and may represent potential sites important for medication development. Results from PPI studies should still be confirmed by different methodologies and/or identified across multiple species, and researchers should filter out interactions that have not been validated. Methods designed to block or disrupt PPIs are potentially promising for elucidating their functional roles. MS-based strategies allow for absolute

quantification of proteins and provide accurate measurement of protein complex levels and detection of even small changes within PPI networks (Marcilla and Albar, 2013; Wepf *et al*, 2009). Complete reference interactome maps of PPIs will not only provide biological insight into cellular organizational principles but also generate novel hypotheses and advance basic experimental research in various disciplines. Importantly, elucidation of novel mechanisms by which alcohol regulates complexes of interacting proteins will enable significant contributions to the field of addiction research.

### Structural Proteomics to Study Alcohol-Sensitive Ion Channels

Several families of brain proteins that are sensitive to modulation by ethanol have been defined in the past few years, and this allows a molecular and even atomic level analysis of the alcohol binding sites on these target proteins (Harris *et al*, 2008). Prominent among these targets are ion channels, which catalyze neurochemical reactions and form the supportive structural environment for neurotransmission to occur. For example, important alcohol-sensitive ion channels include potassium channels (especially GIRK), ligand-gated ion channels (including glutamate, especially NMDA), and the pentameric ligand-gated ion channels (pLGIC) (Howard *et al*, 2011). There has been considerable progress in defining sites of alcohol actions on several pLGIC, including  $\gamma$ -aminobutyric acid type A (GABA<sub>A</sub>), glycine (Gly), and nicotinic receptors (Blednov *et al*, 2011; Borghese and Harris, 2012; Borghese *et al*, 2003; McCracken *et al*, 2010, 2013; Murail *et al*, 2012; Sauguet *et al*, 2013). Importantly, genes for five of the pLGIC receptor subunits (GlyR  $\alpha$ 3, GABA<sub>A</sub>  $\gamma$ 1,  $\rho$ 1,2, and  $\alpha$ 2) have been linked with human alcohol dependence (Gorini *et al*, 2011; Han *et al*, 2012; Ittiwut *et al*, 2012; Mayfield *et al*, 2008; Xuei *et al*, 2010), but very little is known about specific roles of these subunits in alcohol actions. Indeed, as noted above, conventional gel-based and shotgun proteomic techniques have limited capability in detecting low abundance integral membrane proteins like native ion channels, because of various technical problems including solubilization, enzymatic digestion and recovery, and separation of hydrophobic peptides from transmembrane (TM) domains. However, a recent study described a method for the deep amino-acid sequencing of native brain GABA<sub>A</sub>Rs using high-resolution MS (Chen *et al*, 2012b). Starting from a small sample amount, the researchers were able to obtain high peptide coverage for 12 GABA<sub>A</sub>R subunits. A label-free quantitative MS analysis was also applied to three separate brain samples to determine the relative abundances of 11 GABA<sub>A</sub>R subunits. In another study (Vogel *et al*, 2009), MALDI-ToF MS was used to identify tryptic fragments corresponding to both native disulfide bonds (superfamily-specific Cys-loop and GlyR-specific bond) in the extracellular domain (ECD) of GlyR  $\alpha$ 1. This evidence confirmed the disulfide bond formation for both pairs of cysteines in

the ECD of GlyR  $\alpha$ 1, which had been predicted by homology modeling.

Understanding high-resolution 3D structures of alcohol-sensitive ion channels can simplify the design and development of new structure-based drugs. Indeed, such structural studies by X-ray crystallography and nuclear magnetic resonance spectroscopy have a crucial role in pharmaceutical research (Kim and Doyle, 2010). For example, a critical advance in our understanding of the molecular interaction of alcohol on protein comes from explorations of the bacterial pLGIC GLIC, which provided the first X-ray crystal structure of ethanol bound to a pLGIC (Sauguet *et al*, 2013). Emerging structural evidence indicates that ethanol occupies water-filled cavities in proteins, particularly between  $\alpha$ -helices, and this requires hydrogen bonding with the hydroxyl group of the alcohol and usually a serine or threonine in the protein as well as weak hydrophobic interactions (Cheng *et al*, 2008; Harris *et al*, 2008; Sauguet *et al*, 2013). However, crystallographic and spectroscopic methods are complex, lack dynamicity, require high protein concentrations, and can be challenging depending on the size of proteins and/or their inability to form crystals. Therefore, there is a strong interest in alternative techniques for the detection of small proteins at low concentrations. Structural proteomics is the application of protein chemistry and modern mass spectrometric approaches to important biological questions such as the characterization of protein structures and complexes, and the in-depth identification of PPIs. Importantly, the use of structural proteomics can help to decipher the detailed structure of interfaces between proteins and the interactions between proteins and ligands (Serpa *et al*, 2012). Therefore, the application of such techniques can provide valuable information on putative druggable targets. Structural proteomics uses different complementary methods in combination, including chemical protein modification, chemical crosslinking, covalent labeling, limited proteolysis, photoaffinity, and hydrogen/deuterium exchange (Kaur and Chance, 2012), all followed by mass spectrometric analysis. These MS-based technologies are emerging as new tools to study the highly dynamic process of ligand binding to ion channels, and have been utilized to shed light on the interactions between a protein and its ligand in absence of high-resolution crystallography data on the protein of interest. As such approaches cannot provide 3D structural information, they are usually combined with theoretical computations, modeling, and other biochemical methods. For example, limited proteolysis coupled to MS on reconstituted GlyR  $\alpha$ 1 homopentamers identified proteolytic cleavages within proposed TM domains, postulated to fold as bilayer-spanning  $\alpha$ -helices, and also identified unexpected membrane-associated regions in the N-terminal domain (Leite *et al*, 2000). These proteolysis data were later integrated with additional biochemical evidences to construct novel topological models (Leite and Cascio, 2001). Further studies showed homology modeling and molecular dynamics simulations to construct models of the

