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General Introduction
1.1 A World View on Alcoholism

Ethanol, commonly alcohol, is among the most widely abused substances and has played central roles in many cultures throughout the world. The World Health Organization (WHO) estimates there are approximately 2 billion people worldwide who consume alcoholic beverages, 76.3 million of which have a diagnosable alcohol abuse disorder (Global Status Report, 2004). From a public health perspective, alcohol consumption is one of the major risk factors for burden of disease and social harm in both developed and developing countries (Murray and Lopez, 1996; WHO, 2002); expressly, it is the leading risk factor for disease burden in low mortality developing countries and the 3rd largest in developed countries (Global Status Report, 2004). In Europe, alcohol is the 3rd most important risk factor for morbidity and premature death after smoking and raised blood pressure, and is of more consequence than high cholesterol and obesity, three times more important than diabetes and five times more important than asthma (WHO, 2002). Alcohol is attributed to 1.8 million deaths (3.2% of total) and a loss of 58.3 million (4% of total) disability adjusted life years (DALYs; WHO, 2002). Consequently, alcoholism presents as one of the most costly health problems.

Recently, per capita consumption has dramatically increased in China (Cochrane et al., 2003) and Eastern Europe (Rehm et al., 2003). Patterns of drinking also appear to be changing in the west (Jackson et al., 2006; Webb et al., 1996), including Australia, where a dramatic increase in binge drinking and hazardous/harmful drinking has been documented in young women (Jonas et al., 2000). Consequently, alcohol-related disorders and brain damage continues to be an important area of research.
1.2 Alcohol Consumption: How much is too much?

Alcohol has a biphasic effect on the body, in that its effects change over time (Holdstock and de Wit, 1998). Ethanol acts as a central nervous system depressant, which in small amounts causes a mild euphoria and removes inhibitions. In large doses it causes drunkenness (blood alcohol level [BAL] of approx. 0.1%), leading to slurred speech, blurred vision, ataxia or coordination problems and impairment in attention and/or memory (American Psychiatric Association, 1994). Alcohol poisoning, coma and death can occur after extreme levels are consumed (BAL=0.55% will kill approximately half of those people affected). However, regular heavy drinkers can tolerate somewhat higher levels than non-drinkers (First and Tasman, 2004). Death can also be caused by asphyxiation after vomiting, a frequent result of over-consumption. Unlike withdrawal from some other drugs/intoxicants such as the opioids, withdrawal from chronic alcohol consumption can produce delirium tremens that can be fatal (DeBellis et al., 2005). Guidelines put forth jointly by the U.S. Department of Agriculture and the U.S. Department of Health and Human Services (1990) define moderate drinking as no more than one drink a day for most women, and no more than two drinks a day for most men. The risk of drinking compared with non-drinking, appears to begin increasing significantly at an intake around 3 drinks per day and continue to rise thereafter for:

- Cancers of the oral cavity and pharynx; oesophagus; larynx; breast; liver; colon; and rectum
- Liver cirrhosis
- Essential hypertension
- Chronic pancreatitis
- Injuries and violence (Global Status Report, 2004).
Furthermore, any alcohol consumption during pregnancy carries a heavy risk of permanent mental and physical defects in the child, known as fetal alcohol spectrum disorder (Mukherjee et al., 2006).

1.3 Chronic Alcohol Use Disorders

Alcohol abuse is a chronic disease in which a person refuses to give up drinking even though it is negatively impacting their lives, i.e. neglect of important family and work obligations. The American Psychiatric Association has developed strict criteria for the clinical diagnosis of alcohol abuse; as stated in the Diagnostic and Statistical Manual of Mental Disorders, 4\textsuperscript{th} Edition (DSM-IV), alcohol abuse is a maladaptive pattern of substance use leading to clinically significant impairment or distress, as manifested by one (or more) of the following, occurring within a 12-month period:

- Recurrent substance use resulting in a failure to fulfill major role obligations at work, school, home (e.g., repeated absences or poor work performance related to substance use; substance-related absences, suspensions, or expulsions from school; neglect of children or household)
- Recurrent substance use in situations in which it is physically hazardous (e.g., driving an automobile or operating a machine when impaired by substance use)
- Recurrent substance-related legal problems (e.g., arrests for substance-related disorderly conduct)
- Continued substance use despite having persistent or recurrent social or interpersonal problems caused or exacerbated by the effects of the substance (e.g., arguments with spouse about consequences of intoxication, physical fights)
- The symptoms have never met the criteria for Substance Dependence for this class of substances (American Psychiatric Association, 1994).
If left untreated, alcohol abuse may progress to alcohol dependence, though, some alcohol users abuse alcohol for long periods without developing dependence. Alcohol dependence is a chronic, progressive, and potentially fatal disease, defined by the *DSM-IV* as a maladaptive pattern of substance use, leading to clinically significant impairment or distress, as manifested by three (or more) of the following, occurring at any time in the same 12-month period:

- Tolerance, as defined by either of the following:
  a) A need for markedly increased amounts of the substance to achieve intoxication or desired effect,
  b) Markedly diminished effect with continued use of the same amount of substance.
- Withdrawal, as manifested by either of the following:
  a) The characteristic withdrawal syndrome for the substance,
  b) The same (or a closely related) substance is taken to relieve or avoid withdrawal symptoms.
- The substance is often taken in larger amounts or over a longer period than was intended,
- There is a persistent desire or unsuccessful efforts to cut down or control substance use,
- A great deal of time is spent in activities to obtain the substance, use the substance, or recover from its effects,
- Important social, occupational or recreational activities are given up or reduced because of substance use,
- The substance use is continued despite knowledge of having a persistent or recurrent physical or psychological problem that is likely to have been, caused or exacerbated by the substance (American Psychiatric Association, 1994).
A number of alcohol-induced psychiatric conditions may precipitate from the abovementioned disorders. They are classified by DSM-IV as follows:

- Alcohol-induced anxiety disorder
- Alcohol-induced mood disorder
- Alcohol-induced persisting amnestic disorder
- Alcohol-induced persisting dementia
- Alcohol-induced psychotic disorder (with delusions and/or hallucinations)
- Alcohol-induced sexual dysfunction
- Alcohol-induced sleep disorder
- Alcohol-induced intoxication
- Alcohol-induced intoxication delirium
- Alcohol-related disorders not otherwise specified (NOS)
- Alcohol withdrawal
- Alcohol withdrawal delirium (American Psychiatric Association, 1994).

Most substance-induced psychotic symptoms are considered to be short lived and to resolve with sustained abstinence, however, the guidelines described by DSM-IV are challenged by practical difficulties in distinguishing between substance-induced and independent psychoses. Ultimately, the ideal psychiatric nomenclature will define syndromes on the basis of established etiology and/or pathophysiology.
1.4 Neuropharmacological Action of Ethanol

The complexity and multitude of ethanol’s effects paradoxically rely on the simplicity of its chemical structure (Figure 1.4.1; Fadda and Rossetti, 1998). The hydroxyl group provides a dipole that can form hydrogen bonds with elements of membrane phospholipids (Barry and Gawrisch, 1994). The aliphatic end of the ethanol molecule provides a lipophilic group that can interact with non-polar domains of macromolecules, thus giving ethanol a dual-edged physico-chemistry that governs its interaction with biological substrates (Fadda and Rossetti, 1998).

