Proteomics Approach in the Study of the Pathophysiology of Alcohol-Related Brain Damage

Izuru Matsumoto*

Discipline of Pathology, University of Sydney, NSW 2006, Australia

*Corresponding author: Discipline of Pathology, University of Sydney, NSW 2006, Australia. Tel: +61-2-935-13319; Fax: +61-2-935-13429; E-mail: izuru@med.usyd.edu.au

Current contact address: Department of Psychiatry, Asahiyama Hospital, 4-3-33 Futagoyama, Cyu-o-ku, Sapporo 064-0946, Japan. Tel: +81-11-641-7755; Fax: +81-11-631-5512; E-mail: izuru@med.usyd.edu.au.

Abstract — Aims: Chronic, excessive drinking of alcohol can induce brain damage in the regions important for neurocognitive function. Some of the damage are permanent while some are apparently reversible. It is our aim to understand the molecular mechanisms underlying alcohol-induced and/or related brain damage, particularly of that observed in ‘medically uncomplicated’ (without hepatic cirrhosis or Wernicke-Korsakoff Syndrome [WKS]) alcoholics. Methods: A high-throughput proteomics technology has been applied to several ‘alcohol-sensitive’ brain regions from uncomplicated and hepatic cirrhosis-complicated alcoholics to understand the mechanisms of alcohol-related brain damage at the level of protein expression. Results: It was clearly demonstrated that each brain region reacts in significantly different manner to chronic alcohol ingestion. Apparent abnormalities in vitamin B1 (thiamine)-related biochemical pathways were observed in several brain regions, such as the dorsolateral prefrontal cortex, genu (a frontal part of the corpus callosum) and cerebellar vermis in uncomplicated alcoholics, suggesting that the reduction of this important nutritional component might be associated with brain damage even without the signs of WKS. In addition, in the two different subregions of the corpus callosum (genu and splenium [a posterior part of the corpus callosum]) and the cerebellar vermis, significant differences in protein expression profiles between uncomplicated and complicated alcohics with hepatic cirrhosis were identified, suggesting that hepatic factors such as ammonia have significant additive influences on brain protein expression, which might lead to the structural changes and/or damage in these brain regions. Furthermore, in the hippocampus, significant change of the level of glutamine synthetase expression was observed, suggesting once again the importance of ammonia as a cause of brain damage in this region. Conclusions: Although our data did not show any evidence of “direct” alcohol effects to induce the alteration of protein expression in association with brain damage, high-throughput neuroproteomics approaches are proven to have a potential to dissect the mechanisms of complex brain disorders.

NEUROCOGNITIVE DYSFUNCTION AND BRAIN DAMAGE IN ALCOHOLICS

Excessive chronic alcohol consumption, caused by addictive behaviours in alcoholic patients, is closely related to macroscopic shrinkage of the brain, reduced viability of neuronal cells (neurons and glial cells) and axonal degradation. These structural abnormalities are presented as cognitive dysfunction of alcoholics in clinical setting (Bethesda, 2000). Alcohol-related structural changes and/or damage of the brain, and cognitive dysfunction are partially reversible after a substantial period of abstinence (Harper and Kril, 1985; Jernigan et al., 1991; Kril et al., 1997) although residual deficits persist even in long-abstinent alcoholics (Pfefferbaum et al., 1998, 2006a, 2006b; O’Neill et al., 2001; Harper and Matsumoto, 2005).

Significant evidence suggests that cognitive deficit is one of the core features of alcoholism with 50–80% of alcoholic patients presenting the symptoms (Bates et al., 2002). Various cognitive functions such as verbal skills, visual-spatial performance, verbal memory and set-shifting flexibility (the ability to change problem-solving strategies in response to changing requirements) were widely affected (Glenn et al., 1993). Although there has not been enough clinical evidence, abnormalities in cognitive functions can severely damage the quality of life of the patients, prolong the recovery process and negatively affect the prognosis (Bates et al., 2002).