homopentameric  $\alpha 1$ -subunits of the GlyR TM domain to compare the binding of ethanol molecules to the channel in open and closed states (Cheng *et al*, 2008).

In another example, structural proteomic techniques combining chemical crosslinking and MS have recently been applied to the analysis of human P2X1 receptors expressed in HEK293 cells, providing insight into phosphorylation, modeling, and highlighting the importance of conformational change for channel activation (Roberts *et al*, 2012). Similarly, a hybrid structural approach was used to analyze ligand binding by the human 5-HT<sub>4</sub>, G-protein-coupled receptor for serotonin (Padayatti *et al*, 2013).

In the past decade, structural proteomic techniques have been increasingly used to overcome the restrictions inherent to traditional biophysical methods. In the next future, structural proteomic experimental data, integrated with computational modeling, will provide more specific evidences and reliable results that will be progressively used to support new drug design for the treatment of brain diseases.

### Proteomics to Study miRNA Regulation of Gene Expression

The majority of the RNA in a cell does not code for proteins but is non-coding regulatory RNA that orchestrates the function of the cell. miRNAs are small, non-coding oligonucleotides involved in post-transcriptional regulation of gene expression. They are considered as key regulators of a broad spectrum of cellular functions and may thus have a role in many of the widespread genomic changes induced by chronic alcohol consumption. Recent studies have revealed that miRNAs have critical roles in regulating diverse biological processes such as neuronal differentiation, developmental timing, synapse function, and neurogenesis (Le *et al*, 2009; Papagiannakopoulos and Kosik, 2009; Shen and Temple, 2009). With regard to alcoholism, miR-9 was found to promote splice variations in the mRNA coding for the pore-forming  $\alpha$ -subunit of the BK channel, a large-conductance calcium- and voltage-activated potassium channel (Pietrzykowski *et al*, 2008). Alcohol exposure caused an increase in miR-9 expression, which then results in a rapid degradation of one splice variant of the  $\alpha$ -subunit, causing reorganization of transcripts to form an alcohol-resistant BK channel.

Mature miRNAs regulate biological processes by associating with the RNA interference silencing complex to induce translational inhibition and/or degrade target mRNA transcripts. Although a major challenge, identification of miRNA targets is crucial for understanding the biological roles of miRNAs. Target genes are defined by short sequences in their 3' untranslated regions that are complementary to a given miRNA, and although bioinformatic tools have improved the unbiased prediction of miRNA binding sites, different algorithms produce divergent results with high false-positive rates. Experimental evidence showed that each individual miRNA may down-regulate mRNA levels, but multiple miRNAs can also work

together on an individual mRNA to alter its protein output (Nunez and Mayfield, 2012; Wu *et al*, 2010).