![Ethanol Molecule Diagram](http://dopamine.chem.umn.edu/chempedia/index.php/Breath_Analyzers)

Prior to the 1970s, the central effects associated with alcohol abuse were thought to be a result of altered membrane fluidity, or a change in the composition of membrane lipids (Davis and Wu, 2001; Seeman, 1972). It is now thought that the actions of alcohol have a biological basis related to ethanol’s selective interaction with, or modulation of neurotransmitter function. Almost all of the important pathophysiological targets for ethanol appear to be specific membrane proteins responsible for mediating signal transduction (Weight, 1992). These targets include...
certain ion channels, transporters, neurotransmitter receptors, G proteins and enzymes that produce second messenger molecules (Diamond and Gordon, 1997).

The \( N \)-methyl-D-aspartate (NMDA) receptor is a ligand-gated ion channel and a major receptor for glutamate, the principal excitatory neurotransmitter in the brain. Activation of this receptor promotes calcium influx through the ion channel, which in turn, regulates signal transduction (Higgins and George, 2007). It is well established that acute ethanol exposure inhibits the excitatory action of glutamate at the NMDA receptor (Lovinger et al., 1989). Non-NMDA receptors, such as kainate and \( \alpha \)-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors, have also been shown to be sensitive to inhibition by ethanol (Martin et al., 1995; Lovinger, 1993). \( \gamma \)-Aminobutyric acid (GABA) is a major inhibitory neurotransmitter in the brain, activating \( \text{GABA}_A \) and \( \text{GABA}_B \) receptors. The \( \text{GABA}_A \) receptor is an oligomeric protein complex consisting of a receptor-operated chloride channel and specific allosteric sites for benzodiazepines and barbiturates (Olsen and Tobin, 1990). Interestingly, there is a cross-tolerance between ethanol, benzodiazepines and barbiturates (Suzdak et al., 1986) and benzodiazepines are helpful in treating the alcohol withdrawal symptoms (Diamond and Gordon, 1997). Generally, ethanol is thought to potentiate the inhibitory action of the \( \text{GABA}_A / \text{benzodiazepine} \) receptor complex (Ticku and Burch, 1980), though ethanol’s action on this receptor varies between brain regions (Criswell et al., 1993; Proctor et al., 1992) and these regional sensitivities are poorly understood. Molecular studies have determined the \( \text{GABA}_A \) receptor complex as a multi-gene family and a variety of subunits have been cloned (\( \alpha-, \beta-, \gamma-, \delta-, \varepsilon-, \theta-, \rho- \); Whiting et al., 1999). \( \text{GABA}_A \) receptors appear to be comprised of multiple combinations of these various subunits, thus changes in subunit
composition may account for regional differences in alcohol-sensitivity (Diamond and Gordon, 1997). A majority of the studies on the effects of ethanol have focused on the GABA_A receptor however, ethanol has also been reported to modulate the metabotropic receptor, GABA_B (Allan et al., 1991).

There is mounting evidence to suggest that ethanol can also act on other cellular proteins to indirectly change the function of ion channels residing in the membrane (Pandey, 1998). In general, it is thought that cyclic adenosine 3’, 5’–monophosphate (cAMP) and the phosphoinositide (PI) signal transduction pathways may be the intracellular targets of ethanol action (Pandey, 1998). Protein kinases have also been suggested as targets of ethanol action (Tao and Ye, 2002; Stubs and Slater, 1999). Protein kinases can phosphorylate ion channels, which in turn can modulate the activity of the channel or regulate the receptor’s sensitivity to ethanol (Pandey, 1998). Many reviews are available highlighting current opinion of ethanol’s pharmacology; the specific actions ethanol exerts at various neurotransmitter receptors, channels, transporters, and signaling cascades (Diamond and Gordon, 1997; Davis and Wu, 2001; Chandler, 2003).
1.5 Alcohol-related Cognitive Dysfunction

The core symptoms of alcoholism can be divided into two categories:

- Cognitive Dysfunction (e.g. deficits in attention, memory and executive function)
- Substance Dependence (e.g. tolerance, withdrawal, loss of control and craving)

Cognition is the ability to guide thought and action in accord with internal intentions. Convergent lines of evidence suggest that cognitive deficits are a core feature of alcoholism. A recent review paper indicated that 50-80% of alcoholic patients show cognitive decline (Bates et al., 2002). Cognitive changes include deficits in abstract problem solving, spatial and verbal learning, memory function and perceptual motor skills (Harper and Matsumoto, 2005). Glenn and colleagues applied several batteries of neuropsychological tests to alcoholic patients. Verbal skills, visual-spatial performance, verbal memory, and set-shifting flexibility (which is typified by the ability to change problem-solving strategies in response to changing requirements) were all affected in both male and female alcoholics (Glenn et al., 1993). Although precise evidence is still lacking, it is generally agreed that the presence of cognitive impairment can reduce quality of life and affect the treatment process and prognosis (Bates et al., 2002). However, some of these cognitive changes appear to be reversible after prolonged abstinence (O’Neill et al., 2001b; Pfefferbaum et al., 2006a; Pfefferbaum et al., 2006a; Pfefferbaum et al., 2006b).

This thesis will focus on brain regions specifically vulnerable to alcohol-induced damage underlying cognitive dysfunction. Therefore, substance dependence and
associated reward pathways will not be discussed in this thesis.

1.5.1 The Prefrontal Cortex (PFC) & Cognition

The principle functional/anatomical subdivisions of the PFC are the orbitofrontal and dorsolateral regions. The orbitofrontal cortex appears to be important in evaluating the reward value of stimuli as demonstrated by its reciprocal connections with brain regions that are involved in emotional processing (amygdala), memory (hippocampus) and higher-order sensory processing (temporal visual association areas), as well as projections to the dorsolateral prefrontal cortex (dIPFC; Brodmann’s Area 9; BA9; See Figure 1.5.1.1). By contrast, dIPFC is primarily involved in working memory. The dIPFC receives reciprocal inputs from brain regions, such as the hippocampus, association areas and parietal cortex, which provide information about the context of current stimuli for higher-order sensory processing. As a consequence, actions are planned and implemented with connections to motor regions (basal ganglia, premotor cortex, and supplementary motor area), and to the cingulate cortex resulting in motor control and performance monitoring, respectively. Hence, the PFC plays a crucial role in cognitive control as its reciprocal projections connect to numerous brain regions involved in cognitive functions.

The dIPFC is complex and heterogeneous, with subregions varying in cytoarchitecture and connectivity to primary, multimodal association areas and subcortical nuclei. Structurally, the dIPFC conforms to the structural plan prevailing throughout the neocortex. The general cortical architecture is commonly described as a 6-layered region (see Figure 1.5.1.2), distinguishable from one another by the relative
proportions of different kinds of neuronal cells. The two principal neuronal cell types are stellate cells and pyramidal cells. Approximately 75% of cortical neurons are pyramidal cells, found in layers II-VI and constitute the bulk of projection neurons (Peters et al., 1997). The axons of pyramidal neurons project into the white matter and provide excitatory projections to other cortical regions and subcortical structures. By contrast, stellate cells are interneurons, located in the middle cortical layers and provide axons that project locally within the cortical grey matter.

