SYSTEM BIOLOGY OF ALCOHOLISM: UNDERSTANDING OF THE CONSEQUENCES OF THE METABOLISM IN BRAIN CELLS

SYSTEM BIOLOGY OF ALCOHOLISM: UNDERSTANDING OF THE CONSEQUENCES OF THE METABOLISM IN BRAIN CELLS

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Abbreviations List:

AB= Alcoholic brains;

Ach=Acetylcholine;

ACN = Acetonitrile;

ADHD = Attention Deficit Hyperactivity Disorder

CB=Control brains;

CC= Corpus callosum;

CpG = Cytosine and Guanine separated by phosphate (the target of DNA methyltransferases);

Ch= Choline;

CN= Caudate nucleus;

COMT=Catechol-O-methyl transferase;

CREB= cAMP response element-binding protein;

DA= Dopamine;

DAT = Dopamine Transporter;

DBH= Dopamine-β-hydroxylase;

DDC= DOPA decarboxylase;

DHB= 3,4-dihydroxybenzylamine;

DOPAC=3,4-dihydrophenylacetic acid;

DND1 =Dead-end protein homolog 1

DS = Dorsal striatum;

DTT = Dithiothreitol;

EAAT = Excitatory Amino Acid Transporter;

ESI = Electro spray ionization;

FASD = Foetal Alcohol Spectrum Disorder;

GABA= (γ) Aminobutyric acid

GAD = Glutamate decarboxylase,

GLAST = Glutamate and Aspartate Transporter;

GLT = Glutamate Transporter;

Glu = L-glutamate;

GS = Glutamine synthase;

GSH = Glutathione;

HDC = Histidine decarboxylase;

HFBA = Heptafluorobutyric acid;

His = Histamine;

HP= Hippocampus

HVA = Homovanilic acid;

5HT = 5-hydroxytryptamine (serotonin);

IPG = Immobilized pH gradient;

LC-MS/MS = Liquid chromatography /tandem mass spectroscopy;

MALDI = Matrix-assisted laser desorption/ ionization;

MOWSE = Molecular weight search;

MRM = Multiple reactions monitoring;

NAc = Nucleus accumbens;

NE = Norepinephrine, noradrenaline;

NPM = Nucleophosmin

NSC = Neural stem cell

P = Putamen;

PFC = Prefrontal cortex;

PMI = Post-mortem interval;

PPPase = Pyridoxal phosphate phosphatase;

SDS-PAGE = Sodium dodecyl sulphate – polyacrylamides gel electrophoresis;

TFA = Trifluoroacetic acid;

TH = Tyrosine hydroxylase/tyrosine 3-monooxygenase;

SLC = Solute Carrier family of membrane transporter proteins.

TPH = Tryptophan hydroxylase;

Tryp = Tryptophan;

Tyr = Tyrosine;

VS = Ventral striatum;

VTA = Ventral tegmental area;

WB = Western blotting;

WKS = Wernicke-Korsakoff Syndrome

Summary

The addictive drug ethanol represents a serious universal health and social problem. Alcohol abuse and dependency have been increasing in many countries worldwide. Chronic use of alcohol leads to metabolic abnormalities and damage to the brain, which can result in severe cognitive dysfunction and addiction. Areas of the brain such as prefrontal cortex (PFC), the white matter, the hippocampus (HP), the cerebellum and the striatum are particularly vulnerable to the effects of alcohol. The striatum (ST), a region of brain closely associated with addiction mechanisms, is commonly divided into two regions, dorsal (DS) and ventral striatum (VS); the DS comprising caudate nucleus (CN) and putamen (P) while the VS is constituted mainly by nucleus accumbens (NAc). Neuroimaging analysis indicates that microstructural degradation is occurring in alcohol-affected brains and the abnormalities may be correlated with altered locomotor activity, aggressive behavior, changes in reward/aversion-related learning, deficient motor coordination, disturbances in procedural learning and memory. The NAc is strongly implicated in drug addiction but current evidence suggests that DS has also a significant role in this process. One of the aims of the project is to carry out comparative study of the sub-regions of human alcoholic striatum using two omics technologies (proteomics and metabolomics).

There were no differences in the levels of dopamine (DA), 3,4-dihydrophenylacetic acid (DOPAC), serotonin (5HT), homovanillic acid (HVA), 5-hydroxyindoleacetic acid (HIAA), histamine, L-glutamate (Glu), γ-aminobutyric acid (GABA), tyrosine (Tyr) and tryptophan (Tryp) between the DS (CN and P) and VS (NAc) in control brains. Choline (Ch) and acetylcholine (Ach) were higher and, norepinephrine (NE) is lower, in the VS Overall. Alcohol-affected ST had lower levels of neurotransmitters except for Glu (30% higher in the alcoholic ventral striatum). Ratios of DOPAC/DA and HIAA/5HT were higher in alcohol-affected ST indicating an increase in the DA and 5HT turnover. Glutathione was significantly reduced in all three regions of alcohol-affected ST. The pattern of changes of 13 neurometabolites in alcoholic sub-regions relative to their respective control was similar. In order to investigate correlations between the alcohol-related changes in metabolites and and altered protein expressions, the author has selected a single sub-region of ST for a global proteomics study. By analysing CN tissues, 25 unique proteins were found to be differently expressed in alcohol-affected tissue

relative to control. From the identified proteins two were dopamine-related proteins and one a GABA-synthesizing enzyme glutamate decarboxylase (GAD) 65. Two proteins that are related to apoptosis and/or neuronal loss (BiD and amyloid-β A4 precursor protein-binding family B member 3) were increased. These results suggest that neurotransmitter metabolism and systems possibly related to neuroprotective mechanisms in both the DS (CN and P) and the VS (NAc) are significantly influenced by long-term heavy alcohol intake associated with alcoholism. Amongst the mechanisms mediating the effects of alcohol oxidative stress may have produce a particularly significant impact and could make a strong contribution to the microstructural damage.

It has been known that alcohol can have a particularly damaging effect on the PFC and the HP regions of the fetus/developing/adolescent brain. The change imparted at those stages of development could be irreversible resulting in lasting deficits in a range of personality traits and impacting decision making, memory and learning. The HP is a significant place of neurogenesis and a source of neural stem cell (NSC). These can differentiate into neurons, astrocytes and oligodendrocytes and constitute building blocks of the developing central nervous system. They can also contribute to brain repair at later stages of development and, possibly, even in adulthood. On current evidence, alcohol reduces neurogenesis but little or no tangible information is available on the actual biochemistry and/or the fate of NSC.

In the present experiments, the NSC obtained from rat embryos were exposed to various concentrations of ethanol (25 to 100 mM) for up to 96 hours. The cell numbers were found to be reduced in the presence of ethanol but only at the higher concentrations (50 and 100 mM). There were no apparent dramatic changes in the morphology of the cells but the numbers of neuron-like (MAP2-positive) cells were reduced by ethanol in a dose-dependent manner. In proteome analysis of alcoholic cells, a total of 28 proteins were altered in 50 mM ethanol relative to ethanol-free control. Of these proteins some were constituents of cytoskeleton, others were involved in transcription/translation, energy metabolism, signal transduction and oxidative stress. Two of the proteins identified as altered were nucleophosmin (NPM) and dead-end protein homolog 1 (DND1). These were further studied by immunological techniques in cultured neurons and astrocytes. NPM decreased and DND1 increased in both alcohol affected neurons and astrocytes cells.

Interactome (protein-protein interaction) is a new branch of system biology, playing an important role in regulating various biological mechanisms including DNA replication & transcription, enzyme-mediated metabolism, transmembrane trafficking, signal transduction and cell cycle control. It is known that proteins function often if not always in the context of larger complexes and an adequate understanding of protein function requires at least some knowledge of their interactions, whether real or potential. In immunological analysis (Western blot), chronic excessive alcohol decreased NPM by 43% and increased DND1 by 80% in post-mortem alcoholic human PFC relative to healthy controls. Interactome was performed using the protein extract of human postmortem PFC by shot gun proteomics. NPM showed apparently specific interactions with 55 proteins and the associated proteins belong to cell growth (4), cell structure (4), metabolism (18), oxidative stress response (5), signalling (8), apoptosis and DNA damage (2), epigenetics (2), transcription (7) and transmembrane proteins (5) classes. DND1 interacted with 24 proteins; related to cell growth (1), cell structure (2), metabolism (13), oxidative stress (3), signalling (3) and transcription (2). The results show that alcohol alters a very large number of proteins capable of dramatically altering protein homeostasis in alcoholic cells. Thus the ethanol exposure modifies a wide range of mechanisms needed for the function of cells – and this includes NSC's eventually leading to the production of seriously impaired cells; *inter alia*, the processes of development, differentiation and repair using such cells by ethanol-exposed brains will result in abnormalities such as those typically encountered in the fetal alcohol spectrum disorder and/or in alcoholism later in life.

CHAPTER 1

INTRODUCTION

Alcoholism can very well be labelled "a biopsychosocial disease" to do justice to its multiple causes and multiple impacts on both individual and society. Approximately 90% of Australians have tried alcohol by the age 14 and consumed a standard drink (10 g alcohol) by the age of 16 (Annonymous, 2010). The rate of binge drinking is about 40% among Australian teenagers and its impact is probably best illustrated by everyday news (Annonymous, 2010). Early age (adolescent) onset of this type of alcohol consumption has been linked to the development of dependence in adult life (Grucza et al., 2008). Alcoholism has two types of symptoms: behavioral (addiction) and physiological. Behavioral symptoms include greater tolerance to alcohol, the presence of withdrawal symptoms upon cessation of alcohol intake, inability to control the desire to drink ("craving"), spending excessive time figuring out how to obtain alcohol and a reduction in social, recreational or working activities. The physiological symptoms can be broken down into two major categories: symptoms of acute and symptoms of chronic alcohol use. Acute use of alcohol causes several health problems such as poor balance, slurred speech and impaired peripheral vision. Long-term use of alcohol causes nutritional deficiencies, problems with balance and walking, liver disease (including cirrhosis and hepatitis), decreased immunity to infections, gastrointestinal inflammation and cognitive dysfunction. Cognitive symptoms include memory loss, impaired planning skills, loss of verbal fluency and learning ability.

Chronic use of alcohol not only alters the cellular physiology of the body but also causes specific organ (liver and brain) damage.

A person's susceptibility to alcoholism–related brain damage can be associated with their age, gender, drinking history and nutrition. The degree of the brain damage and related neurobehavioral deficits varies from brain region to brain region. Areas of the brain that are especially vulnerable to alcohol–related damage are the cerebral cortex and the subcortical areas such as the limbic system, the thalamus, the hypothalamus, the basal forebrain (Oscar–Berman 2000), the hippocampus (Ryabinin, 1998) and the cerebellum (Sullivan 2000). Volumetric magnetic resonance imaging (MRI) studies have consistently revealed reduced grey matter volumes in alcoholics. Significantly smaller volumes have been identified in the subcortical grey

matter, dorsolateral frontal and parietal cortices and the medial temporal lobe (Jernigan et al., 1991). Several studies on older alcoholic adults have documented that alcohol reduces the hippocampal volumes as well as verbal and spatial memory impairments (Grant, 1987; Munro et al., 2000). Pathological (Harper et al., 1985; Kril et al., 1997) and MRI studies (Pfefferbaum et al., 1992) showed that the white matter (WM) atrophy is related to maximum daily alcohol consumption and there are decreases in WM volume (Schweinsburg et al., 2001) including corpus callosum (CC) (Pfefferbaum et al., 2006; Pfefferbaum et al., 2006). The shrinkage and/or volume change is greater in the genu of the CC followed by the other sub-regions like the body and the splenium. Alcohol-induced volume reduction and disruption of the structural integrity of the CC could result in slowing of information processing and interhemispheric transfer, leading to deficits and/or abnormalities of visuo-spatial ability, gait, balance and working memory (Pfefferbaum et al., 2006; Pfefferbaum et al., 2006). Moreover, slight reductions in the volume of grey matter have also been shown in the putamen and the caudate nucleus (Norman et al., 2009). Striatal structural alteration and abnormal metabolism were shown in a child prenatally exposed to drugs including methamphetamine and these structural changes were accompanied by deficits in cognitive functions (Roos et al., 2014, Chang et al., 2007). Pathogenic mechanisms underlying the micro structural changes of the brain regions, only some of which appear to be reversible, are largely unknown.

Most of our attention has been on the problems of alcohol abuse and alcohol dependence in the adult; however, consequences of alcohol on fetus/unborn and/or newborn baby has been neglected and the disorders are collectively called Fetal Alcohol Spectrum Disorder (FASD). The prevalence of FASD might be as high as 1 to 5% in young school children in the USA (May et al., 2009; Lipton et al., 2004), which is apparently higher than the prevalence rate in Australia (Sokol et al., 2004). A study with FASD in indigenous Australians revealed that 540 out of 614 children under the age of 12 have signs of primary and secondary disabilities associated with FASD (Plevitz et al., 2009). The prevalence of FASD is 2 to 3 times as high as that of non-indigenous children (Harris and Bucens, 2003; Bower et al., 2000).

Those who get used to drinking regularly while young may find it difficult to stop when they reach the age when reproduction normally takes place. Regular heavy drinking (4 or more standard drinks in one sitting at least once weekly) or binge drinking (more than 5 drinks in one

sitting), particularly in the first trimester of pregnancy, is strongly associated with FASD-related characteristics (Elliott et al., 2008). Impairments associated with FASD are often a reflection of underlying structural changes in the brain, as evidenced above by changes in the corpus callosum (Spadoni et al., 2007). An MRI might also reveal decrease in brain size, damage to the basal ganglia, or reduced size of the cerebellum (Spadoni et al., 2007). Alcohol related brain deficits can manifest as either structural changes such as microcephaly, agenesis of the corpus callosum or cerebellar hypoplasia; or functional deficits affecting behaviour and cognition (Spadoni et al., 2007). The molecular mechanisms of the impact of alcohol on developing brain are still being uncovered.

The hippocampus (HP) is a site of neural stem cell production and neurogenesis and if these are impaired, whether pre- or postnatally it will result in an abnormal behavior later in life (McKay, 1997, Lledo et al., 2006). Neural stem cells (NSC) are pluripotent cells capable of differentiating into neurons, and glia including astrocytes and oligodendrocytes. The NSC play a pivotal role in the development and maturation of the CNS and migrate first to form specific brain regions while, later in the life, the "adult neurogenesis" may represent a response to functional demands or a replacement for damaged cells (Lledo et al., 2006). NSC proliferation, differentiation, cell migration and integration into the context of the tissue are affected by abiotic (eg. drugs, alcohol, antipsychotic agents etc) factors (McKay, 1997, Lledo et al., 2006). Administration of moderate doses of ethanol for 6 weeks has been shown to reduce the number of new neurons by two thirds and increase (by 227–279% relative to controls) the rate of cell death in the dentate gyrus (Herrera et al., 2003). Experiments using low to high concentration of ethanol in 35–40 days old rats indicated that adolescent brain is particularly sensitive to acute ethanol and displays, in addition to other changes, significantly decreased neurogenesis (Crews et al., 2006). Prenatal exposure to ethanol has long-lasting depressive effects on postnatal neurogenesis in the subgranular zone of the dentate gyrus and such abnormal neurogenesis may have a serious impact on brain health in later life (Redila et al., 2006; Klintsova et al., 2007). Longer exposures, such as a 4-day binge model, have been reported to result in reduced cell proliferation and impaired survival of NSC (Morris et al., 2010; Nixon and Crews, 2002). Moreover, ethanol at high concentration (50 mM) affects both the proliferation of NSC and gliogenesis, leading to concomitant changes in the glial phenotype; it has been suggested that ethanol can alter intrinsic cellular mechanisms of stem cells eventually impacting on the functional characteristics of both

neurons and glial cells (Vemuri et al., 2005). For example, such cells may have impaired genomic or biochemical makeup caused by remodeling of chromatin via ethanol-induced changes in acetylation and deacetylation of histones H3 and H4. This may happen in various brain regions [prefrontal cortex, nucleus accumbens, and striatum (MacDonald and Roskams, 2008; Wit, 2010)] and the existence of such damaged cells may possibly contribute to functional deficits in those regions (Liyanane et al., 2014; Stragier et al., 2015; Bonnaud et al., 2016).

Researchers have been interested not only in the genes that could contribute to the development of alcoholism but are increasingly looking into the functional activities of the proteins encoded by the genes. Many genetic approaches including QTL (quantitative trait locus) have been applied to study the polymorphisms in genes that may increase susceptibility to alcoholism. However, this search has been slow because the traditional molecular biology approaches are focused on the quantification of mRNA levels and generally target one or a few genes. The recent developments in system biology including DNA microarray studies have allowed for a nonbiased, simultaneous measurement of the expression of thousands of genes that are related to the 'downstream' effects of alcoholism (Xu et al., 2001; Kerns et al., 2005; Thibault et al., 2000; Lewohl et al., 2000). However, this generated a bounty of microarray data regarding the biological effects of ethanol, and to make the most effective use of this wealth of information, it will require the identification of the corresponding proteins. There are several reasons why studying the proteins produced by a cell can be more useful than examining mRNA expression in trying to understand the processes contributing to the cell's normal and pathological functioning. Firstly, RNA expression often does not correlate with protein expression (Celis et al., 2000). Secondly, miRNA (MicroRNA) interferes with the protein expression at the translation level. Thirdly, the activity of a protein could be significantly modulated by post-translational modification, which cannot be detected at the level of RNA. Fourthly, proteins are normally much more stable than RNA not only in cells, but particularly in the isolated body fluids and postmortem tissues. Thus, the study of proteins seems absolutely crucial for elucidating the cellular role of gene products apart from presenting a potential gold mine for understanding the cellular mechanism in the stem cell.

High-throughput proteomics technology is rapidly achieving recognition as a complimentary and superior approach to examine global changes in protein expression in complex biological system

and, this technology is also very useful for novel targets for medications, biomarker development and the understanding of the molecular mechanism of different diseases. Using proteomics approach, Lewohl et al., (2004) examined postmortem superior frontal cortex in alcoholism versus control tissue and identified 182 proteins which were differentially expressed in alcoholics; of these, 139 showed significantly lower expression, 35 showed significantly higher expression, and 8 were "new" or had disappeared in alcoholics. Proteomic based differential protein expression has been studied in postmortem human alcoholic in the prefrontal gray and white matter (Alexander-Kaufman et al., 2006; Alexander-Kaufman et al., 2007a), cerebellar vermis (Alexander-Kaufman et al., 2007b), sub-regions of the CC (Kashem et al., 2007; Kashem et al., 2008; Kashem et al., 2009a) and the hippocampus (Matsuda-Matsumoto et al., 2007) and the striatum of alcoholic rat brain (Kashem et al., 2012). Screening of more than 7000 proteins in various regions of brains mostly in the neocortex and HP, author has identified 238 proteins that were associated with alcohol induced abnormal metabolic disorders. According to their putative functions of these proteins can be classified as cytoskeletal (16%), metabolic (42%), oxidative stress-related (15%), signaling (22%) and apoptosis-related (5%). These results suggest that alcohol alters a very large number of proteins expressed in the cell thus almost certainly altering concomitantly the very mechanism of the cell homeostasis.

It has been estimated that at least 80% of proteins function in the form of complexes. The protein-protein interaction (PPI) (i.e. interactome) plays an important role in regulating various biological mechanisms including DNA replication & transcription, enzyme-mediated metabolism, transmembrane trafficking, signal transduction and cell cycle control (Berube et al., 2005). PPI is crucial for neurotransmission in the brain; for example whole release mechanism of neurotransmitter molecules depends on a precise and complex regulation of PPI (Chang and Min, 2011). As for other examples, ion channel or neurotransmitter transporter proteins located in the synaptic membrane have been regulated by complex protein interactions (Jackson and Robinson 2016). Identification and characterization of the PPI is important for our understanding of the metabolic processes that occur during normal neurotransmission and might provide new insights into cell function adaptations in the presence of alcohol. Therefore the study of interactome will certainly deepen and improve our understanding of diseases and, importantly, could provide the basis for new therapeutic approaches.

The traditional approach of biomarker discovery which usually focuses on one or a few potential candidates at a time is often ineffective for clinical diagnosis. Alcohol is a multifactorial addictive drug which affects many biological processes. It interacts with DNA, RNA and proteins and the result is an alteration of metabolism in the whole body. Several traditional alcohol biomarkers such as gamma-glutamyl transferase (GGT), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and carbohydrate-deficient transferin (CDT) have been used for many years. Out of the identified 238 proteins, some proteins such as tyrosine hydroxylase, dopamine-β-hydroxylase, catechol-O-methyltransferase, DOPA decarboxylase, tryptophan hydroxylase, glutamate decarboxylase-2, glutamate synthetase, N-acetylaspartate-glutamate synthetase, glutamate carboxypeptidase II, histidine decarboxylase, glutathione peroxidase, glutathione synthase, glyoxalase-1, apolipoprotein A-I, triosephosphate isomerase, isocitrate dehydrogenase, phospholipase D, SNAP-25, stathmin, sirtuin-2, DJ-1, α-synuclein, protein phosphatase, DARPP-32, CREB, apoptic death agonist (Bid) and cathepsin D have the greatest potential to become biomarkers for alcoholism and alcohol-related disorders. However, using other recently developed techniques such as metabolomics point to subtle changes in genes, transcripts or protein levels that can cause substantial changes in the levels and dynamics of metabolites and many studies reported identification of metabolite-based biomarkers for neurological disorders including Alzheimer disease (AD) and Parkinson disease (PD) (Nicholson et al., 2012; Xia et al., 2013; Kaddurah-Daouk and Krishna, 2009; Trushina et al., 2013). Alcohol alters the microstructure of the brain and causes brain damage and early detection of these changes would be the most effective way to improve the clinical outcome of the disease. Current advances in analytical technologies promise quantification of biomarkers from even small amounts of bio-specimens using non-invasive or minimally invasive approaches, and facilitate high-throughput analysis required for real time applications in clinical diagnosis. Thus emerging metabolomics provides a powerful platform for discovering novel biomarkers and biochemical pathways to improve our understanding of early diagnosis of alcoholism and FASD.

During the past eight years author carried out research (including the studies contributing to author's Master of Science degree) focusing on differential protein expression in various postmortem human brain regions such as the genu, body, splenium of CC, the HP and the striatal tissue of rats exposed to chronic alcohol. However, this provided no tangible information on system biology of the addiction-regulating brain region striatum. Moreover, alcohol is

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particularly damaging to young brain; yet, has very limited knowledge of how alcohol impacts on the developing brain tissue, particularly at the cellular and molecular level. Given the fundamental importance of neural stem cells in the process of brain development, studies on rat neural stem cells (rNSC) cultured in the presence of alcohol were also included in the present set of projects.

The main objectives of the studies discussed in the thesis are the following:

1. To study global protein expression in selected sub regions of the striatum from human post mortem alcoholics.
2. To study differential expression of metabolites in the sub regions of alcoholic human postmortem striatum.
3. To identify differential protein expression profiles in alcoholic rNSC.
4. To study interactomes of selected proteins (in this case those of NPM and DND-1 as the two proteins most altered both by alcohol-treated neural stem cells and in human alcoholic brain tissue) in human post-mortem prefrontal cortex tissues.

CHAPTER 2

Metabolomics of Neurotransmitters and Related Metabolites in Most-mortem Tissue from the Dorsal and Ventral Striatum of Alcoholic Human Brain

Abstract

This chapter presents a report on changes in neurotransmitter metabolome and protein expression in the striatum of humans exposed to heavy long-term consumption of alcohol. Extracts from post mortem striatal tissue (dorsal striatum; DS comprising caudate nucleus; CN and putamen; P and ventral striatum; VS constituted by nucleus accumbens; NAc) were analysed by high performance liquid chromatography coupled with tandem mass spectrometry. Proteomics was studied in CN by two-dimensional gel electrophoresis followed by mass-spectrometry. Proteomics identified 25 unique molecules expressed differently by the alcohol-affected tissue. Two were dopamine-related proteins and one a GABA-synthesizing enzyme GAD65. Two proteins that are related to apoptosis and/or neuronal loss (BiD and amyloid-β A4 precursor protein-binding family B member 3) were increased. There were no differences in the levels of dopamine (DA), 3,4-dihydrophenylacetic acid (DOPAC), serotonin (5HT), homovanillic acid (HVA), 5-hydroxyindoleacetic acid (HIAA), histamine (His), L-glutamate (Glu), γ-aminobutyric acid (GABA), tyrosine (Tyr) and tryptophan (Tryp) between the DS (CN and P) and VS (NAc) in control brains. Choline (Ch) and acetylcholine (Ach) were higher and norepinephrine (NE) lower, in the VS. Alcoholic striata had lower levels of neurotransmitters except for Glu (30% higher in the alcoholic ventral striatum). Ratios of DOPAC/DA and HIAA/5HT were higher in alcoholic striatum indicating an increase in the DA and 5HT turnover. Glutathione was significantly reduced in all three regions of alcohol-affected striatum. Author concludes that neurotransmitter systems in both the DS (CN and P) and the VS (NAc) were significantly influenced by long-term heavy alcohol intake associated with alcoholism.

Introduction

As dopamine (DA) has been considered a key neurotransmitter in mechanisms leading to addiction, it seems natural that DA systems in brain have been studied in relationship to alcoholism for some time. The most powerful associations between alcohol dependence and genes involved in dopamine metabolism has been reported for genes *DRD2* (TaqI A polymorphism of *ANKK1* gene) and *DRD4* encoding corresponding dopamine receptors D2 and D4 (Mota et al., 2012, Creemers et al., 2011, Zwaluw et al., 2011). Significant associations between alcoholism and polymorphisms in genes for enzymes monoamine oxidase (Whitfield et al., 2000) catechol-O-methyl transferase (*COMT*) (Sery et al., 2006, Voisey et al., 2011, Wang et al., 2001), dopamine-β-hydroxylase (DBH) (Weinshenker et al., 2000), and a serotonin transporter (*SLC6A4*);(Zwaluw et al., 2011) have also been found; however, despite many studies in the literature, no satisfactory understanding of neurochemical mechanisms underlying alcoholism has been achieved to date.