As miRNAs may regulate their targets at the translational level without affecting mRNA levels (Bhattacharyya *et al*, 2006; Filipowicz *et al*, 2008; Pillai *et al*, 2005), proteomic techniques are being increasingly used to understand miRNA biology. MS-based proteomics has the power to identify several core components of the canonical miRNA processing pathway and the PTMs involved in relative regulatory mechanisms. In addition, quantitative proteomic approaches are emerging as tools for the identification of genuine miRNA targets and to understand the contribution of translational repression by allowing direct determination of changes in protein expression. The measurement of mRNA levels is not ideal for the identification of miRNA targets and should be carefully used to draw conclusions about the proteome from analysis of the transcriptome for several reasons. First, mRNA and protein expression levels do not necessarily correlate (Chen *et al*, 2002; Griffin *et al*, 2002; Gygi *et al*, 1999; Vogel and Marcotte, 2012). Second, the simultaneous quantification of absolute mRNA and protein abundance has demonstrated discrepancies between half-lives of mRNAs and proteins (Schwanhäusser *et al*, 2011). Third, even the efficiency of miRNA-mediated gene silencing can be reduced by PTMs in Argonaute/RISC complex, suggesting that the simple presence of miRNA does not necessarily decrease protein production (Huang *et al*, 2013; Leung *et al*, 2011). Therefore, the use of MS-based proteomic techniques allows more in-depth investigations of the effects of miRNAs on the proteome. Current quantitative proteomic technologies like 2D-DIGE, iTRAQ, and SILAC have advantages and limitations but can be combined to reveal the full spectrum of miRNA targets. As growing evidence suggests that alcohol exposure changes miRNA expression profiles (Pietrzykowski *et al*, 2008; Miranda *et al*, 2010; Lewohl *et al*, 2011; Gorini *et al*, 2013b), future studies are expected to increasingly utilize proteomics to investigate how specific alcohol-sensitive miRNA can affect their targets at the translational level by accurately measuring protein levels under different conditions.

### Systems Biology and Integration of Proteomics with Complementary Approaches

Alcohol abuse is a complex-trait disorder involving a number of pathways and inducing negative effects on multiple tissues. At the molecular level, the effects of alcohol are in part due to changes in DNA, RNA, miRNAs, proteins, and metabolites, whereas at the systems level, alcohol affects many organs, biochemical or signaling pathways, and other biological processes. Genetic factors that influence the risk for alcoholism originate from multiple genes, but the contribution of each gene may be quite small (Gorini *et al*, 2011; Mayfield *et al*, 2008; Phillips and Belknap, 2002).

Even though microarrays have improved our comprehension of complex cellular function, their reliance on hybridization kinetics results in several technical limitations

(ie, knowledge of sequences probed, cross-hybridization, narrow dynamic range of signal). New methodologies, collectively termed next-generation sequencing, are free of the limitations inherent to microarrays. Sequencing the genome (DNA-Seq), the transcriptome (RNA-Seq), or the miRNome (miRNA-Seq) allows the detection of the entire population of RNA molecules in a cell or living tissue, thus providing information on gene structure, alternative splicing events, expressed single-nucleotide polymorphism (SNPs) (Mane *et al*, 2009; Tang *et al*, 2009; Walter *et al*, 2009), and transcript size, while also quantifying the absolute abundance of genes, with greater sensitivity and dynamic range than the competing microarray technology (Mortazavi *et al*, 2008). Nevertheless, it is evident that approaches to the study of gene regulation purely based on gene sequence would not be sufficient to explain alcohol-induced pathogenesis. Detailed whole genome information can be combined with other experimental, clinical, and/or phenotypic data to provide increased understanding of basic biological processes and a more integrated view of cellular function and regulatory networks.

There is general agreement that proteomic and transcriptomic studies deliver different albeit complementary information about gene regulation. Although the genome can be comprehensively arrayed, proteomic approaches can offer incomplete information on protein expression, PPIs, and PTMs that genomics cannot yet provide. Over the past decade, alcohol research has been continuously reshaped by increasingly sophisticated genetic tools. Selected lines, recombinant inbred strains, linkage scan, association studies, QTLs, microarray expression, SNP maps, and other tools have been used to extensively dissect alcohol dependence in humans and ethanol response in animal models. As a result, a number of databases with alcohol-related chromosome regions and candidate genes are available for diverse model organisms as well as for *Homo sapiens*. With such blooming of resources, one may wonder whether and how much these massive databases are converging and/or overlapping with proteomic-based findings. Comparing different databases is not always appropriate, because of diverse experimental designs, protocols, cutoffs, and procedures that could potentially bias any tentative of data synthesis. For example, a comparison of microarray studies should take account of distinct platforms (ie, chip versions), brain regions and/or species, with related differences in the background of assessed genes. In addition, it is possible that a number of matches among different data sets would occur by chance, although some extent of the overlapping genes/proteins should represent true positives when their statistical significance is high. Furthermore, the candidate genes identified can often differ in their direction and magnitude of change across studies. On the other hand, correspondence among data sets and previous alcohol-related gene/protein expression work adds support to and strengthens the results from combined proteomic studies, helping with the interpretation of massive lists and offering a molecular

‘zoom’ function that can direct the focus to promising candidate molecules.