Figure 1.5.1.1: Brodmann area 9 (BA9), part of the dorsolateral prefrontal cortex (dPFC), is primarily involved in working memory. The BA9 is highlighted on the rostral view of a gross brain (A; http://psychology.wikia.com/wiki/Brodmann_area_9) and coronally on a MRI scan (B) above.
Figure 1.5.1.2: A Brightfield photomicrograph of the six cortical layers (1-6) and white matter (WM) of normal human BA9 (part of the dIPFC). Scale bar = 100 µm. (Figure obtained from Maldonado-Aviles et al., 2006).
1.5.2 The Cerebellum & Cognition

Like the cerebrum, the cerebellum consists of two hemispheres (see Figure 1.5.2.1). Three functional regions can be distinguished: the centrally located vermis and the lateral and intermediate zones in each hemisphere. The cerebellar cortex has three layers; from the outer to inner layer, these are the molecular, Purkinje, and granular layers (see Figure 1.5.2.2). Essentially, the cerebellar cortex modulates information flow through the deep nuclei. There are several reciprocal pathways between the cerebellum and cerebral cortex. The cerebellum receives input via pontine nuclei from the parietal cortex, the prefrontal cortex and the superior temporal sulcus and projects back to the same regions via the thalamus. These afferent and efferent projections imply a possible role of the cerebellum in the modification of information, which is projected from the cortex to the cerebellum and sent back to the cortex.

Although the cerebellum is primarily involved in motor control and co-ordination, it is increasingly recognized for its role in various aspects of cognitive and sensory functioning (Martin et al., 2003). Such functions are thought to include learning, cognitive processing of words, anticipatory planning and making time-based judgments. Neuroanatomical studies demonstrate that the superior cerebellar hemispheres are well connected to frontal and prefrontal areas (Schmahmann and Pandya, 1997) and functional neuroimaging has shown that prefrontal cortical and contralateral cerebellar activations occur in tandem (Diamond, 2000). The corticopontocerebellar and cerebellothalamocortical circuits underlie a wide range of neuropsychological processes that mediate not only traditional cerebellar functions, such as motor control, but also perceptual motor tasks, executive functions, and learning and memory, all of which are compromised by alcoholism (Parks et al.,
2002; Sullivan, 2003). Accordingly, alcohol–induced damage to the cerebellum could indirectly affect neurocognitive functions normally attributed to the frontal lobe (Martin et al., 2003).

Figure 1.5.2.1: The human cerebellum or “little brain”.

Image retrieved from http://ahsmail.uwaterloo.ca/~kin356/ltm/cerebellum.html)

Figure 1.5.2.2: Cellular organization of the cerebellar cortex.

Neurons are arranged in three layers, the molecular, Purkinje and granular cellular layer.

Image from http://web.lemoyne.edu/~hevern/psy340/lectures/psy34...
1.6 Neuropathological Changes in the Chronic Alcoholic Brain

1.6.1 Brain ‘Shrinkage’

Perhaps the most notable of abnormalities in the brains of alcoholics is the presence of brain shrinkage (see Figure 1.6.1.1; Harper and Kril, 1990) and indeed both neuroimaging and postmortem studies have indicated cortical and subcortical cerebral atrophy (Harper and Kril, 1994; Pfefferbaum et al., 1992b). Brain weight studies show that a group of ‘uncomplicated’ alcoholics (i.e., alcoholics with no concomitant neurological disease including commonly associated nutritional and metabolic disorders), had a significantly reduced brain weight compared to controls (1352g versus 1433g respectively; Harper and Kril, 1993a). The degree of brain atrophy was reported to correlate to the rate and amount of alcohol consumed in a lifetime (Harding et al., 1996). Brain atrophy can be more accurately defined by expressing brain volume as a proportion of intracranial volume or the pericerebral space (PICS; Harper et al., 1984). The PICS in a group of chronic alcoholics (drinking 30-80g alcohol per day) is shown in Figure 1.6.1.2.
Figure 1.6.1.1: Brain shrinkage of the alcoholic brain as seen by increased ‘dark’ areas - note the red arrows corresponding to similar areas in the alcoholic brain (B) compared to the normal control brain (A). Ventricular enlargement of the alcoholic brain (B) can also be seen (depicted by blue arrows) compared to the normal control brain (A), indicating shrinkage of the alcoholic brain.

Figure 1.6.1.2: There is a significant increase in PICS values in the three groups of chronic alcoholics (*p<0.01; **p<0.001). Changes in moderate drinkers are not significant. A significant shrinkage in uncomplicated alcoholics indicates the presence of alcohol-specific brain damage (Data obtained from Harper and Kril, 1994).
1.6.2 White Matter Changes

The documented reduction in brain weight and volume of chronic alcoholics is largely accounted for by loss of white matter volume rather than a loss of cortical tissue (see Figure 1.6.2.1; Harper et al., 1985; de la Monte, 1988) and some of this damage appears to be reversible given a prolonged period of abstinence (see Figure 1.6.2.2; Harper et al., 2003; Carlen et al., 1978; Bartsch et al., 2007). CT and magnetic resonance imaging (MRI) observations of reduced white matter volumes in alcoholics (Pfefferbaum et al., 1992b; Pfefferbaum et al., 1988) have been confirmed by quantitative pathological studies (Harper, 1998a) and have been shown to correspond to an absolute increase in ventricular size, which is approximate to the amount of cerebral white matter tissue lost (de la Monte, 1988). In a volumetric study, Kril et al. demonstrated a negative correlation between white matter volume and maximum daily alcohol intake (Kril et al., 1997b).

![Diagram](image_url)

Figure 1.6.2.1: Brain shrinkage is largely accounted for by loss of white matter volume rather than a loss of cortical tissue (*p<0.05; Data obtained from Harper, 1998a).
Figure 1.6.2.2: This proton MR Spectroscopy study revealed significant morphometric edge flow indicating early brain regeneration in the brains of uncomplicated alcoholics after short term abstinence (adapted from Bartsch et al., 2007).

There is some evidence to suggest that alcohol-related white matter loss is more pronounced in the frontal lobes (Jernigan et al., 1991), which may help explain the high incidence of cognitive dysfunction observed in alcoholics. Brain volume analyses revealed that white matter in alcoholics complicated with WKS is most markedly reduced in the prefrontal region (Kril et al., 1997b). The corpus callosum is also significantly reduced in alcoholics (Harper and Kril, 1988), especially in alcoholics who have experienced nutritional deficiencies (Ferrer et al., 1986). Using 1H magnetic resonance spectroscopy (MRS), Schweinsburg et al., showed a significant reduction of the metabolite N-acetyl aspartate in frontal white matter of
recently detoxified alcoholics (Schweinsburg et al., 2001). White matter atrophy is also commonly reported in the cerebellar vermis in chronic alcoholics (Harper and Kril, 1993b) and white matter loss in the vermis appears to contribute to ataxia in chronic alcoholics (Barker et al., 1999).