Previouly author studied more than 7000 expressed proteins in various regions of alcoholic post-mortem human or rat brains and identified 238 proteins that have been associated with alcoholism; these have been categorized into cytoskeletal (28%), metabolic (42%), oxidative stress (15%), signalling-related (22%) and apoptosis-related (5%) proteins (Kashem et al., 2007, Kashem et al., 2008, Kashem et al., 2009a, Matsuda-Matsumoto et al., 2007, Matsumoto et al., 2007). The functional profiles of the altered proteins (enzymes in particular) have indicated that alcohol could interfere with glucose metabolism, tricarboxylic acid cycle, and other crucial metabolic pathways associated with thiamine, lactate, glutathione, fatty acid/lipase as well as the metabolism of signalling compounds such as dopamine, serotonin and glutamate (Kashem et al., 2007, Kashem et al., 2008, Kashem et al., 2009a). In order to follow up in more detail how the changes in expression of the functional proteins that author's research has already identified may translate into specific changes in neurochemistry of neurotransmitters and related metabolites author is now expanding this approach by adding metabolomic techniques to his investigations. Given that biochemical and physiological changes underlying development of drug addiction have often been traced to the striatum, author selectes this part of the brain as the main region of interest in the present study.

The striatum has been conventionally divided into caudate nucleus (CN) and putamen (P) but, more recently, a third sub-region has been recognized (Heimer, 2000) comprising mainly nucleus accumbens (NAc) at the ventral confluence of CN and P. Striatum receives two major inputs: axons of excitatory pyramidal glutamatergic neurons from the cerebral cortex and an array of mainly dopaminergic, serotoninergic and cholinergic fibres from the brain stem. While the dorsal striatum (DS: CN and P) receives most of its direct cortical input from sensorimotor (P) or association (CN) cortex (Russo and Nestler, 2013), ventral striatum (VS: NAc) is targeted mainly by the areas of the cerebral cortex concerned with motivational or emotional activities and include insula, temporal lobe, anterior cingulate gyrus, parts of prefrontal cortex (PFC) and hippocampus (Dichter et al., 2012). Dopaminergic innervation of the VS originates mainly in the ventral tegmental area (VTA) of the brainstem and, to a lesser extent, in the medial substantia nigra (Andrisani, 1999, Hjelmstad et al., 2013). The DS receives its dopaminergic input from substantia nigra, pars compacta. Interestingly, the VTA dopaminergic fibres also release glutamate (Chuhma et al., 2009, Trudeau et al., 2014). Functional differences between the dorsal and ventral striatum could be reflected in neurochemical differences; this would agree with authors previous proteomic studies of human brain (Kashem et al., 2009b).

In terms of cytoarchitecture, medium-sized densely-spiny GABAergic neurons constitute the principal neuronal population and the only projection neurons in both dorsal and ventral striatum accounting for 75-80% of all striatal neurons in human brain (Tepper et al., 2004). The remainder is formed by local interneurons, the majority of which are also GABAergic (Tepper et al., 2004, Kreitzer, 2009). There is a small but functionally significant indigenous population of cholinergic interneurons throughout the striatum.

While no sharp boundary between the dorsal and ventral striata can be established on the basis of cytoarchitecture, myeloarchitecture or chemoarchitecture (Heimer , 2009, Prensa et al., 2003), accumulated evidence indicates that there exists a functional heterogeneity within the structures (Prensa et al., 2003, Tran-Nguyen et al., 1996). The VS appears to regulate the overall level of locomotor activity, aggressive behaviour and reward/aversion-related learning (Koob and Volkow, 2010) while DS is more intimately involved in motor coordination and procedural learning (Beeler et al., 2010, Dang et al., 2006). The circuitry involving the dopaminergic input from VTA to NAc seems crucial for processing of incentive-motivational properties of drugs and drug-related cues while other regions such as the amygdala and the hippocampus (HP) are more

likely to contribute to the habitual learning behavior which could also be a part of the addiction-forming mechanism (O'Brien and Gardner,2005). A model of neuronal networking regulating human addiction process as proposed by Volkow *et al.* (2003) includes the following: (a) reward and drug seeking circuits mediated by the NAc, (b) motivation/drive located in the orbitofrontal cortex and the subcallosal cortex, (c) memory and learning mediated by DS, the amygdala and the HP and (d) the cortical control executed from the PFC and the anterior cingulate gyrus (Volkow et al., 2003).

The overall evidence therefore suggests that, even though the VS is anatomically and/or cytoarchitecturally similar to DS, the functional activities related to addiction could be very different and this may be reflected in the regional neurochemical make up, as author has noted in his previous study using proteomics in otherwise apparently homogenous sub-regions of the corpus callosum (Kashem et al., 2009a). Here author looks for such differential neurochemical changes; particularly for those associated with neurotransmitters and cellular signalling.

Materials and Methods

Chemicals

Dopamine hydrochloride (DA), serotonin hydrochloride (5HT), tyrosine (Tyr), tryptophan (Tryp), homovanillic acid (HVA), 3,4-dihydroxyphenylacetic acid (DOPAC), 5-hydroxyindole-3-acetic acid (5-HIAA), norepinephrine (NE), γ-aminobutyric acid (GABA), L-glutamic acid (Glu), histamine (His), choline (Ch), acetylcholine (Ach), glutathione (GSH), heptafluorobutyric acid (HFBA) and internal standard 3,4-dihydroxybenzylamine (DHB) were purchased from Sigma–Aldrich (St. Louis, MO, USA). HPLC grade methanol and formic acid were also supplied by Sigma–Aldrich and ultrapure water was obtained from a Milli-Q Gradient water system (Millipore, Bedford, MA, USA). All consumables for gel electrophoresis were obtained from Proteome Systems Ltd (Australia) and IPG (immobilized pH gradient) strips were from Biorad Lab Pty Ltd (Australia).

Human brain tissue

In this study, author used post-mortem brains from 12 males. The samples came from 6 control and 6 alcoholic brains. Mean age in groups, alcoholics and controls was 59 years (cf. Table 1 for further details). All post-mortem human brains were obtained from the NSW Brain Bank (University of Sydney). The tissue samples were taken from the caudate nucleus (CN), the putamen (P) and the nucleus accumbens (NAc). The groups were, as far as possible, matched for age and pH (except for the post-mortem interval; PMI, of 3 alcoholic samples). Post-mortem examination of the alcoholic brains revealed that there were no complications from Wernicke-Korsakoff Syndrome (WKS) in any of the alcoholic brains used in the present study. Clinical history of the patients indicated that patients did not suffer from any additional psychiatric or neurological diseases unrelated to their alcoholic condition. Death of the patients was not directly caused by any known dysfunction of the central or peripheral nervous system. Alcoholic cases fulfilled the criteria of the Diagnostic and Statistical Manual of Mental Disorders IV (DSM-IV) for alcohol dependence (Annonymous 1994). Alcohol consumption data were obtained from medical records or from reports by the next of kin. Ethics approval for human tissue use was obtained from the Sydney South West Area Health Service.

Table 1. Patients Demography

Brain Bank Code	Age (Yrs)	PMI (hrs)	Brain pH	Cause of Death
Control brains				
650	37	14.5	6.46	Cardiomyopathy
582	50	30	6.37	Coronary artery
583	59	40	6.53	Ischaemic heart disease
442	63	24	6.94	Atherosclerotic coronary heart
603	73	38.5	6.28	Acute myocardial infraction
278	74	10	6.22	Respiratory arrest
Mean ± SEM	**59 ± 6**	**26.2 ± 5.0**	**6.50 ± 0.11**	
Alcoholic brains				
332	41	54	6.70	Epilepsy & chronic alcoholism
330	50	60	6.75	Chronic airflow limitation
597	59	35	6.57	Coronary artery thrombosis
533	63	25.5	6.21	Ischaemic heart disease
313	70	62	6.82	Cardiomyopathy
512	73	43.5	6.59	Coronary artery atheroma
Mean ± SEM	**59 ± 5**	**46.7 ± 5.9**	**6.61 ± 0.09**	

Preparation of standards and calibration curves for liquid chromatography/tandem mass spectrometry (LC–MS/MS)

Standard stock solutions were prepared in a 0.1M formic acid solution to assist dissolution and minimize oxidation of labile samples (Gonzala et al., 2011). These solutions were stored at −20°C until use. Standard curves were prepared by adding analytes (Tyr, Tryp, DA, DOPAC, NE, 5HT, GABA, Ch, Ach, His, 5HIAA, HVA, Glu, GSH and internal standard DHB to a 0.1% formic acid solution. A constant amount (500 pg/μl) of internal standard was added to all standard solutions to produce a fixed concentration.

Sample preparation for LC-MS/MS

The brain samples were weighed and homogenized at 1 mg per 50 μL of 0.1M formic acid (Xu et al., 2011, Wei et al.,2014). The internal standard (DHB) was then added to 500 pg/μL final concentration. The suspension was sonicated and centrifuged at 16,000 x g for 20 min at 4°C. The supernatant was filtered and diluted within the range of calibration curve.

Table 2. The analytical parameters of metabolites[a] estimated in the extracts of postmortem human brain by LC-MS/MS

Analytes	RT (min)	Cone voltage (v)	Quantitation transition	Confirmation transition
Glu	0.49-0.51	25	148.3>84.1 (15)[b]	148.3>130.1 (10)
GABA	0.64-0.66	35	104.0>0.1 (10)	104.3>58.1 (25)
Chl	0.7-0.71	20	104.3>87.1 (10)	104.3>69.1 (15)
GSH	0.76-0.77	12	308.096>75.99 (20)	308.096>179.048 (26)
NE	0.83-0.85	12	170 >106.9 (20)	170 >1152.0 (8)
Ach	1.03-1.07	25	146.2>87.1 (15)	146.2>43.1 (15)
His	1.18-1.19	10	112.2 >95.1 (20)	112.2 > (8)
Tyr	1.51-1.52	18	182.1>136.0 (14)	182.1>165.0 (10)
DA	1.56-1.58	20	154.2>137.1 (10)	154.2>91.0 (25)
DOPAC	1.99-2.04	20	169.1>123.1 (10)	169.1>77.1 (30)
5HIAA	2.16-2.21	30	192.2>146.2 (25)	192.2>91.2 (35)
5-HT	2.25-2.26	18	177.2>160.2 (10)	177.2>132.1 (20)
HVA	2.50-2.55	22	183.2>137.2 (10)	183.2>122.0 (25)
Tryp	2.63-2.64	18	205.1>188.1 (10)	205.1>146.0 (18)

[a]Abbreviations: Dopamine (DA), serotonin (5HT), tyrosine (Tyr), tryptophan (Tryp), homovanillic acid (HVA), 3,4-dihydroxyphenylacetic acid (DOPAC), 5-hydroxyindole-3-acetic acid (5-HIAA), norepinephrine (NE), γ-aminobutyric acid (GABA), L-glutamic acid (Glu), histamine (His), choline (Ch), acetylcholine (Ach), glutathione (GSH), [b]Collision energy (eV) is given in brackets.

Analysis of metabolites

Chromatographic analyses have been performed in an Acquity UPLC™ system (Waters, Milford, MA, USA), using a BEH C_{18} column (150 mm × 2.1 mm; Waters), with 1.7 μm particle size (Gonzala et al., 2011, Xu et al., 2011). The mobile phase for elution was a gradient established between solvent A (0.05% formic acid in water + 1mM HFBA) and solvent B (methanol) at a flow rate of 200 μL/min. Gradient conditions were: 0.0–1.0 min, 5–50% B; 1.0–2.0 min, 50–95% B; 2.0–3.0 min, 95% B; and back to 5% B in 1.0 min. Flow rate was

$0.2 \, \mathrm{mL \, min^{-1}}$, injection volume $5 \, \mu\mathrm{L}$ (in partial loop mode), the column temperature was maintained at 25-27°C and the sample temperature was at 4°C.

Mass spectrometric detection was carried out using a Waters Acquity TQD tandem quadrupled mass spectrometer (Waters, Manchester, UK) (Gonzala et al., 2011). The tandem mass spectrometer was operated in multiple reactions monitoring (MRM) mode and Q1 and Q3 quadruples were set at unit mass resolution. The instrument was operated using electro spray ionization (ESI) source in positive mode. ESI parameters were capillary voltage 3.0 kV, extractor voltage 3 V, source temperature 120°C, desolvation temperature 350°C, cone gas flow 80 L/h and desolvation flow 600 L/h (both gases were nitrogen). Collision-induced dissociation was performed using argon as the collision gas at the pressure of 4×10^{-3} mbar in the collision cell. The MRM transitions as well as the cone voltages and collision energies are shown in Table 2. Data acquisition was performed using MassLynx 4.0 software with QuanLynx program (Waters). Protein assay was performed by the Bradford method using bovine serum albumin as a standard (Bradford, 1976).

Preparation of tissue extracts for proteomics and two-dimensional gel electrophoresis:

Protein extraction and gel analysis were done as previously described in detail (Kashem et al., 2008, Hjelmstad et al., 2013, Kashem et al., 2009a). Briefly, 100 mg of tissue was homogenised, sonicated (3x) and centrifuged at 16000 x g for 20 min at 15°C. The supernatant was reduced and alkylated in 5 mM tributyl phosphine and 10 mM acrylamide monomer at room temperature for 2 h. The reaction was quenched using 10 mM DTT (dithiothreitol). Acetone (five times of extract volume) and citric acid (20 mg) and the resulting precipitate centrifuged at 2500 x g, 15 min at 15°C. The pellet was air dried, resuspended and stored at -80°C before isoelectric focusing. Following protein estimation, 2-DE was conducted as previously described using SDS-PAGE (sodium dodecyl sulphate - polyacrylamide gel electrophoresis); (Kashem et al., 2012, Kashem et al., 2009a, Kashem et al., 2008, Kashem et al., 2007, Kashem et al., 200b).

Image analysis, Matrix-assisted laser desorption/ionization (MALDI)-mass spectroscopy and protein identification

A total of 24 gels (duplicate runs for each sample) were scanned using a flatbed scanner (UMAX, USA). The images were analysed by Phoretix 2D Expression software (Nonlinear Dynamics Ltd, UK) and quantified (spot area x optical density, background subtracted). Following log transformation and normalization, one-way analysis of variance (ANOVA, p <

0.05) was performed to identify significant differences (spot volume) between alcoholic and control brains.

The spots so identified were de-stained using 25 mM NH4HCO3/50% (v/v) acetonitrile (ACN) for 3 x 15 min at 37°C. The gel portions were then dehydrated using 100% ACN and digested with 12.5-ng/ml trypsin (Roche, sequencing grade, Germany) buffer {25 mM NH4HCO3/0.1% TFA (trifluoroacetic acid)} for 45 min at 4°C and incubated a further 3 hours at 37°C. The peptide mixtures were purified using C-18 purification tips (Eppendorf, Germany), eluted onto a MALDI sample plate with 3 µL of matrix solution {5 mg/mL solution of α-cyano-4-hydroxycinnamic acid in 70% (v/v) ACN/0.1 %(v/v) TFA} and allowed to air dry. The samples were analyzed using Qstar XL Excell Hybride MS system (AB Applied Biosystems, Foster City, CA, USA) in positive reflector mode, with delayed extraction. The data from mass spectra were matched to the Swiss-Prot database (http://www. matrixscience.com/) using criteria based on a MOWSE (Molecular Weight Search) score (>64, human database) with matched isoelectric pH (pI), molecular weight values (estimated from 2D gels) and sequence coverage.

Statistical analyses

Each sample was measured twice and each group contained 12 data points. All data were statistically analysed using single factor ANOVA. Accordingly, the mean of the two groups is considered to be statistically significant only if $p < 0.05$. Pearson correlation was performed on the estimates of metabolite levels and on the values of agonal factors.

Protein extraction and Western blot (WB) analysis:

Striatum protein extractions were performed according to the method previously described by Kashem (Kashem et al., 2007, Kashem et al., 2008, Hargreaves et al., 2009). Protein were separated and transferred to PVDF (polyvinylidene difluoride) membranes according to a protocol described earlier (Kashem et al., 2012). The membranes were blocked with 5% skim milk and incubated with primary antibodies [all primary antibodies obtained from Santa cruiz and dilutions were ranged from 300-500)] solution. Secondary antibodies (anti-mouse and anti-rabbit IgG; Sigma) were added according to the manufacturer's instructions and the resulting spots were visualized using an ECL (enhanced chemiluminescence) full spell system (GE Healthcare, Australia). The intensity of spots was quantified by commercially available Bio-rad software (Bio-rad, Sydney, Australia).

Results

General

Control brains (CB) and alcoholic brains (AB) were matched for age, brain pH and post-mortem interval time (PMI) (Table 1). The only significant difference between alcoholic brains and control brains was in PMI but, even there, the overlap between the two groups was quite large. Available evidence suggest that PMI has no significant effect on GABA, amino acid and biogenic amine contents of brain tissue (Korpi et al., 1987, Spokes et al., 1980), and, additionally, previously published data indicated that agonal factors had no effect on the expression of proteins, including those acting as enzymes in neurotransmitter metabolism, in several regions of post-mortem human brain (Kashem et al., 2007, Kashem et al., 2008, Kashem et al., 2009). As Pearson correlation statistical test showed no significant difference between agonal factors and metabolite expression author assumed that the data in this study are free from the effects of agonal factors.

Regional Variations in Metabolite Levels

Analyses of the metabolites in sub-regions (CN, P and NAc) of striatum in control brains showed no significant differences in the levels of metabolites from one sub-region of striatum to another except for NE, Ch and Ach contents (Table 3). In the control brains striatum, Ch and Ach were, resp. 58% and 70% higher, and NE 40% lower, in the NAc relative to the DS (represented as mean of the values in CN and P, resp.; Table 3).

Changes in Neurotransmitter and Metabolite Levels in Alcoholic Striatum: Catecholamines

The levels of DA, NE, DOPAC, HVA and their precursor amino acid Tyr were significantly decreased in all subregions of alcoholic brains striatum compared to control brains (Table 4). DA reduction was similar in all sub-regions (~ 60%). The decline rates of other catecholamine metabolites appeared somewhat variable but the regional variability was not statistically significant (Table 4).

The increase in the ratio of DOPAC/DA observed in alcoholic brains was significantly higher in the VS (76%) than in the DS (~ 18%);(Table 4). Similarly, the increase in HIAA/5HT ratio was higher in the NAc (34%) than in the DS (Table 4).

Table 3. Region specific levels of metabolites and their ratios in the striatal regions of post mortem human brains (non-alcoholic controls)

Metabolites	Caudate N.	Putamen	N. accumbens	P value
DA	32.45 ± 3.38	29.56 ± 0.67	30.13 ± 3.40	0.75
DOPAC	17.65 ± 0.36	17.55 ± 1.13	16.04 ± 0.39	0.24
5HT	1.42 ± 0.05	1.32 ± 0.04	1.31 ± 0.05	0. 6
NE	1.98 ± 0.44	1.89 ± 0.09	1.15 ± 0.09	0.002*
HVA	31.24 ± 1.63	40.60 ± 3.85	28.85 ± 1.63	0.055
HIAA	5.44 ± 0.38	6.51 ± 0.76	4.89 ± 0.66	0.09
Histamine	6.22 ± 0.77	4.95 ± 0.29	6.31 ± 0.62	0.189
Choline†	3.24 ± 0.25	4.26 ± 0.61	5.86 ± 0.93	0.04*
Acetylcholine	0.97 ± 0.08	1.16 ± 0.17	1.81 ± 0.14	0.003*
Tyrosine	756 ± 33	788 ± 45	820 ± 33	0.58
Tryptophan	220 ± 16	244 ± 17	260 ± 16	0.22
Glutamate†	23.3 ± 2.0	26.9 ± 1.9	23.5 ± 1.7	0.58
GABA	742 ± 38	790 ± 72	811 ± 53	0.68
Glutathione†	9.57 ± 1.69	11.85 ± 2.17	11.34 ± 1.98	0.694

Ratios of metabolite levels

	Caudate N.	Putamen	N. accumbens	P value
DOPAC/DA	0.57 ± 0.05	0.59 ± 0.04	0.58 ± 0.08	0.2
HVA/DA	1.03 ± 0.17	1.37 ± 0.13	1.01 ± 0.13	0.95
HIAA/5HT	3.83 ± 0.25	5.00 ± 0.71	3.75 ± 0.22	0.5
ACh/Ch **(x1000)**	0.30 ± 0.03	0.34 ± 0.10	0.38 ± 0.10	0.83
GABA/Glu **(x1000)**	32 ± 4	31 ± 3	35 ± 3	0.65

The levels are expressed as nmole/g tissue (or † μmole/g tissue) ± SEM (n =6). Asterisks (*) mark where the values vary among the regions at $P < 0.05$ level, at least.

Preliminary estimation using Western blotting (WB) suggested that the expression of dopamine biosynthesis enzyme tyrosine hydroxylase (TH) was lower (Fig. 1) but the decreases in other three enzymes of the catecholamine metabolic pathway: DOPA decarboxylase (DDC), catechol-O-methyltransferase (COMT) and dopamine-β-hydroxylase (DBH), were variable and the apparent differences did not reach statistical significance (Fig 1; see also the section on proteomics in control brains below).

Expression of two other proteins potentially linked to dopamine and/or addiction mechanisms, namely DARPP-32 (dopamine- and cAMP-regulated phosphoprotein of 32 kDa) and CREB (cAMP response element-binding protein) were significantly decreased (Fig. 1) in alcoholic relative to control brains (Kashem et al., 2009, Pandey et al., 2004).

Changes in Neurotransmitter and Metabolite Levels in Alcoholic Striatum: Indolamines

Serotonin (5HT), HIAA and Tryp contents in alcoholic brains striatum were significantly decreased compared to the control brains striatum (Table 4; cf. also (Kashem et al., 2009, Pandey et al., 2004). The HIAA depletion in the alcoholic CN was higher (57%) relative to other regions (~ 38%; Table 4). Additionally, the expression of tryptophan hydroxylase (TPH), the key enzyme of serotonin biosynthesis, was decreased (44%) in the CN of AB (Fig 1, cf. also (Sun et al., 2012, Jacobsen et al., 2012). The increase in HIAA/5HT ratio was higher in the NAc (34%) than in the DS (Table 4).

Changes in Neurotransmitter and Metabolite Levels in Alcoholic Striatum:

Indolamines

Serotonin (5HT), HIAA and Tryp contents in alcoholic striatum were significantly decreased compared to the control brains striatum (Table 4; cf. also (Kashem et al., 2009, Pandey et al., 2004). The HIAA depletion in the alcoholic CN was higher (57%) relative to other regions (~ 38%; Table 4). Additionally, the expression of tryptophan hydroxylase (TPH), the key enzyme of serotonin biosynthesis, was decreased (44%) in the CN of alcoholic brains (Fig 1). The increase in HIAA/5HT ratio was higher in the NAc (34%) than in the DS (Table 4).