A number of proteins described in the alcohol-related proteomic studies reviewed here are encoded by genes reported to be differentially expressed following excessive alcohol consumption or dependence in different species (Gorini *et al*, 2011). As an example, the ERGR database described above (Guo *et al*, 2010a) provides data integration and candidate gene selection based on multiple data sets and organisms. When comparing their top 57 consolidate genes with the 28 genes listed in Table 1, only *Gfap* and *Snca* are common. However, all the genes in Table 1 have been previously reported as related to alcohol consumption, dependence, or genetic predisposition by individual studies listed in the ERGR database or by more recent ones (Mulligan *et al*, 2011; Ponomarev *et al*, 2012).

Rather than detecting all the relevant players involved in a biological process, it is likely that every individual expression study would identify distinct complementary subsets of biologically relevant genes/protein along with some false positives and with at least partial overlap across different studies. Therefore, a more practical way to compare and combine findings from multiple sources in a meaningful way implicates the analysis of pathways involved.

Systems biology is a relatively new scientific discipline, which aims to investigate complex biological processes by integrating data from different sources and using mathematical modeling to predict functions of biological systems (Aderem, 2005; Auffray *et al*, 2003; Kirschner, 2005; Liu, 2005; Westerhoff and Palsson, 2004). A fundamental concept in system biology is represented by functional ‘modules’, which are the building blocks of a biological system or process (ie, different species of interacting molecules within a cell). These modules are evolutionary conserved, interact with other molecules, and have discrete functions that arise from interactions among their components (ie, proteins, DNA, RNA, miRNAs, and smaller molecules), although these functions cannot be predicted by studying the properties of the isolated components. Modules are not fixed structures; their components can belong to different modules at the same time and they are also interconnected. The interactions of these interconnected modules in turn generate a higher layer of functions at another biological level (Guo and Zakhari, 2008). Modules are organized in hierarchical structures to form subsystems and systems. A biological system can consist of many interconnected modules that operate synergistically to execute the functions of a biological process. Organisms have developed evolutionary mechanisms to keep their biological systems functionally stable (Hartwell *et al*, 1999).

Single-discipline approaches are limited as they can only offer a fragmented view of a very complex picture. This highlights the advantage of systems approaches where we can focus on the more complex modules as opposed to individual genes. Moreover, it is possible that some effects of alcohol may be caused or accompanied by changes in coregulation that are invisible to single gene-, protein-, or

miRNA-based analyses. Integrative approaches are thus essential in providing a global picture of the disturbances leading to alcohol addiction or related disorders. As an example, weighted gene coexpression network analysis (WGCNA) is a bioinformatics tool for the study of coexpression patterns from high-throughput data (Langfelder and Horvath, 2008; Zhang and Horvath, 2005). The correlation values between genes indicate expression level similarities or differences, and WGCNA analysis can identify modules of interconnected genes showing over-represented patterns of coexpression. These modules can be treated as single units and related to external information used as a trait (ie, alcohol phenotypes) via simple measures (ie, correlation). This method was recently applied to the identification of epigenetic modifications in human alcoholic brain (Ponomarev *et al*, 2012) and to miRNA and proteomic data from a mouse model of alcohol dependence (Gorini *et al*, 2013b) (see below).

The application of comprehensive, integrative approaches will define the future of alcohol research (Rodd *et al*, 2007; Tabakoff *et al*, 2009; Tapocik *et al*, 2013) and promises to be fruitful in elucidating the mechanisms underlying alcohol addiction. Genetic and biological studies in animal models or post-mortem human brain, with epidemiological and clinical surveys on human subjects, should be integrated with genomic data. Current technologies have the potential to dissect the effects of alcohol exposure on DNA (genome, epigenome), RNA (transcriptome), protein (proteome), metabolites (metabolome), and glycans (glycome) to generate meaningful amounts of data (Guo and Zakhari, 2008). Those distinct yet complementary pieces of information can be integrated using bioinformatics (Abul-Husn *et al*, 2009; Reker and Malmström, 2012), computation, and mathematical modeling to bring a systems approach to alcohol research.

However, the application of such integrative methods is complicated by several intrinsic limitations that still need to be solved. First, computational models of systems-wide properties should be mainly used to make predictions and provide rationale for experimentation, and not to draw definitive conclusions. Second, it could be nearly impossible to accurately model certain gene–environment interactions that occur in living cells and organs. Indeed, addiction research is being continuously reshaped by increasingly sophisticated genetic tools. Nevertheless, we may not be able to decode the genetics of dependence until we reach a better understanding of how genes interact with environmental variables to influence alcohol responses and their related behaviors (Gorini *et al*, 2011; Mayfield *et al*, 2008). In addition, the bioinformatics field is advancing rapidly and is typically conducted by relatively specialized subgroups of researchers. Continuous advances in the field and low standardization require constant devotion and adaptation to newly developed software and tools; general accessibility remains limited because not all bench-based biologists can become computer programmers. *In silico* analyses are reshaping the idea of ownership of data, and

multidisciplinary teamwork is often necessary to overcome technological difficulties.