Generally, the pattern of cognitive deficits is suggested to be characterized with prefrontal cortical nature. More recently, much wider brain regions/paths/circuits including cerebellothalamicortical and cerebropontocerebellar systems have been indicated to be involved in the symptoms of alcoholics (Sullivan 2003). In addition, the hippocampus has been indicated as one of the responsible regions to generate cognitive dysfunction (Matsumoto et al., 2007). Furthermore, a recent study using diffusion tensor neuroimaging (DTI) identified microstructural damage in the corpus callosum (CC) (Pfefferbaum et al., 2006a). Interestingly, the deficits of cognitive functions such as attention and executive function were shown to be correlated with the subtle structural abnormalities identified in this region (Pfefferbaum et al., 2006a). Disruption of the above-mentioned brain regions can be suggested as underlying mechanisms of alcohol-related neurocognitive deficits, either by abnormalities of individual nodes or by disconnection and interruption of selective circuitry.

Patterns of alcohol-related brain damage appear to be affected by lifetime alcohol consumption even without medical complications (uncomplicated alcoholics) although underlying mechanisms are still largely unknown. When alcoholics are medically complicated, damage appears to be more widespread precipitating more neurocognitive dysfunction. Wernicke–Korsakoff syndrome (WKS) is one of the most important complications affecting the brains of alcoholics, which is caused by thiamine shortage/deficiency (Harper and Matsumoto, 2005). Another important medical complication in alcoholics is liver dysfunction. Alcohol is primarily metabolized in the liver and liver capacity to eliminate neurotoxic metabolites from the blood can be compromised by the chronic effects of alcohol abuse (Tarter and Edwards, 1986). When studying the effects of alcohol on CNS, these two factors, nutritional deficiency and neurotoxic effects caused by hepatic factors, need careful attention. Understanding the effects of these complications may provide more explanations for the mechanisms underlying alcohol-specific brain damage and related cognitive impairment.
NEUROPROTEOMICS IN ALCOHOL-SENSITIVE BRAIN REGIONS

Potential of high-throughput proteomics analyses

High-throughput approaches, such as cDNA microarray and proteomics, are particularly useful when investigating multifactorial, complex diseases, i.e. psychiatric illnesses, as systematic analyses of whole sets of genes and proteins expressed are possible. Recent studies have employed these technologies to investigate psychiatric illnesses such as schizophrenia and alcoholism (Choudhary and Grant, 2004; Fan et al., 2004; Lewohl et al., 2004; Alexander-Kaufman et al., 2006, 2007a, 2007b; Clark et al., 2006, 2007; Kashem et al., 2007; Matsuda-Matsumoto et al., 2007). cDNA microarray provides a snapshot of a tissue’s mRNA profile, potentially detecting changes in gene sets in the state of disease. However, this analysis is limited to the cellular component, i.e. is largely restricted to analyses of the brain grey matter. In addition, critically speaking, mRNA is not the functional endpoint of gene expression (Anderson and Seilhamer, 1997; Gygi et al., 1999).

Proteomics technology is an approach to examine and identify protein dynamics of biological pathways and multifactorial disease processes. Because these techniques can analyse the final end products of gene, they provide more functional insight. Recent works in our laboratory have focused onto high-throughput proteomics approaches using two-dimensional electrophoresis (2-DE)-based proteomics to isolate differentially regulated protein expression in the above-mentioned brain regions from human alcoholic brains (Alexander-Kaufman et al., 2006, 2007a, 2007b; Kashem et al., 2007; Matsuda-Matsumoto et al., 2007). We have performed our analyses using two carefully selected alcoholic groups (>80 g of ethanol/day), and results were compared to control (<20 g of ethanol/day): alcoholics who are medically uncomplicated (without WKS and hepatic cirrhosis) and alcoholics complicated with hepatic cirrhosis. Expression profile comparisons between these two groups have contributed to the development of some insight on the possible effects of hepatic dysfunction in the brain. All brain materials were provided from the New South Wales Tissue Resource Centre (NSW TRC; www.braindonors.org), situated within the University of Sydney and Central Sydney Area Health Service Australia (Sarris et al., 2002; Harper et al., 2003). All alcoholic cases fulfilled the criteria of the Diagnostic and Statistical Manual of Mental Disorders Edition IV (DSM IV) of the American Psychiatric Association for alcohol abuse. Cases were matched for age, sex, post-mortem interval and brain pH.