Table 4. Regional variations in alcohol-induced changes, expressed as % decrease (or † % increase) of metabolite levels in the regions of striatum

Metabolites	Caudate	Putamen	N. Accumbens	P value
DA	54.0 ± 4.2	62.4 ± 3.1	61.3 ± 7.9	0.51
DOPAC	47.1 ± 10.2	57.1 ± 6.7	36.8 ± 3.7	0.188
5HT	36.9 ± 9.1	44.3 ± 4.7	53.2 ± 2.7	0.202
NE	63.0 ± 7.0	41.5 ± 5.1	50.8 ± 4.9	0.056
HVA	41.9 ± 9.4	50.3 ± 9.1	47.5 ± 2.4	0.7373
HIAA	57.0 ± 3.7	36.5 ± 3.8	39.5 ± 4.5	0.0056**
Histamine	34.1 ± 8.4	28.7 ± 5.3	33.3 ± 9.0	0.872
Choline	24.8 ± 4.8	25.9 ± 5.1	13.5 ± 2.5	0.11
Acetylcholine	31.9 ± 7.4	34.8 ± 12.9	51.1 ± 6.5	0.322
Tryptophan	18.8 ± 7.5	19.3 ± 4.9	22.9 ± 4.5	0.861
Tyrosine	18.8 ± 8.8	20.3 ± 6.7	20.1 ± 5.1	0.987
Glutamate†	$+ 6.9 \pm 1.7$	$+ 4.4 \pm 0.8$	$+ 30.0 \pm 9.0$	0.0068**
GABA	14.7 ± 2.9	13.5 ± 2.7	15.5 ± 4.3	0.911
Glutathione	67.8 ± 5.5	34.7 ± 9.1	58.1 ± 6.8	0.0163*

Changes in the ratios of metabolite levels

	Caudate	Putamen	N. Accumbens	P value
DOPAC/DA†	$+ 17.1 \pm 3.9$	$+ 19.8 \pm 6.5$	$+ 76.4 \pm 6.5$	0.0002**
HVA/DA†	$+ 25.6 \pm 5.8$	$+ 41.9 \pm 10.7$	$+ 52.3 \pm 5.05$	0.074
HIAA/5HT†	$+ 9.3 \pm 3.8$	$+ 14.4 \pm 5.5$	$+ 34.1 \pm 8.3$	0.0296*

The values are decreases or increases (in %) relative to the absolute values obtained in control brains and shown in Table 3. All decreases were statistically significant at $P < 0.01$ except for tyrosine and tryptophan where the statistical significance was at $P < 0.05$ level. Asterisks mark where the magnitudes of decrease/increase varied among regions (* $P < 0.05$, ** $P < 0.01$).
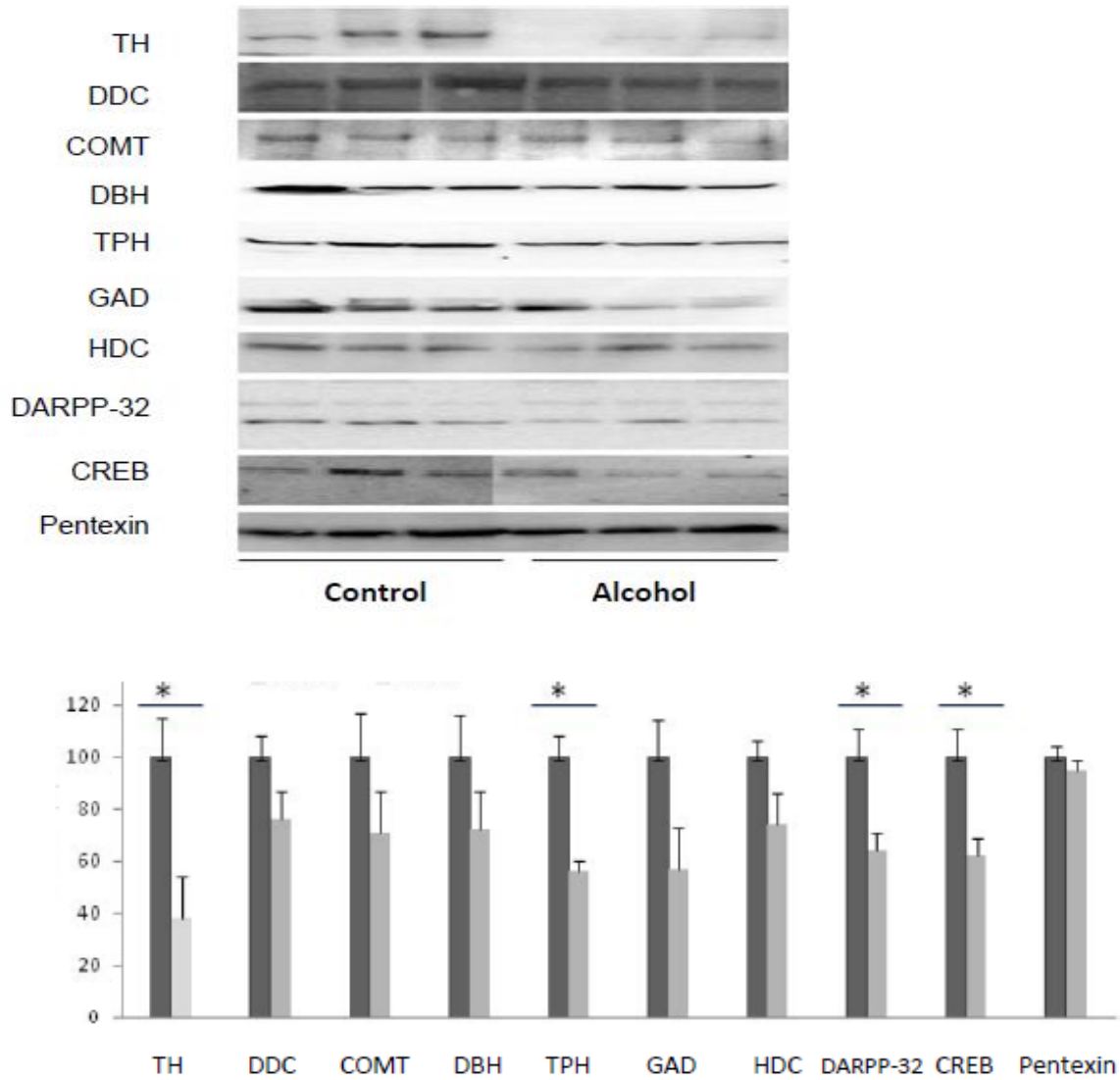
Legend for Figure 1.

Total protein was extracted from the caudate nucleus of both control and alcoholic tissues and Western blotting (top) was performed using antibodies as specified in Materials and Methods. Bands were digitized and quantified; the controls were normalized as 100 (bottom). Columns represent the mean ± SE ($n = 3$ per group; *significantly different from control at $P < 0.05$).

Changes in Neurotransmitter and Metabolite Levels in Alcoholic Striatum:

Acetylcholine

Choline and acetylcholine contents were significantly decreased in alcoholic brains striatum (Table 4) and the degree of depletion appeared variable from one subregion to another but these variations did not reach statistical significance (Table 4).

Changes in Neurotransmitter and Metabolite Levels in Alcoholic Striatum:

Amino Acids

No significant changes in GABA levels were observed between striata of alcoholic brains and control brains (Table 4). Glu content was significantly higher in the VS (30%) relative to the DS (5%) (Table 4). Glutamate decarboxylase (GAD), the enzyme of GABA synthesis, was lower in the CN of alcoholic brains (Fig. 1 and Table 5, see below).

Changes in Neurotransmitter and Metabolite Levels in Alcoholic Striatum:

Histamine

Histamine (His) content was significantly lower in alcoholic brains and the rates of depletion were similar in all regions (Table 4). Preliminary estimations of histidine decarboxylase (HDC), an enzyme that synthesizes histamine, have indicated that its expression was not significantly decreased in AB (Fig. 1).

Glutathione (GSH)

Free radical scavenging molecule GSH content was lower in alcoholic brains. The reduction was highest in CN (67%) followed by the NAc (58%) and P (34%) (Table 4). The only enzyme related to GSH that was found to be significantly decreased in the CN of alcoholic brains was glutathion-S-transferase Mu 3 (cf. the next section on proteomics and Table 5).

Expression of proteins in the caudate nucleus studied by proteomics

The average 2D-gels showed 570 spots in the samples from control brains and 550 spots in the samples of alcoholic brains. Subsequent analysis identified 25 unique proteins which were differentially expressed in the CN of alcoholic and control brains (Table 5). The proteins directly linked to DA included TH and COMT, an enzyme of catecholamine metabolism. One other protein linked to neurotransmitter metabolism, a GABA-synthesizing enzyme GAD65, was also decreased in the alcohol affected tissue. The decrease in TPH indicated by Westerb blotting studies was not, however, supported by the proteomics.

Changes in some of the proteins found to be differentially expressed in the CN of alcoholic and alcoholic brains could be related to oxidative stress, tissue damage and/or neurodegenerations (e.g. glutathione-S-transferase Mu 3, synaptosomal-assocaciated protein 25, amyloid-β-precursor protein-binding family B member 3, BH3-interacting domain death agonist BiD; Table 5).

Table 5. Differentially expressed proteins in the alcoholic dorsal striatum relative to controls

Protein	Access. No.	pI/mass (kDa)	MOWSE score	Change
Tyrosine hydroxylase (TH)	P07101	5.90/59	78	- 3.67
Pyridoxal phosphate phosphatase (PPPase)	Q96GD	6.12/32	80	- 2.06
Catechol O-methyltransferase (COMT)	P21694	5.29/24	93	- 2.56
Serine/threonine protein phophatases/ Protein phosphatase 1B	O75688	4.95/53	52	2.22
Glia maturation factor beta	P60983	5.16/17	89	- 2.04
Intermediate filament family orphan 1	Q0D215	4.83/63	33	- 5.06
Glyoxalase domain-containing protein 2	A6NK44	6.08/17	137	- 2.19
Lys-63-specific deubiquitinase BRCC36	P46736	5.59/37	152	1.10
Synaptosomal-associated protein 25	Q53EM2	4.66/24	74	- 1.62
Proactivator polypeptide	P07602	5.06/60	44	4.50
Glutamate decarboxylase 2/GAD65	Q05329	6.45/65	61	- 3.28
N-Acetylaspartyl-glutamate synthase A	Q81XN7	6.21/43	78	2.99
GTPase NRas	P01111	5.01/21	72	- 3.43
NF-kappa-B inhibitor beta	Q15653	4.70/38	23	2.81
Guanine nucleotide-binding protein G(i) subunit alpha-2	P04899	5.34/30	76	- 1.80
Protein-tyrosine phosphatase 1B	P18031	5.88/51	50	1.40
Phosphoserine phosphatase	P78330	5.53/25	41	- 4.31
Glutathione S-transferase Mu 3	P21266	5.37/27	68	- 1.50
Beta-synuclein	Q16143	4.41/14	71	- 1.59
Putative oncomodulin-2	P0CE71	4.11/12	127	- 1.52

Hippocalcin	Q5U068	4.96/23	120	- 2.08
BH3-interacting domain death agonist (BiD)	P55957	5.27/22	85	2.20
Transforming acidic coiled-coil-containing protein 1	O75410	4.86/16	93	- 2.35
Interferon alpha-1/1	P01562	5.32/22	67	- 1.49
Amyloid beta A4 precursor protein-binding family B member 3	O95704	5.97/54	21	1.99

Tissue samples came from the caudate nucleus. The change is expressed as n-fold increase or (-) decrease. Accession number is for Swiss-Prot database, pI signifies the value of isoelectric point. MOWSE score, molecular weigth search score, a form of protein identification.

Discussion

General

To the best of author's knowledge, this is the first study using the LC-MS/MS technique to obtain information on the effects of long-term heavy alcohol intake on the levels of neurotransmitters and related metabolites in striatal regions of human brain. The major findings in this study are the following: First, lower levels of Tyr, Tryp, His, Ch, Ach, DA, 5HT, HVA, HIAA and GSH in alcoholic brains compared to control brains. Second, variation, in some cases, of the depletion rates from one part of striatum to another, suggests that neurotransmitter metabolism in the striatum does not have the same sensitivity to chronic alcohol in all subregions (Table 4). Third, the ratios of DOPAC/DA and HIAA/5HT were, in alcoholic brains higher in the VS relative to the DS. Thus it appears that both the VS and DS are sensitive to the effects of chronic alcohol intake. The susceptibility of DS to the chronic excessive alcohol intake is also in accordance with the observation of differences between alcoholi and control brain in terms of expression of several important proteins in the CN (a part of DS), as revealed by proteomics (Table 5) and WB (Fig. 1).

Regional variations in the striatum of Control brains - differential sensitivity of DS and VS to alcohol:

In control brains, Ch and Ach levels were higher and the NE level was lower in the NAc relative to the dorsal striatum suggesting that either the density of noradrenergic and cholinergic innervations, their synaptic activities or, simply the metabolic rate of Ch, Ach and NE in the VS differs from that in the DS. The above findings are compatible with other published data; for example a comparative study of cholinergic neurons in sub-regions of the striatum revealed that NAc has at least 30% more cholinergic neurons than the dorsal striatum (Meredith et al., 1989). The cholinergic interneurons of the VS have large dendritic arbors that connect many cell bodies including the core and shell subdivisions of the NAc. Cholinergic neurons have been shown to play an important role in the modulation of both food and drug intake including aversive responses to foods or drug-related addictive behaviours (Avena and Rada, 2012, Morilak et al., 2005). The stress-related neurotransmitter norepinephrine (NE) (Morilak et al., 2005) is lower in the VS relative to DS (Table 3).

The levels of catabolic products of DA and 5HT and their ratios in the DS did not differ significantly from those in NAc. This is consistent with the morphologic similarities of DS and the VS. Region-specific variable metabolite levels in the rat striatum have, however, been previously reported (Laplante et al., 2012, Schilman et al., 2010); it is, therefore, possible that more subtle differences will be revealed by future studies using larger numbers of human brains.

In our earlier proteomic study it is noted that free radical scavenging (GSH metabolism) and pentose phosphate pathways were dominantly expressed in the splenium of corpus callosum (CC) relative to the genu of CC and, the higher activities of these two pathways might have been linked to the region-specific sensitivity to the alcohol. Considering the present results in the context of the previous evidence and in analogy with previous conclusions, author propose that the neurochemistry of the NAc is different from that of DS in terms of alcohol-sensitivity of metabolic pathways associated with noradrenergic, dopaminergic and cholinergic neurotransmission and these differences may contribute to the pathophysiology of alcoholism and alcohol addiction (Dobbs and Mark, 2012, Mark et al., 2011).

Alcoholism-associated region-specific changes in neurotransmitter metabolism

Previous studies have preferred NAc as the striatal region most important for the mechanisms of addictive behaviour, however there are indications that the DS may also be involved in addiction-related mechanisms (Volkow et al., 2003, Porrino et al., 2004, Volkow et al., 2004) even if this may involve its connections to VS (Nummenmaa et al., 2012). Specifically, alleviation of alcohol and nicotine addiction after a cerebrovascular incident in the human the DS has been reported (Muskens et al., 2012). In the present study author has noted that the contents of most of the neurometabolites in the DS and theVS are similar (Table 3) but alcohol-induced expression patterns, in particular the relative changes (Table 4), in alcoholic regions (CN, P and NAc) are more variable. These results are reminiscent of those of Tran-Nguyen et al (Tran-Nguyen et al., 1996) who found that the administration of the dopamine-depleting toxin 6-hydroxydopamine into the DS and the VS produced a heterogeneous response in terms of changes in monoamine content in the regions.

Thus DA in the DS might be involved in the addiction-related process analogous to that which exists in NAc and it is possible that both parts of the striatum contribute to drug craving/relapse and/or habitual learning involved in substance abuse (Everitt and Robbins, 2013,

Lee et al., 2013, Natori, et al., 2009). This suggestion is strongly supported by the differences between alcoholic and control brains identified by proteomics in the CN (a part of DS), particularly the reduced expression of DA-related proteins (Table 5).

Glu content in alcoholic brains compared to control brains was significantly increased in NAc (30%) relative to DS (Table 4). This is in contrast to the other signalling molecules but similar findings have been made before; Glu level has been reported as increased in the prefrontal cortex of alcoholic patients relative to control and glutamate/glutamine ratio has been proposed as a biomarker for alcohol-dependence (Hermann et al., 2012, Koob, 2013). L-Glutamate is the principal excitatory neurotransmitter in the mammalian CNS and, as such, would be expected to play the central role in a whole spectrum of brain functions (Bennett and Balcar, 1999, Sery et al., 2015). While short term exposure to alcohol decreases glu activity, long term exposure has the opposite effect (Koob, 2013, Valenzuela, 1997).

Alcohol and oxidative stress

Alcohol has been shown to induce oxidative stress and result in low levels of GSH in the brain cells; in fact, oxidative stress may be one of the most important mechanisms mediating alcohol-induced brain damage. It is, therefore, quite significant that, in this study auhtor observed that glutathione, the free radical scavenging molecule, was reduced by up to 60% in alcoholic tissues (Table 4). This is similar to the reductions in GSH reported by others (Lee et al., 2014). The depletion of glutathione (GSH) would seem to correlate with alcohol-induced reduction in glutathione biosynthesis enzymes as detected previously in author's proteome studies in alcoholic post-mortem human and rat brain (Kashem, et al., 2007, Kashem, et al., 2008, Kashem, et al., 2009a); cf. decrease in glutathione S-transferase Mu 3 in the alcoholic CN in the present study (Table 5). In this context, the decreases in synaptosomal-associated protein 25 and β-synuclein (Table 5) may indicate loss of synaptic contacts and the increase in apoptosis-associated protein BiD (Table 5) might indicate apoptotic loss of neurons caused by oxidative stress. The apparent regional variation in the effect of alcohol on GSH levels (Table 4) merits additional study.

Alcohol Induced Alteration of Neurotransmitter Biosynthesis Pathways

The regionally-variable elevations of Glu content (but no clear change in the content of GABA) as observed in alcoholic brains relative to the control brains are of potential interest, given that a significant component of the ethanol effect on brain metabolism is exerted through a subtype of GABA(A) receptor (Rae et al., 2014) or by NMDA receptors (for a review see (Spanagel., 2009). GABA is synthesized from glutamate via pyridoxal 5'-phosphate dependent glutamic acid decarboxylase (L-glutamate-carboxy-1-lyase, EC 4.1.1.15; GAD). The deficiency of GABA would be consistent with low level of GAD (McBride et al., 1990) and/or deficit of its cofactor (Kashem et al., 2012).

In fact, even when the data in Table 4 were analysed by ANOVA over six independent data groups i.e. including three regions of control striatum and three regions of alcohol-affected striatum, the decreases were not statistically significant at $P = 0.05$ (not shown). GABA/Glu ratio in alcoholic relative to control brain might appear regionally variable; it is 39% in the VS compared to ~ 19% in the DS (Table 4) but, again, these variations were not statistically significant. Proteomics data show reduced expression of GAD65 in the alcoholic CN (a part of DS) compared to the control but this may not be enough to cause a significant deficiency in GABAergic inhibitory system (no significant difference detected by WB; Fig. 1).

Alcohol and histamine are metabolized through a common enzymatic pathway (aldehyde reductase) and administration of ethanol has been shown to decrease His concentration in mice (Brabant et al., 2010); the present findings could be a result of reduced expression of His biosynthesis enzymes HDC (Fig. 1).

Serotonin precursor amino acid Tyr, serotonin, 5HIAA and serotonin metabolic key enzymes tryptophan hydroxylase (TPH) and DDC have all been reported as decreased in alcoholic tissues (McBride et al., 1990, Vilpoux et al., 2009) indicating that long-term alcohol intake could suppress serotonin biosynthesis. Transgenic mice (TPH–2 deficient) displayed 60–80% lower 5HT and increased capacity of ethanol consumption (Jacobsen et al., 2012). Other studies have shown that reduced levels of brain 5HT are associated with increased ethanol intake in both rodents and primates (Sachs et.al., 2014, Higley et al., 1996, Murphy et al., 1982). It has been proposed that 5HT deficiency (e.g., reduced levels of the 5HT metabolite, 5-HIAA) be used as a biomarker of alcoholism (Sachs et al., 2014, Borg et al., 1985).

Dysregulation of catecholaminergic system has been reported in alcoholism (Kohnke et al., 2002). In the present study we observed that DA and its precursor amino acid Tyr and catabolic products, NE, DOPAC and HVA content were all decreased in regions of alcoholic brains relative to their respective controls (Kashem et al., 2012, Sun et al., 2012, Rothblat et al., 2001); these results suggested that alcohol either disturbed and/or damaged DA innervations leading to reduced biosynthesis. If the lower content of DA is caused by higher catabolism, then the alcoholic tissues might be expected to show higher levels of DOPAC, NE and HVA but this has turned out not to be the case in the present study, their contents were actually lower in the alcoholic regions. Moreover, the preliminary data have not shown any statistically significant changes in the two most important catecholamine catabolising enzymes: catechol-O-methyltransferase and dopamine-β-hydroxylase (DBH);(Fig. 1), while more comprehensive 2D-gel proteomic actually indicated reduced expression of COMT in alcoholic brains, at least in the CN (Table 5) Genetic studies of human populations indicated that both high- and low- activity COMT alleles could be associated with alcoholism and the enzyme could also be regulated epigenetically (Sery et al., 2006, Voisey et al., 2011, Wang et al., 2001). Thus, the reasons for the decreases in the content of NE, DOPAC and HVA in alcoholic tissues could be caused by lower availability of the precursor molecule DA or it might be a result of deficient of biosynthesis.

Expression of DA biosynthesising enzymes and proteins associated with DA signalling [TH, pyrophosphate phosphatase (PPP), DARPP-32] have been found altered in the striatum of a beer-drinking rat model of alcoholism (Kashem et al., 2012, McBride et al., 2009) and this seems consistent with the decreased expressions of DARPP-32 indicated by the present study (Weinshenker et al., 2000, McPherson and Lawrence, 2007).

It is interesting to note that the preliminary WB quantification also indicated a decrease in the expression of CREB in the alcoholic tissue. CREB regulates expression of many proteins in brain including TH, TPH, GAD, choline acetyl transferase, DBH, adenylyl cyclase and glutamine synthase (GS) (Asanuma et al., 1996, Lonze and Ginty, 2002). Given that CREB activity is regulated by multiple phosphorylations and its immunological estimations may require special care (Platenik et al., 2005), the present finding would seem to be worth of a more thorough study using a larger sample.

\Concluding Remarks

The present study has identified significant changes in neurotransmitter levels in all regions of striatum in alcoholic brain. There are significant decreases in the levels of several neurotransmitters (dopamine, noradrenaline and acetylcholine) and related metabolites. This is accompanied by similar changes in enzymes (at least in the caudate nucelus) involved in the neurotransmitter metabolism. Additionally, there is a significant decrease in the levels of antioxidant agent glutathione in all parts of striatum in alcoholic brains. This may further exacerbate the harmful effects of chronic heavy alcohol intake on brain tissue. Author has also identified changes to several proteins not directly related to the function and metabolism of neurotransmitters; these may reflect more general aspects of the damage caused by long-term heavy drinking and should be investigated in greater detail in more extensive studies. Despite a small number of brains used in the present study (and the protein data limited to the dorsal striatum only), the findings may add to a more comprehensive model of how alcohol and alcoholism affect neurotransmitter signalling in striatum and lay more solid foundation for the understanding of the relationship between alcohol addiction and alcohol-related disorders.

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CHAPTER 3

Exposure of rat neural stem cells to ethanol affects cell numbers and alters expression of 28 proteins

Abstract

Rat embryonic neural stem cells (rNSC) were exposed to ethanol (25 to 100 mM) for up to 96 hours. Ethanol induced oxidative stress and the cell numbers were lower at 50 and 100 mM ethanol with neuron-like (MAP2-positive) cells particularly affected. Proteomics revealed 28 proteins as altered by ethanol. Some were constituents of cytoskeleton, others were involved in transcription/translation, signal transduction and oxidative stress. Nucleophosmin (NPM1) and dead-end protein homolog 1 (DND1) were further studied by immunocytochemical techniques in cultured neuron and astrocytes. In the case of DND1 (but not NPM1) ethanol induced similar changes in both types of cells. Given the critical role of the protein NPM1 in cell proliferation and differentiation, its reduced expression in ethanol-exposed rNSC could explain the lower cells numbers. However, considering the known effects of ethanol on brain tissue, comparison of the present findings with data on the effects of the anaesthetics sevoflurane, propofol and ketamine on stem cells, as available in the literature, suggests that ethanol may be cytotoxic to rNSC by activating GABA(A) receptors and inhibiting NMDA receptors. Thus the ethanol exposure may profoundly alter protein composition and functioning - including proliferation and survival - of rNSC, resulting in abnormalities which, if translated to humans, could contribute to the molecular and cellular mechanisms underlying the foetal alcohol spectrum disorder and/or alcoholism later in life.

Introduction

Development and maturation of brain tissue includes both pre- and postnatal neurogenesis (Giedd et al., 1999, Anderson et al., 2000, Anderson et al., 1993). The rate of neurogenesis varies as a function of brain maturity (Dennis et al., 2006) but may also be influenced by anxiety, depression as well as by drug and alcohol intake (Cowen et al., 2008).

Neural stem cells (NSC) can generate neuron, astrocytes and oligodendrocytes in the central nervous system (CNS; (McKay, 1997). NSC first migrate to form specific brain regions while, later in the life, during the process of adult neurogenesis, may respond to functional demands or serve as a replacement for damaged cells (Imitola et al., 2004). The normal cell generation may become compromised if the damage to the tissue is too great or the initial insult continues such as in an ongoing stress. The latter would include chronic drug use and alcoholism and, indeed, animal models have demonstrated that alcohol is toxic to the neuron-generating regions in brain (Geil et al., 2014). Even the blood ethanol levels not exceeding 330 mg/dl (app. 72 mM) have been shown to decrease neurogenesis in adolescent rats (Nixon and Crews, 2002, Herrera et al, 2003). In this context, it is of interest to note that blood alcohol level in Indian-American (indigenous) mothers who gave birth to offspring with fetal alcohol syndrome (fetal alcohol spectrum disorder; FASD) was reported at 80 mM (Kvigne et al., 2012); it is known that 100 mM alcohol can significantly influence human neurogenesis (Louis et al., 2017). In another study, low to moderate doses [(0.095% (21 mM) to 0.18% (40 mM)] of ethanol were shown to reduce the number of new neuron by two thirds and significantly increased the rate of cell death in the dentate gyrus (Herrera et al., 2003). Administration of ethanol to 35–40 days old rats indicated that the neurogenesis in the adolescent brain is particularly sensitive to ethanol (Crews et al., 2006, Redila et al., 2006). Moreover, prenatal exposure to ethanol may further compromise postnatal neurogenesis and this may have an additional impact on mental health later in life (Redila et al., 2006, Klintsova et al., 2007).

Production of neuron proceeds in four stages; proliferation of NSC, differentiation, migration and selective neuronal death/survival. Certain proportion of cells will succumb to programmed cell death (apoptosis); this is an integral part of the differentiation process and may accompany even adult neurogenesis. Ethanol interferes mainly at stages one and four (Teteno et al., 2006, Campbell et al., 2014); it inhibits NSC proliferation and increases the cell death. Longer exposures (a four-day binge model) result in reduced cell proliferation and impaired survival (Morris, et al., 2010). Moreover, ethanol at 50 mM concentration can affect both the proliferation of NSC and gliogenesis (glial phenotype); ethanol can alter intrinsic cellular mechanisms of NSC, eventually impacting on the structural and

functional characteristics of both neuron and glial cells (Vemuri et al., 2005). Such changes occur in prefrontal cortex, nucleus accumbens, and striatum (MacDonald and Roskams, 2009, Witt, 2010) thus potentially contributing to alcohol-induced functional deficits in those regions (Liyanage et al., 2014, Stragier, et al., 2015, Bonnaud et al., 2016).

Protein composition is of key importance to the understanding of normal cellular functions and their changes in disease. The technique of proteomics can thus serve as a convenient indicator of the state of health of a biological system. Specifically, proteomic analyses of human brain tissue have identified changes in 238 proteins as associated with alcoholic disorders (Kashem et al., 2007, Matsuda-Matsumoto, 2007, Matsumoto et al., 2007, Kashem et al., 2008, Kashem et al., 2009a,Kashem et al., 2012, Kashem et al., 2016).

Proteomic analysis of human neural stem cells (hNSC) detected thousands of proteins at the differentiation stage (review: vanHoof et al., 2012) but little is known how this proteom is influenced by ethanol. The aim of the present study was to investigate *in vitro* changes in the proteome of rat embryonic neural stem cells (rNSC) following a chronic exposure to the concentrations of alcohol which could be encoutered *in vivo* during heavy (or very heavy) drinking. Firstly, we looked at whether ethanol applied for 96 hours at concentrations 25, 50 and 100 mM can influence the numbers of cultured rNSC and whether such changes are accompanied by an increased lipid peroxidation indicating the presence of oxidative stress. Secondly, we used a proteomic approach to study the protein composition of rNSC exposed to 50 mM ethanol for 96 hours. Four of the proteins found to be significantly changed (two increased and two decreased) by the exposure to ethanol were estimated by Western blotting to verify the validity of the proteomic analysis. Additionally, two of the proteins strongly affected by the ethanol exposure (nucleophosmin; NPM1 and dead end homolog1; DND1; both classified as "nuclear proteins" and therefore deemed as potentially involved in the regulation of protein synthesis as well as cell proliferation and cell survival were selected for further studies by both Western blotting and immunocytochemistry, using primary cultures of neuron and astrocytes derived from rat brains.

