



GENERAL DISCUSSIONS & CONCLUDING REMARKS

I. Matsumoto, K. Alexander-Kaufman, T. Iwazaki, M. A. Kashem and H. Matsuda-Matsumoto, (2007) “*Central Nervous System Proteomes in Alcohol and Drug Abuse and Dependence*”, *Expert Review of Proteomics* 4(4): 539-52 – For Complete Article refer to Appendix V

7.1 General Discussion: Molecular Brain Mapping

Characterizing the brain's regional proteomes provides a foundation for the detection of proteins that may be involved in disease-related processes. The work presented in Chapter 3 of this thesis, demonstrates the first normative proteomic comparisons of soluble fractions of adjacent grey and white matter of a single cytoarchitecturally defined area (BA9) and of two adjacent regions of frontal white matter (BA9 and CC body). The results described herein highlight the importance of correct tissue sampling, i.e. proper separation of regional white matter, as heterogeneity in the respective proteomes was demonstrated. Furthermore, they highlight the necessity for future molecular brain mapping studies.

7.2 General Discussion: Alcohol-related Brain Damage Studies

Comparisons to the Other Important Brain Regions

Proteomic analyses of two other brain regions important in alcohol-related brain damage and associated cognitive dysfunction were recently published. The alcohol-related neuropathology of the corpus callosum and hippocampus and the differential protein abundances isolated in these brain regions in alcoholics are briefly introduced below. Finally, the major findings in these studies are compared to the BA9 region and cerebellar vermis studies described in the thesis.

7.2.1 Corpus Callosum Proteomes from Human Alcoholics

An MRI study of the CC of patients with alcohol abuse suggested that volume reduction is greatest in the genu, followed by the body and splenium (Pfefferbaum et al., 2006a). Neuroimaging, particularly DTI, and post-mortem studies have repeatedly shown that alcohol and/or other drugs of abuse (cocaine, marijuana) cause microstructural degradation in specific tracts of white matter, particularly in the CC (Gruber and Yurgelum-Todd, 2005; Lim et al., 2002; Matochik et al., 2005; Pfefferbaum et al., 2006a). Structural disruption and morphological alterations in the CC causes the information processing and interhemispheric transfer time to slow down, leading to deficits and/or abnormalities of visio-spatial performance, gait, balance and working memory (Pfefferbaum et al., 2006a; Pfefferbaum et al., 2006b; Pfefferbaum and Sullivan, 2005). Pathogenic mechanisms underlying these structural changes, some of which appear to be reversible, are again largely unknown.

Proteomic analyses were conducted on two sub-regions of the CC, the genu and splenium. Using similar brain cases to those described in this thesis (Chapter 2, page 50-51), Kashem and colleagues isolated changes in the relative abundance of 43 proteins in the splenium (Kashem et al., 2007) and 50 proteins in the genu (Kashem et al., unpublished observation, 2007). The identified proteins were important in a number of metabolic pathways, lipid peroxidation, oxidative stress, energy and vitamin cascade pathways, signaling and apoptosis. Although changes in 21 proteins were common between the two CC regions, the sub-regions appeared to react quite differently to chronic alcohol exposure. Of the identified splenium and genu proteins, almost half (46% and 44% respectively) were specifically regulated in alcoholics with liver cirrhosis. Accordingly, the authors concluded that the presence of hepatic

cirrhosis may have synergistic effects on the CC proteome. In cirrhosis patients, various neurotoxic agents are released into the blood due to liver impairment, including ammonia, free radicals and alcohol (Felipo and Butterworth, 2002; Lieber, 1997). Ammonia is transported from liver to the brain directly and can alter at least 16 different enzymatic pathways, the most important of which is glutamate dehydrogenase (Felipo and Butterworth, 2002). Changes in the levels of glutamate carboxypeptidase, a free glutamate-producing enzyme were identified in both CC sub-regions of complicated cases (Kashem et al., 2007; Kashem et al., unpublished observations, 2007). An MRS study previously reported an alteration in glutamate contents in the brains of hepatic cirrhosis patients (Sawara et al., 2004). Ammonia induces an increase in glutamate levels, which can activate NMDA receptors and induce neuronal nitric oxide synthase, leading to increased production of NO (Chan and Butterworth, 2003). High levels of NO can increase the cellular burden of oxidative stress, which is an important mechanism proposed to underlie HE (Chan and Butterworth, 2003). Although none of the complicated cases demonstrated clear clinical or pathological evidence of HE, all were diagnosed with hepatic cirrhosis and minor elevations of blood and/or CNS ammonia may have precipitated the alterations seen in the protein profiles of these cases.