Microscopically, there are no obvious white matter lesions in the cerebral hemispheres of uncomplicated alcoholics (Harper, 1998a) and studies of lipid profiles have revealed only minor alterations, if any at all (Harper et al., 1991; Olsson et al., 1996). The subtle nature of alcohol-related white matter changes in the cerebral hemispheres are borne out by other physical and chemical analyses of white matter (Harper, 1998a). No significant differences in the relative density of frontal, parietal and occipital white matter were found in alcoholics and age- and sex-matched controls (Harper et al., 1987b). It has been suggested that reversible white matter loss is caused by changes in hydration however neurochemical (Trabert et al., 1995) and imaging studies (Mann et al., 1993) have refuted this hypothesis. A history of heavy alcohol consumption was associated with an acceleration of age-related myelin-loss in histologically normal brain tissue, however no influence on the myelin composition or the ratio of myelin and myelin-like membranes was documented (Wiggins et al., 1988). Ultrastructural studies of the effects of alcohol on an experimental model of the developing rat optic nerve revealed a permanent reduction in the relative thickness of myelin sheaths (Phillips et al., 1991) potentially causing significant neurological dysfunction. Although the nature of alcohol-related white matter loss has not yet been elucidated, it is likely that this disruption involves changes in both myelination and axonal integrity (Harper and Matsumoto, 2005).
1.6.3 Neuropathological Changes Underlying Alcohol-Related Cognitive Dysfunction

This thesis will focus on brain regions specifically vulnerable to alcohol-induced damage and associated cognitive dysfunction, the dlPFC and the cerebellar vermis. Neuropathological changes in these regions in alcoholics are described below. For a summary of quantitative neuropathological data from different groups of alcoholic patients, refer to Table 1.6, page 27.

1.6.3.1 The Dorsolateral Prefrontal Cortex (dlPFC)

Disorders of the frontal lobe are frequently encountered in alcoholics, even in the absence of neurological complications. Regional diversity of cytoarchitecture and function has been well described within the frontal lobes and past studies suggest that neuronal loss in alcoholics is not widespread, but restricted to functionally discrete areas (Kril et al., 1997b). Although the volume of the cerebral cortex appears to be intact in alcoholics, more detailed quantitative studies have shown some abnormalities (Harper et al., 1987a; Harper and Kril, 1989; Kril and Harper, 1989). A significant decrease in neuronal density was documented in the superior frontal cortex of alcoholics, but not in the adjacent primary motor, inferior temporal or anterior cingulate cortices (Harper et al., 1987; Kril and Harper, 1989; Pappin et al., 1993). Kril et al. later confirmed the loss of neurons from the frontal cortex using unbiased quantitative methods and reported a 15-23% neuronal loss in the frontal association cortex of alcoholics, with no pathology found in the motor cortex (see Figure 1.6.3.1.1; Kril et al., 1997b).
Analysis of the pattern of neuronal loss from the superior frontal cortex in alcoholics revealed that large pyramidal neurons, with a somal area greater than 90\(\mu\)m were selectively lost (Harper and Kril, 1989). It appears that the neuronal population(s) selectively lost are those without calcium-binding proteins, i.e. non-GABA-ergic pyramidal neurons, as numbers of calbindin, calretinin and parvalbumin-immunoreactive neurons are unaltered in alcoholics (Kril et al., 1997b). A previous study also reported shrinkage of neurons (reduction in the mean area of the cell body) in the superior frontal cortex as well as in regions where no neuronal loss has been documented, i.e. cingulate and motor areas (Kril and Harper, 1989). The size of the neuronal body is thought to positively correlate to the extent of dendritic arborization, i.e. the larger the body, the larger the dendritic field (Harper and Kril, 1990). Indeed, there is a significant reduction in the basal dendritic arbors of layer III pyramidal neurons in the superior frontal and motor cortices of chronic alcoholic patients when compared with matched controls (Harper and Corbett, 1990). A decrease in the density of dendritic spines was also shown in layer V pyramidal neurons in uncomplicated alcoholics (Ferrer et al., 1986).

Neuropsychological assessments of neurologically normal alcoholics have reported selective alterations of frontal executive functions, such as planning or problem-solving ability. An alcoholic-selective frontal dysfunction is supported by functional imaging studies; glucose hypometabolism has been reported in the mediofrontal cortex (Dao-Castellana et al., 1998; Adams et al., 1993). Neuronal loss and a reduction in basal dendritic arborisation were described in the superior frontal cortex of alcoholics (Harper and Kril, 1994). These pathological findings may help explain the high incidence of cognitive dysfunction observed in alcoholics.
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1.6.3.1.1: A 22% neuronal loss was documented in the superior frontal gyrus of uncomplicated alcoholics (*p<0.001), with no loss was reported in the adjacent primary motor cortex (Data obtained from Harper and Kril, 1994).

1.6.3.2  The Cerebellar Vermis

Excessive drinking can lead to functional and structural brain changes and one region that appears to be particularly vulnerable is the cerebellum. Pathological changes in the cerebellum are commonly reported in alcoholism and acute and chronic alcohol consumption produce profound impairments in cerebellar function. In autopsy studies, approximately 40% of alcoholics show signs of cerebellar degeneration (Torvik and Torp, 1986). This can be recognized in vivo using MRI (Pfefferbaum and Rosenbloom, 1993) and the changes are characterized by a general shrinkage or atrophy of the cerebellar foliae, particularly in the anterior superior cerebellar vermis (see Figure 1.6.3.2.1; Pfefferbaum and Rosenbloom, 1993; Harper, 1998a; Andersen,
Microscopically there is significant loss of Purkinje nerve cells in the vermis (Harper, 1998a) and reduced dendritic arbor (Ferrer et al., 1984; Pentney, 1982). White matter atrophy is also commonly reported in chronic alcoholics (Harper and Kril, 1993b) and white matter loss in the vermis appears to contribute to ataxia in chronic alcoholics (Baker et al., 1999). Other histological studies have reported a reduction in the volume of the molecular and medullary layers of the vermis (Phillips et al., 1987; Torvik and Torp, 1986). Using proton magnetic resonance spectroscopy, Parks and colleagues showed a significant reduction of N-acetylaspartate, a putative marker of neuronal integrity, and choline-containing compounds in the cerebellar vermis of chronic alcoholics (Parks et al., 2002), suggesting that the vermis is uniquely sensitive to alcohol’s effects.

It has been proposed that many of the pathological changes described in the cerebellum in alcoholics may be consequent to other common alcohol-related medical complications, particularly thiamine deficiency (Baker et al., 1999; Phillips et al., 1990). Alcoholics are often thiamine deficient (Majumdar, 1980; Majumdar et al., 1981), the most devastating neurological complication of which is the Wernicke-Korsakoff’s Syndrome (WKS; Kril, 1996). Cerebellar degeneration is seen in approximately one third of alcoholics with WKS (Harper, 1983; Victor et al., 1989) as well as in non-alcoholic WKS (Kril, 1996). Hence, the relative contribution of alcohol toxicity and thiamine deficiency to cerebellar degeneration is debatable (Martin et al., 2003), although it should be noted that many alcoholics without overt WKS also have deficient or marginal thiamine status (Damton-Hill and Truswell, 1990). The involvement of thiamine in cerebellar disease is supported by a study which demonstrated a correlation between serum thiamine level and cerebellar volume on
MRI, which held true for thiamine levels within the normal range (Maschke et al., 2005).