The data we present may contribute to a better understanding of how heavy alcohol drinking (particularly during pregnancy) alters the brain structure and function.

Materials and Methods

Neural stem cell culture

Rat neural stem cells (rNSC) were prepared from 14-day-old (E14) rat embryos as described earlier (Tateno, et al., 2006, Kashem et al., 2009). Animal experimental procedures were approved by the University of Sydney Animal Ethics Committee (AEC Protocol Number: 2013/5742). The telencephalon was separated and blood vessels and meningae were removed while the tissue was submerged in ice-cold Hank's balanced salt solution (HBSS; Invitrogen, CA USA). After sectioning, the telencephalon tissue was incubated with 0.05% trypsin solution for 30 min, triturated with a glass pipette and filtered through 70 µm nylon cell strainer (Falcon, USA). The resulting cell suspension was centrifuged (300 g for 5 minutes at 4°C). The viability of the cells in the pellet was assessed by trypan blue (Invitrogen). The cells were seeded in complete neurobasal medium [(NBM, Invitrogen) + 2% B27 supplement (Invitrogen), + 0.5 mM L-glutamine + 20 ng/ml recombinant human fibroblast growth factor 2 (FGF-2) + 20 ng/ml recombinant human epidermal growth factor (EGF, Life Tech)] at a density of 1 x 10^6 cells per mL in culture dishes coated with poly-L-ornithine (Sigma). The dishes were maintained for a week at 37°C in a 5% CO_2 atmosphere. For differentiation, the cells were washed and cultured in differentiating media (all components of complete neurobasal media except FGF-2 and EGF).

Neuron and astrocytes

The cortex tissue was obtained at the embryonic age (E) of either E15 (neuron) or E20 (astrocytes) and treated as above. Following recommendations by available culture protocols (e.g. https://www.thermofisher.com/au/en/home/references/protocols/cell-culture) the cells were cultured either in neurobasal medium (NBM, Invitrogen) + 2% B27 supplement (Invitrogen), + 0.5 mM L-glutamine with antibiotics (neuron) or in serum/antibiotics containing DMEM/F-12 (astrocytes) for 2 weeks at 37°C in humidified 5% CO_2/95% air conditions. While most of the protocols usually recommend brain tissue harvested at embryonic ages E17 or E18 for the neuronal cultures and brains from later embryonic stages or neonatal rats for the preparation of astrocytic cultures (Hansson, 1984) we used E15 for the neuronal cultures (cf. also (Dichter, 1978) and E20 for the astrocytic culture with satisfactory results, as indicated both by the appearance of the cells and expression of the characteristic markers (Fig. 3 and 4).

Ethanol treatment and sample collection

The cultured cells were exposed to ethanol (0, 25, 50 and 100 mM) added directly to the differentiation neurobasal media. In order to reduce the loss of ethanol by evaporation (Rodriguez et al., 1992), author used a closed system (closed 25 mL vessel) and changed the media every 24 hours

(Rodriguez et al., 1992, Eysseric et al., 1997). The ethanol exposure lasted for up to 96 hours. For proteomics, the cells were washed twice with ice-cold phosphate-buffered saline (PBS) and collected in 200 μL solubilising buffer (7 M urea, 2 M thiourea, 1% C7bZO and 40 mM Tris-HCl, pH 10.4; Sigma). After sonication (3 times for 5 sec each) the samples were stored at -80°C (Kashem et al., 2009, Ahmed et al., 2012).

Estimation of cell numbers and immunofluorescence

Cells on coverslips were fixed with 4% paraformaldehyde for 15 min, followed by washing with PBS and exposure to 0.3% Triton-X100 for 5 min. After washing with PBS, the cells were incubated (blocked) with 5% normal horse serum for 1 hour, followed by anti-microtubule-associated-protein 2 antibody (MAP2, mouse, 1:1000; Sigma) for 2 hours. The cells were further incubated in mouse IgG FITC labelling reagent (1:1000, Invitrogen) for 45 min and subsequently washed with PBS. Following the application of DAPI (for 10 min) cells were observed under Leica SPE2 confocal microscope (Leica Microsystem). The images were captured and the cells with DAPI-labelled nuclei were counted (about 150 per image) using Image J software (manual option). Whole coverslip contained, typically 200 to 300 cells or fewer in ethanol treated cultures. (cf. legend of Fig. 1 for further details).

For immuno-histochemistry of neuron and astrocytes, the cells were fixed, treated with Triton X-100 and blocked with horse serum as described above (immunofluorescence) and incubated with a mixture of neuron- or astrocyte-specific marker antibodies. For neuron, anti-microtubule-associated-protein 2 (MAP2, mouse and rabbit, 1:1000; Sigma) was used as a marker while anti-glial fibrillary acidic protein (GFAP, mouse and rabbit, 1:1000; Sigma) was applied to label astrocytes. The cells were further incubated with anti-rabbit nucleophosmin (NPM1) or anti-mouse dead end protein homolog 1 (DND1; Santa Cruz Biotech, Australia) for 2 hours. Anti- rabbit and/or anti-mouse secondary antibodies conjugated with CF-568 and/or CF488 were treated for 45 min and then washed with PBS. The cells were observed under confocal microscope (Leica SPE2) and the intensity of the fluorescence was quantified by image J software (Shin et al., 2009, Nguyen et al., 2010).

Lipid peroxidation assay

Acohol treated and untreated cells were homogenised in 0.5 mL of 1% KCl solution and supernatant was collected after centrifugation at 16,000 g x 10 min at 4 ^{0}C. Aliquots of 0.25 mL of the homogenates were mixed with double volume (0.50 mL) of 15% acetic acid containing thiobarbituric acid (0.5% (Garcia et al., 2005). The mixture was heated to 95 ^{0}C for 15 min and, after cooling, centrifuged at 5000 g x 5 min to remove any precipitated matter. The absorbance of the supernatant was measured at 532 nm. The malondialdehyde concentration was calculated from the following formula: absorbance/1.56 x 105; (where 1.56 x 105 M^{-1}cm^{-1} is the malondialdehyde extinction coefficient).

Statistical analyses and presentation of data

Statistical evaluation of differences between alcohol-free (controls) and alcohol-treated cells was done by ANOVA (Tukey multiple comparison test). Both the statistics and the graphical representation of data (Figs. 1, 3 and 4) was done using GrapPad Prism software version 7.02 except for Fig 2 where BioRad software was used. For details see the figure legends.

Proteomics

The cells (exposed to ethanol for 96 hours; cf. the section on "Ethanol treatment and sample collection" above) were trypsinized, extracted with urea buffer (7 M urea, 2 M thiourea and 1% C7bZO and 40 mM tris-HCl) and the proteomics performed as detailed earlier (Kashe et al., 2007, Kashem et al., 2008, Kashem et al., 2009a, Kashem et al., 2012, van Nieuwenhuijzen et al., 2010), Kashem et al., 2016). The extracted samples were pelleted at 16,000x g for 20 min at 15 °C; the supernatant was reduced and alkylated with 5 mM tributyl phosphine and 10 mM acrylamide monomer at room temperature for 2 hours. The reaction was quenched by 10 mM dithiothreitol (DTT). The citric acid was used to acidify the samples to approximately pH 5. The acetone-precipitated pellets were air dried and resuspended in 0.3 mL of buffer consisting of 7 M urea, 2 M thiourea and 1% C7bZO. The final extracts were stored at -80 °C before isoelectric focusing.

The protein was quantified (Bradford, 1976) using BSA (Sigma-Aldrich, Castle Hill, NSW, Australia) as a standard. Details of isoelectric focusing, electrophoresis and staining have been published Kashe et al., 2007, Kashem et al., 2008, Kashem et al., 2009b, Kashem et al., 2012, van Nieuwenhuijzen et al., 2010), Kashem et al., 2016). A total of 12 gels (duplicates for each sample, n=3) were scanned using a flatbed scanner (UMAX). The images were analysed by Phoretix 2D Expression software (Nonlinear). Averaged gels were created for each alcohol treated/control group and averaging parameters were set at 70%. Protein spots were evaluated as volumes (spot area x optical density) with the image analysis software and compared between groups with statistical analysis (ANOVA, $P<0.05$). The false discovery rate (FDR) was calculated as described by Storey (Storey and Tibshirani, 2003). The protein spots of interest were cut from the gel, de-stained using 25 mM NH_4HCO_3/50% (v/v) acetonitrile (ACN) for 3 min × 15 min at 37 °C and digested with 12.5 ng/mL trypsin (Roche, sequencing grade), The peptides were purified using C_{-18} purification tips (Eppendorf). Matrix (5 mg/mL solution of α-cyano-4-hydroxycinnamic acid in 70% (v/v) ACN/0.1% (v/v) TFA) mixed samples were analysed by Qstar XL Excll Hybride MS system (AB Applied Biosystems) in positive reflector mode, with delayed extraction.

The spectra from MALDI-TOF were searched against the Swissprot protein databases using the MASCOT search engine (http://www.matrixscience.com/). Positive protein identification was performed

based on a MOWSE score (> 54, rattus database) with matched isoelectric pH (pI) and molecular weight (MW) values (estimated from 2D gels) and sequence coverage.

Western blot analysis

Alcohol treated (96 h) brain cells were collected (n=3) and protein was extracted by previous described techniques (Kashem et al., 2012). Western blot analysis was performed as described previously (Kashem et al., 2009b, Kashem et al., 2012, Kashem et al., 2016). Protein separated (20 µg) by SDS-PAGE were transferred to PVDF (polyvinylidene difluoride) membranes (Kashem et al., 2009) blocked with 5% skim milk and incubated with primary antibodies [(NPM, DND1, heat shock protein, heterogeneous nuclear ribonucleoproteins-C (hnRNP-C), enolase and synaptosome associated protein-29 (SNAP)] solution. Secondary antibodies (anti-mouse and anti-rabbit IgG, Sigma) were added and the spots were visualized using an ECL (enhanced chemiluminescence) full spell system (GE Healthcare, Australia). The intensity of the spots was quantified by Biorad software (Bio-Rad, Sydney, Australia).

Results

Exposure (96 hours) to the lowest ethanol concentration (25 mM) had no significant ($P > 0.05$) impact on either cell numbers or morphology but the higher concentrations (50 or 100 mM) reduced cell growth (Fig 1a). MAP-2 positive cells appeared to be reduced in numbers at 50 and 100 mM ethanol relative to control (Fig 1a). The reduction in cell numbers was quantified by counting the cell nuclei stained by DAPI. DAPI-positive cells were reduced in numbers by 38% and 52% ($P < 0.05$) at 50 and 100 mM ethanol relative to control (Fig. 1b). Lipid peroxidation analysis (Fig. 1b) revealed that the exposure of the cells to ethanol induced oxidative stress compared to controls (at 50 mM about 1.6-fold and at 100 mM about 2.1-fold).
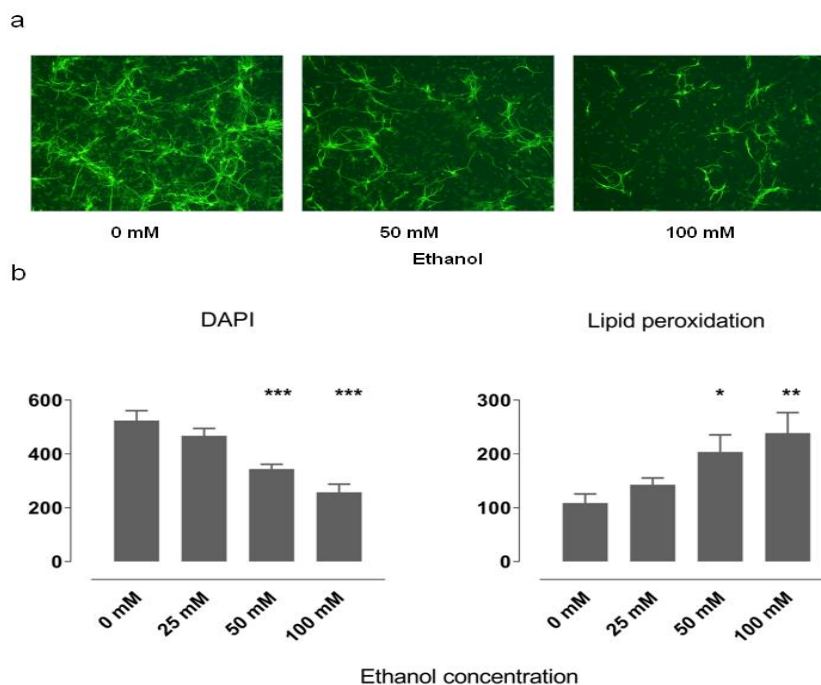

Fig. 1. Immunofluorogenic visualization of rat neural stem cells treated with various concentration of alcohol at 96 h of incubation (a). Fig.1a is MAP-2 positive cells growth. Fig.1b shows the numbers of DAPI stained nuclei and the extent of lipid peroxidation in the cells after 96 hours of ethanol exposure. The scales are in arbitrary units and the columns are means ± SD (n = 3). The data were analysed by ANOVA (Tukey multiple comparison test) and found significantly different from control (0 mM ethanol) at * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

In the proteomics, typical 2-DE gels displayed 550 spots (six gels from controls and six gels from ethanol-treated samples) and more than 92% of all spots were matched across the two groups. Analysis revealed that 40 protein spots were differentially expressed in ethanol-exposed cells relative to the ethanol-free controls ($P < 0.05$; ANOVA); 17 protein spots (~ 43%) showing an increased expression while the remainder were decreased. From the differentially expressed protein spots, author has positively identified 28 proteins using MALDI-TOF (Table 1) while the remaining ones were not identifiable because of low expression of proteins. Functionally, the identified proteins could be classified as structural (two proteins, ~ 7%), intracellular metabolism (six proteins, ~ 21%), nuclear (seven proteins, ~ 25%), related to oxidative stress (six proteins, ~ 21%), signal transduction (four proteins, ~ 14%) and no specific class (three proteins, ~ 11%). Among the identified proteins, nine were increased (including two out of six metabolic, three out of seven nuclear and three out of six oxidative stress-related proteins) while the remaining ones were decreased (including both structural, four out of six metabolic, four out of seven nuclear, three out of six oxidative stress-related and all four signalling proteins).

Heat shock protein (HSP), synaptosomal associated protein-29 (SNAP-29), enolase (α) and heterogeneous nuclear ribonucleoprotein C (hnRNP C) which were altered in ethanol-exposed rNSC (Table 1) were assessed by Western blotting (Fig 2). These analyses did not contradict the outcomes of the estimations obtained by the proteomic studies (Table 1) and were not pursued further. Nucleolar phosphoprotein (nucleophosmin, NPM1) and dead end homolog1 (DND1) which were selected for immunocytochemical analyses in cultured neurons and astrocytes were also estimated by Western blotting in those preparations (Fig 3 and Fig 4).

Immunofluorescence indicated somewhat higher expressions of NPM1 at 25 mM ethanol, especially in neuron (cf. also Western blotting in Fig 3d and 3f), but a lower expression in astrocytes exposed to 100 mM ethanol (Fig. 3e). The immunofluorescence was detected particularly over the nuclear area though author also noticed the NPM staining in cytosolic area at the lowest concentration (25 mM) of ethanol (Fig. 3c).

In the case of DND1, immunocytochemical analysis revealed that the expression significantly increased both in neuron and astrocytes at 25, 50 and 100 mM ethanol (Fig 4). Western blotting was consistent with the immunocytochemistry, indicating that DND1 protein expression increased in both neuron and astrocytes at the higher (50 and 100 mM) concentration of ethanol (Fig 4).
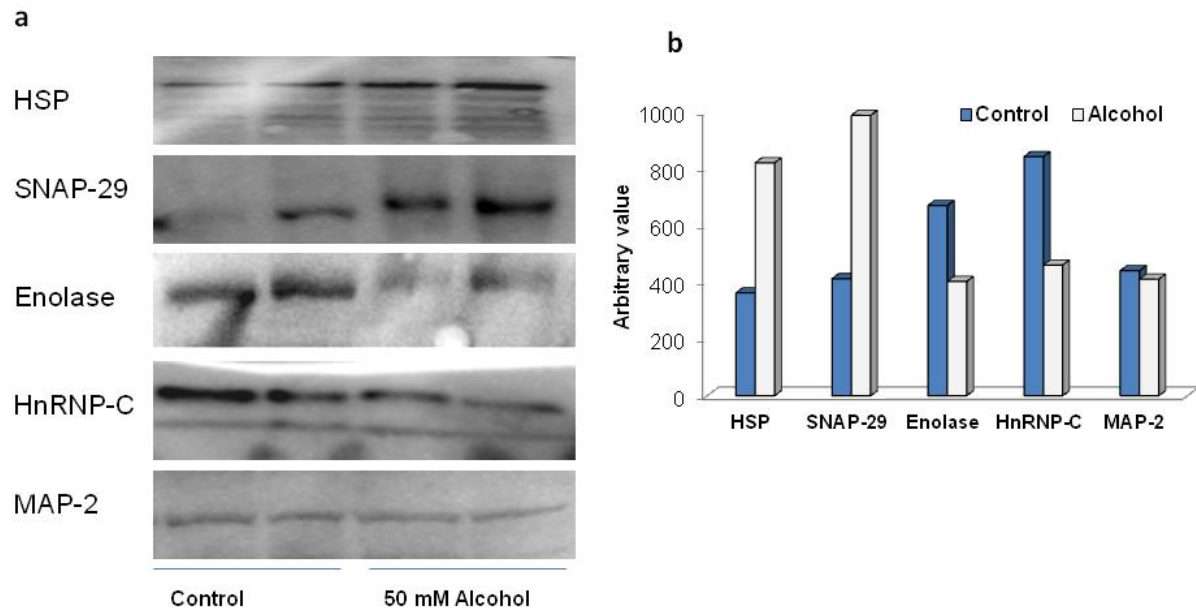
Fig 2 Total protein was extracted from the neural stem cells from both control and 50 mM alcohol-exposed cells and western blotting was performed on selected proteins identified as altered by alcohol-exposure by proteomics. MAP-2 was used as an internal control. Bands were digitized and evaluated by Bio-Rad software. Fig 2b represents average values of two bands.
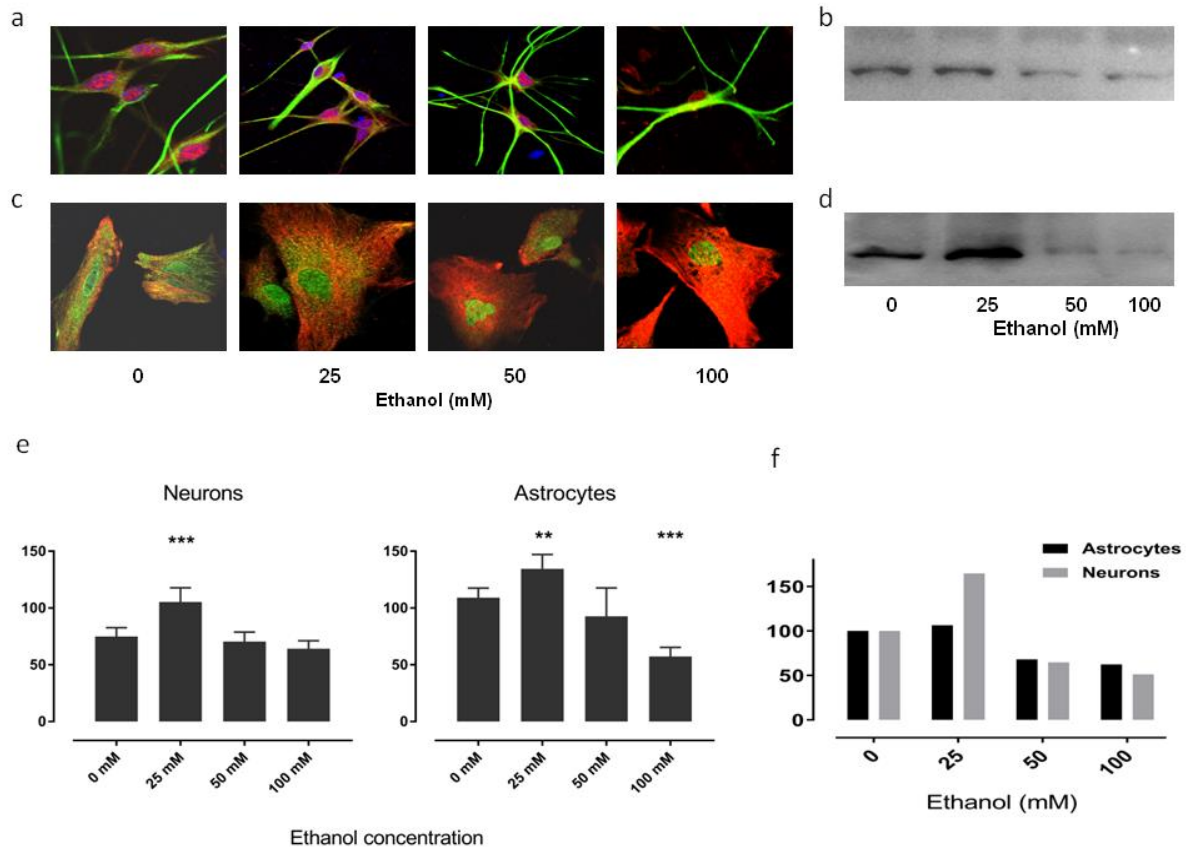
Fig. 3 Immunocytochemical visualization of the expression of NPM1 in neuron (a) and astrocytes (c) exposed to various concentrations of alcohol for 96 hours. The green colour of the Fig 3a is MAP-2 and red colour is NPM1 protein; in astrocytes (c), green is NPM1 and red is GFAP. Intensity of fluorescence was estimated for 9 or 10 cells by Image J software (e). The scales (e) are in arbitrary units and the columns are means ± SD (n = 9-10). The data were analysed by ANOVA (Tukey multiple comparison test) and those significantly different from control (0 mM ethanol) are marked by asterisks as ** $P < 0.01$ and *** $P < 0.001$. Total protein was extracted from cells exposed to 50 mM alcohol for 96 h and subjected to western blotting using an antibody against NPM1 protein (neurons: b, astrocytes: d). Bands were analysed by Bio-rad software; Fig 3f shows average of the two bands shown in b and d, normalized so as the control (0 mM ethanol) equals 100.
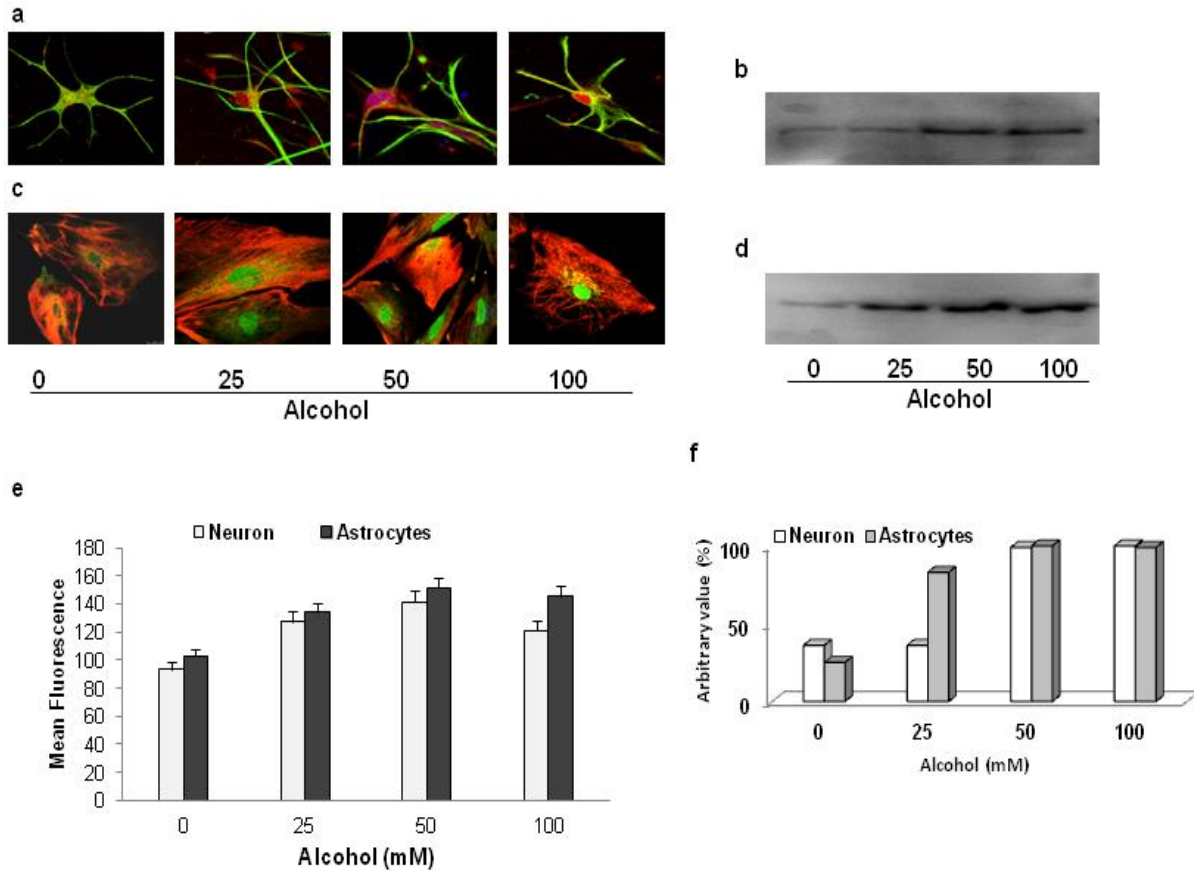
Fig. 4. Immunocytochemical visualization of the expression of DND1 in neuron (a) and astrocytes (c) exposed to various concentrations of alcohol for 96 hours. The green colour of the Fig 4a is MAP2 and red colour is DND1 protein; in astrocytes, green is DND1 and red is GFAP. Intensity of fluorescence was estimated for 10 or 11 cells by Image J software (e). The scales (e) are in arbitrary units and the columns are means $\pm$ SD (n = 10-11). Total protein was extracted from cells exposed to 50 mM alcohol for 96 h and subjected to western blotting using an antibody against DND1 protein (neurons: b, astrocytes: d). Bands were analysed by Bio-rad software.