In addition to the use of proteomic technologies, several researchers have already applied integrative approaches to the study of alcoholism by combining data obtained with different techniques. Briefly, Park and co-workers (2004) integrated their proteomic analysis of brains from B6 vs D2 mice with information on PPIs taken from web-based databases and built a linkage map to reveal the inter-relationship of the alcohol-responsive proteins between different species (Park *et al*, 2004). In another study, an attempt to determine anxiety factors contributing to alcoholism was made by testing a transgenic mouse model for anxiety using three combined approaches: QTL analysis, microarray analysis, and shotgun proteomics of synaptosomes (Sikela *et al*, 2006). In their analysis of the DAT proteome, Maiya and co-workers (2007) identified DAT's PPIs, and also evaluated the biological significance of these proteins as a group. This was accomplished by determining expression levels of the genes encoding these proteins using computerized searches of existing databases of gene expression. The analysis found that the correlation between the expression levels of the genes encoding the various interacting proteins was greater than would be predicted by chance alone, suggesting common regulatory mechanisms and excluding experimental artifacts. Another group collected large-scale data sets for alcohol dependence and ethanol response in five different organisms and integrated these data with PPI databases to identify candidate alcohol-sensitive genes (Guo *et al*, 2010a).

QTL studies have shown that differences in alcohol-associated sensitivity is, in general, under genetic control. Complex or quantitative traits are influenced by multiple genomic regions, referred to as QTLs. To date, many studies mapping QTLs associated with human diseases and complex traits have uncovered new loci and provided unexpected insights into the biology of diseases. For alcohol-associated behaviors, rodents have been used extensively to study alcohol-related phenotypes and the behavioral genetics of alcohol's action (Crabbe *et al*, 2010). Crosses between inbred strains are useful and valuable tools for determining which chromosomal regions control these genetic differences in alcohol-associated sensitivity. However, these loci account for only a small fraction of the total genetic variation associated with alcohol-induced effects and do not map to individual genes. Indeed, QTL regions are often very broad and contain many genes. Therefore, protein data provide additional information in search for the differentially expressed genes that explain phenotypic differences between strains. An innovative integrative proteomic approach has been used to search for quantitative trait genes (Fei *et al*, 2011). In this study, striatal synaptosomes from two mouse strains (alcohol-preferring B6 vs alcohol-avoiding D2) were compared, and differentially expressed proteins with coding regions within QTL regions were considered as 'quantitative trait proteins'. Differentially expressed protein groups were mapped to the

genome, and 83% of them had members with coding sequences that fell within QTLs previously found between these strains, particularly within the replicable alcohol preference QTLs. This study demonstrated the utility of combining a proteomic data set with genetic and transcriptomic data between two strains of mice.

An integrative approach was used to combine data from miRNA and proteomic profiling in cerebral cortices and midbrains from C57BL/6J mice subjected to a CIE-2BC paradigm (Gorini *et al*, 2013b). A systems approach to data analysis combined miRNA and protein differential expression, miRNA and protein coexpression networks, miRNA target predictions, PPIs, and gene annotations to unveil crucial neurobiological alterations associated with alcohol dependence. The analysis identified modules of coexpressed miRNAs with a high correlation for CIE drinking phenotypes. In addition, some of these coexpressed miRNAs were predicted to target genes encoding differentially expressed proteins. Furthermore, certain proteins were in turn negatively correlated with these miRNA modules. Overall, the results of this comprehensive study provide an interpretation of differential expression data of individual miRNAs and proteins in terms of the relative contribution to alcohol dependence, and represent a model of how alcohol research can benefit from the application of system biology approaches to integrate complementary techniques.

Integrative approaches offer the best potential for advancing our knowledge of the complex mechanisms by which alcohol affects the brain and other organs. Continually evolving methodologies are essential for understanding the pathogenesis of alcohol dependence and for identifying therapeutic targets for treatment and prevention of the deleterious effects of this addictive substance.

## BIOMARKERS OF ALCOHOLISM

The successful treatment of most diseases relies heavily upon early detection. Biomarkers with diagnostic and prognostic value are critical to the alcohol field. Most individuals with alcohol dependence or alcohol use problems evade detection until severe medical, legal, or social consequences arise. An obvious method to reveal acute alcohol consumption is to measure alcohol concentration in body fluids or breath, and small inexpensive instruments are routinely used for these purposes by law enforcement, medical, and security personnel. However, a reliable retrospective examination of alcohol intake across days or weeks remains more challenging. Indeed, the short half-life of alcohol in the blood after cessation of drinking eliminates the feasibility for using blood alcohol as a biomarker. Nevertheless, modern noninvasive wearable biosensors are capable to continuously measure transdermal alcohol vapor electrochemically, estimating when drinking has occurred and recording the drinking schedule (Sakai *et al*, 2006; Swift, 2003). In addition, biomarkers of alcohol intake with longer ranges of assessment have been