2-DE-based proteomics

A method of 2-DE-based proteomics was employed in the studies described herein. 2-DE-based proteomics consists of multiple steps including protein extraction from the samples and protein separation according to the isoelectric charge and molecular weight (MW) of the proteins. Each spot in the resultant gels corresponds to a single protein or protein fragment. Gel images are digitalized for PC-based image analysis to identify differentially expressed protein spots. Protein spots of interest are excised, digested with proteases and then subjected to Matrix Assisted Laser Desorption/Ionisation Time-of-Flight (MALDI-TOF) for protein identification. Detailed methodol-

ogy is described in our recent articles (Alexander-Kaufman et al., 2006, 2007a, 2007b; Clark et al., 2006, 2007; Iwazaki et al., 2006, 2007; O’Brien et al., 2006; Kashem et al., 2007; Matsuda-Matsumoto et al., 2007). 2-DE-based proteomics is a relatively simple and cost-effective technique that allows the detection and comparison of proteomes. A common drawback of 2-DE-based proteomics is the difficulty in analysing very high- and low-MW proteins and membrane-associated insoluble proteins, which often are functionally important ones in CNS diseases.

Analysis of the dorsolateral prefrontal cortex

The dorsolateral prefrontal cortex (DLPFC) plays a crucial role in cognitive control as its reciprocal projections connect to a number of brain regions involved in cognitive functions, particularly executive function mostly represented by working memory. Neuroimaging and neuropathological studies have reported a significant volume reduction of this brain region of alcoholic subjects, white matter in particular (Harper and Kril, 1985; de la Monte, 1988; Pfefferbaum et al., 1988, 1992; Harper, 1998). In the grey matter, the number of pyramidal neurons, dendritic spine density and dendritic arborization (Harper and Corbett, 1990) are reported to be reduced (Harper and Kril, 1994). These structural abnormalities of the DLPFC, both grey and white matters, may explain the high incidence of cognitive dysfunction observed in alcoholics.

Our proteomics of the DLPFC grey matter identified differential expression of ∼80–100 protein ‘spots’ in uncomplicated alcohics and complicated alcohics. Fifty-four protein spots out of these were identified as 44 different proteins using MALDI-TOF (Alexander-Kaufman et al., 2007a). In the white matter of this region, ∼40–50 spots were differentially regulated in both alcoholic groups compared to control. Out of these, we have identified 28 different proteins (Alexander-Kaufman et al., 2006, 2007a). In this region, most of the protein spots identified to be differentially expressed were common to both alcohol groups. This might indicate that there are not much significant effects of hepatic dysfunction on the protein expression in this brain region.

In both grey and white matter of the DLPFC, two thiamine-dependent proteins, transketolase and pyruvate dehydrogenase E1β-subunit, were identified to be differentially expressed in both groups of alcohics (Alexander-Kaufman et al., 2006, 2007a). Reduced activity of transketolase was previously demonstrated in the prefrontal cortex of alcohols with hepatic cirrhosis but without WKS (Lavoie and Butterworth, 1995). Our proteomics data indicated abnormalities in thiamine-dependent cascade not only in the brains of hepatic cirrhosis-complicated alcohics but also in the brains of ‘neurologically uncomplicated’ alcohics. Further, our data suggest that thiamine deficiency directly affects the DLPFC, both grey and white matter, causing alcohol-related brain damage and cognitive impairment. Finally, our proteomics results indicate that a reduction in thiamine availability may exist long before the onset of clinical signs of WKS in this brain region. This emphasizes the importance of thiamine supplementation to reverse deficiency condition and therefore potentially prevents damage in this important brain region, which will lead to the improvement of cognitive dysfunction.
A large number of metabolism-related enzymes, in particular the ones related to energy transduction [glycolysis and the tricarboxylic acid (TCA) cycle], were identified to be differentially regulated. Two enzymes of the non-oxidative branch of the pentose–phosphate pathway (PPP), thiamine-dependent transketolase and transaldolase, are known to reversibly link this pathway to glycolysis. Expression of both of these enzymes was found to be altered in both alcoholic groups (Alexander-Kaufman et al., 2006, 2007a). The connection of these pathways, via common intermediates, may afford cells ‘compensatory shifts’ in energy metabolism whilst under stress. Disturbance of energy metabolism, which might be primarily caused by the abnormalities in the PPP, may lead to an energy deficit, causing a loss of viability and cell death in the DLPFPC of the alcoholics. It should be noted that a number of previous studies have reported glucose hypometabolism in the frontal regions of chronic alcoholics (Adams et al., 1993; Dao-Castellana et al., 1998; Goldstein et al., 2004).