Table 1. Alcohol induced differential expression of proteins in rNSC exposed to 50 mM ethanol for 96 h.

Protein Class	Fold change (p/q values)	Accession no	PI/Mass (kDA)	Protein Name	Function
Structural Protein	-1.68 (0.048/0.095)	P47942	5.95/62	Dihydropyrimidinase related protein-2 (DRP-2)	Involves neural development, polarity, axon growth and guidance, migration and remodelling of the cytoskeleton.
	-1.56 (0.028/0.065)	Q62952	5.96/62	DRP-3	
Metabolic protein	-2.98 (0.018/0.025)	P04764	5.0/47	Enolase (α)	Glycolytic enzyme works as marker of stress in cellular system.
	-1.98 (0.024/0.045)	Q02589	5.5/40	ADP-ribosylarginine hydrolase	Regulates G1-S phase of cell cycle and regulates DNA stability.
	-1.76 (0.0161/0.027)	Q68FS2	5.4/46	COP9 signalosome complex subunit 4	Involves in various cellular & developmental processes including signalling.
	2.88 (0.0032/0.026)	P54921	5.04/40	Soluble NSF attachment protein (α)	Required for vesicular transport between the endoplasmic reticulum and the Golgi apparatus
	5.92 (0.0012/0.016)	Q9JI56	4.66/29	SNAP-29 protein (synaptosomal associated protein-29)	The protein encoded by this gene binds tightly to multiple syntaxins and is localized in intracellular plasma membrane.
	-1.88 (0.0014/0.022)	Q8VHK7	5.2/26	Hepatoma-derived growth factor	Heparin-binding protein, with mitogenic activity for fibroblasts. Acts as a transcriptional repressor and involves in proliferation, angiogenic, and neurotrophic activity.
Nuclear protein	-1.68 (0.0022/0.036)	P13084	5.1/32	Nucleolar phosphoprotein (nucleophosmin, NPM-1 or B-23)	Involved in diverse cellular processes such as ribosome biogenesis, centrosome duplication, protein chaperoning, histone assembly, cell proliferation, and regulation of p21/p53.
	1.75 (0.0033/0.046)	Q8IYX4	6.9/38	Dead end homolog protein 1	RNA-binding protein that positively regulates gene expression by prohibiting miRNA-mediated gene suppression.
	-3.74 (0.0024/0.039)	Q8VHV7	5.32/45	Heterogeneous nuclear ribonucleoprotein H	These proteins are associated with pre-mRNAs in the nucleus and appear to influence pre-mRNA

	-2.69 (0.0032/0.026)	P07910	5.4/33	Heterogeneous nuclear ribonucleoprotein C	processing and other aspects of mRNA metabolism and transport. hnRNP C heavily involved in the alternative splicig mechanisms
	-2.70 (0.0327/0.081)	Q63413	5.42/49	Spliceosome RNA helicase Bat-1	Involved in nuclear export of spliced and unspliced mRNA.
	1.80 (0.012/0.047)	Q68FR9	5.02/35	Translation elongation factor-1 (δ)	Regulates induction of heat-shock-responsive genes through association with heat shock transcription factors and direct DNA-binding at heat shock promoter elements
	1.53 (0.042/0.067)	Q3T1J1	5.12/16	Translation initiation factor 5A-1	Involve in cell proliferation and regulation of apoptosis.
Oxidative stress	3.5 (0.01/0.04)	P34058	5.37/90	Heat shock protein (HSP-90)	Molecular chaperone, promotes maturation and maintenance the structure of the proteins and control signal transduction.
	4.44 (0.012/0.047)	P55063	5.3/70	Heat shock protein (HSP-70)	
	1.88 (0.016/0.037)	P63039	5.23/60	Heat shock protein (HSP-60)	
	-2.65 (0.0079/0.042)	Q6AXV9	6.14/25	Glutathione S transferase	Exhibits glutathione-dependent thiol transferase activity
	-1.68 (0.002/0.0205)	P28480	5.8/60	T-complex protein 1 subunit	Molecular chaperone; assists the folding of proteins upon ATP hydrolysis.
	1.63 (0.007/0.040)	Q5VLR5		BWK4	Cell redox homeostasis
Signalling protein	-1.68 (0.0015/0.029)	P62138	4.95/53	Serine/threonine protein phosphatase (PP-1)	PP-1 is essential for cell division, and regulates glycogen metabolism, and protein synthesis. It is associated with over 200 regulatory proteins and dephosphorylates 100 of biological targets.
	-2.2 (0.0012/0.0197)	Q32PX6	6.9/21	Ras homolog gene family, member G	GTPase activity
	-2.2 (0.0013/ 0.018)	P50399	5.5/50	RAB GDP dissociation inhibitor (β)	Regulates the GDP/GTP exchange reaction of most Rab proteins by inhibiting the dissociation of GDP from them.

	-1.82 (0.0013/ 0.018)	P50398	5.2/50	RAB GDP dissociation inhibitor (α)	Regulates the GDP/GTP exchange reaction of most Rab proteins by inhibiting the dissociation of GDP from them.
Not classified elsewhere	1.91 (0.001/0.0197)	Q9GZS3	7.0/33	WD repeat-containing protein 61	Component of the polymerase associated factor and is implicated in regulation of development and maintenance of embryonic stem cell pluripotency.
	-1.86 (0.007/ 0.0177)	Q62952	5.95/61	Collapsin response mediator protein 4 (CRMP-4)	Plays a role in axon guidance, neuronal growth cone collapse and cell migration
	-1.49 (0.0014/0.0247)	Q5RKH6	5.5/75	Protein OS-9	May bind terminally misfolded non-glycosylated proteins

Discussion

Inhibition of glutamate NMDA receptors (Lovinger et al., 1990) and/or activation of a subtype of GABA receptors (Spanagel., 2009, Rae et al., 2014, Rae et al., 2014a) parallel immediate effects of alcohol-drinking and probably account for much of the short-term response and consequences (acute alcohol intoxication followed by "morning after") (Davidson et al., 1995, Hoffman, 1995). In contrast to the receptor-mediated acute effects, longer exposures to ethanol may trigger additional mechanisms leading to profound changes in the living cells and tissues. Apart from resulting in cytotoxicity, chronic alcohol can specifically influence protein synthesis and cell proliferation, particularly in the developing brain (Berg et al., 2013, Ming and Song, 2011). The present study reports changes in the protein composition of cells cultured from developing rat brain following a four-day exposure to high concentrations of ethanol.

As author did not specifically investigate neural stem cell functions and properties; the "rNSC" culture represents, strictly speaking, merely a primary culture of fetal brain tissue at certain stage of development. However, the methodology that author used is known to produce > 95% NSC (Kashem et al., 2009) and the results in Fig. 1a are, therefore, consistent with significant cytotoxic effects of alcohol on the fetal rat brain-derived stem cells. This is further underscored by the increased lipid peroxidation in the presence of alcohol (Fig 1b). Lipid peroxidation has been used as a marker of oxidative stress associated with cytotoxicity in a range of brain cells and tissues (Stastny, et al., 2004, Garcia et al., 2008, Rangel-Lopez et al., 2015). Therefore, the current data suggest that oxidative damage is a potentially significant component of the ethanol-related cytotoxicity leading to the loss of cells cultured from embryonic (E15) rat brain tissue.

Given that ethanol interferes with the NSC proliferation and increases the cell death (Tateno, et al., 2006, Campbell et al., 2004) the remainder of the following discussion will focus on proteins potentially involved in the cell proliferation/differentiation, oxidative stress and nucleus-associated mechanisms.

Cell Proliferation and Differentiation

Hepatoma-derived growth factor (HDGF) was significantly influenced by ethanol exposure. HDGF has been linked to hepatocellular carcinoma (HCC) as a multi-functional protein involved in cell proliferation, angiogenesis and anti-apoptosis (Enomoto et al., 2015). HDGF overexpression promotes the proliferation of HCC (Enomoto et al., 2015a) and/or gliomas (Zhang et al., 2012) while the reduction in HDGF expression inhibits the proliferation of HCC cells. Knocking down of nuclear HDGF expression in the cells of human glioblastoma multiformis induces apoptosis (Hsu et al., 2012). By analogy, the

decreased expression of HDGF in ethanol-exposed cells could explain lower proliferation rates of brain cells early in the development.

Two isoforms of dihydropyrimidinase-related protein-2 and 3 (DRP-2 and DRP-3) were reported to be lower in ethanol-exposed cells (Alexander-Kaufman et al., 2006) The DRP family of proteins, also called the collapsin response mediator proteins (CRMP), has been implicated in the development of the CNS particularly in axon guidance and cell migration. Lower DRP-2 could, therefore, disturb neural development and plasticity (Nakata et al., 2003) and this is supported by reports of DRP-2 being decreased in other conditions involving abnormal development such as schizophrenia and Down's syndrome (Johnston-Wilson et al., 2000). Alternatively, the decreased DRP-2 levels could be a result of proteolysis activated by cytosolic Ca^{2+} increased by the ethanol-exposure (Luo, 2014); cf. also the effect of ethanol on Ca^{2+}-permeable NMDA receptors (Davidson et al., 1995).

ADP-ribosylarginine hydrolase (ADPRH) removes mono-ADP-ribose moieties from arginines in cellular proteins. Mutation in the *ADPRH* gene alters G_1 stage of the cell-cycle and a reduced ADPRH in NSC exposed to ethanol could lead to altered G_1-S phase thus decreasing the cell proliferation (Kato et al., 2011).

NPM1, a nucleolar and nucleoplasma-localized protein (Grisendi and Pandolfi, 2005, Qing et al., 2008, Pfister and D'Mello, 2015), may bind to unduplicated centrosomes thus altering Cyclin E/CDK2 activity which, in turn, regulates G_1/S transition (Okuda et al., 2000). NPM1 has been shown to foster survival of proliferating cells (Lindstrom, 2011, Box et al., 2016), therefore the lower cell proliferation in the present study may have been caused by the lower expression of NPM1 induced by ethanol and/or its metabolites (see also below in the section on "Nuclear Activities and Protein Synthesis").

Changes in Oxidative Stress Defence Pathways

Ethanol may generate free radicals [review (Luo, 2014)] *in vivo* either through its metabolism (e.g. via the oxidation to aldehyde) or by interfering with radical scavenging systems. Indeed, long-term chronic ethanol induced lipid peroxidation and depleted GSH levels in human striatum (Kashem et al., 2016). Reduced expression of glutathione transferase (GST) in the present study is also reminiscent of results obtained in human postmortem brain tissue of heavy drinkers (Kashem et al., 2007, Kashem et al., 2008, Kashem et al., 2009, Kashem et al., 2012, Kashem et al., 2016). The ethanol-produced reactive oxygen species are eliminated by GST activity (Peter and Chiramel, 2013) and low expression of GST reduces the protection of neurons against oxidative insults.

The glycolytic enzyme α-enolase also acts as a neurotrophic factor (Takei et al., 1991), a heat-shock protein (HSP48) and a hypoxic stress protein (review: Diaz-Ramos et al., 2012). Glycolytic enzymes including α-enolase are enriched in apoptotic cells and α-enolase deficit could compromise the

hypoxia tolerance (which may include nonglycolytic mechanisms (Diaz-Ramos et al., 2012, Subramanian and Miller, 2000).

Ethanol increases the presence of SNAP25 and SNARE complex (Fatemi et al., 2001, Tafoya et al., 2006) and these changes may indicate a defensive action against the effects of ethanol (Varodayan et al., 2011).

Heat shock protein 70 (hsp70) expression is regulated by environmental stresses including ethanol exposure (Mandrekar et al., 2008, Gorini et al., 2013). Upregulations of hsp70 mRNA, hsp70 protein and heat shock factor-1 (HSF1) have all been reported in the liver of mice exposed to acute ethanol (Varodayan et al., 2011, Gorini et al., 2013). Acute and chronic ethanol induces HSP genes such as *hsp70*, *hsp90*, and glucose-regulated protein 78 (*GRP-78*), via HSF-1 activation in neuronal cells (Tunici et al., 1999). HSP contributes to cell survival via binding to other proteins thus preventing the protein misfolding (Borges and Ramos, 2005, Verghese et al., 2012).

Nuclear Activities and Protein Synthesis

The heterogeneous nuclear protein (hnRNP) corresponds to a family of multifunctional RNA-binding nuclear proteins (review: Geuens et al., 2016). Two of these proteins, hnRNP H and hnRNP C were significantly reduced in the ethanol-exposed rNSC. The protein hnRNP H may be linked to human cancers while protein C of the hnRNP family is best known for its role in pre-mRNA alternative splicing (Geuens et al., 2016). Alternative splicing is of interest in alcoholism since the glutamate transporter EAAT1 (GLAST), reported as severely affected by chronic drinking (review: Sery et al., 2015) but see (Alshehri et al., 2017, Kryger and Wilce, 2010), exists in many alternate splicing forms (Lee and Pow, 2010). EAAT1 (GLAST) is a key protein in glutamatergic neurotransmission which is known to be perturbed by ethanol (Spanagel, 2009); the conflicting data (Alshehri et al., 2017, Kryger and Wilce, 2010, Ramondini et al., 1992, Flatscher-Bader and Wilce, 2005) could be explained by a changed pattern of its alternate splicing in ethanol-affected tissue. The protein hnRNP C has been linked to breast cancer (Anantha et al., 2013) but, perhaps more importantly in the context of FASD, its abnormal expression has been associated with birth defects in mammals including humans (Zhang et al., 2008, Sari et al., 2010).

"Helicase" is a group of enzymes which can unwind double helices of nucleic acids (Umate et al., 2011). Spliceosome RNA helicase Ddx39b is involved in the nuclear export of mRNA and its reduced expression in ethanol-exposed cells could have a severe impact on the RNA translation (Schwer and Meszaros, 2000).

The DND1 (increased by ethanol in both neuron and astrocytes, Fig 4) can block miRNA-mediated gene suppression. DND1 plays a key role in the primordial germ cell (PGC) survival and migration (Kedde and Agami, 2008) and its defects also cause sperm sterility, inducing testicular germ cell tumours in mice (Youngren et al., 2005, Zhu et al., 2011). DND1 inactivation in mice leads to

sterility at birth stage (Bhattacharya et al., 2007). DND1 interacts with several mRNAs including mRNAs of both anti- and pro-apoptotic factors, BCLX and BAX, respectively. Transcripts of pluripotency factors, cell cycle regulators and apoptotic factors associated with DND1 have been reported in DND1 recombinant study in human embryonic stem cells (Zhu et al., 2011).

Exposure to high concentrations of ethanol decreased NPM1 in rNSC and perturbed its expression in both neuron and astrocytes (Table 1, Fig 3). This might in part explain the lower cell numbers of the ethanol-exposed cells (Fig. 1) since NPM1 is involved in DNA replication, recombination, transcription and repair (Grisendi and Pandolfi, 2005, Colombo et al., 2005) as well as elsewhere (Okuwaki et al., 2001, Abe et al., 2018). This interpretation is also in agreement with reports that the knockout of NPM1 in mice leads to unrestricted centrosome duplication, genomic instability and impaired ribosome biogenesis (Grisendi and Pandolfi, 2005, Chiarella et al., 2013). In addition, NPM-induced p21 expression activates gene transcription by de-repressing p300–CREBBP (CREB-binding protein; (Lill et al., 1997, Kasper et al., 2011)) and can regulate p53-mediated apoptosis under the conditions of cellular stress (Abbas and Dutta, 2009). Thus, the normal level of NPM1 expression could very well be a critical factor in determining the healthy proliferation and differentiation of the NSC (cf. also above under "Cell Proliferation and Differentiation").

Summary and concluding statements

The main finding of the study is the large extent of changes in the expression of proteins in cultured embryonic cells (including neural stem cells) exposed to alcohol. Altered expression of so many proteins that are crucial for the normal cell proliferation and survival, should it occur following a similar exposure to alcohol in vivo, would not be without profound consequences for brain development.

More specifically, the present study resulted in the first observation of changes in DND1 and NPM1 expressions linked to ethanol exposure. Given the roles of NPM1 in cell proliferation, the lower expression of NPM1 could, in part, account for the reduction in neural stem cell numbers following the exposure to ethanol; the changes in the NPM1 expression may be less important in the cultured neuron and astrocytes, though, where NPM1 was actually increased at 25 mM ethanol and only moderately decreased at the highest (100 mM) ethanol concentration.

The present observations may help to identify novel mechanisms by which chronic alcohol exposures in utero exert their cytotoxic effects. It should be obvious that existence of such mechanisms does not rule out the role of GABA(A) and/or NMDA receptors in the actions of alcohol including the neurotoxicity (Spanagel, 2009, Rae et al., 2014, Rae et al., 2014a, Davidson et al., 1995, Hoffman, 1995). Ethanol would interact with the neurotransmitter receptors regardless of the length of the exposure and, as recent studies with the anaesthetics propofol, sevoflurane and ketamine suggest, such interactions would be cytotoxic for brain stem cells (Sikker et al., 2015, Wang et al., 2015, Qiu et al., 2015). These data

merely suggest that additional mechanisms involving many more protein species (particularly those 28 protein molecules identified by the present study) could be involved in the process.

The findings of the study are of particular significance for the understanding of mechanisms underlying alcoholism-related conditions such as the foetal alcohol spectrum disorder, especially in relation to alcohol interference with the earliest stages of brain development. Given that neurogenesis may occur in adult brains, too, the present results could also be relevant for the understanding of changes in adult alcoholic brains.

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CHAPTER 4

A Interactome Study of Alcohol-altered Nuclear Proteins Nucleophosmin (NPM; B-23) and Dead End Homology Protein 1 (DND1) in Human Postmortem Prefrontal Cortex

Abstract

Long term-exposure of human brain tissue to excessive intake of alcohol is accompanied by major neurochemical changes; over two hundred proteins have been reported to alter their levels of expression and several neurotransmitter systems have been found to be affected. Studies using animal brains and cultured cells show similar patterns of alcohol-related changes. Author used post mortem brain tissue to investigate effects of chronic alcohol on two nuclear proteins mucleophosmin (NPM) and dead end homology-1 (DND1). Both these proteins are likely to be involved in the regulation of transcription and translation but they could interact with many additional systems. Using western blotting author has found that, in the tissue from the human prefrontal cortex exposed to chronic excessive alcohol, NPM was decreased by 43% and DND1 increased by 80%, compared to controls. The author then applied proteomic methodologies to establish with which proteins NPM and DND1 can interact. NPM showed apparently specific interactions with 55 proteins, classified as related to cell growth (4), cell structure (4), metabolism (18), oxidative stress response (5), signalling (8), apoptosis and DNA damage (2), epigenetics (2), transcription (7) or as transmembrane proteins (5). DND1 interacted with 24 proteins; related to cell growth (1), cell structure (2), metabolism (13), oxidative stress (3), signalling (3) and transcription (2). Therefore, NPM and DND1 can influence a broad spectrum of mechanisms which are fundamental for normal functioning of brain cells. Particularly striking are the numerous associations of the two proteins with metabolic enzymes, including those of NPM with Na^+,K^+-ATPase and DND1 with 4-aminobutyrate (GABA) transaminase. Any perturbations of these proteins could impact the signal conduction and synaptic transmission; Na^+,K^+-ATPase provides energy for neuronal activity by maintaining the Na^+ and K^+ transmembrane gradients and drives neurotransmitter-inactivating transporters. GABA transaminase metabolizes and regulates the most important inhibitory neurotransmitter GABA.

Introduction

Protein-protein interactions

The human genome consists of more than 30,000 coding genes capable of transcribing in excess of 500,000 distinct proteins of which about 10,000 can be produced by the cell at any given time (Berggard et al., 2007). It has been estimated that over 80% of proteins function as complexes. Protein-protein interaction (PPI) is a key characteristic of all known forms of life both at the level of individual cells and protein molecules and in the function of the organism as a whole. Specific PPIs enables the system to constitute and balance the vast array of biochemical and biophysical reactions necessary to support the life. PPIs play an important role in regulating various biological mechanisms including DNA replication & transcription, enzyme-mediated metabolism, transmembrane trafficking, signal transduction and cell cycle control (Berube et al., 2005). Identification and characterization of PPI provides important clues as to the biological functions of proteins both in health and disease.

PPIs can be studied *in vivo, in vitro* and *in silico*. The *in vivo* techniques such as the hybridization of two yeast cells (Y2H, Y3H) require the use of living cells. *In silico* studies utilize methodologies of theoretical chemistry; protein molecules are constructed in virtual spaces and their potential to interact is evaluated under a variety of simulated conditions. Author has opted for an *in vitro* approach, using a method based on a combination of co-immunoprecipitation (coIP) and mass spectrometry (MS), because of its high sensitivity to detect a broad spectrum of PPI's in a complex biological sample (Khacho et al., 2008, Ma et al., 2012, Huang and Kim, 2013). In this approach an antibody against a protein in the PPIs which author wishes to investigate is immobilized by crosslinking to the commercially available protein A/G beads and allowed to bind the specific antigen along with the interacting species. After removing non-specifically bound proteins by repeated washing, the co-immunoprecipitated interacting partners can be eluted from the beads by using a succession of low and high pH media. The eluted proteins are separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), digested by proteases, and identified using tandem MS (MS/MS; Huang and Kim, 2013). The technique is highly sensitive; for example, over 70 proteins have, in this way, been shown to be associated with a glutamate receptor protein (Genda et al., 2011).

Protein expression in brain cells and tissues exposed to alcohol

In previous studies author has analysed, using proteomics, various regions of alcoholic human and rat brains (human: Kashem et al., 2007, Matsuda-Matsumoto et al., 2007, Matsumoto et al., 2007, Kashem et al., 2008, Hargreaves et al., 2009, Kashem et al., 2009a, Kashem et al., 2016; rat: Kashem et al., 2012) and have identified 238 (out of 7000 detected proteins) proteins as being altered in alcohol-affected brain tissue. These proteins were functionally classified as cytoskeletal (16%), metabolic (42%), oxidative stress-related (15%), signaling (22%) and apoptosis-related (5%). In a recent study (Kashem et al., 2018) author has detected 28 proteins which were altered in alcohol exposed rat neural stem cells, neurons and glia; these can also be classified as metabolic (21%), cytoskeletal (10%), signal transduction (14%), nuclear (such as transcriptional factors; 24%), molecular chaperones and stress-related (21%) proteins as well as some (10%) with no identified role (Kashem et al., 2018). From the above studies, it seems obvious that alcohol perturbs the cell physiology and, in the process, alters the levels of certain proteins. Among those found to be altered by the alcohol exposure six were nuclear proteins and transcription factors but none were proteases implying that alcohol may have a greater impact on the synthesis rather than the degradation of proteins. Author focused on two of the nuclear proteins: nucleophosmin (NPM) and dead end homology-1 (DND1) (Kashem et al., 2018).

Nuclear proteins nucleophosmin (NPM) and dead end homology-1 (DND1)

NPM has been reported to regulate, or, be involved in, histone chaperon activity, ribosome biogenesis & transportation, genomic stability & DNA repair, endoribonuclease activity, centrosome duplication during cell cycle, regulation of ARF-p53 tumor suppressor pathway, RNA helix destabilizing activity, and inhibition of caspase-activated DNase and prevention of apoptosis (Lindstrom, 2011). Its expression was decreased 1.68 fold in the alcohol-exposed cells (Kashem et al., 2018). NPM has three variants; the variants NPM1 and NPM3 have been reported as playing important roles in chromatin decondensation and remodelling (Lindstrom, 2011). A recent study using FLAG-tag-based affinity purification coupled with LC-MS/MS, Haga et al. (2013) identified 106 proteins as potential partners of NPM in Ewing's sarcoma cells (Haga et al., 2013).

DND1 binds to miRNA-targeting sequences of mRNAs, inhibiting miRNA-mediated repression which plays the key role in the primordial germ cell survival and migration during the early embryonic development in chicken (Aramaki et al., 2009). By performing ribonucleoprotein

immunoprecipitation followed by RT-PCR, pluripotency factors (OCT4, SOX2, NANOG, LIN28), cell cycle regulators (TP53, LATS2) and apoptotic factors (BCLX, BAX) were found to be specifically associated with the HA-DND-1 ribonucleoprotein complex (Zhu et al., 2011). Ethanol affected proliferation of stem cells as well as their transformation, leading to an astrocytic-like phenotype; this might be interpreted as an indication of alcohol inducing abnormal astrocytes in the developing brain and potentially persisting until adulthood (Kashem et al., 2018). Such cells may display an early "tolerance" to alcohol and/or impaired genomic or biochemical make up. This could be a result of chromatin remodeling involving acetylation of histones H3 and H4 as reported by Witt (2010).

Aims of the study

The chief aims of the present study are two fold. First, we shall investigate the levels of expression of two nuclear proteins (NPM and DND1) in human postmortem brains exposed to excessive amounts of alcohol for a number of years. Second, if the expressions of NPM and DND1 are altered in the alcohol exposed brains we shall determine their interactomes in non-alcoholic brain tissue. We shall be using samples of brain tissue taken from the prefrontal cortex (PFC). PFC may be the most highly developed and differentiated part of the human brain (Teffer and Semendeferi, 2012) and the changes in this area have been previously implicated in aetiologies of mental diseases (reviews: Weinberger 1987; Nanitsos et al, 2005; Teffer and Semendeferi, 2012). The results will add a new element to our investigations of how chronic alcohol affects the human brain structure and function at the molecular level.