Lipid peroxidation impairs the physical properties of a cellular membrane by altering the membrane lipids or fatty acids (Natarajan et al., 1993). This process is regulated by lipases. Phospholipase D (PLD) was induced in the two sub-regions of the CC in both alcoholic groups. PLD has a high affinity to ethanol and in the presence of ethanol, generates phosphatidylethanol (PEth) instead of phosphatidic acid (PA; Frohman et al., 1999; Nishida et al., 1997). PEth, a unique phospholipid, is readily

incorporated into cell membranes and increases membrane fluidity (Frohman et al., 1999). PEth also inhibits phospholipase C (PLC), which is important for cell growth, proliferation and cytosolic Ca^{+2} regulation (Hubbard and Hepler, 2006; Nishisa et al., 1997). Therefore, altered membrane lipids and a disruption in membrane integrity/permeability may underlie alcohol-related CC damage through this mechanism.

Transaldolase and glycerol 3-phosphate dehydrogenase are enzymes involved in thiamine/energy biosynthesis and were found to have decreased abundance in both alcoholic groups in the genu (Kashem et al., unpublished observation, 2007) with no changes seen in the alcoholic splenium (Kashem et al., 2007). It is interesting to note that this is the first time thiamine cascade abnormalities have been observed in the genu of alcoholics and that these changes are occurring in a sub-region specific manner. Again, it is possible that a low thiamine status has wider impact on the brain than previously suggested by neuropathological studies.

7.2.2 The Hippocampal Proteome of Human Uncomplicated Alcoholics

Another brain region sensitive to alcohol effects is the hippocampus. Acute ethanol exposure significantly suppresses memory and learning, in particular short-term memory and visuospatial recognition, of which both are closely associated with hippocampal function (Ryabinin, 1998). In contrast to the prefrontal cortex and cerebellar vermis, no apparent loss of neurons has been observed in this brain region (Harding et al., 1997). Past studies using human material have revealed a reduction in

astrocyte number (Korbo, 1999) and increased TUNEL staining in astrocytes, suggesting possible DNA damage (Ikegami et al., 2003), but no morphological findings indicating neuronal damage have been reported thus far. It is possible that alcohol affects the hippocampus at more functional level, i.e. changes in neuronal circuitry and/or connection rather than visible morphological changes.

Due to the limitations of brain tissue availability, this proteomics study was conducted on the hippocampus of uncomplicated alcoholic brain only. Changes in the relative abundance of 17 proteins were identified in the uncomplicated alcoholic hippocampus (Matsuda-Matsumoto et al., 2007). These proteins were categorized into five functional groups; metabolic (52%), signaling (24%), oxidative stress (12%), vesicle trafficking (6%) and cytoskeletal (6%). One of the proteins differentially regulated in the alcoholic hippocampus was glutamine synthetase (GS). In the CNS, GS is an enzyme predominantly located in astrocytes (Suarez et al., 2002). The main function of GS is to protect neurons against toxicity from ammonia and glutamate (Suarez et al., 2002). GS converts glutamate into glutamine by taking up excess ammonia (Suarez et al., 2002). A reduction in GS activity in the CNS has been reported in patients with HE and in animal models of HE (Girard et al., 1993; Lavoie et al., 1987). Although the alcoholic cases analysed in this study had no clinical and pathological findings of either liver cirrhosis or HE, chronic alcohol consumption may induce mild liver dysfunction and a subsequent elevation of ammonia in the CNS, in turn altering GS protein abundance. Morphological and functional alterations in astrocytes in the hippocampus have been demonstrated in animals chronically treated with alcohol (Franke et al., 1997; Tagliaferro et al., 2002). This result is consistent

with previous studies, suggesting that chronic alcohol exposure induces selective dysfunction of astrocytes in the hippocampal region.

7.2.3 Differential Response of Each Alcohol-Sensitive Brain Region to Chronic Alcohol Consumption

After comparing the abovementioned studies with those described in this thesis, the respective protein abundance profiles were generally quite different, although there was some overlap. Most interesting was the observed difference in protein abundance in what is ordinarily considered as part of the same white matter compartment, the BA9 white matter, and the genu and splenium of the CC. This indicates that different brain regions are individually sensitive to alcohol-related neuronal changes or damage at least at the level of protein expression. This different reactivity may be due to the heterogeneity of white matter proteomes as seen in Chapter 3, part 2 (page, 95).