Analysis of the cerebellum

Cerebellar degeneration observed in 40% of alcoholics (Torvik and Torp, 1986) is characterized by a general shrinkage or atrophy of the anterior superior cerebellar vermis (Pfefferbaum and Rosenbloom, 1993; Harper, 1998; Andersen, 2004). Microscopically, significant Purkinje nerve cell loss (Harper, 1998; Andersen, 2004) and reduced dendritic arbor are prominent features (Pentney, 1982; Ferrer et al., 1984). White matter atrophy is also commonly reported in the vermis of chronic alcoholics (Harper and Kril, 1993). The superior cerebellar hemispheres are directly connected to prefrontal cortex areas (Schmahmann and Pandya, 1997). It is becoming increasingly recognized for the role of cerebellum in numerous aspects of cognitive function (Martin et al., 2003). Such functions include learning, cognitive processing of words, anticipatory planning and making time-based judgements. Therefore, alcohol-induced damage to the cerebellum may indirectly affect neurocognitive functions normally attributed to the frontal lobe (Martin et al., 2003).

Proteomics successfully isolated ~40–70 protein spots in the cerebellar vermis of uncomplicated and cirrhosis-complicated alcoholics (Alexander-Kaufman et al., 2007b). MALDI-TOF identified 51 of these protein spots as 40 different proteins. Interestingly, in significant contrast to the DLPFPC, nearly half of the isolated proteins were unique to the hepatic cirrhosis-complicated group, indicating that specific effects of liver dysfunction might be occurring onto the protein expression in the vermis. These proteins may be selectively vulnerable to changes in hepatic function. It has been previously suggested that hepatic dysfunction-induced factors significantly affect the extent and degree of Purkinje cell damage in the vermis that may be associated with alcohol-related cognitive impairment (Phillips et al., 1990). Further studies of these proteins are warranted to substantiate these findings.

Three thiamine-dependent enzymes, transketolase, dihydrolipoamide dehydrogenase and pyruvate dehydrogenase E1 β-subunit, were identified to be altered in their expression in the cerebellar vermis not only in cirrhosis-complicated alcoholics but also in the brains of ‘neurologically uncomplicated’ alcoholics. It has been well known that the cerebellar vermis is particularly sensitive to thiamine deficiency (Lavoie and Butterworth, 1995; Baker et al., 1999). A postmortem study demonstrated reduced activities of transketolase and pyruvate dehydrogenase in the cerebellar vermis from alcoholic patients with WKS (Butterworth et al., 1993). Reduced activities of transketolase were identified in the more widespread area of the brains including the cerebellum and prefrontal cortex, of alcoholic patients even without WKS (Lavoie and Butterworth, 1995). A significant reduction in the number and neuronal size of the Purkinje cells in this region has been repeatedly reported. There neuropathological changes might be induced mainly by thiamine shortage/deficiency. As aforementioned, a shortage of thiamine possibly affects glycolysis and the TCA cycle. As expected, a large number of enzyme proteins associated with these two pathways were identified to be differentially regulated. Previously, hypometabolism of glucose was demonstrated in the cerebellar vermis of alcoholics with cerebellar degeneration (Gilman et al., 1990). As found in the DLPFPC, a disturbance in thiamine-dependent enzyme levels together with changes in glycolytic and TCA-related enzymes suggests that a derangement in energy metabolism may be an important factor in cerebellar vermis damage.