Materials and methods

Human Brain Tissue

Post-mortem human brains were obtained from the NSW Brain Bank (University of Sydney). Samples were grouped as (i) control (< 20 g of ethanol/day) and (ii) alcoholics (> 80 g of ethanol/day) (Table 1). Post-mortem examination of the alcoholic brains indicated that there were no apparent complications from Wernicke–Korsakoff Syndrome (WKS) in any of the alcoholic brains used in the present study. Alcoholic cases fulfilled the criteria of the Diagnostic and Statistical Manual of Mental Disorders IV (DSM-IV) for alcohol dependence (Anonymous, 1994); the relevant data having been obtained from medical records or from reports by the next of kin. The estimations of NPM and DND1 levels in control and alcoholic prefrontal cortices (PFC) were done by Western blotting while the interactome studies were performed according to the procedures specified in the immunoisolation section, using prefrontal cortex (PFC) from control subjects. Ethics approval for the use of human tissue use was granted by the Sydney South West Area Health Service (Protocol no X13-0379).

Protein extraction

Protein extractions were performed according to the method previously described by Kashem (Kashem et al., 2007, Kashem et al., 2008, Hargreaves et al., 2009, Kashem et al., 2009a, Kashem et al., 2009b, Tang et al., 2009, van Nieuwenhuijzen et al., 2010). In brief, the tissues samples were extracted by urea buffer (7 M urea, 2 M thiourea and 1% C7bZO, 40 mM tris-HCl) and tissue extract was pelleted at 16,000 g for 20 min at 15 °C. The supernatant was reduced and alkylated with 5 mM tributyl phosphine and 10 mM acrylamide monomer at room temperature for 2 hours. The reaction mixture was quenched using dithiothreitol (DTT) at 10 mM. The citric acid was used to acidify the samples to approximately pH 5. The acetone-precipitated pellet was air dried and resuspended in buffer consisting of 7 M urea, 2 M thiourea and 1% C7bZO. Total protein was quantified according to Bradford (1976) using BSA as a standard.

Western blot (WB) analysis

The WB analysis was used to identify and estimate the NPM and DND1 proteins. Analogous analysis used to identify the immunoprecipitated (IP) protein is described in the immunoisolation

section on SDS-PAGE and In-gel Digestion below. First, we separated the extracted proteins by SDS-PAGE as described previously by Kashem et al. (2016). The proteins were then transferred to PVDF (polyvinylidene difluoride) membranes according to a protocol described earlier (Kashem et al., 2012). The membranes were blocked with 5% skim milk and incubated with primary antibodies [(NPM, DND1,(Santa cruiz)] solution. Secondary antibodies (anti-mouse and anti-rabbit IgG; Sigma) were added according to the manufacturer's instructions and the resulting spots were visualized using an ECL (enhanced chemiluminescence) full spell system (GE Healthcare, Australia). The intensity of spots was quantified by commercially available Bio-rad software (Bio-rad, Sydney, Australia).

Immunoisolation of DND1 and NPM Associated Protein

Immunoisolation of NPM and DND1 associated proteins was performed according to protocol described by Pierce CrossLink immunoprecipitation kit (Thermo Scientific, Rockford, USA). Acetone precipitated protein was resuspended in CHAPS and 0.5% sodium deoxycholate buffer. For pre-cleaning of the lysate, 80 µL of the control agarose resin slurry was added to 1 mg of extracted proteins followed by incubation at $4^{\circ}C$ for one hour and passaged through a specialized Pierce spin column. The flow through the spin column, which can be capped and plugged with a bottom plug for incubation or unplugged to remove the supernatant by centrifugation at 1000 g for 1 minute, was used for immunoisolation. The protein A/G agarose slurry (20 µL) was washed twice with 200 µL PBS buffer, and incubated with 100 µL NMP and DND-1 antibody prepared in PBS (10 µL NPM or DND1 antibody +85 µL H_2O+5 µL 20X PBS) at $25^{\circ}C$ for 30 min on a mixer. In parallel, 100 µL of rabbit and mouse IgG peroxidase secondary antibody with the same concentration of IgG as that of NPM/DND1 antibody was similarly prepared as the negative control. The supernatant was discarded and the beads were washed three times with 300 µL PBS, followed by incubation with 50 µL disuccinimidyl suberate (DSS) solution (2.5 µL 20 X PBS+38.5 µL H_2O+2.5 mM DSS in DMSO) at $25^{\circ}C$ for 45–60 min on a mixer. After removing the supernatant the beads were washed three times with 50 µL of 100 mM glycine (pH 2.8), followed by three wash with PBS. About 500 µg of pre-cleaned protein extract was passed through the columns (anti rabbit-NPM, negative control (rabbit -IgG), anti-mouse-DND1 and negative control (mouse-IgG). The columns were washed with washing buffer five times and eluted with acidic buffer. The elution was neutralized with high concentration of Tris-HCl buffer.

SDS-PAGE and In-gel Digestion

 The eluted proteins were resuspended in SDS-loading buffer (150 mM NaCl, 50 mM Tris-Cl, pH 7.5), the mixture was heated at 95ºC for 5 min and duplicate samples were prepared from protein mixture of 6 PFC protein extracts and pooled samples were loaded onto 12% SDS-PAGE (n=3). One set of samples was stained with Coomassie Brillant Blue (CBB) and other set was used in Western blotting to confirm the presence of NPM- and DND1-protein immuniprecipitaed (IP) complexes. The IP positive lanes were cut into 2-4 mm pieces and processed according to a previously described proteomics protocol (Kashem et al., 2007, Kashem et al., 2008, Kashem et al., 2009a). In brief, gel bands were excised and de-stained using 25 mM NH_4HCO_3/50% (v/v) acetonitrile (ACN) overnight at room temperature. The gel portions were then dehydrated using 100% ACN. Each gel piece was incubated with 12.5 ng/mL trypsin (Roche, sequencing grade) buffer (sodium bicarbonate buffer) for 45 min at 4 °C. After several washings the gel piece was incubated at 37 °C overnight in 25 mM NH_4HCO_3/0.1% trifluroacetic acid buffer. The peptide mixtures were purified from the supernatant using C-$_{18}$ Eppendorf purification tips.

 Nano-HPLC MS/MS Analysis

Purified peptides were resuspended in 0.1% formic acid (FA) and separated using an Eksigent nanoLC Ultra 2D Plus system coupled to an AB SCIEX (Framingham MA) TripleTOF 6600 mass spectrometer. Peptides were initially loaded onto a 300 μm × 10 mm I.D. ProteCol™ C18 P-120 3 μm HPLC trap column (SGE Analytical Science, Ringwood, Australia) in 2% MeCN, 0.1% FA at 10 μL/min for 10 mins. Peptides were then eluted by altering the composition of the mobile phase from 97% buffer A (0.1% FA) to 40% buffer B (99.9% MeCN, 0.1% FA) over 95 mins at 300 nL/min directly onto an in-house packed 30 cm × 75 μm I.D. ReproSil-Pur 120 C18 AQ-3 μm column (Dr Maisch, Ammerbuch-Entringen, Germany) for separation immediately prior to injection. The TripleTOF 5600 was operated in data independent acquisition mode with the top 15 precursor ions (fulfilling the criteria of charge state between +2 and +5, minimum intensity of 120 cps, and m/z between 350 and 1250) selected for MS/MS. Selected ions were entered into an exclusion list for 12 s after 1 occurrence. For MS scans, a mass window of 350–1500 m/z and maximum accumulation time of 250 ms were used, while MS/MS scans were conducted using a mass window of 100–1500 m/z, accumulation time of 200 ms, resolution set

to unit and with the options for rolling collision energies and adjustment of collision energies. Raw MS/MS data were converted to Mascot generic format (mgf) files using Peakview 2.2 (AB Sciex Aust. Pty Ltd). Resulting mgf files were used to identify proteins using Mascot search engine 2.2.07 (Matrix Science) with setting up the parameters: carbamidomethylation on cysteine, plus variable methionine oxidation, peptide mass tolerance at 20 ppm and fragment mass at 0.6 Da and using Uniprot's human (*Homo sapiens*) database.

Results

Western blotting

Protein extracts from prefrontal cortices (PFC) of human alcoholic and control (non-alcoholic) brains were analysed for NPM and DND1 proteins by quantitative western blotting and immunoisolation. NPM was 43% lower in the alcoholic tissue compared to the controls while the content of DND1 was 80% higher in the alcoholic brain extracts than in those from control brains (Fig 1 a-d).
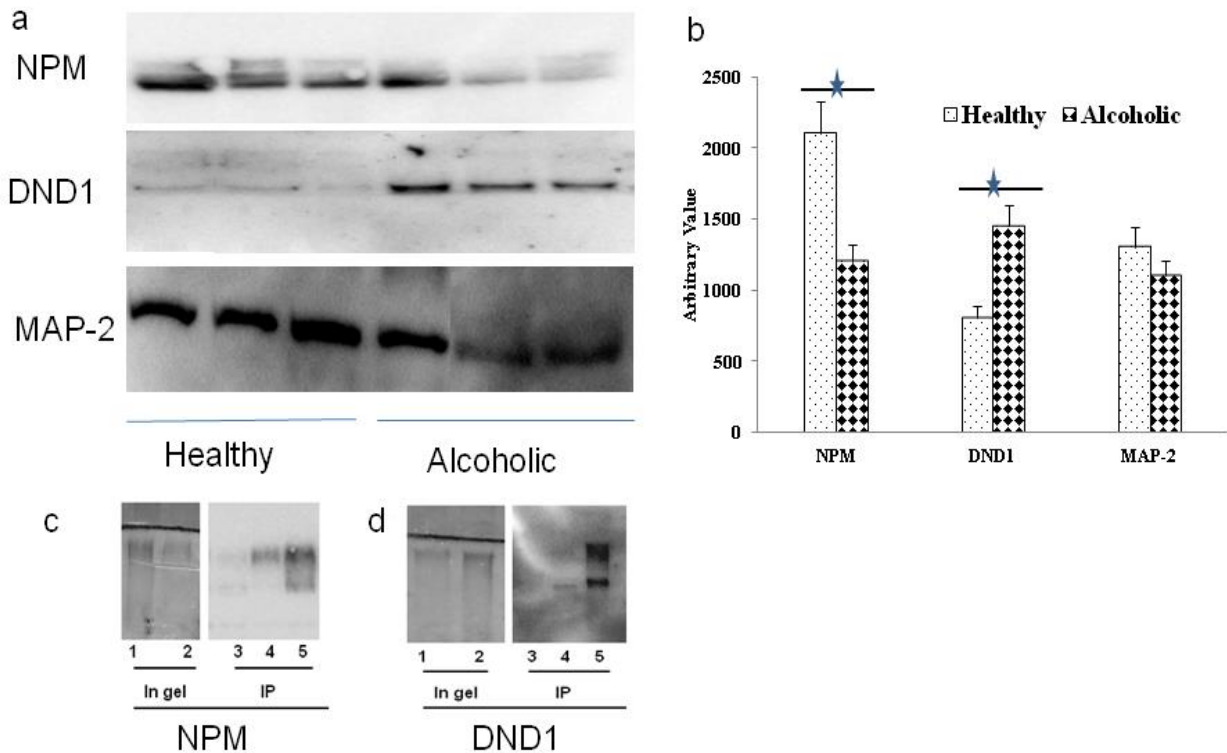

Figure 1.

Western blot of NPM and DND1and MAP-2 (internal control) (1a,b) in the prefrontal cortex tissue of healthy and alcoholic postmortem human brain. Three samples are prepared from the six PFC protein extracts. Proteins were immunoanalysed and the bands in 1a were digitized; average values (n=3) plus SE are plotted in 1b (--*---, significant at 5%). Fig 1c and 1d shows the immunoisolation of NPM and DND1 proteins (n=3). Lane 1 (IgG of either rat or rabbit) and lane 2 (either NPM or DND1) are in gel CBB

staining of NPM and DND1. Immunoisolated protein complex were further confirmed through western blotting (lanes 3 and 5). Lanes 3, and 5 are anti-IgG and primary antibody (anti-NPM and anti-DND1 antibodies) immunoislated protein complex respectively. Lane 4 is PFC protein extracted (original sample) that treated with either anti-NPM or anti-DND1 antibodies, respectively.

Interactome

Author has identified 65 proteins in anti-rabbit NPM samples and 10 proteins in a negative control experiment (only rabbit IgG); these 10 proteins were eliminated as not uniquely related to NPM. The remaining 55 proteins were considered specifically bound by NPM (Table-1). Similarly, a total of 30 bound proteins with 6 non-specifically bound proteins were detected in anti-mouse DND1 samples and negative control (mouse IgG), respectively. Therefore, after the deduction, 24 proteins were considered as specifically interacting with DND1 (Table 2).

NPM associated proteins can be categorized as being involved in cell growth (4 protein; 7%), cell structure (4 proteins: 7%), metabolism (18 proteins; 33%), oxidative stress (5 proteins; 9%), signalling (8 proteins: 16%), apoptosis & DNA damage (2 proteins; 3%), epigenetics (2 proteins; 3%), transcription (7 proteins; 13%) and as transmembrane structures (5 proteins; 9%). Similarly, the twenty four proteins identified as DND1-associated can be classified as related to cell growth (1 protein; 4%), structure (2 proteins; 8%), cell metabolism (13 proteins; 54%), oxidative stress (3 proteins; 12.5%), signalling (3 proteins; 12.5%) and transcription mechanisms (2 proteins; 8%).

Table-1. NPM co-IPed proteins from the prefrontal cortex of human post mortem brain lysates identified

by nano-HPLC–MS/MS of in-gel tryptic digests (see Fig. 1C) using the programmes MASCOT data based.

Protein Class	Protein Name	No of opeptide match	Accession no	Function
Cell division	Cyclin-Y-like protein 1	4	Q8N7R7	Regulation of cyclin-dependent protein serine/threonine kinase activity
	Cell division Cycle protein 20	3	Q12834	Acts as a regulatory protein interacting with several other proteins at multiple points in the cell cycle.
	Myocyte-specific enhancer factor 2B	3	Q02080	A transcription factors and control gene expression. Important regulators of cellular differentiation and play a critical role in embryonic development.
	Protocadherin-16	4	Q96JQ0	Calcium-dependent cell-adhesion protein and involves in neuroprogenitor cell proliferation and differentiation
Cell-structural proteins	Protein shroom 3	3	Q8TF72	Controls cell shape changes in the neuroepithelium during neural tube closure.
	Ankyrin-3	2	Q12955	Participates in the maintenance targeting of ion channels and cell adhesion molecules at the nodes of Ranvier and axonal initial segments.
	Synaptopodin	4	Q8CC35	Modulating actin-based shape and motility the dendritic spines in the telencephalic neurons.
	Desmocollin-2	3	Q02487	It is a cadherin-type protein that functions to link adjacent cells.
Metabolic proteins	Glucose-6-phosphate isomerase	5	P06744	Plays neurotrophic factor (Neuroleukin) for spinal sensory neurons.
	Isocitrate dehydrogenase (IDH)	4	P48735	Deficiency causes several brain tumors including astrocytoma, oligodendroglioma and glioblastoma multiforme.
	3-phosphoglycerate dehydrogenase	6	P26718	Carbohydrate metabolic enzyme.
	Malate dehydrogenase	3	P40925	Involved in gluconeogenesis
	Acyl-CoA dehydrogenase	4	P11310	Involves in beta-oxidation and lipid

	(medium)			metabolism
	Adenosylhomocysteinase	3	P23526	Activates methyl cycle and plays a critical role in the modulation of the activity of various methyltransferases
	Glutamate dehydrogenase	6	P54385	Catalyzes reversible inter-conversion of glutamate to α-ketoglutarate and ammonia
	Propionyl-CoA carboxylase (β)	4	P05165	Catalyses the carboxylation reaction of propionyl CoA in the mitochondrial matrix.
	UFM1-protein ligase-1	4	O94874	Plays a role in the unfolded protein response, mediating the ufmylation of multiple proteins in response to endoplasmic reticulum stress
	26S protease regulatory subunit 4	3	P62191	The 26S protease is involved in the ATP-dependent degradation of ubiquitinated proteins.
	Retrotransposon like –protein 1	2	B9EK54	Expressed imprinted gene that is highly expressed at the late fetal stage in both the fetus and placenta and essential for maintenance the fetal capillaries.
	Titin OS	2	Q8WZ42	Key component in the assembly and functioning of vertebrate striated muscles
	Fukutin-related protein	2	Q9H9S5	This protein is involved in the pathway protein glycosylation, which is part of protein modification.
	NACHT-domain and WD repeat-containing protein 1	4	Q149M9	May play a role in the control of androgen receptor (AR) protein steady-state levels.
	Low–density lipoprotein receptor-related protein 4	5	O75096	Plays a key role in the formation and the maintenance of the neuromuscular junction (NMJ), the synapse between motor neuron and skeletal muscle.
	Peptidyl-prolyl cis-trans isomerase	2	P62937	Peptidyl prolyl *cis/ trans* isomerases (PPIases) are ubiquitous enzymes that catalyse the *cis/trans* isomerisation of peptide bonds preceding proline in peptides and proteins.
	WD repeat-containing protein 63	4	Q81WG1	Testis development protein
	WD repeat-containing protein 87	4	Q6ZQQ6	Testis development protein

Oxidative stress	Heat shock 71 kDa protein	7	P11142	Acts as a repressor of transcriptional activation
	Stress 70 protein	6	P38646	Implicated in the control of cell proliferation and cellular aging. May also act as a chaperone activity.
	78 kDa glucose regulated protein	5	P20029	Prevent misfolding and promote refolding of proteins. Proper assembly of unfolded polypeptides generated under stress conditions in the mitochondrial matrix.
	60 kDa heat shock protein	4	P10809	
	NKG2-D type integral membrane protein	5	P26718	Provides both stimulatory and costimulatory innate immune responses on activated killer (NK) cells, leading to cytotoxic activity.
Signaling protein	Phosphoglycerate kinase-1	2	P00558	Involves in glycolytic enzyme and may play a role in sperm motility
	cAMP-dependent protein kinase	4	P17612	Phosphorylates a large number of proteins in the cytoplasm and nucleus and activates cAMP, and involves in cell proliferation and differentiation and regulation of microtubule dynamics.
	Sarcoplasmic Ca ATPase	3	O14983	Involves in reuptake of cytosolic Ca^{2+} into the sarcoplasmic reticulum.
	Ribosomal protein S6 kinase (alphas 3)	4	P51812	Activation of the transcription factors CREB1, ETV1/ER81 & NR4A1/NUR77 and involves in cellular proliferation, survival and differentiation, and repressing pro-apoptotic function of BAD and DAPK1.
	Rho GTPase-activating protein 29	4	Q5PQJ5	Activator of Rho-type GTPases,
	TBC1 domain family member 22A	2	Q8WUA7	May act as a GTPase-activating protein for Rab family protein(s)
	Phostensin	4	Q6NYC8	Phostensin interacts with protein phosphatase 1 (PP1).
	Guanin nucleotide-binding protein (G-β)	5	P62873	Involves as a modulator or transducer in various transmembrane signaling systems.
Trans-membrane protein	Na^+/K^+ transporting protein ATPase	6	P05023	Na^+/K^+-ATPase is an integral membrane protein responsible for establishing and maintaining the electrochemical gradients of Na and K ions across the plasma membrane.
	Phospholipid transporting protein ATPase	5	Q9Y2Q0	Acts as aminophospholipid translocase at the plasma membrane

				in neuronal cells.
	Transitional endoplasmic reticulum ATPase	4	P55072	Necessary for the fragmentation of Golgi stacks during mitosis and for their reassembly after mitosis.
	Coatomer subunit (γ-1)	2	Q9Y678	Binds to dilysine motifs and reversibly associates with Golgi non-clathrin-coated vesicles in protein biosynthetic and transporting process.
	Golgi in subfamily A membrane 4	2	Q13439	May play a role in delivery of transport vesicles containing GPI-linked proteins from the trans-Golgi network through its interaction with MACF1.
Epigenetic regulation	DNA (cytosine-5)-methyltransferase 3B	3	Q9UBC3	Required for genome-wide de novo methylation and is essential for DNA methylation.
	Adenosylhomocysteinase 3	5	Q96NH2	Multifaceted cellular regulator & coordinates several essential cellular functions including regulation of epithelial $HCO3^-$ and fluid secretion, mRNA processing and DNA replication.
Transcriptional machineries	NSF1 cofactor p47	3	Q9UNZ2	Reduces the ATPase activity and necessary for the fragmentation of Golgi stacks during mitosis.
	Spermatid perinuclear RNA-binding protein	3	Q96S19	Involved in spermatogenesis and sperm function. Plays a role in regulation of cell growth. Binds to double-stranded DNA and RNA.
	Mediator of RNA polymerase II transcription subunit 16	2	Q9Y2X0	A coactivator involves in the regulation of transcription of all RNA polymerase II-dependent genes.
	Structure specific endonuclease subunit SLX1	3	Q9BQ83	The structure specific endonuclease (SLX1) involves in DNA secondary structures generated during DNA repair and recombination.
	Putative polycomb group protein ASXL1	4	Q8IXJ9	Involved in transcriptional regulation mediated by ligand-bound retinoic acid receptors and peroxisome proliferator-activated receptor gamma.

	Eukaryotic translation initiation factor 5A-1	5	P63241	mRNA-binding protein involved in translation elongation. Involved in actin dynamics and cell cycle progression, mRNA decay and probably in a pathway involved in stress response and maintenance of cell wall integrity.
	ELM2 and SANT domain containing protein 1	4	Q6PJG2	Transcription factor and regulation of transcription from RNA polymerase II promoter
Apoptosis and DNA damage	p-53-induced death domain-containing protein1	6	Q9HB75	A key adapter protein that links to p53 apoptosis pathway and activation of the transcription factor NF-kappa-B.
	DNA damage binding protein 2	3	Q92466	A protein complex that is responsible for repair of UV-damaged DNA.

Table-2. DND1 co-IPed proteins from the prefrontal cortex of human post mortem brain lysates identified

by nano-HPLC–MS/MS of in-gel tryptic digests (see Fig. 1D) using the programmes MASCOT data based.

Protein Class	Protein Name	No of peptide match	Accession no	Function
Cell division	Cell division control protein 6	6	P09119	Regulator of DNA replication and plays important roles in the cell cycle particularly S phase of mitosis
Structural Protein	Actin related protein 2	5	P61160	Activating nucleation-promoting factor (NPF) mediates the formation of branched actin networks.
	Vimentin	8	P08670	Vimentins are class-III intermediate filaments found in various non-epithelial cells, especially mesenchymal cells.
Metabolic protein	Malate dehydrogenase	5	P40925	Involves in gluconeogenesis
	Fructose–bis phosphate aldolase	5	P04075	Plays a key role in glycolysis and gluconeogenesis.
	Succinyl-CoA:3-ketoacid coenzyme A transferase-1	5	P55809	Key enzyme for ketone body catabolism and transfers the CoA moiety from succinate to acetoacetate
	Phosphoglycerate mutase 1	4	P18669	Carbohydrate metabolic protein
	Isocitrate dehydrogenase	5	P48735	Deficiency causes several brain tumors including astrocytoma, oligodendroglioma and glioblastoma multiforme,
	Pyruvate dehydrogenase E1	4	P11177	The pyruvate dehydrogenase complex catalyzes the overall conversion of pyruvate to acetyl-CoA and CO_2,
	Cytosolic non-specific-dipeptidase	4	Q96KP4	Hydrolysis of dipeptides, preferentially hydrophobic dipeptides including prolyl amino acids
	4-aminobutyrate amino-transferase	5	P80404	Conversion of gamma-aminobutyrate and L-beta-aminoisobutyrate to succinate semialdehyde and methylmalonate semialdehyde, respectively.
	Fumarate hydratase	6	P07954	Carbohydrate metabolism
	Aldehyde dehydrogenase	4	P05091	Detoxification of alcohol metabolites acetaldehyde also

				involved in the metabolism of corticosteroids, biogenic amines, neurotransmitters, and lipid peroxidation.
	Aceyl-CoA acetyl transferase	3	9BWD1	Lipid metabolic process and plays a major role in ketone body metabolism
	ATP synthase	4	P06576	Mitochondrial membrane ATP synthase produces ATP from ADP in the presence of a proton gradient across the membrane.
	Ubiquitin-conjugating enzyme E2	6	Q9ULV8	Acts as an E3 ubiquitin-protein ligase, which accepts ubiquitin from specific E2 ubiquitin-conjugating enzyme
Oxidative stress	Heat shock 71 kDa protein 4	4	P11142	Acts as a repressor of transcriptional activation and inhibits the transcriptional coactivator activity of CITED1 on Smad-mediated transcription.
	Na^+ channel protein type 10	5	Q6R2W3	RNA polymerase II transcription factor activity
	ATP-binding cassette subfamily A member 13	4	O14678	ABC-type metal ion transport system permease component
Signaling protein	Guanin nucleotide-binding protein (beta)	5	P18872	Adenylate cyclase-modulating G-protein coupled receptor signalling pathway
	Pyruvate kinase	4	P14618	Glycolytic enzyme that catalyzes the transfer of a phosphoryl groupfrom phosphoenolpyruvate (PEP) to ADP, generating ATP.
	Phosphoglycerate kinase	3	P00558	Glycolytic enzyme and acts as a polymerase alpha cofactor protein and play a role in sperm motility
Transcription machineries	Zinc Finger protein 257	4	Q9Y2Q1	Regulation of transcription
	Elongation factor 1-alpha 1	4	P68104	Promotes the GTP-dependent binding of aminoacyl-tRNA to the A-site of ribosomes during protein biosynthesis

Discussion

General: Changes in protein composition of brain tissue chronically exposed to alcohol

Practically all conceivable biological phenomena involve interactions of proteins. Identification and mapping of these interactions is fundamental to the understanding of how cells, organs and organisms function, reproduce, grow, develop and die. In previous proteomic studies author detected 238 proteins as having been altered in human and animal brains following chronic exposure to alcohol. Additionally, a recent metabolomic study of human alcoholic striatum suggested that long term excessive intake of alcohol can have a potent effect on several neurotransmitter systems and may even adversely impact the brain defences against oxidative stress and neurotoxicity (Kashem et al., 2016). All these findings represent a significant body of neurochemical evidence correlating with the common notion that a chronic exposure to large doses of alcohol profoundly alters the homeostasis of brain tissue and that such changes underlie the abnormal brain development, cognitive deficits, addiction and other manifestations of alcoholism in humans.