Where both uncomplicated alcoholics and alcoholics with liver cirrhosis were compared, hepatic factors appear to affect the proteomes of the cerebellar vermis and the two sub-regions of the CC, genu and splenium most significantly. It is still unclear which hepatic factors are important however, there seems to be a link to ammonia in these brain regions. Indeed in the hippocampus of uncomplicated alcoholics, ammonia sensitive, astrocyte-specific protein, GS was suggested to play an important role in the dysfunction of this brain region (Matsuda-Matsumoto et al., 2007).

A disruption in energy metabolism, perhaps related to thiamine deficiency, was observed in the prefrontal (BA9) grey and white matter, cerebellar vermis and genu of

the CC, but not in the splenium. Although the prefrontal regions and CC are not typically associated with WKS neuropathology, the effects of a low thiamine status may simply be more widespread than once believed and this may alter protein abundance in these regions before the clinical onset of WKS symptoms. Notably, abnormalities in the thiamine and energy pathways were isolated in both alcoholic groups, including cases of 'neurologically uncomplicated' alcoholics.

Considering that the regions studied are all important for the maintenance of normal cognitive function, even subtle changes in protein expression may cause changes in neuronal plasticity, generating a basis for enhanced neurotoxicity. Maintenance of liver function and ammonia levels and sufficient thiamine supplementation (Harper et al., 1998) even at the early stages of alcoholism will greatly contribute to preventing further brain damage.

7.3 Technical Considerations

7.3.1 General Limitations of Proteomics Technology

While proteomics has many advantages for the high-throughput global quantification of protein abundance, the limitations associated with 2D-GE based proteomics have recently become more apparent. A common limitation of the 2D-GE approach is the difficulty analysing very high- and low- MW proteins and hydrophobic, insoluble proteins, which often are of particular interest in disease. Unfortunately, in the CNS, as most neurotransmitter receptors and transporters are membrane-bound insoluble proteins and have very small MWs, they present as difficult targets to analyse. A number of steps may be taken to help minimise these shortcomings. A pre-fractionation step (which may include ultracentrifugation and/or enrichment steps, for example column chromatography) may be necessary to help simplify complex protein mixtures and improve the resolution of low abundant proteins (Fountoulakis and Langen 1997). Additionally, pre-fractionation allows increased amounts of protein to be loaded onto the gel, thus facilitating the detection of lower abundant proteins (Gorg et al. 2002). Using a series of narrow pH ranges (eg, 3-5, 4-7 and 6-7) for first dimension protein separation may also improve resolution of lower abundant proteins (Prabakaran et al., 2004).

Currently, the most common and simple gel staining method, colloidal Coomassie Blue, is often not sensitive enough to visualize changes occurring in low abundant proteins. Although other staining methods such as silver staining and new highly sensitive fluorescence dyes have superior detection sensitivities, they are often not compatible with mass spectrometry identification techniques (Scheler et al. 1998).

Although this area of high-throughput science is in its relative infancy, recent advances have made these techniques more sensitive and reliable. The application of laser capture microdissection (LCM) to dissect homogenous cell populations from the sections of brain (Emmert-Buck et al., 2000) will improve the specificity of proteomics analyses. To improve detection sensitivity, differential 2D fluorescence gel electrophoresis (2DIGE; Unlu et al., 1997) has been recently developed. To minimize the time consuming process and subjectivity of gel image analysis, software with more automated functions are being introduced (Beranova-Giorgianni, 2003). Furthermore, although there are still technical and financial problems for liquid chromatography/mass spectrometry (LC/MSMS; Dosemeci et al., 2006) and SELDI-TOF (Khwaja et al., 2006), these technologies are expected to be further developed as simple detection tools to identify low abundant proteins in smaller samples.

Post-translational modifications, including phosphorylation, oxidation and glycosylation, generate tremendous diversity, complexity and heterogeneity of gene products, and their determination is one of the main challenges in proteomics research (Jensen, 2004). This is particularly important as proteins are often identified as multiple spots on a gel and using standard proteomic approaches it is difficult to discriminate whether they are different isoforms. MS is the logical solution because of its ability to capitalize on the intrinsic property of molecular mass (Kiernan, 2007). Combining MS with affinity-based enrichment and extraction methods and multidimensional separation technologies will greatly enhance the systematic investigation of post-translationally modified proteins in proteomics (Jensen, 2004).