Analysis of the genu and splenium of the CC

The CC, the largest white matter structure, is regulating various cognitive functions dependent upon its five anatomical subfields. The most anterior part is called genu and posterior part, splenium. Higher order cognitive information from the prefrontal cortex is predominantly transmitted from one hemisphere to another through these two regions (de Lacoste et al., 1985; Lantamia and Rakic, 1990; Aboitiz et al., 1992). A MRI study has shown the largest volume reduction in the genu within the CC of alcoholic patients while lesser, but significant changes were also observed in the splenium (Pfefferbaum et al., 2006a). DTI studies have clearly demonstrated alcohol-related microstructural degradation/disintegrity in the CC (Pfefferbaum et al., 2006a). These microstructural abnormalities in the CC can be associated with cognitive dysfunction due to disturbance of information processing and interhemispheric transfer (Pfefferbaum and Sullivan, 2005; Pfefferbaum et al., 2006a, 2006b).

The above-mentioned two sub-regions of the CC were subjected to proteomics analyses. Approximately 40-50 protein spots were isolated in both alcoholic groups in the splenium (Kashem et al., 2007) and the genu (Kashem et al., 2008). Forty-six percent (20 proteins) and 44% (22 proteins) were regulated in the splenium and genu of only alcoholics complicated with hepatic cirrhosis, respectively. This clearly indicates that hepatic factors may have synergistic effects in both regions of the CC. In the patients with hepatic cirrhosis, a number of neurotoxic agents including ammonia are released into the blood (Lieber, 1997; Felipo and Butterworth, 2002). Ammonia is transported into the CNS and alters a number of different biochemical pathways, including glutamate-related cascades (Felipo and Butterworth, 2002). Alteration in glutamate contents in the brains of the patients with hepatic cirrhosis was reported (Sawara et al., 2004). In our proteomics studies, the change of glutamate carboxypeptidase, a free glutamate-producing enzyme, was identified in both the splenium (Kashem et al., 2007) and genu (Kashem et al., 2008) of complicated alcoholics. It is possible that even slight elevations
of blood and/or CNS ammonia may precipitate in changing the protein expression observed only in complicated alcoholics.

Another important finding of our proteomics studies in this region (both genu and splenium) is that expression of phospholipase D (PLD) was increased in both alcoholic groups. PLD produces phosphatidylethanol (PETH) in the presence of ethanol through its high affinity to ethanol (Nishida et al., 1997). PETH is incorporated into cell membranes resulting in increased membrane fluidity (Frohman et al., 1999). It is therefore possible that the composition of membrane lipids is altered causing disruption in membrane integrity/permeability in the CC of alcoholics.

Proteins of two enzymes involved in thiamine/energy biosynthesis, such as transaldolase and glycerol 3-phosphate dehydrogenase, were demonstrated to be differentially regulated in the genu of both alcoholic groups (Kashem et al., 2008), without any alteration observed in the splenium (Kashem et al., 2007). Our proteomics results first identified the abnormalities of the thiamine cascade in the CC of alcoholics occurring in a sub-region-specific manner. Shortage of thiamine might exist in alcoholic brains even without the signs of WKS, affecting much wider areas than previously suggested.

Analysis of the hippocampus
The hippocampus is known as one of the brain regions that play key roles in cognition such as short-term memory and visuospatial recognition (Ryabinin, 1998). These cognitive functions are sensitively affected by alcohol administration. Although macroscopic volume reduction in the hippocampus has been demonstrated in alcoholic patients (De Bellis et al., 2000; Nagel et al., 2005; Beresford et al., 2006), there have been no neuropathological studies that identified neuronal damage in this brain region (Harding et al., 1997). Several other studies have suggested potential damage in glial cells (both astrocytes and oligodendrocytes) in the hippocampus (Franke et al., 1997; Korbo, 1999; Tagliaferro et al., 2002; Ikegami et al., 2003; Okamoto et al., 2006). Alcohol-related damages in glial cells may result in changes in neuronal circuitry rather than visible morphological changes in this important brain region.