Figure 1.6.3.2.1: Alcohol-related cerebellar changes are characterized by a general shrinkage or atrophy of the cerebellar foliae, particularly in the anterior superior cerebellar vermis (circled in A and B).
Table 1.6: Summary of quantitative neuropathological data from different groups of alcoholic patients (Table adopted from Harper and Matsumoto, 2005)

<table>
<thead>
<tr>
<th>Region and Effect</th>
<th>Uncomplicated Alcoholic</th>
<th>Chronic WE</th>
<th>Korsakoff Psychosis</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain shrinkage (↑ pericerebral space)</td>
<td>36%</td>
<td>77%</td>
<td>77%</td>
<td>Harper and Kril, J Neurol Neurosurg (1985)</td>
</tr>
<tr>
<td>↓ Cortical neuronal dendrites</td>
<td>81%</td>
<td>N/A</td>
<td>N/A</td>
<td>Harper and Corbett, J Neurol Neurosurg Psychiatry (1990)</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>Kril, et al., Neuroscience (1997)</td>
</tr>
<tr>
<td>↓ Mamillary body neuronal number</td>
<td>98%</td>
<td>53%</td>
<td>32%</td>
<td>Harding, et al., Brain (2000)</td>
</tr>
<tr>
<td>↓ Thalamic neurons (mediodorsal)</td>
<td>100%</td>
<td>52%</td>
<td>36%</td>
<td>Harding, et al., Brain (2000)</td>
</tr>
<tr>
<td>↓ Thalamic neurons (ant. principal)</td>
<td>100%</td>
<td>86%</td>
<td>47%</td>
<td>Harding, et al., Brain (2000)</td>
</tr>
<tr>
<td>↓ Basal forebrain neurons</td>
<td>100%</td>
<td>76%</td>
<td>79%</td>
<td>Cullen, et al., Neurol Neurosurg Psychiatry (1997)</td>
</tr>
<tr>
<td>↓ Median raphe neurons</td>
<td>95%</td>
<td>30%</td>
<td>N/A</td>
<td>Baker, et al., Metabolic Brain Diseases (1996)</td>
</tr>
<tr>
<td>↓ Cerebellar vermis Purkinje cells</td>
<td>95%</td>
<td>57%</td>
<td>N/A</td>
<td>Baker, et al., Neuroscience (1999)</td>
</tr>
</tbody>
</table>
1.7 Pathophysiology of Alcohol-Related Brain Damage

The precise pathophysiologic mechanisms responsible for alcohol-related brain damage are not well known and remain subject to much speculation (Butterworth, 1995). Alcoholics frequently suffer concomitant diseases, thus several concurring factors may contribute to their neuropathology. Many alcoholics are malnourished, and vitamin deficiencies are frequently encountered, particularly vitamin B1 or thiamine (Damton-Hill and Truswell, 1990). The most frequently encountered manifestation of thiamine deficiency is Wernicke-Korsakoff’s Syndrome (WKS), which encapsulates an acute, potentially reversible Wernicke encephalopathy (WE) progressing to a chronic phase, Korsakoff’s amnesic psychosis (KP). Cirrhosis of the liver is also present in many chronic alcoholics, as well as poly drug abuse, psychiatric diseases and other pathologies such as head injuries, epilepsy and cerebrovascular disease (Kril, 1995). Thus there is still debate as to whether alcohol per se causes brain damage as there is difficulty delineating lesions caused by alcohol itself and those caused by the above-mentioned alcohol-related factors, principally thiamine deficiency and severe hepatic dysfunction (Harper and Kril, 1994). These key alcohol-related factors concerning the pathophysiologic mechanisms are introduced below.
1.7.1 Mechanisms Underpinning Alcohol-Specific Brain Damage

Ethanol’s metabolites, acetaldehyde and fatty acid ethyl esters may directly damage the brain by their accumulation or by forming adducts with brain proteins (Nakamura et al., 2003; Bora and Lange, 1993). Although the contribution of ethanol’s metabolic products to neurodegeneration cannot be ruled out, these mechanisms are in stark contrast to the fact that it is the withdrawal from chronic alcohol consumption rather than chronic intoxication that is harmful to neurons (Fadda and Rossetti, 1998). As the withdrawal syndrome seems to be the central event for the onset of pathological alterations in the brain, withdrawal excitotoxicity is the primary hypothesis for the pathophysiology of ethanol-related brain damage (Fadda and Rossetti, 1998).

As previously discussed, it is well established that acute ethanol exposure inhibits the excitatory action of glutamate at the NMDA receptor (Lovinger et al., 1989), and enhances GABA inhibitory action at the GABA\_A/Benzodiazepine receptor complex (Ticku and Burch, 1980). Prolonged, chronic exposure to ethanol, however, leads to neuroadaptive changes that up-regulate NMDA receptor function (Grant et al., 1990) and reduce GABA\_A receptor function (Brailowsky and Garcia, 1999). These changes counteract the acute action of ethanol, and the overall effect is one of neuronal hyperexcitability and a characteristic withdrawal syndrome when the inhibitory effect of ethanol is withdrawn (Wilce et al., 1994).

Several mechanisms of neurotoxicity are associated with the stimulation of glutamate receptors and some or all can contribute to ethanol-induced neurodegeneration (Fadda and Rossetti, 1998). Excessive activation of glutamate receptors leads to an increased influx of Ca\(^{2+}\). In addition, Ca\(^{2+}\) concentrations are increased in neurons following
depolarisation through voltage-gated Ca\(^{2+}\) channels (Randall and Tsien, 1995). Evidence suggests that ethanol-induced up-regulation of Ca\(^{2+}\) channels (Messing et al., 1986) may contribute to excitotoxicity and many features of the alcohol-withdrawal syndrome, including neuronal hyperactivity and life-threatening seizures (Messing and Diamond, 1997). The increase in cytosolic Ca\(^{2+}\) is closely coupled to Ca\(^{2+}\) uptake by mitochondria and results in the production of reactive oxygen species (ROS) – superoxide anion (\(\cdot O_2^-\)), hydroxyl radical (\(\cdot OH\)) and hydrogen peroxide (\(H_2O_2\); Coyle and Puttfarcken, 1993; Tsai et al., 1998). Oxygen radicals can attack proteins, DNA and lipid membranes, thereby disrupting cellular functions and integrity (Coyle and Puttfarcken, 1993). Concurrently, free radicals can cause the release of glutamate by inhibiting its transport process, thereby enhancing neurotoxic mechanisms (Tsai et al., 1998). The investigation of acute and chronic effects of ethanol at the molecular level have suggested cell damage in the brain may result from the formation of these ROS, lipid peroxidation products, nitric oxide, a decrease of neurotrophins and hyperhomocysteinaemia (Bleich et al., 2003; Bleich et al., 2004; Brooks, 2000; Ikegami et al., 2003). Interestingly, DNA fragmentation has been detected in the human superior frontal cortex and in the CA1 subfield of the hippocampus using a TUNEL assay. These TUNEL-positive cells may indicate DNA damage induced by ethanol-related overproduction of ROS (Ikegami et al., 2003).