identified. These biomarkers measure alcohol consumption indirectly, by detecting tissue damage or other physiological reactions to heavy drinking over time. For example, liver tests are available but may not identify abuse problems until advanced stages of the disease are present. This is further complicated by the fact that liver damage is not present in all alcoholics. For example, it has been reported that two-thirds of individuals with alcohol problems had normal liver function upon admission for treatment (Sobell *et al*, 1999). Development of a reliable molecular blood test for alcohol dependence and heavy drinking would be a milestone in the diagnosis and ultimate treatment of the disease (Freeman *et al*, 2010; Mayfield and Harris, 2009).

Carbohydrate-deficient transferrin (CDT) is one of the most specific serum marker of chronic, heavy alcohol use (Kasinathan *et al*, 2004; Reynaud *et al*, 2000). CDT has proven to be a useful diagnostic aid for suspected alcohol abuse and has received Food and Drug Administration approval. However, the low sensitivity of the CDT test in the general population makes it an unreliable candidate for predicting either heavy alcohol use or for diagnosing alcohol abuse and/or dependence (Aertgeerts *et al*, 2001; Alte *et al*, 2004; Neumann and Spies, 2003). Sensitivity of the CDT test varies from ~20 to 85% depending on the population being studied (Koch *et al*, 2004). The highest sensitivity rates were found in patients recruited from alcohol treatment programs, whereas some of the lowest sensitivities were observed in subjects in general medical settings. Thus, the accuracy or predictive value of the CDT test varies depending on the prevalence of heavy alcohol use in the population studied and is marginal in populations with a low prevalence of heavy alcohol use (Koch *et al*, 2004; Lijmer *et al*, 1999; Neumann and Spies, 2003). Other factors such as age and gender have also been shown to reduce CDT sensitivity (Conigrave *et al*, 2002; Montalto and Bean, 2003). Specificity also varies by population, and though initial studies suggested that CDT tests were highly specific, subsequent studies indicate a more complex picture as several biological factors have been shown to increase CDT levels. Moreover, the test in its current form is rather cumbersome, time consuming, and expensive (Javors and Johnson, 2003; Rosalki, 2004). Another serum marker that has been tested frequently is  $\gamma$ -glutamyltransferase (GGT). GGT is much less specific than CDT because GGT is also increased in non-alcohol-related liver diseases. The overall sensitivity of GGT and CDT are similar, and greater sensitivity has been reported by the combined measurement of GGT and CDT (Montalto and Bean, 2003). A third marker that has been tested is erythrocyte mean corpuscular volume, but this marker has a variable sensitivity and is nonspecific (Hock *et al*, 2005; Neumann and Spies, 2003; Schwan *et al*, 2004). Recently developed biomarkers include serotonin metabolites (Beck *et al*, 2007) and alcohol metabolites (Litten *et al*, 2010). Interestingly, ethyl glucuronide (EtG) (Borucki *et al*, 2007; Hoiseth *et al*, 2007; Kissack *et al*, 2008) can be detected in scalp or non-head hair up to

several months after drinking has stopped (Bendroth *et al*, 2008; Litten *et al*, 2010; Pianta *et al*, 2013). Such properties designate EtG as promising alcohol metabolite biomarker, with potential use for forensic purposes, for the monitoring of abstinence among individuals convicted of driving while intoxicated (Wurst *et al*, 2008b) and women who are at risk for drinking during pregnancy (Pragst and Yegles, 2008; Wurst *et al*, 2008a).

To date, no clinical test is reliable enough on its own to support a diagnosis of active alcohol dependence or abuse in the general population. Indeed, traditional blood biomarker tests for detecting alcohol use have not been universally accepted or generally adopted in clinical practice because of their low accuracy, sensitivity, and specificity (Freeman and Vrana, 2010; Litten *et al*, 2010), as they are influenced by several factors including age, gender, and also on the population being studied (Mayfield and Harris, 2009). Combining two or more tests has been shown to improve predictive values for determining recent heavy use (Anton and Moak, 1994; Anton *et al*, 2002), indicating that monitoring more than a single marker may be critical as has been reported for cancer (Rhodes *et al*, 2004).

Biomarker development strategies involve the identification of proteins that differ in abundance between alcoholics and non-alcoholics and which can be easily measured to assess whether a person has been drinking alcohol recently or is an alcoholic. High-throughput technologies such as proteomics and genomics increase the possibility of discovering biomarker panels or signatures with the potential to be more sensitive and specific (Bearer *et al*, 2010; Torrente *et al*, 2012). Studies involving non-human primates might be promising as they do not present some of the limitations associated with the human subjects, such as inconsistent self-reporting of alcohol intake, variations in diet, and other individual differences between subjects. As basic scientists discover alcohol consumption biomarker signatures that reflect either alcohol intake or alcohol-induced organ damage, clinicians will ultimately provide the tools to translate the findings to patient populations and provide insight into how these markers may be included in routine alcohol abuse testing.