In recent experiments using neural stem cells (Kashem et al., 2018) author has observed that several days of exposure to 50 mM alcohol significantly affects expression of nearly 28 important proteins; these included NPM and DND1. NPM is involved in early brain development, cell proliferation and embryonic morphogenesis (Grisendi et al., 2005, Pfister and D'Mello, 2015, Xian et al., 2016) while DND1 may also play part in the male reproductive process (Aramaki et al., 2009, Youngren et al., 20015, Zhu et al 2011). In the present study, the levels of both NPM and DND1 have also been found to be altered in the adult brain alcoholic tissue. Rather than delving into how the long-term alcohol exposure might impact any specific mechanism directly involving the two proteins in cell physiology, the remaining discussion focuses on associations of these two macromolecules with other proteins ("interactomes") as they have been detected in the present study. More to the point, it examines how the altered expressions of NPM and DND1 in alcoholic tissue could perturb the cell and tissue functions via indirect effects mediated by their interactomes.

In the following discussion the proteins are broadly categorized according to the functional and structural classes as specified in Tables 1 and 2 but only the proteins and categories potentially involved in mechanisms most likely to be affected by alcoholism are included.

a. Proteins interacting with nucleophosmin (NPM)

1. Proteins involved in the cell cycle and cell division

Of the 55 proteins interacting with NPM, 4 (7%) are classified as being involved in cell cycle, e.g. cyclein Y like protein 1 and cell division cycle protein 20 (Cdc20). In G_1 phase of the cell cycle, centrosomes serve as the main microtubule-organizing centre and, as such, their presence and normal functioning are crucial for the process of cell replication. A failure of microtubule organization would lead to an inability to form proper mitotic spindle resulting in a malfunction of cell division (Pfister and D'Mello, 2015). The G_1/S transition is regulated by cyclin E/cyclin-dependent kinase 2 (CDK2) phosphorylation and Okuda et al. (2000) demonstrated that NPM is bound to unduplicated centrosomes and alters cyclin E/CDK2 activity (Okuda et al., 2000). The complex between Cdc 20 and NPM has been reported to be involved in mitosis and it binds to APC/C (Anaphase-promoting complex or cyclosome). This complex is an essential component of the mechanism responsible for the clustering of synaptic vesicles at the active zone of the presynaptic membrane (Gieffers et al., 1999, Wang et al., 2016a). Thus any alteration in the Cdc-NPM complex may adversely impact on the mechanism of neurotransmitter release and could, together with the changed metabolism and decreased levels of neurotransmitters (Kashem et al., 2016), further add to the corruption of synaptic signalling in alcoholism (Spanagel, 2009).

Cyclin Y-like protein 1 (CLYP1) is another example of a protein identified as a partner of NPM. It is able to interact with Cyclin B/CDK1 complex (Jiang et al., 2009) suggesting that alcohol could interfere with the stem cell division. This process may involve Wnt signaling as indicated by a report of CLYP1-mediated mitotic Wnt signaling in the murine embryonic development and in the function (proliferation) of stem/progenitor cells (Zeng et al., 2016).

Myocyte enhancer factor-2 protein is a transcription factor regulating cell differentiation and is, therefore, likely to be involved in embryonic development (Pon and Marra, 2016).

2. Structural proteins.

Several structural proteins (four or 7%) have been detected as partners of NPM. Protocadherin (Pcdhs) is from a cadherin superfamily of homophilic cell-adhesion proteins. A cluster of Pcdhs

proteins has been detected throughout the neuronal soma, axons, and dendrites (Junghans et al., 2008, Hulpiau and van Roy, 2009). Ankyrin binds to various other membrane and structural proteins such as vimentin, microtubule-related proteins and spectrin. It plays a key role at the axonal initial segment of both central and peripheral neurons (Lambert et al., 1997).

3. Signalling proteins

Signaling proteins such as kinases, ATP-driven transport proteins and several metabolic enzymes were also contributing to the NPM interactome. Author cannot be certain whether the NPM interactions with some of these enzymes are direct or whether NPM binds another protein which, in turn, is a part of a greater complex or aggregate containing the above species. For example malate dehydrogenase binds αB-crystallin and the complex interacts with HSP70 (Mattoo and Goloubinoff, 2014, Park and Seo, 2015) which could then bind NPM (Table-1). Such a triprotein complex could be important in alcohol-exposed tissue because it is a part of the cellular chaperone network of HSP's that may act by preventing the aggregation of selected proteins under stress conditions by maintaining them in a folding-competent state and facilitating their liberation from aggregates in an active form via an ATP-dependent chaperone activity (Clerico et al., 2015, Park and Seo, 2015). The issue of protein complexes/aggregates may have to be addressed in greater detail by future studies involving additional proteins and their interactions (and perhaps misfoldings) in alcoholic brain tissue.

4. Proteins related to metabolism and energy production

If the expression and/or function of isocitrate dehydrogenase (ICD, Table-1), a key regulating enzyme of the NADH-producing TCA cycle, is affected by the low levels of NPM, the main source of energy and reducing power in the cells may be seriously compromised. This adverse status could be further compounded by simultaneous perturbations of another NPM-associated protein glutamate dehydrogenase (GDH, Table-1) which catalyzes the reversible interconversion of glutamate to α-ketoglutarate and ammonia (α-ketoglutarate is a substrate of another key TCA-regulating enzyme α-ketoglutarate dehydrogenase). Moreover, GDH expression itself is reduced by long-term excessive alcohol (Kashem et al., 2016). This could cause chronic malfunction of the TCA cycle resulting in deficient production of NADH (and ATP) thus further exacerbating the vulnerability of the brain tissue to oxidative stress caused by lower levels of GSH (Kashem et al 2009; Kashem et al., 2016).

Another component of the NPM interactome is Na$^+$/K$^+$-ATPase. This enzyme is particularly important in brain tissue; it maintains the ionic gradients across the neuronal membranes which are a *conditio sine qua non* for the maintenance of resting potentials as well as the generation and propagation of action potentials. The enzyme also provides the driving force - and interacts with - the normally very efficient glutamate transport mediated by the highly abundant transporters GLT1 and GLAST; these molecules may contribute to a functional complex in the astrocytic plasma membrane which not only transports glutamate but also regulates metabolism and blood flow (Pellerin and Magistretti, 1997, Abe and Saito, 2000;, Nanitsos et al., 2004, Moussa et al., 2002, Moussa et al., 2007, Bauer et al., 2012, Robinson and Jackson, 2016, Langer et al., 2017, reviews: Robinson et al., 2003, Sery et al., 2015). Increased expression of GLAST is one of the largest neurochemical changes linked to chronic alcohol exposure (Flatchscher-Bader et al., 2008, reviews Spanagel, 2009, Sery et al., 2015, but see also Alshehri et al., 2017) and any perturbation of the apparently NPM-associated enzyme Na$^+$/K$^+$-ATPase in alcoholic brain tissue would almost certainly compromise the normal function of glutamate transport and would have major consequences for the function and survival of brain neurons (Sheldon and Robinson, 2007).

The glycerolaldehyde-3-phosphate dehydrogenase (GAPDH), a protein with links to the control of reactive oxygen species (ROS), nuclear trafficking and signalling is known to be altered in brain tissue chronically exposed to alcohol (Kashem et al., 2012). It should not, therefore, be surprising that GAPDH has emerged among the proteins interacting with NPM. In response to oxidative stress, GAPDH undergoes S-nitrosylation followed by association with E3 ubiquitin-protein ligase (SIAH-1). The GAPDH-SIAH-1 complex moves from cytoplasm to the nucleus, where it participates in the regulation of gene expression and apoptosis (Tristan et al., 2011). Furthermore, Lee et al., (2012), using primary neural cultures, noted that the NPM can protect against N-methyl-D-aspartate (NMDA)-mediated excitotoxicity by inhibiting the SIAH1-GAPDH death cascade (Lee et al., 2012). Eukaryotic translation elongation factor 1 delta (EEF1D) is also known to interact with SIAH-1 and, as such, it acts as its negative regulator (Wu et al., 2011). Author has recently shown that alcohol increases EEF1D expression in NSC (Kashem et al., 2018) and the presently identified links of EEFD1 with alcohol exposure sensitive expression of NPM further underscores its importance in complex mechanisms by which cells respond to the presence of alcohol.

5. Response to oxidative stress

Alcohol-exposure of rat neural stem cells, cultured neurons and glia (Kashem et al., 2018) resulted not only in lower levels of NPM but also in significantly altered expressions of several forms of heat shock proteins (HSP) such as HSP 71, HSP 70 and HSP 60. All these, plus HSPA5 (78 kDa glucose-regulated protein precursor), have now been identified as contributing to the NPM interactome in human brain tissue (Table-1). NPM and the interacting HSP's may be linked to protective mechanisms mitigating oxidative damage (Liu et al., 2012) and their altered expressions in the alcohol-treated (Kashem et al., 2018) cells could reflect a response to the oxidative stress associated with alcohol exposure. Present data suggest that the response could involve interactions between NPM and HSP. Such interpretation appears plausible, given that HSP are known to interact with other proteins while promoting resistance to oxidative stress in the process. For example, interaction of glucose 6 phosphate dehydrogenase (G6PD) with phosphorylated homo-oligomers of heat shock protein beta-1 (HspB1) has been shown to result in a significant stimulation of oxidoresistance (Arrigo, 2013).

6. Proteins involved in transcription, translation, epigenetics and neuronal survival

Out of the 55 protein molecules associated with NPM about 41% belong to the class of DNA/RNA regulating proteins such as eukaryotic initiation factor (eIFs), ELM2 and SANT domain containing protein 1, spermatid perinuclear RNA-binding protein (SPRBP), retrotransposon-like protein 1, mediator of RNA polymerase II transcription subunit 16, structure-specific endonuclease subunit SLX1, eukaryotic translation initiation factor 5A-1, 26S protease regulatory subunit 4, Sp110 nuclear body protein, DNA damage-binding protein 2 (DDB2), adenosylhomocysteinase and DNA methyltransferases 3B (DNMT3B (Table 2). The protein eIFs is involved in the initiation phase of the eukaryotic translation process and promotes cell viability and cell growth (Wang et al., 2016b). It is a modulator of the protein synthesis and it is involved in the progression of the cell cycle, cellular proliferation and apoptosis (Sharma et al., 2016). This factor is also thought to facilitate protein synthesis by participating in the nuclear export and plays an important role in chaperon activity (Aitken and Lorsch, 2012). The gene *SLX1* encodes a protein that is an important regulator of genome stability. This protein represents the catalytic subunit of the SLX1-SLX4 structure-specific endonuclease, which can resolve DNA secondary structures during repair and recombination processes (Fricke and Brill, 2003; Coulon

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et al., 2004). The SPRBN is expressed at high levels in testis, ovary and brain; a mutant of this gene, if expressed in the sperm flagellum, causes defective motility of the sperm (Pires-daSilva et al., 2001) but its role in brain is unknown.

Adenosylhomocysteinase regulates methylation cycle and a deficient expession of this protein has been linked to hypermethioninemia (Palmer and Abeles, 1979). As DNA methyltransferases (DNMT's) use S-adenosyl methionine (SAM) for donation of the methyl group to DNA and the enzymes are strongly expressed during early embryo development, any alterations of the activity of adenylhomocysteinase could have an impact on a wide range of mechanisms involving DNA methylation from early differentiation to adult epigenetics (Maresca et al., 2015, Saitou and Miyauchi, 2016, Tognini et al., 2015). NPM may also interact with the human DDB2 protein which is involved in transcription and cell cycle regulation (Ghosh et al., 1996). Deficient expression of DDB2 could, in turn, be causally linked to a reduction in p21 protein which is a potent inhibitor of cyclin-dependent kinase (Kisielewska et al., 2009). The expression of p21, which, as the present data suggest, might be dependent on NPM, activates gene transcription by de-repressing p300–CREBBP (CREB-binding protein) and regulates p53-mediated apoptosis under cellular stresses (Abbas and Dutta, 2009); p53 involves transcriptional activation of pro-apoptotic genes and inhibits the anti-apoptotic genes. For example, p53 transcriptionally activates expression of BID and BAX (Chipuk et al., 2004) and induces apoptosis (Kashem et al., 2009a). These findings, therefore, indicate a potential relationship between NPM abundance and neuronal survival. This is interesting because NPM has also been linked with death induced domain protein 1 (PIDD) a protein acting as an effector of p53-dependent apoptosis (Berube et al., 2005) and recently shown to be aberrantly expressed in alcohol-affected brain tissue (Kashem et al., 2017). Thus, the NPM expression level could be a critical factor in determining the life and death of neurons in brain chronically exposed to alcohol.

b. Proteins interacting with dead end homology-1 (DND1)

1. Are DND1 and associated proteins critical for the survival of dividing cells?

The changes in DND1 expression presently seen in the human PFC (Fig 1) are in line with our recent findings that, following a four-day long exposure to alcohol, DND1 levels significantly increase in the cultured rat neural stem cells, neurons and astrocytes (Kashem et al., 2018). What could be the significance of an increased expression of DND1 in alcohol-exposed brain tissue?

The RNA-binding protein DND1 plays a key role in the primordial germ cell survival and migration at early embryonic development stage (Kedde and Agami, 2008) and this might be related to what author has observed in the neural stem cells (Kashem et al., 2018). The increase in DND1 expression could reflect, for example, a compensatory response by the transcription and/or translation apparatus of developing cells to potentially deleterious effects of alcohol. Such interpretation would seem to be strengthened by the observations suggesting that DND1 function is essential to maintain viable germ cells; its defects are known to produce sperm sterility and induce testicular germ cell tumours in mice (Youngren et al., 2005, Zhu et al., 2011). In fact, inactivation of DND1 in mice leads to progressive loss of germ cells and eventual male sterility (Bhattacharya et al., 2007). But what could one expect in the adult alcoholic brain tissue expressing abnormally high levels DND1?

Previous reports have shown that DND1 protects 3'-untranslated regions (UTR's) against miRNA-mediated gene suppression (Kedde and Agami, 2008). This is probably related to the key role of DND1 in the primordial germ cell survival and migration (Kedde and Agami, 2008). In the context, it is interesting that the present study has identified cell division control protein 6 (CDC6) as being part of the DND1 interactome (Table 2). CDC6 protein is essential for initiation and regulation of DNA replication and localizes to the cell nucleus at cell cycle phase G1 (Wlodarchak and Xing, 2016); further translocation of this protein being directed via phosphorylation by CDKs (Kisielewska et al., 2009). What seems, however, the most important is that CDC6 can promote DNA hyperreplication inducing a senescence response similar to that of oncogene activation; its deregulation has been discussed as a serious risk of cancer (Borlado and Mendez, 2008). This is an intriguing suggestion since deregulation of CDC6 in the presence of such highly abnormal levels of DND1 as observed in the present study seems quite probable. If this indeed triggers the process of cancerous growth, the actions of DND1 (present in excess) could be instrumental in further spreading the cancer given the similarities between the migrating embryonic cells and the metastatic cancer (Kedde and Agami, 2008, Zhu et al., 2011). To author's knowledge, links between alcoholism and brain tumours have not been systematically explored; above considerations indicate that research in this direction could be very worthwhile.

2. Proteins related to carbohydrate metabolism

In the DND1-protein "interactome", more than half of the proteins are related to the metabolism of carbohydrates. DND1 is usually thought to be involved in transcription so it is somewhat

intriguing why it should display such an apparent involvement in carbohydrate metabolism. One plausible explanation is that alcohol causes aggregation of many proteins and if any of the aggregated species possesses a DND1 binding epitope, all of such proteins may appear as "interacting" with DND1. Alternatively, as heat shock proteins have an ability to bind number of proteins and, since, HSP's are binding partners of DND1, the bound proteins may also appear as DND1 partners.

3. Miscellaneous DND1-associated proteins: Role of DND1 in cell differentiation and apoptosis?

Number of transcripts such as encoding pluripotency factors (OCT4, SOX2, NANOG, LIN28), cell cycle regulators (TP53, LATS2) and apoptotic factors (BCLX, BAX) have been previously detected by RIP (ribonucleoprotein immunoprecipitation) technique as interacting with DND1 (Zhu et al., 2011). Author has now identified zinc finger protein 257 and elongation factor 1-1 (α) in the postmortem brain tissue (Table 2) as interacting partners of DND1. The elongation factor 1 (α) is involved in the recruitment of aminoacyl-tRNA to the A-site of the 80S ribosome at protein synthesis (Itagaki et al., 2012, Rehman et al., 2012) and it also plays a central role in the nuclear export process (Khacho et al., 2008). Additionally, DND1 interacts with many mRNAs including those of both anti- and pro-apoptotic factor, BCLX and BAX (Zhu et al., 2011). It should be noted that Zhu et al., 2011 used embryonic stem cells while the present study is looking at a proteomic DND1 interactome and does so, for the first time, using adult human brain tissue (Zhu et al., 2011).

Concluding Remarks

The above interactions and the proposed mechanisms in which they may participate are based on both current and previous observations and, combined with the detected changes in the expressions of the two nuclear proteins NPM and DND1; they come some way towards explaining the damage to brain tissue which accompanies severe alcoholism. The presented discussion of possible interactions and their consequences is not intended to be exhaustive; this would be beyond the scope of the discourse. Rather, it offers examples of pathways which could be involved in the deleterious effects of long term excessive consumption of alcohol on brain ranging from synaptic malfunction and neurodegeneration to an increased risk of cancer.

Author should be aware of the limitations of the "interactomic" approach as discussed e.g. by Robinson and Jackson (2016). The proteins detected as "interacting" may do so only after the solubilization of the tissue and may not necessarily have a ready access to each other in vivo. This could be either because of their fixed separate locations in membranes or because they exist in distinct cells and/or compartments. In the case of nuclear proteins DND1 and NPM, however, it would be difficult to argue against genuine in vivo interaction with proteins known to be involved in DNA- or RNA-dependent mechanisms taking place in the nucleus or its immediate vicinity. Other findings, such as the multiple interactions with metabolic proteins are more difficult to justify but possible explanations are mentioned in the text.

It seems also obvious that the conclusions of the present study do not directly imply that the changes in DND1 and NPM are "pimary" consequences of alcohol exposure; changes in their levels may have resulted from altered levels/functions of their interacting proteins or, indeed, through any other mechanism linked to alcoholism.

In conclusion, the present study offers an extensive set of data which will be useful in future studies targeted at nuclear proteins such DND1 and NPM and their role in changes taking place in brain tissue exposed to excessive long term consumption of alcohol.

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CHAPTER 5

General Discussion

Impact of Alcohol in the Young

A. Impacts on Developing Brain:

i. Impact on Adolescent Brain: Evidence suggests that adolescent brain is particularly sensitive to alcohol relative to adult. Individuals who start to drink prior to 14 years of age are 4 times more likely to progress to addiction than those who do not commence using of alcohol until the age of 21 (Hingson et al., 2002, Grant,1998). Animal models revealed that alcohol intake in adolescence significantly increases the risk for alcohol-related problems and alcohol-dependence in later life (Füllgrabe, et al., 2007) and increases alcohol drinking 2-3 fold compared to an average adult drinker who would have started consuming alcohol later in life (Füllgrabe, et al., 2007, Siegmund, et al., 2005). The molecular mechanism of alcohol involvement in this latent dynamic effect has not been adequately documented.

Various avenues of research have demonstrated that alcohol exposure disrupts motor coordination, causes sedation and social disinhibition in adolescence (Spear, 2000, Spear, 2014, Spear, 2015). Experiments in Linda Spear's lab have demonstrated that adolescent rats are weak in digestion with alcohol relative to adult rats, and high level of alcohol is associated with more brain damage in their prefrontal cortex region (important for decision making) and in the brain regions related to working memory such as hippocampus (Spear, 2000). Repeated exposure to alcohol in adolescent rats suggested that long-term chronic alcohol is likely to be responsible for dramatic damage in several brain areas, including regions associated with learning (basal forebrain) and language acquisition (neocortex). Adolescents who have recently recovered from an alcohol dependence disorder revealed weak performance on verbal and non-verbal memory relative to a control group with no history of alcohol dependence (Brown et al., 2000), and had reduced volume in the hippocampus (Tapert and Schweinsburg, 2004).

Studies in animal models suggest that synaptic neurotransmission generally develops in utero or very early after birth. It does require further fine tuning post partum, through, and this process (including a degree of "rewiring") may continue well into adolescence (Wolff, 1984). Neurotransmitter dopamine is thought to be intimately involved with the reward system and undergoes pronounced developmental changes during adolescence (Spear, 2014, Spear, 2015).

The density of dopaminergic connections to the prefrontal cortex continues to increase during the life (Lambe et al., 2000; Rosenberg and Lewis, 1994; Tunbridge et al., 2007). Dopamine synthesis and its turnover, and projection of dopamine neurons from prefrontal cortex to the striatum are increased from adolescence to adulthood (Andersen et al., 1997; Teicher et al., 1993), and this shift of dopamine balance between prefrontal and subcortical structures may be, at least in part, due to synaptic "pruning" taking place in the neocortex (Bourgeois et al., 1994). The GABAergic neurotramission undergoes profound development during adolescence. In rodent (rats), neuron fibers from the basolateral amygdala continue to form connections with GABAergic interneurons in the prefrontal cortex throughout periadolescence (Cunningham et al., 2002, Wolff, 1984). In non-human primates, GABAergic inputs to pyramidal cells undergo changes during the perinatal period and adolescence (Akil and Lewis, 1992; Cruz et al., 2003) in concert with continued maturation of behavioral patterns mediated by the prefrontal cortex (Cruz et al., 2009). The input to GABAergic interneurons in the prefrontal cortex appears to decrease strongly from human adolescence to adulthood (Lewis et al., 2005, Spear, 2000). The various neurotransmitter changes occurring during adolescence are in synchrony with the anatomical changes seen in the prefrontal cortex and other brain regions during this stage, as well as maturation of cognition and behavior and the emerging increased risk for psychopathology (Paus et al., 2008). Alteration of dopamine and reward circuitry are critical to assigning value and reinforcing behaviors, such as social interaction, food consumption, romantic behaviors, novelty seeking, and alcohol and other drug intake (Spear, 2009), while ongoing refinement of inhibitory neurotransmission has broad implications for information processing and modulation of brain activity. Thus the alcohol consumption and vulnerability of the brain to alcohol's harmful effects may result in cognitive deficits and other problems that persist far beyond adolescence. Furthermore, the long-term behavioral changes that follow alcohol exposure during adolescence may involve subtle changes in neuronal connections and any potential deficits so caused may come to the surface at much later stages of life, including the old age.

The neurogenesis continues in the postnatal and adult brain in at least two regions close to the lateral ventricles [subventricular zone (SVZ)] and in the dentate gyrus of the hippocampus [subgranular zone (DGZ)] (Ming and Song, 2011, Fuentealba et al., 2012, Urban and Guillemot,

2014). Evidence suggests that the rate of neurogenesis varies with the age of the individual but is also influenced by environmental factors like anxiety, depression, drugs and, in particular, by alcohol. Pre- and/or postnatally impaired neurogenesis has been reported to result in abnormal behavior in later life and during adulthood. Prenatal exposure to ethanol can have long-lasting depressive effects on postnatal neurogenesis in the subgranular zone of the dentate gyrus and the abnormalities of this neurogenesis could be linked to deficits in cognitive function in later life (Winocur et al., 2006, Klintsova et al., 2007). The above evidence implies that the early exposure to ethanol can lead to latent changes in brain which may resurface at a later age, even in adulthood. Author has performed proteomics in 96 hour ethanol exposed rat neural stem cell (NSC) and identified 28 proteins that showed changed expression; these have been classified into following groups: metabolic proteins (13%), cytoskeletal (7%), proteins involved in signal transduction (7%), nuclear proteins including transcriptional factors (20%), molecular chaperones and stress related proteins (35%), and other (18%) (Kashem et al., 2018). Interestingly, nuclear protein nucleophosmin (NPM) expression was found to be decreased 1.8 fold in alcoholic exposed cell. NPM is located in the nucleolus and it is transported from nucleolus to nucleoplasm during serum starvation and to the cytoplasm by anticancer drugs. It regulates the following metabolic functions such as histone chaperon activity, ribosome biogenesis and transportation, genomic stability and DNA repairing, endoribonuclease activity, centrosome duplication during cell cycle, regulation of ARF-p53 tumor suppressor pathway, RNA helix destabilizing activity, inhibition of caspase-activated DNase and has been reported to prevent apoptosis (Qing et al., 2008). Ethanol at high concentration (50 mM) affected proliferation of stem cells and their transformation, leading to an apparently abnormal astrocytic phenotype; this phenotype alteration indicates that alcohol can modify intrinsic cellular mechanisms of stem cell which might in part be responsible for subsequent defective astroglial and neuronal functions (Vemuri et al., 2005). Thus, the cells that develop early "tolerance" to intoxicating environment might have impaired genomic or biochemical make up. Significantly, this may be a result of alcohol-related remodeling of the chromatin through acetylation of histones H3 and H4 in various brain regions (prefrontal cortex, nucleus accumbens, and striatum) as reported by many researches (MacDonald et al., 2009, Wit, 2010). This impaired neurons continue to develop, will get incorporated into the structure of brain and may confer abnormal structural and functional characteristics on whole brain regions.

ii. Impact on Fetal Brain: Alcohol induces anatomical (mainly, but not only neuroanatomical) and cellular changes in both rodents and humans. Neuroimaging of mice pups revealed that alcohol exposure during the gestation period results in malformations analogous to those observed in human FASD (Fetal Alcohol Spectrum Disorder), including facial dysmorphia and defective brain development. For example, exposure of mice to alcohol on gestational day (GD) 7 produces defects in the cerebral cortex, hippocampus and basal ganglia (Godin et al., 2010); while alcohol use on gestational day 8 produces neuronal death in the hindbrain and loss of ganglia, and abnormalities in fiber tracts (Parnell et al., 2009). Significant neural loss in the cortex, brainstem, and subcortical structures of offspring of Macaque monkeys exposed to ethanol during early pregnancy has been reported (Farber et al., 2010; Mooney and Miller, 2009). Loss of cortical neuron in offspring following ethanol exposure during gestation or in the postnatal period has been similar in the rodent (Schneider et al., 2011, Thompson et al., 2009). Neuroimaging of the human brain has revealed that significant reduction of the volume of cerebral cortex, cerebellum, hippocampus and the corpus callosum as observed in adult alcoholics are paralleled by similar changes in brain of FASD children (Biran et al., 2012).