It is likely that ethanol affects a number of signaling cascades and transcription factors, which in turn results in distinct gene expression patterns. Matsumoto and colleagues showed that during ethanol withdrawal, immediate early gene (IEG) expressions increase in the rat brain (Matsumoto et al., 1993). IEGs encode proteins that form transcription factors; c-fos and c-jun form transcription factor AP-1 and zif
encodes a protein that forms Egr. DNA binding activity of transcription factors AP-1 and Egr have been shown to increase in the rat cerebrum, hippocampus and cerebellum during ethanol withdrawal (Beckmann et al., 1997). Excitotoxic accumulation of intracellular Ca\(^{2+}\) may lead to the observed increase in IEG, and this has been suggested as the first step in a cascade of gene expression underlying ethanol-related plasticity (Beckmann et al., 1997; Beckmann et al., 1994).

Although alcohol-related white matter damage has been well documented, the underlying mechanisms remain elusive. Whether alcohol affects myelin, axonal integrity or both is currently unclear. Using DNA microarray technology, genes encoding myelin-related proteins were found to be decreased in the superior frontal cortex of human alcoholic subjects (Mayfield et al., 2002; Lewohl et al., 2001a). Yet, results at this stage are inconclusive.

1.7.2 Thiamine Deficiency and Brain Damage

Wernicke’s encephalopathy (WE) is an acute neuropsychiatric disorder resulting from thiamine deficiency (vitamin B\(_1\)) and a significant portion of cases have a history of heavy drinking or alcoholism (Harper, 1979). Clinically, patients may demonstrate a “classical triad” of symptoms; altered mental state, oculomotor abnormalities and ataxia. However, all three signs may not be present and diagnoses are often missed (75-80%) pre-mortem (Harper, 1983; Kril, 1996; Sechi and Serra, 2007). In most untreated cases of WE (80%), the patient will develop a permanent amnesic stage, Korsakoff’s psychosis (KP; Sechi and Serra, 2007). This disorder is principally characterised by severe memory defects, in particular a striking loss of working
memory that accompanies relatively little loss of reference memory (Victor et al., 1971). There appears to be considerable variation in the incidence of the WKS in different countries (Harper and Kril, 1990; Sechi and Serra, 2007). In developed countries, majority of WKS cases are in people who misuse alcohol, although there is no obvious correlation between prevalence and per capita consumption of alcohol (Harper et al., 1995). Other factors related to the prevalence of WKS include diet, national thiamine fortification programmes (i.e. bread flour) and drinking habits (Harper et al., 1995).

Brain cells require thiamine as a co-factor for several enzymes that are essential to the cells’ metabolism and functioning (Langlais, 1995). Three important enzyme systems involved in energy metabolism in the brain require thiamine. Two of these systems, α-ketoglutarate dehydrogenase and the pyruvate dehydrogenase complex play important roles in energy production. The third thiamine-dependent enzyme, transketolase, is a key component of the pentose phosphate pathway (PPP), which is responsible for generating ribose for nucleic acid production and reducing equivalents for energy transduction. This pathway is also essential for the maintenance and synthesis of myelin (Langlais, 1995). Thiamine deficiency leads to reduced activities of these enzymes with consequent impairment of cellular energy metabolism and deleterious effects on the viability of brain cells (Martin et al., 2003; Sechi and Serra, 2007).

In alcoholics, several factors may contribute to thiamine deficiency. Firstly, inadequate nutrition due to poor eating habits may cause low thiamine availability. However, alcohol can also influence thiamine absorption from the gastrointestinal
tract, the storage of thiamine or uptake into cells, and the utilisation of thiamine by the cells (Martin et al., 2003). In neurons and glia cells, thiamine is converted (phosphorylated) to its active form thiamine pyrophosphate (TPP). Ethanol directly affects brain thiamine phosphorylation/dephosphorylation mechanisms (Butterworth, 1995). Findings of alcohol-related decreases in TPP synthesis (Rindi et al., 1986) and increased TPP hydrolysis (Rao and Butterworth, 1994; Laforenza et al., 1990), suggest that chronic alcoholic consumption results in severely diminished brain concentrations of TPP. Indeed activities of all three TPP-dependent enzymes are significantly reduced in brain in alcoholic patients with WKS (Butterworth et al., 1993). Some studies have shown that in WKS, transketolase binds TPP less avidly than in control subjects (Mukherjee et al., 1987; Blass and Gibson, 1977), thus suggesting a genetic component predisposing alcoholics or people at risk of alcoholism to be more affected by thiamine deficiency than non-alcoholics (Martin et al., 2003). Yet, no consistent correlation between determined transketolase variants and thiamine deficiency has been found. Thus it is thought that several genetic defects may combine with environmental factors to generate this phenotype, and these genetic defects become clinically important when the diet is deficient in thiamine (Sechi and Serra, 2007).

Quantitative studies have shown significant abnormalities in the cerebral cortex, white matter and cerebellum, that are similar, but more severe than those described in alcoholic patients (Harper and Kril, 1985; Harper and Kril, 1989; Harper et al., 1988; Harper and Blumbergs, 1982; Phillips et al., 1990). Yet, these macroscopic and microscopic features depend on the stage and severity of WE (Harper and Butterworth, 1997). Approximately half of patients have symmetrical, greyish
discolouration, congestion and petechial haemorrhages, mainly in the periaqueductal grey matter, mamillary bodies and medial thalamus (Victor et al., 1971). The medial dorsal thalamus is involved bilaterally in 100% of patients and the superior vermis is affected in a third of cases (Victor et al., 1971).

The question of relative importance of thiamine deficiency and alcohol toxicity (or its metabolites) in the pathogenesis of alcohol-related brain damage is yet to be clarified. However, experimental studies have suggested that the onset and progression of clinical disease and severity of pathological changes is worse in animals with thiamine deficiency than in animals with thiamine deficiency alone (Phillips, 1987).

1.7.3 Liver Dysfunction and Brain Damage

Malnutrition, regardless of its cause, can lead to liver damage (Lieber, 2003). As most alcoholics are malnourished, clinicians originally thought that malnutrition, rather than alcohol, was responsible for alcohol-related liver injury. However, it has since been established that alcohol itself can precipitate liver damage even in well-nourished people (Lieber, 1992). Normal brain function depends on several aspects of normal liver function. The liver not only supplies certain nutrients, which the brain cannot produce itself, but plays a crucial role in cleansing the blood of neurotoxins. As a result, elevated, circulating neurotoxins may enter the brain where they can exert a number of damaging effects that interfere with normal neurotransmitter function, motor function and cause morphological changes to astrocytes (Butterworth, 2003).
The liver is the primary site of alcohol metabolism and its capacity to eliminate neurotoxic metabolites from the blood can be compromised by the chronic effects of alcohol abuse (Tarter and Edwards, 1986). Chronic liver disease, be it alcoholic or non-alcoholic in etiology, results in cognitive dysfunction and, ultimately, may itself cause brain lesions (Butterworth, 1995). Severe liver dysfunction may lead to hepatic encephalopathy (HE), neuropsychiatric syndrome, which causes cognitive dysfunction as well as motor disturbances and in its most severe form can be fatal (Butterworth, 2003; Walton and Bowden, 1997). HE is precipitated by the liver’s inability to remove blood-borne neurotoxic agents, including ammonia and manganese (Butterworth, 2003). Ammonia adversely affects both neurons and astrocytes, however only astrocytes are equipped to eliminate ammonia (through a reaction mediated by glutamine synthetase; Butterworth, 2003). Alzheimer type II astrocytosis is a pathological hallmark of HE where astrocytes undergo a characteristic morphological change (Kril et al., 1997a), i.e. large pale nucleus, prominent nucleolus, glycogen accumulation and margination of chromatin (Norenberg, 1981). Accumulation of ammonia in the brain results in a redistribution of cerebral blood flow and metabolism and deleterious effects on nerve signal transmission mediated through several neurotransmitter systems, leading to severe central nervous system dysfunction including brain edema, convulsions and coma (Felipo and Butterworth, 2002; Butterworth, 2003). Patients with chronic liver failure also demonstrate elevated blood manganese (normally cleared from the body by the hepatobiliary system) and as a result, this metal can enter the brain and deposit in the globus pallidus and associated brain structures (Butterworth, 2003). Brain manganese elevation was shown to cause a selective loss of pallidal dopamine D2 receptors (Mousseau et al., 1993) as well as inducing Alzheimer type II changes in astrocytes.
1.7.4 Synergistic Effects of Alcohol, Thiamine Deficiency and Alcoholic Liver Disease