Future goals of alcohol biomarker research include the development of reliable blood tests for diagnosing alcoholism in the general population. The development of such tests would have important implications and would benefit the society, with increased detection, treatment, and prevention programs. Furthermore, unbiased detection of variable amounts of alcohol consumption can affect employment and forensic areas (Anton, 2010). A test for recent heavy drinking could be implemented in future drug screenings before/during employment or for obtaining licenses, especially for airline pilots, taxi, truck, and school bus drivers. Notably, the availability of a blood assay will lead to a faster and wider spread detection of alcoholism, with subsequent prompt treatment to disrupt the progression of the disease.

## CLINICAL IMPLICATIONS

In the past century, alcoholism was mainly perceived as a moral failing and usually treated with long-term non-medical therapies involving correction of related behaviors, cognitive behavioral therapy, psychological counseling, and mutual support groups (ie, Alcoholics Anonymous) (Leggio and Addolorato, 2010). Fortunately, current thoughts and attitudes about alcohol-use disorders are considerably different. Despite some rooted reticence in considering alcoholism strictly as a medical problem, alcohol and other addictive substance disorders are now more widely recognized as brain diseases, similar to other clinical psychiatric disorders (Addolorato *et al*, 2012). There is an increased awareness about genetic predisposition to alcoholism and long-lasting changes in neurotransmission systems associated with different phases of addiction. Consequently, treatment has progressed from social and behavioral therapies to complementary pharmacotherapies attempting to disrupt the mechanisms underlying this condition (Jupp and Lawrence, 2010). In the near future, the pharmacological treatment of alcohol-related disorders will gradually be integrated into the general health-care system. Ideal therapeutic approaches should combine both pharmacological and psychosocial treatments (Swift, 2007), given that the etiology of alcoholism includes genetic, neurobiological, psychological, and environmental factors (Koob, 2006). However, as diagnosis of alcoholism can be biased and problematic (Leggio and Addolorato, 2010), treatment trials are increasingly adopting biomarkers to help in the evaluation of standard interventions and new medications (Anton, 2010; Torrente *et al*, 2012). Biomarkers have the potential to serve as trait markers of alcohol-related phenotypes, whose identification and categorization will characterize models for the multiple subtypes of alcohol dependence (Mayfield and Harris, 2009). Clinicians will then be better able to identify patients who are likely to respond positively or negatively to specific drugs and treatments. Therefore, biomarkers are expected to have a key role in future personalized protocols for the treatment of alcohol abuse (Litten *et al*, 2010).

## FUTURE RESEARCH DIRECTIONS

As the initial results of the ENCODE project have been released, it is becoming clear that large portions of non-coding DNA are involved in regulating the expression of coding genes, and that gene regulation is far more complex than previously imagined (Dunham *et al*, 2012; Pennisi, 2012). Rapidly maturing DNA- and RNA-sequencing technologies are currently more comprehensive in covering whole genomes than proteome profiling. Nevertheless, the proteome exhibits unique complexity and dynamicity, and next-generation sequencing approaches still lack the ability to fully decode some key layers of gene expression regulation and network biology, such as PTMs. Therefore, proteomic analyses are gaining an indispensable complementary role

in the study of complex diseases, and the list of techniques for studying proteomes is growing. Increasing evidence of limited correspondence between protein and mRNA levels requires protein, miRNA, and mRNA profiling to be applied in parallel, along with supportive biochemical and analytical techniques to fully understand gene regulation mechanisms underlying the neuronal adaptations related to dependence. Thus, development of novel proteomic strategies should be focused on complementing current transcriptomic techniques. Unbiased MS-based methods are rapidly becoming faster and more comprehensive (Altelaar *et al*, 2013), and are expected to be further improved to generate more sensitive and reproducible data. A better characterization of PTMs will be achieved through the development of more sensitive, refined, and improved methodologies and analytical tools. Indeed, modification-specific proteomics, which enables selective enrichment, purification, separation, and identification of various PTMs, will allow increasingly detailed analysis and quantitation of PTMs within proteins (Chung *et al*, 2013; Engholm-Keller and Larsen, 2013; Jensen, 2000; Zhao and Brasier, 2013) and will help elucidate their role in complex biological diseases like alcoholism. A reduction in the quantity of material required and analysis time necessary to obtain relevant proteomics data, while maintaining rigorous quality control, is also desirable.