Our proteomics study of this important region was conducted only on the uncomplicated alcoholics. Approximately 20 proteins were identified to be differentially expressed in the uncomplicated alcoholics compared to normal control (Matsuda-Matsumoto et al., 2007). When these proteins were compared to those identified in other regions (Alexander-Kaufman et al., 2006, 2007a), such as cerebellar vermis (Alexander-Kaufman et al., 2007b; Kashem et al., 2007), and animals administered other abusive or neuroleptic drugs (O’Brien et al., 2006; Iwazaki et al., 2007), differentially regulated proteins specific to this region of alcoholics were isolated. One such protein was glutamine synthetase (GS) that is an enzyme predominantly located in astrocytes (Suarez et al., 2002). GS plays a significant role in CNS to protect neurons from toxicity induced by glutamate and ammonia (Suarez et al., 2002) by converting glutamate into glutamine by taking up excess amount of ammonia (Suarez et al., 2002). The reduction in GS activity in the brains of patients with hepatic encephalopathy (HE) and in animal models of HE was previously reported (Girard et al., 1993). Although there were no alcoholic cases with clinical signs of HE included in our study, mild hepatic dysfunction and a sub-

SUMMARY AND CONCLUSIONS
Although a small portion of the differentially expressed proteins are overlapped between the above-described alcohol-sensitive brain regions, using 2-DE-based proteomics it became evident that each brain region would react to chronic alcohol ingestion in a quite different manner at least at the level of protein expression. It was quite surprising to find significant region-specific expression profiles of proteins within the different white matters (DLPFC white matter, genu and splenium of the CC) while we know that the histological components of the white matter are almost the same between different brain regions. This may indicate the different sensitivity and vulnerabilities of each white matter to alcohol-related structural changes including brain damage. Our proteomics study using the white matters (the DLPFC and the body of CC) from a normal human brain identified significant heterogeneity of white matter from the aspect of protein expression profiles (unpublished observation) that may explain the bases of this difference.

When both uncomplicated alcoholics and complicated cases with hepatic cirrhosis were compared, it was indicated that hepatic-induced factors might have some influences on the protein expression profiles of the cerebellar vermis, genu and splenium most significantly. When liver function declines by excessive alcohol consumption, the levels of a number of neurotoxic chemicals rise in the blood with some of them entering into the brain through BBB. Out of these chemicals, Butterworth has been proposing that ammonia is one of the most important neurotoxins to cause brain damage (Butterworth 2003a, 2003b). Our proteomics data indicated some link to ammonia in these brain regions, particularly in the hippocampus, where even in the uncomplicated case, ammonia-sensitive, astrocyte-specific and only enzyme that can detoxify ammonia, GS was identified to be differentially regulated. Further studies are warranted to confirm this by using the complicated cases. Although precise brain regions affected by these hepatic factors have not been well determined yet, using sensitive high-throughput analyses such as proteomics, it is possible that much wider areas of the brain will be identified to be affected by these factors. Therefore, it has to be stressed that improvement of liver function, lowering ammonia levels even at the early stages of alcoholism, will greatly contribute to preventing further brain damage.

Thiamine shortage/deficiency and a secondary disturbance of energy metabolism may affect the protein expression pattern in the DLPFC grey and white matter, cerebellar vermis and genu, but not in the splenium. Although the most vulnerable brain regions to thiamine deficiency are the mammillary bodies, mediodorsal thalamic nuclear and cerebellar vermis (Victor et al., 1971; Vortmeyer et al., 1992; Baker et al., 1999), it is also important to note that areas of damage might be much wider than classically indicated areas, involving the CC and the cerebral cortex (Victor et al., 1971). The effects of thiamine shortage may be more widespread than once believed resulting in alteration of the protein expression profiles in these regions even before the clinical onset of WKS symptoms. This is reasonable considering the fact that thiamine transport occurs at
the level of BBB mainly by passive diffusion. Shortage of thiamine may affect various brain regions that are implicated in cognitive function even without significant neuropathological changes. Because of its known cause, supplementation of the diet by thiamine for alcoholic cases even without the signs of WKS should be considered as one of the most important means to prevent brain damage (Chikritzhs et al., 2001).

Recent studies to understand the mechanisms underlying alcohol-related brain damage have identified the pharmacological and biochemical actions of ethanol per se in the CNS. Chronic effects of these alcohol actions will lead to brain damage and cognitive dysfunction. More importantly, thiamine deficiency and hepatic dysfunction-induced neurotoxins can cause further damage in much wider areas of the brain. Each different brain region possibly shows different sensitivity/vulnerability to these three factors, ethanol, thiamine and hepatic effects. When studying the human alcoholic brain where multiple factors play roles, using high-throughput methodologies, identification and discrimination of the effect of each factor on expression profiles become possible and should be carefully considered.

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