Alterations seen in astrocyte parameters and monoamine neurotransmitters in the brains of alcoholic cirrhotics who died in hepatic coma (Mousseau et al., 1993; Rao and Butterworth, 1994), could result in modifications in amino acid and monoamine trafficking between neurons and astrocytes (Butterworth, 1993). Such alterations could contribute to alcohol-related brain damage by, for example, maintaining extracellular glutamate concentrations at potentially toxic levels (Butterworth, 1993), thus acting synergistically with the effects of thiamine deficiency and leading to cell death via an excitotoxic mechanism mediated by the NMDA receptor (Butterworth, 1995). Astrocytes are also involved in thiamine phosphorylation/dephosphorylation mechanisms (Butterworth, 1995). Thus, thiamine homeostasis in the CNS may also rely on intact neuron-astrocytic-metabolic interactions. Studies of the effects of chronic alcohol consumption in both humans (Rao and Butterworth, 1994) and experimental animals (Laforenza et al., 1990) reveal alterations of the activities of these enzymes in brain. These and other potential synergistic mechanisms whereby ethanol/thiamine interactions and liver disease may contribute to the phenomenon of alcoholic brain damage are shown in Figure 1.7.4.
Figure 1.7.4: Synergistic mechanisms whereby the direct neurotoxic effects of ethanol/acetaldehyde, thiamine deficiency and liver disease may contribute to the phenomenon of “alcohol-related brain damage” (Figure obtained from Butterworth, 1995).

There is increasing evidence to suggest that alcoholic brain damage and related cognitive dysfunction results from a combination of metabolic and toxic insults, which include direct neurotoxic effects of alcohol/acetaldehyde, the effects of thiamine deficiency and of liver disease. Synergetic combinations of these factors could to be implicated in neuronal dysfunction and, ultimately, brain damage resulting from chronic alcoholism.
1.8 Alcohol-related Brain Damage: Current Molecular Data

Several studies have applied cDNA microarrays to human alcoholic frontal cortex demonstrating a number of alterations in gene expression, including those which were classified into myelin-related, synaptic, mitochondria-related, signal transduction, intracellular metabolism, protein trafficking and apoptosis (Lewohl et al., 2001a; Liu et al., 2006; Mayfield et al., 2002). Changes in gene families encoding mitochondrial proteins, the ubiquitin system, and signal transduction were shown in the temporal cortex of alcoholics (Sokolov et al., 2003). These results suggest that multiple pathways may be important for neuropathology and altered neuronal function observed in alcoholism (Mayfield et al., 2002). However, changes in myelin-related genes and mitochondrial genes were suggested to be particularly important underlying causes in white matter damage and oxidative-stress related cellular injury respectively (Lewohl et al., 2001a).

Since the advent of high-throughput methodologies, several new hypotheses have been proposed based on the identification of new gene set associations to alcohol-related brain damage and adaptive neuronal plastic changes. An example of which is human neuronal protein 22 (hNP22). A significant increase in hNP22 gene expression was discovered in the prefrontal cortex of the alcoholic brain using differential display (Fan et al., 2001); this finding again was later substantiated using cDNA microarray (Fan et al., 2004). Furthermore, increased hNP22 protein levels were confirmed using western blotting and immunohistochemistry (Depaz et al., 2003). hNP22 is an actin- and tubulin-binding protein which may be involved in neuronal plasticity, playing a role in alcohol-induced loss of the pyramidal neurons, reduced dendritic arbor and secondary adaptive changes in the brain circuitry. Other studies have employed
western blotting and/or immunohistochemical approaches to examine specific protein changes in the human alcoholic brain. Examples of suggested altered proteins include neuronal nitric oxide synthase (nNOS; Gerlach et al., 2001), Type I & VIII adenyl cyclase (AC; Yamamoto et al., 2001), and GABA_A (Lewohl et al., 2001b; Dodd and Lewohl, 1998). However, there has been little consistency in human studies to date.

1.9 Proteomics Defined

The term ‘proteomics’ was first described by Marc Wilkins in the mid-1990s (Wilkins et al., 1996) and has prompted the scientific community to re-emphasise the importance of protein analysis of a cell, tissue, or organism as a whole as a complement of the genome (Marcus et al., 2004). Specifically, proteomics refers to the large-scale analysis of ‘the PROtein complement of genOME’ (Swatton et al., 2004). The proteome is the complete set of proteins produced by an organism under a certain set of conditions at a given time. Comprehensive proteomics investigations provide a unique global perspective on how proteins interact, their localization, modifications and in particularly the regulating changes affecting their expression, function and activity (Figure 1.9.1; Marcus et al., 2004). Thus, changes in the proteome provide a snapshot of the cell in action. It is important to emphasise that proteins are not merely downstream expressions of genes. The proteome is expressed as a constant state of protein metabolism and is dependent on multiple factors. Given the occurrence of differential splicing, post-translational modifications and complex protein-protein interactions, the proteome is a highly dynamic and intricate system. Importantly, proteomics allows the analysis of proteomes under various physiological and pathological conditions, which enables the identification of biomarkers and
improves the understanding of disease mechanisms at the molecular level. Subsequently, this may aid in the development of molecular diagnostics, targeted therapies and personalized medicines (Choudhary and Grant, 2004).

Figure 1.9.1: Overview of the variety of research fields in proteomics. Proteomics today represents a substantial, interdisciplinary area in research, combining the fundamental natural sciences such as biology, chemistry and biochemistry. The main goal of proteomics is to obtain a comprehensive overview about the processes in a cell, tissue or organism to be able to explain the regulatory mechanisms in detail, which will inevitably help explain the causes of diseases (adapted from Marcus et al., 2004).