With a significant shift in proteomics' throughput and pricing, in the next five years we expect a surge in publications where these methods will be applied to the study of neurobiology of addiction. Although previous studies have mostly reported changes engendered by alcohol consumption and exposure, low-cost access to an improved proteomics will lead to detailed studies where proteomes from discrete brain subregions will be profiled with an increased sample number and during different time points after withdrawal. Indeed, recent evidences in rodent models have suggested that, at least for cocaine or nicotine, a longer withdrawal corresponds to a greater drug-seeking, which may imply greater potential for relapse vulnerability (Gipson *et al*, 2013; Ben-Shahar *et al*, 2013). Long-lasting changes in the proteome that persist after protracted withdrawal and likely relate to relapse liability would be of high interest to translational scientist.

Many proteomic studies reviewed above provide lists of proteins with limited practical value. In the next few years, the required better avoidance of false positives will be reached with a combination of appropriate statistical filtering, extensive downstream functional analyses, experimental and computational validations with luciferase assays, immunoblotting, RT-qPCRs, and viral-mediated translational activation or suppression. This will restrict the number of candidate druggable targets and will hopefully lead to improved therapies for alcoholism.

Furthermore, the use of quantitative proteomic approaches to characterize targets of miRNAs has provided new tools for the study of miRNA biology. A number of miRNA targets important for other diseases are being

discovered, allowing for functional analyses of miRNA-mediated gene regulation and potentially leading to the development of novel therapeutic approaches. Thus, an increase in proteomic analyses aimed to understand the role of non-coding RNAs is expected in the near future (Grosshans and Filipowicz, 2008; Huang *et al*, 2013; Li *et al*, 2012), along with next-generation-based proteomics to enable an in-depth, multifaceted view of the proteome.

Some of the reviewed studies offer glimpses of innovative integrative methods that may become popular in the next years. The application of emerging systems biology approaches to the neurobiology of alcohol addiction can be technologically challenging. Nevertheless, significant progress has already been made and the impact of comprehensive systems biology in the study of alcohol-related disorders promises to be ground-breaking. Innovative efforts to combine proteomics with other complementary postgenome platforms and integrate resulting information will accelerate its application in clinical practice and maximize its capabilities. These approaches are founded on the concept that alcohol-perturbed protein and gene regulatory networks differ from their normal counterparts (Hood *et al*, 2004; Weston and Hood, 2004). A better understanding of protein and gene regulatory networks underlying the neurobiology of alcohol addiction will pave the road for novel drug development strategies. Indeed, these networks share key nodal points that could represent more effective targets for therapeutic interventions. Systems approaches require the availability of reliable large and disparate data types from diverse technological platforms. Therefore, investigators need interdisciplinary skills to expand their research to new technological levels to include, for example, epigenomic, metabolomic, glycomic, modifomic, and other complementary proteomic approaches (Guo and Zakhari, 2008). Bioinformatics, computation, statistical analysis, and mathematical modeling are all crucial to integrate complex data sets generated through these techniques and to extract their global biological or clinical significance. Academic researchers would need better access to high-throughput facilities for DNA arrays, sequencing, genotyping, and various proteomic platforms. Development of more accessible databases and analysis software is also expected to allow more researchers to use integrative approaches. Advancing technological innovations will require standardization as well as collaborative crosstalk and multidisciplinary teamwork between different scientific communities.

Ultimately, we will decipher the molecular mechanisms underlying alcohol-use disorders and provide the foundation for their future prognosis, diagnosis, and treatment. In the case of alcoholism, investigators should find ways to prioritize the use of systems approaches. The boundaries between basic research and clinical applications of proteomics and systems biology are fading (Weston and Hood, 2004). These approaches will have a major role in creating a predictive, preventive, and personalized approach to medicine for addiction disorders. Proteomics is still a

relatively immature technology and will require intensive efforts in the development of future strategies. Increasingly sophisticated proteomic methods are currently being developed in search of novel therapeutic targets and for better multiparameter diagnostics on alcohol-perturbed proteins and/or metabolites.

Snapshots of individual proteomes will likely be part of the development of personalized medicine of the future (Chen *et al*, 2012a). Over the next decades, when diagnostic tools will be more predictive and therapies more preventive, new cost-efficient and integrated technologies will allow individuals to have relevant portions of their genomes and proteomes sequenced; routine procedures for assessing health, disease status, and future disease risks will be available for each individual and will likely include multiparameter informative molecular diagnostics via blood analysis (Bailey, 2009; Hood *et al*, 2004; Mayfield and Harris, 2009). Improved proteomics, together with new technologies, will ultimately contribute to the design of preventive drugs for individuals with a genetic or psychosocial predisposition for alcoholism and offer a breakthrough in addiction treatment.

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