7.4 Postmortem Human Brain Tissue – Technical Considerations

In studies using post-mortem human brain tissue, the conditions of brain collection, in particular agonal state and post-mortem interval conditions are very important as they may profoundly impact results. Yet, there is still little basic data accumulated on this issue. Although theoretically any delay could cause artefacts from postmortem autolysis (Mahy, 1993), there is considerable evidence suggesting that the majority of brain enzymes are stable postmortem, even when RNA is degraded (Stan et al., 2006). Furthermore, these enzymes appear to remain associated with particular structures whose integrity are well maintained during postmortem storage (Hardy and Dodd, 1983). Brain pH is thought to be a better indicator of tissue integrity than PMI (Stan et al., 2006), a variable that was well matched between the cases (See Table 2.2.1, pages 50-51).

In our laboratory, all diseased and normal control samples were matched according to the age, sex, PMIs and brain pH as closely as possible to minimize these effects. In addition, post-hoc comparisons were performed to determine any correlation between these factors and protein spot volume. However, there is a great shortage of human brain tissue with accurate clinical and neuropathological histories of alcohol and drug abuse and researchers are ultimately reliant on the number of cases available from these 'brain banks'. The TRC is a reliable resource for such cases and is supported by the NHMRC and the National Institute of Alcohol Abuse and Alcoholism (NIAAA), USA. However, with the advancement of genomic and proteomic technologies, human brain tissue collections of alcohol and drug abuse/dependence with reliable clinical/pathological data on a much larger scale will be imperative (Harper et al., 2003a; Harper et al., 2003b).

7.5 Validation Studies & Future Directions

The application of proteomics holds great potential for understanding normal brain physiology as well as mechanisms underlying disorders such as alcoholism. Such studies may lead to the identification of biomarkers useful for diagnosis, selection of the treatment regimes and prediction of prognosis for individuals with these complex disorders. However, proteomics is still in its relative infancy and no standardised validation methodologies have been described.

Previously, authors have used 1D immunoblot assays to validate their abundance data without consideration of the possible existence of multiple spots per protein/antibody. Although there are commercially available antibodies for many of the proteins listed in these studies, many of the protein spots identified here could be variants/isoforms; indeed some of these proteins were identified as ‘precursors’ and ‘fragments’. The protein identification step in these studies required a significant MOWSE score and an identified peptide sequence coverage greater than 20% of the whole protein. Although our sequence coverage was generally much better than 20%, there was still some debate as to which subunit/isoform of the proteins we had identified. Without further detailed characterization of one or all of these proteins, immunoblot validation studies would not necessarily help. A number of authors have recently omitted such ‘validations’ from their articles, in a variety of different journals, including specific proteomics journals (Bajo et al., 2002; Witzmann et al., 2003; Zhan et al., 2003; Lewohl et al., 2004; Paulson et al., 2004; Vitorino et al., 2004; Clark et al., 2006; Iwazaki et al., 2006; Clark et al., 2007; Iwazaki et al., 2007). Although, immunoblot analyses of some of the proteins would help strengthen these studies, this still does not deal with the likelihood of false positives and negatives. To address this issue, the

false discovery rate (FDR) was calculated for each study. Other technologies which have been used to cross-validate findings include immunohistochemistry. Although this has the added advantage of illustrating specific cellular expression patterns, as with immunoblotting, the lack of specificity of many of the antibodies makes this technique unreliable for validation purposes.

A number of methods, including N-terminal sequencing and amino acid composition analysis of hydrolyzed spots can be used in conjunction with MS to help confirm protein identity (Fountoulakis and Langen 1997). Moreover, quantitative real time polymerase chain reaction can be used to directly compare gene transcript levels with protein abundance levels. Finally, ES-MS tandem mass spectrometry (MS-MS) is a valuable tool for the confirmation and expansion of results obtained by MALDI-TOF-MS (Lubec et al. 2003).

Based on the work described in this thesis, a number of future studies can be proposed. These studies would endeavor to overcome the limitations described herein to confirm protein abundance data and help explain the changes identified in the context of alcohol-related brain damage.

Future studies related to the pathophysiology of alcohol-related brain damage may include:

- Expand these alcohol-related brain damage studies to include patients diagnosed with Wernicke's Encephalopathy and Korsakoff's Psychosis to demonstrate more pronounced changes in thiamine-dependent enzymes etc.
- Expand these studies to include more recovered alcoholics to isolate which proteins underlie white matter regeneration.
- Conduct downstream analyses on thiamine-related enzymes, including characterizing the different variants of transketolase identified in the BA9 grey matter of alcoholics.
- Apply a metabolomics approach to examine the effect of ethanol on the major metabolic pathways in the brain, as isolated by the IPA software tool.
- Apply a genomic approach, i.e. microarray and SNP to ascertain whether protein abundance is altered as a result of a genetic predisposition to alcoholism

Some general neuroproteomics studies could include:

- Optimisation of 2DIGE technologies for use with human postmortem brain in combination with prefractionation steps and narrow IPG pH ranges to identify more low abundant proteins.
- Generate a molecular map of the normal healthy human brain, by the use of laser micro-dissected, cyto-architecturally defined brain regions.
- Develop other MS analytical methods to improve the sensitivity and accuracy of protein detection and identification.