One of the main approaches used in proteomic investigations combines protein separation by high-resolution two-dimensional gel electrophoresis (2D-GE) with Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) as well as tandem mass spectrometry (MS/MS). 2D-GE separates
proteins according to two independent properties in two discrete steps. The first
dimension separation separates proteins according to their net electrical charge or pI
(Isoelectric focusing) and second dimension separation sorts proteins by molecular
weight (MW; SDS-PAGE). Protein spots resolved from 2D-GE are then identified
using mass spectrometry (MS), a complementary approach routinely used in
combination with proteomics.

Proteomics is rapidly achieving recognition as a complimentary and perhaps superior
approach to examine global changes in protein expression in complex biological
systems. This can be attributed to important technological advances in 2D-GE and
protein mass spectrometry, which have made proteome approaches described above
more sensitive, versatile, reproducible and high-throughput. Such advances include
the application of immobilized pH gradient (IPG) strips, isoelectric focusing (IEF),
computerized imaging, spot detection/isolation, protein digestion protocols and the
development of mass-spectrometry-based protein digestion systems (Vercauteren et
al., 2004). Collectively, these advancements have created an integrated technology
that allow the simultaneous analysis of multiple proteins in a cell region or system and
can provide quantitative data regarding changes in corresponding expression levels
and/or posttranslational modifications that may be associated with disease in a highly
automated fashion. By making such a high throughput analysis available, it may be
possible to detect and identify more functional proteins related to alcohol-induced
brain damage or plasticity.

A major difference of proteomics from the previous protein analytical techniques is
that proteomics investigates proteins on a large, potentially automated, scale
Perhaps because of previous ‘one-by-one’ approaches, there has been little progress towards elucidating the molecular mechanisms of alcohol-induced brain damage. In retrospect, this may be expected when examining proteins of a complex system in isolation. As a result of technological advancement in expression profiling techniques, mass spectrometry and software development, a fundamentally different approach to study molecular mechanisms of alcohol-related diseases and brain damage is emerging.

### 1.9.1 Proteomics in Molecular Brain Mapping - Neuroproteomics

Understanding the human brain is perhaps the most significant challenge of medical research today and proteomics is fast becoming a popular investigative approach. Protein identification using mass spectrometry (MS) combined with the unique resolving power of 2D-GE is the most developed platform for large-scale analysis of gene products from biological samples (Herbert et al., 2001). These conventional proteomics techniques have been used widely in neuroscience for protein profiling and differential expression analyses (Choudhary and Grant, 2004). These techniques allow visualization, quantification and identification of specific therapeutic biomarkers and might help unravel the complex dynamics of protein expression in normal healthy brains. To date, only a handful of studies have applied these proteomics technology to study regional differences in protein expression in normal brains. These analyses include comparisons of the left and right temporal cortices (Chen et al., 2002), the cerebellum and frontal lobes (Wang et al., 2005) and protein profiling of the hippocampus (Yang et al., 2004).
1.9.2 Neuroproteomics in Psychiatric Disease and Alcoholism

Because the expression levels of genes and their protein products, which are responsible for a wide variety of functions in the CNS, are altered by the long-term use of abusive substances including alcohol, it is difficult to capture the complete pathogenic mechanisms using targeted or gene/protein candidates approaches. High throughput technologies such as cDNA microarray and proteomics, are particularly useful when targeting potentially multi-factorial diseases, i.e. psychiatric illnesses, as they enable researchers to systematically analyse whole sets of genes and proteins expressed in a cell, tissue or organ. Thus far, these technologies have been used predominantly to study neurodegenerative diseases (e.g. Alzheimer’s and Parkinson disease) and neurodevelopmental disorders (e.g. Down syndrome), however, more recently, studies have successfully applied these techniques to psychiatric illnesses such as schizophrenia and alcoholism (Choudhary and Grant, 2004; Clark et al., 2006; Clark et al., 2007; Lewohl et al., 2004).

Microarray technology has certainly demonstrated the power of using high throughput analyses to study the pathophysiology of complex disorders. However, analysis by microarray technology is limited to the cellular component, i.e. is largely restricted to analyses of brain grey matter. Transcriptome profiling provides a snapshot of a tissue’s mRNA profile and has the potential to detect changes in genes sets, which can reveal cellular systems affected by disease. Using this method, the altered expression of genes associated with metabolism, stress and myelin proteins was found in the prefrontal cortex of human alcoholic subjects (Lewohl et al., 2001a). Yet, mRNA is not the functional endpoint of gene expression and mRNA abundance has been suggested to be a poor indicator of the levels of corresponding protein (Gygi et al.,
Moreover, proteins are uniquely modified in ways that are not apparent from the gene sequence, i.e. by differential splicing and co- and/or post-translational modifications. As a result, it is speculated that up to a million different protein variations could be translated from 20-30,000 original coding genes and the presence of protein-protein interactions may further complicate results interpreted from such studies. Proteomics is rapidly achieving recognition as a superior approach to examine global changes in protein expression in complex biological systems although most proteomic technologies are still in their infancy. Yet, as these techniques allow the analysis of final end products of genes, they provide more functional insights, which may help understand the complex neuropathophysiology of alcohol-related brain damage.

Proteomics technologies have provided the tools to examine and identify protein dynamics of biological pathways and multifactorial disease processes at the molecular level. Proteomics studies of the human alcoholic brain, however, are limited. The only previously reported study was conducted on pooled brain tissue samples (no separation of grey/white matter; n=4) and isolated 182 significant protein abundance changes in the alcoholic superior frontal cortex (Lewohl et al., 2004). Described in this thesis are studies employing 2D-GE-based proteomics in combination with MALDI-TOF to investigate the protein abundance profiles in the various alcohol-sensitive brain regions from fully characterised alcoholic and control patients. By identifying changes in protein abundance in these important brain regions from alcoholics, hypotheses may draw upon more mechanistic explanations as to how chronic alcohol consumption causes brain damage.
1.10 Hypotheses

The following studies described in this thesis are non-hypothesis driven in nature. From the outset, however, the following assumptions were made;

- Proteomics is a superior approach to examine global changes in protein abundance in complex biological systems and is therefore an important tool for molecular mapping the human brain under normal physiological conditions.

- If morphologic abnormalities observed in the human alcoholic brain contribute to clinical findings of cognitive dysfunction in alcoholism, then abnormally expressed proteins in these regions (dorsolateral prefrontal region and cerebellar vermis) may underlie the molecular and structural abnormalities observed in alcohol-related brain damage.

- The changes we see in the brain probably underlie many of the acute and chronic neurological events associated with alcoholism. Hence investigating the protein abundances surrounding these events may provide a link between structural, pharmacological and gene expression changes.

- A non-hypothesis driven approach is useful to generate new hypotheses to explain mechanisms underlying alcohol-induced brain damage.
1.11 Research Aims

The main aim of this thesis is to characterise molecular mechanisms important in alcohol-related brain damage. In order to achieve this, detailed research aims are listed below.

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

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

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.

IV. To identify proteins with differential abundance in the BA9 white matter proteome of uncomplicated alcoholics and alcoholics with cirrhosis of the liver, relative to matched controls.

V. To identify proteins with differential abundance in the BA9 grey matter proteome of uncomplicated alcoholics and alcoholics with cirrhosis of the liver, relative to matched controls.

VI. To identify proteins with differential abundance in the cerebellar vermis proteome of uncomplicated alcoholics and alcoholics with cirrhosis of the liver, relative to matched controls.

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

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