7.6 Concluding Remarks

To conclude this thesis, the research aims outlined in Chapter 1 will be reviewed.

I. To achieve optimal conditions for protein extraction and applications of proteomics technology suitable for use with postmortem human brain tissue.

Proteomics technology was successfully optimised for use with postmortem human brain tissue. The protocols developed have been used by many other students and visiting scholars and have resulted in the generation of a number of high quality, peer reviewed articles.

II. To examine differences in protein abundance between the BA9 grey and white matter regions of healthy individuals.

In the BA9 region, 31 protein spots showed greater relative abundance in grey matter and 18 spots were more abundant in white matter (ANOVA, $p < 0.05$). MALDI-TOF MS peptide mass mapping post-trypsin digest identified 46 protein spots as 36 different proteins; 23 of which identified proteins were more abundant in grey matter and 13 were more abundant in white matter. These results provide a molecular map of the soluble fraction of this brain region.

III. To examine the normal protein profiles of the BA9 white matter and the body of the corpus callosum in healthy individuals to explore regional differences in white matter proteome.

Seventy-seven protein spots showed significantly increased relative abundance in the CC body and 67 protein spots showed greater abundance in the BA9 white matter ($p < 0.05$, ANOVA). Sixty-four protein spots were subjected to MALDI-TOF MS peptide mass mapping post-trypsin digest for identification, of which, 46 proteins were identified. These results provide evidence for the heterogeneous nature of regional white matter, which must be taken into account by all researchers using these techniques in the study of various brain disorders.

IV.-VI. To identify proteins with differential abundance in the BA9 white matter, grey matter and cerebellar vermis proteome of uncomplicated and liver cirrhosis complicated alcoholics relative to matched controls respectively.

Many significant protein abundance level changes were isolated in the aforementioned brain regions in two groups of alcoholic patients:

In the BA9 white matter study, the differential abundance of 39 protein spots was isolated in uncomplicated alcoholics and 51 protein spots were found in alcoholics with liver cirrhosis. Of these changes, 30 protein spots were common to both groups of alcoholics.

In the BA9 grey matter study, the differential abundance of 84 protein spots was isolated in uncomplicated alcoholics and 107 protein spots were found in alcoholics with liver cirrhosis. Of these changes, 81 protein spots were common to both groups of alcoholics.

In the cerebellar vermis study, the differential abundance of 43 protein spots was isolated in uncomplicated alcoholics and 75 protein spots were found in alcoholics with liver cirrhosis. Of these changes, 41 protein spots were common to both groups of alcoholics.

VII. To identify all proteins with altered levels in the brain regions examined

In the BA9 white matter study, 36 protein spots were identified as 28 different proteins. In the BA9 grey matter study, 54 protein spots were identified as 44 different proteins. In the cerebellar vermis study, 51 protein spots were identified as 40 different proteins. Although many proteins were identified in the various brain regions studied, some proteins did evade identification by MALDI-TOF MS peptide mapping. The development of other MS analytical methods may improve the sensitivity and accuracy of protein identification.

VIII. Generate hypotheses and plan future studies based on these proteomics findings.

During the course of this thesis, a number of hypotheses were generated and future directions proposed.

In summary, several proteins that appear to be altered in various brain regions important in alcohol-related brain damage and cognitive dysfunction were isolated and identified from two groups of alcoholic patients, uncomplicated alcoholics and alcoholics with liver cirrhosis. Some of the proteins identified were common between

studies. A disorder of energy metabolism, perhaps related to thiamine deficiency, seems to be important in the prefrontal grey and white matter and cerebellar vermis, even where there are no clinical or pathological findings of WKS. These studies also suggest that oxidative changes are important in all brain regions analysed. Interestingly, several liver cirrhosis-specific proteins were identified in the vermis, perhaps indicating the effects of liver dysfunction in this brain region. Together these results highlight the complexity of this disease process in that a number of different proteins from different cellular pathways appear to be affected.

Some of the proteins identified have previously been implicated in alcoholism and related disorders, while others may offer new insights into the pathogenic mechanisms underlying alcohol-related brain damage. Further investigation of these proteins, their functions, interactions and disruption in the disease may help decipher the complexities of this disorder and the structural abnormalities observed in the brains of alcoholic patients.