Microstructural alterations of brain regions may be associated with (or even be a result of) altered genetic and/or biochemical make up. Protein expression studies in the corpus callosum (Kashem et al., 2007, Kashem et al., 2008, Kashem et al. 2009a), prefrontal cortex (Alexander-Kaufman et al., 2007) and hippocampus (Matsuda-Matsumoto et al, 2007) in post-mortem samples of human brain revealed that profound changes in the expression of specific protein are directly related to abnormal metabolism known to exist in alcoholic brains. Prolonged heavy alcohol consumption caused major changes in the phenotype in FASD-diagnosed offspring but low to moderate level of alcohol drinking which does not appear to lead to any phenotypic symptoms may nevertheless alter genetic and/or biochemical make up that could gradually result in the appearance of secondary symptoms and/or disabilities. Thus, it seems crucially important to develop early diagnostic tools and find out treatment strategies which could help timely identification of FASD individuals and made early medical intervention possible and effective.

B. Quest for Alcoholic Biomarker for FASD Diagnosis

Ideal clinical biomarker would be a distinct morphological physiological, biochemical, genetic, or molecular trait or substance that is specific to a particular medical condition. In practice,

FASD biomarker may not be fully reliable; like routinely used clinical detection system for conditions such as diabetes and heart disease, biomarkers in psychiatry and, in particular, in FASD would have limitations in clinical diagnosis, perhaps more so than in the somatic medicine (Boksa, 2013).

Chronic and/or heavy alcohol intake during pregnancy causes a wide range of damage to an unborn child and results most often in the following abnormalities in the newborn: low birth weight, small head circumference, developmental delay, various types of organ dysfunction, facial abnormalities, including smaller eye openings, flattened cheekbones and others (Shuler and Schroeder, 2013). Later in life this may extend to attacks of epileptic convulsions, poor coordination/fine motor skills, poor socialization skills, difficulty building and maintaining friendships, learning difficulty, poor memory, poor problem solving capabilities and serious-behavior problem including hyperactivity (Loomes et al., 2008, Paolozza et al., 2015, Hunt and Barnet, 2015). Depending on the amount of alcohol consumption and the frequency of drinking during early stages of pregnancy, still birth, abortion, or preeclampsia may occur (Ornoy and Ergaz, 2010). These may be, however, results of advance stages of alcoholism associated with previous continuing heavy drinking of alcohol.

Effects of alcohol involve molecular alterations at the DNA, RNA, protein and metabolite levels. Current diagnostics and follow up of most of the alcoholic disorders including neurological and neuropsychiatric disorders are based on a set of traditional biomarkers with cluster of symptoms and scales. Several alcohol biomarkers such as activities of gamma-glutamyl transferase, aspartate aminotransferase, alanine aminotransferase, and carbohydrate-deficient transferin have been used for many years. Other biomarkers include mean corpuscular volume, 5-hydroxytryptophol, serotonin metabolite, fatty acid ethyl esters, ethyl sulfate, phosphatidyl ethanol, phosphatidyl ethanolamine and ethyl glucuronide, and biomarkers of alcohol-induced tissue injury, such as those for alcoholic liver disease. Other, include alpha-smooth muscle actin , fibronectin, collagen type I, serum hyaluronate, matrix metalloproteinases. The author opinionis that- an ideal biomarker for alcoholic disorders have the following characteristics: (a) ability to diagnose the abnormality resulting from low-to-moderate levels of drinking over extended periods of time; (b) high sensitivity for detecting abnormalities during pregnancy, (c) high authenticity (low rate of false-positive results), (d) accessibility of biological sample, easy analysis with little amount of sample and simple collection and preparation technique (e)

economical and fast. It is evident that none of the above biomarkers are anywhere near to an adequate sensitivity and specificity required for reliable diagnosis of FASD. At presence fatty acid ethyl esters (FAEEs) in meconium is employed to identify alcohol exposure during pregnancy. However, the biomarker molecules FAEEs do not provide information on the timing, duration and dosage of exposure to alcohol (Litten et al., 2010). Moreover, FAEEs do not provide information on the extent of damage and/or on the mechanism of alcohol-related injury. Thus, there is a significant need for more reliable biomarkers that can be used for FASD diagnosis. Such biomarkers should be related to pathogenicity of alcohol, indicating the level of existing damage and thus helping to plan the treatment.

Author has studied differential protein expression in alcoholic post-mortem human/rat brains (Kashem et al., 2007, Kashem et al., 2008, Kashem et al., 2009a, Alexander-Kaufman et al., 2007, Matsuda-Matsumoto et al., 2007, Kashem et al., 2012) and identified 238 proteins that are associated with alcohol induced abnormal metabolic disorders. Based on the functions of these proteins author selected certain proteins such as tyrosine hydroxylase, dopamine β hydroxylase, catechol-O-methyltransferase, DOPA decarboxylase, tryptophan hydroxylase, glutamate decarboxylase-2, glutamate synthetase, N-acetylaspartate-glutamate synthetase, glutamate carboxypeptidase II, histidine decarboxylase, glutathione peroxidase, glutathione synthase, glyoxalase-1, apolipoprotein A-I, triosephosphate isomerase, isocitrate dehydrogenase, phospholipase D, SNAP-25, stathmin, sirtuin-2, DJ-1, α-synuclein, protein phosphatase, DARPP-32, CREB, apoptic death agonist (Bid) and cathepsin D as those with the greatest potential to become biomarker for alcoholism and alcohol-related disorders.

In addition to protein expression, author has analysed metabolites, extracted from the tissues of the nucleus accumbens, the caudate nucleus and the putamen regions of alcoholic postmortem human brain by liquid chromatography-tandem mass spectroscopy (LC-MS/MS). Initial data suggest that excessive alcohol drinking significantly alters dopamine, serotonin, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-hydroxyindoleacetic acid (HIAA), norepinephrine (NE), histamine, choline, acetylcholine, gamma-aminobutyric acid (GABA), glutamate (Glu) and glutathione and, neurotransmitters particularly DA and 5HT ratio (Kashem et al., 2016). Prenatal alcohol exposure reduces the concentrations of catecholamines, indolamine, and amino acid neurotransmitters (GABA, Glu) in E13 fetal mice brains (Sari et al., 2010). This study suggests that alterations of selective neurotransmitters may be useful for

biomarker of FASD (Sari et al., 2010). However, several other molecules such as polyamines (PA), the naturally occurring di-, tri-, and tetra amines, are closely related to the neuronal activity of the brain via interactions with neurotransmitter receptors (N-methyl-D-aspartate receptor), regulation of substances in degenerating cells and protecting neurons from oxidative damage (Spalloni et al., 2013). In mammals, cellular polyamines (PAs) (putrescine, spermidine and spermine) derive from ornithine. This may be significant because alcohol is known to alter the metabolism of PAs in the cells. Moreover, administration of PA can reduce ethanol consumption in rats genetically predisposed to drinking without producing significant side effects (Bilbeny et. al., 2005). Therefore, it would seem important to study the endogenous concentrations of PA in the brain and other tissue of alcoholic humans and animals in order to better understand the role (and potential benefits) of PA in alcoholism.

C. Impact of Alcohol on Male Reproductive System -Paternal FASD

Those who get used to drinking regularly while young may find it difficult to stop when they reach to the age of reproduction. Birth defects characteristic of FASD have almost universally been thought of as associated with the mother drinking during pregnancy. To date no known safe amount of alcohol or safe time to drink alcohol seems to have been identified for male or female who intend to have a family. It has been virtually assumed that fetal FASD would be prevented if mother abstains from alcohol during pregnancy; however father's contribution to FASD has not been adequately considered. It used to be thought that the offspring is not significantly affetcted by paternal drinking but alcohol consumed by the male can lead to a variety of problems in the male reproductive system particularly in the quality of sperm (Lee et al., 2013). When children of fathers who are drinkers and non-alcoholic mothers are compared with those diagnostic with FASD, the children of the drinking fathers are usually not grossly malformed, but they do have certain intellectual and functional deficits in common, and they are also more likely to be hyperactive (Kim et al., 2014).

Alcohol abuse can have a particularly negative effect on the male reproductive system resulting in infertility, hormonal problems and various sexual disorders. The molecular mechanisms by which alcohol impact on the system at cellular level remains virtually is unknown. Studies on Danish young army man (age 18-25 of 1221 individuals) have shown that regular intake of 5 units (50 ml or 40g ethanol daily; standard drink in Australia is 10 g) alcohol was associated with

a significantly higher levels of the serum free testosterone and sperm level up to 33% lower compared to nondrinkers (Jensen et al., 2014).

Mammalian spermatozoa consist of a head, neck and tail and contains lots of proteins and also have enzymes that for solubilization of the membrane of oozyte. The nucleus of the sperm condenses chromatin with the genetic information. By proteomics study using mature human sperm Wang et al., identified 4675 proteins; of which 227 are testis specific (Wang et al., 2013). Oliva and Castillo suggested that 56% of sperm nuclear proteins have a potential to be involved in epigenetic mechanisms such as chromosome organization, chromatin organization, protein-DNA complex assembly, DNA packaging, gene expression, transcription, chromatin modification and histone modification (Oliva and Castillo, 2011). Chronic alcohol and cocaine is associated with significant decreases various miRNA levels in the testis (Bielawski et al., 2002: Ouko et al., 2009). MiRNAs are small molecules that interfer the protein expression and sperm contained various precursors and mature miRNAs. Several sperm miRNAs such as mmu-mir-133b-3p, -196a-5p, 205-5p, 340-5p,9 29c, 30a, 30c, 32, 193-5p, 204, 375, 5323p, and 698 that author previously reported as altered in fathers sperm due to this high fat diet, stress and obesity (Fullston et al., 2013). Interestingly, mir-133b-3p is up-regulated 3 fold in the sperm of high fat diet father (Fullston et al., 2016). Author recently found that *C. elegans* animals lacking miRNAs, miR-83 (human homologs, hsa-29) and miR-245 (hsa-133) both show a dramatic increase in oxidative stress resistance and lifespan extension (Kashem et al.,unpublished data) and deletion of mir-133 induced dopamine synthesis and activates behavioral aggregation in Locusts (Yang et al., 2014). Moreover, microinjection of mir-34c inhibitor into zygotes inhibited DNA synthesis and significantly suppressed first cleavage division (Liu et al., 2012) and this miRNA is highly abundant in the brain and body tissues where its expression is increased in ethanol-exposed mouse liver (Meng et al., 2012). Another mir-124 is decreased by alcohol (Kim et al., 2014) and injection of this brain developing miRNA into the fertilized eggs resulted in progeny that exhibited a 30% larger body size relative to normal, and this giant phenotype persisted up to F2 generation; after that the body size of the descendants reverted back to normal (Grandjean et al., 2009). Beside the implication of miRNAs on male reproductive system, Author has proposed a specific hypothesis which has been published and is presented in full below.

Hypothesis:

Could Ethanol-Induced Alterations in the Expression of Glutamate Transporters in Testes Contribute to the Effect of Paternal Drinking on the Risk of Abnormalities in the Offspring?

Based on a paper published in Medical Hypothesis (Kashem et al., 2017. Medical Hypothesis, 98: 57-59).

Abstract

It has been known that a preconception paternal alcoholism impacts adversely on the offspring but the mechanism of the effect is uncertain. Several findings suggest that there are signalling systems in testis that are analogous to those known to be altered by alcoholism in brain. Author proposed that chronic alcohol affects these systems in a manner similar to that in brain. Specifically, author hypothesise that excessive alcohol may disturb glutamatergic-like signalling in testis by increasing expression of the glutamate transporter GLAST (EAAT1). Author discuss ways how to test the hypothesis as well as potential significance of some of the tests as tools in the diagnostics of chronic alcoholism.

Background

Presence of apparent cognitive deficits in children fathered by heavily drinking men has been known since antiquity (Ouko et al., 2009). The mechanism(s) by which paternal alcohol-drinking inflicts damage on the offspring remain(s), however, obscure. It has been suggested that the DNA in male gametes affected by alcohol is altered epigenetically, e.g. by an interference in the methylation of DNA cytosine but the evidence, particularly when obtained in humans, is scarce and may be contradictory. Some groups have reported a decrease in the expression and activity of a DNA methyltransferases (Ouko et al., 2009, Bielawski et al., 2002) resulting in a failure of orderly suppression of specific DNA sites in male-contributed alleles (Ouko et al., 2009). In contrast, results of other studies, using male mice exposed to ethanol, implied that an increased methylation of specific DNA loci, e.g. cytosine-rich sequences ("CpG islands") in the promoter region of the *DAT* gene encoding a dopamine transporter, could be at fault (Kim et al., 2014). Disturbed expression of *DAT* has, indeed, been causally linked to an attention-deficit/hyperactivity disorder (ADHD)-like behaviour similar to that observed in the offspring of the ethanol-exposed male mice (Kim et al., 2014); (cf. review of animal models of ADHD (Russel, 2011) and altered expression of *DAT1/SLC6A3* in human ADHD (Šerý et al., 1999). Here authors propose an alternative hypothesis which, in their view, fits better the broad

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spectrum of deficits encountered in the offspring of alcoholic males. It is based on the probable presence in the testis of signalling mechanisms similar to those known to exist in the central nervous system.

The main synaptic transmitters in brain are L-glutamate (excitatory) and GABA (inhibitory); (for a historical review see (Bennett and Balcar, 1999). Both glutamatergic and GABAergic synapses are thought to be strongly involved in mediating the effects of alcohol (Spanagel, 2009, particularly via the NMDA-type of glutamate receptor and the alpha4-subunit containing GABA(A) receptor (Rae et al., 2014, Lovinger et al., 1990, Allgeier, 2002). Both GABAergic and glutamatergic signalling systems, including the two types of receptors mentioned above, are altered in alcoholism (Kashem et al., 2016, Bekdesh and Herison, 2015, Davidson et al., 1995, Burnett et al., 2016). Interestingly, it is not only the synaptic receptors which are affected by alcohol but also the neurotransmitter-inactivating mechanisms that are changed in alcoholic brains, particularly in the case of glutamatergic neurotransmission.

In the central nervous system (CNS), synaptically released L-glutamate is inactivated by several specific transporters located mainly but not exclusively in the plasma membrane of surrounding astrocytes. There are five genes coding for the transporters (reviews: [Zhu and Danbolt, 2013, Sery et al., 2015]) but protein products of two of them predominate: GLAST transporter encoded by *SLC1A3* and GLT1 transporter encoded by *SLC1A2*. GLT1 and GLAST are also referred to as EAAT1 and EAAT2, respectively, particularly when discussing human brains. Both transporters require Na^+ and K^+ transmembrane gradients as the driving force to transport L-glutamate from the extracellular space (reviews: [Zhu and Danbolt., 2013, Sery et al., 2015]). In addition, GLAST acts as a chloride-selective ligand- (L-glutamate-) gated channel and thus has a capability to hyperpolarise GLAST-expressing cells in the presence of L-glutamate [review: Sery et al., 2015]. Both GLAST and GLT are mostly expressed in the central nervous system (Zhu and Danbolt, 2013, Sery et al., 2015); the only other tissue which expresses them in significant quantities is testis (Redecker et al., 2003, Hu et al., 2004, Hu et al., 2004, Takarada et al., 2004, Lee et. al., 2011).

In the testes, GLT1 (EAAT2) has been detected in the interstitial cells but it is located mainly in the seminiferous tubules, both in the Sertoli cells and in the sperm. GLAST is found, apart from the Sertoli and interstitial cells, in the sperm, apparently in its anterior part, possibly concentrated

in the acrosome (Hu et al., 2004, Takarada et al., 2004). The other glutamate transporters that have been detected in the testes are EAAT5 which acts mostly as an L-glutamate-gated chloride channel (Wersinger et al., 2006, Veruki et al., 2006) and EAAT3 (Hu et al., 2004, Takarada et al., 2004).

The precise role of glutamate transporters in the testes is not known. However, L-glutamate is the most abundant free amino acid in testis (Setchell et al., 1967) and is present inside the seminiferous tubules. Moreover, the compartment is separated from the blood stream by a very tight blood-testis barrier formed by tight junctions in the Sertoli epithelium. The presence of L-glutamate (together with several "synaptic" proteins; cf. [Hu et al., 2004] and receptors [(Hu et al., 2004, Takarada et al., 2004)]) sequestered behind such barrier would seem to imply that a signalling apparatus, possibly involving germ cells and sperm, both in their mature and immature forms either dormant or active and mediated by L-glutamate, exists and functions in testes. Glutamate transporters would then provide a regulating ("inactivating") mechanism analogous to that functioning at brain synapses (reviews: [Bekdesh and Herison, 2015, Davidson et al., 1995). Alternatively, L-glutamate transport, mainly by GLAST (EAAT1) and EAAT5 could trigger chloride influx thus hyperpolarizing and activating the sperm (Lee et al., 2011).

The Hypothesis

It has been reported that chronic exposure to large doses of alcohol is associated with significant (several-fold) increases in the expression of GLAST (EAAT1), both in mice and men (Rimondini et al., 2002, Flatcher-Baader and Wilce, 2008). Should a similar overexpression of GLAST (EAAT1) occur in testes under similar circumstances (chronic severe alcoholism), sperm would become much more susceptible to the activation than the sperm of non-alcoholic males possibly leading to a formation of active immature sperm. Subsequent fertilization by such immature sperm would significantly increase the risk of developmental defects in the offspring (Tesarik, 1998).

Evaluation

It should be understood that the developmental defects caused by the paternal alcoholism are distinct from the better known Foetal Alcohol Spectrum Disorder (FASD) and would be entirely independent of the alcoholic status of the pregnant female. The hypothesis does not negate the

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role of epigenetics as a mediator of the effect of paternal alcohol consumption on the offspring; there is ample evidence for such mechanisms from rodent studies (for a review see [Lee et al., 2010]). The present hypothesis adds, however, an extra element to the picture and could help to explain the complexity of the phenomenon (Finegersh and Wilce, 2015).

Testing the hypothesis would present several significant challenges. Firstly, glutamate transporters are expressed in a number of splice variants and the splicing pattern in the testes appears very different from that in the CNS (Veruki et al., 2008). Use of single antibodies against GLAST (EAAT1) or GLT1 (EAAT2) in the testicular tissue could, therefore, easily miss or underestimate the full extent of the changes in GLAST (EAAT1) expression putatively caused by chronic alcoholism. Suitable antibodies against a range of splice variants are available (Lee et al., 2010, Lee et al., 2012) though not yet on commercial basis. The glutamate transporter immunohistochemistry would have to be complemented by in situ hybridization using judiciously selected antisense oligonucleotides to reveal the full extent of the changes and their loci. Secondly, not just the presence but also the function of GLAST (EAAT1), GLT1 (EAAT2) and EAAT5 both in the seminiferous tubules and in the sperm would have to be investigated. Initial approach encountered significant methodological hurdles (Lee et al., 2011) but these can be overcome. Given the proposed hypothetical importance of the transporter molecules in the sperm activation, samples of sperm should perhaps be used directly in this type of studies. Thirdly, a link between the proposed overexpression of GLAST in the testes and/or in the sperm following chronic exposure to alcohol and observed deficits in the offspring would have to be established. This experimental component seems crucial in testing the validity of the hypothesis but may also be the most difficult, probably requiring a large (inter-disciplinary) animal-based approach. In the meantime, GLAST (EAAT1) expression in the sperm of alcoholic men could be looked at and perhaps evaluated as a possible diagnostic test for the severity of chronic alcoholism.

Conclusion and Significance of the Hypothesis

It is proposed that the mechanism of the effect of paternal alcoholism on inborn deficits in the offspring can be at least in part explained by a changed pattern of the expression of glutamate transporters in the testes of alcoholic fathers. This would involve, in particular, overexpression of GLAST (EAAT1) glutamate transporter. Testing the hypothesis presents significant

methodological challenges but none of them seem in principle unsurmountable. Additionally, the tests could yield an important diagnostic tool potentially useful in assessment of chronic alcoholism in men. Should the hyperactivity of glutamate transporter GLAST be an important part of the mechanism, there is extensive pharmacological information on structural requirements of glutamate transporters that could greatly facilitate design and synthesis of specific inhibitors (Balcar, 2002, Bridges and Esslinger, 2005).

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D. Consequence of Alcohol on Energy Metabolism in Brain Cells

In NPM and DND1 interactome studies, 30% of the associated partners of both proteins are related to energy metabolism process. Previously, in proteomic studies, author concluded that 96 (42%) out of 238 proteins could be classified as related to energy metabolism. These findings could be taken as an indication that one of the chief (undesirable) effects of alcohol is a disruption of energy metabolism. It is known that glycolysis, the pentose phosphate pathway, and the tricarboxylic acid cycle (TCA) play an important role in ATP production and maintaining normal synaptic functions in the CNS (Bolaños et al. 2008). Author has previously found the expression of triose phosphate isomerase (TPI), glycerol-3-phosphate dehydrogenase (G3PDH) and glyoxalase 1 (Gly-1) significantly up-regulated in the alcoholic brain (Kashem et al., 2016, Kashem et al., 2012, Kashem et al., 2009b, Kashem et al., 2008). TPI catalyzes reversible interconversion of dihydroxyacetone phosphate (DHAP) and glyceraldehyde phosphate (G3P). G3P is further metabolized for formation of ATP via TCA cycle. One physiological effect of this reaction is the prevention of the DHAP accumulation in the organism, which can otherwise be transformed into methylglyoxylate (MG), a toxic compound that interferes with a variety of metabolic processes (Danise et al., 1999) (Figure 1). It has been found that when red blood cells were incubated with 50 mM glucose for 2 hr, MG and triose phosphates were accumulated and this accumulation of MG was prevented by thiamine supplementation (Thonrnalley et al., 1998). Author identified the thiamine and/or other vitamin biosynthesis enzymes are altered by alcohol (Kashem et al., 2016, Kashem et al., 2012, Kashem et al., 2008). However, G3PDH plays a pivotal role in distinct metabolic functions which include: (1) generation of glycerol-3-phosphate for phospholipid and triglyceride biosynthesis, and (2) conversion of glycerol-3-phosphate to DHAP during gluconeogenesis. Glycerol-3-phosphate dehydrogenase has been identified in the brain (Kashem et al., 2008, Kashem et al., 2012, Leveille et al., 1980) which forms glycerol-3-phosphate during phospholipid synthesis. The increased activity of G3PDH in both the white matter (WM) (Morland et al., 2007) and the body of the CC (Kashem et al., 2009b), suggests that alcohol accelerates the synthesis of phospholipids or induces the reversal of carbon flow

towards DHAP (Figure 1). Previous studies (Kashem et al. 2007; Kashem et al. 2008), TCA cycle first ATP generating enzyme isocitrate dehydrogenase (ICD), has been found to be decreased in the alcoholic CC (Kashem et al., 2009b). The low expression of ICD in the brain cell ceased the maximum capacity to utilize three carbon compounds in the TCA cycle leading to redirection of carbon flow towards DHAP formation, concomitantly, decreasing the rate of ATP production from glucose (Figure 1). Thus, the high level of DHAP activates the accumulation of MG. Author has previously established that the exprression of MG detoxifying enzyme, Gly-1 decreased both in rat striatum and human CC (Kashem et al., 2012, Kashem et al., 2009b, Kashem et al., 2008). The Gly-1 protein expression was increased in early Alzheimer (AD) cases and decreased in late AD (Kuhla et al., 2007). Down-regulation of the Gly-1 in late AD was due to the formation of high level of advanced glycation end products (AGEs) (Kuhla et al., 2007). MG reacts with free amino acids and thiol-molecules to form AGEs and the high level of AGEs contributes to neuronal death and neurodegenerative diseases (Shangari et al., 2005).

Reactive oxygen species (ROS) are cooperatively and synergistically scavenged for and/or degraded by enzyme systems like superoxide dismutases (SOD's), glutathione peroxidase (GPX), glutathione S-transferase (GST) and glutathione reductase (GR) (Yan et al., 2008). I previously observed that GPX expression was decreased in the splenium (Kashem et al. 2007) and, GST(μ) expression was ceased in alcoholic genu (Kashem et al., 2008). The, GST (another isoform) and SOD (Zn^{2+}/Cu^{2+}-dependent in the cytoplasm) were significantly increased in alcoholic tissue compared to control (Kashem et al., 2012, Kashem et al., 2007). Superoxide anions ($O_2{}_{\bullet}{}^-$) are reduced by SOD to form molecular oxygen and hydrogen peroxide (H_2O_2). This H_2O_2 is reduced by GPX through oxidation of two glutathione (GSH) molecules. The oxidized GSH (called glutathione disulfide, GSSG) is subsequently reduced by GR under consumption of NADPH (Berkholz et al., 2008). The low expression of ICD in this tissue might be correlated with this pathway in relation to NADPH supply. Vogel et al., 1999 suggested that redox balancing and oxidative damage prevention are the primary functions of ICD through the supply of NADPH for glutathione (GSH) redox cycling systems. Taken together, it is suggested that alcohol possibly alters the glucose metabolic carbon flow towards MG production, leading to starvation of TCA precursor molecules, and concomitantly,

decreases the ATP production. The starvation energy (ATP) unable to balancing free radicle leading to activates apoptosis signaling pathway (Figure 1).
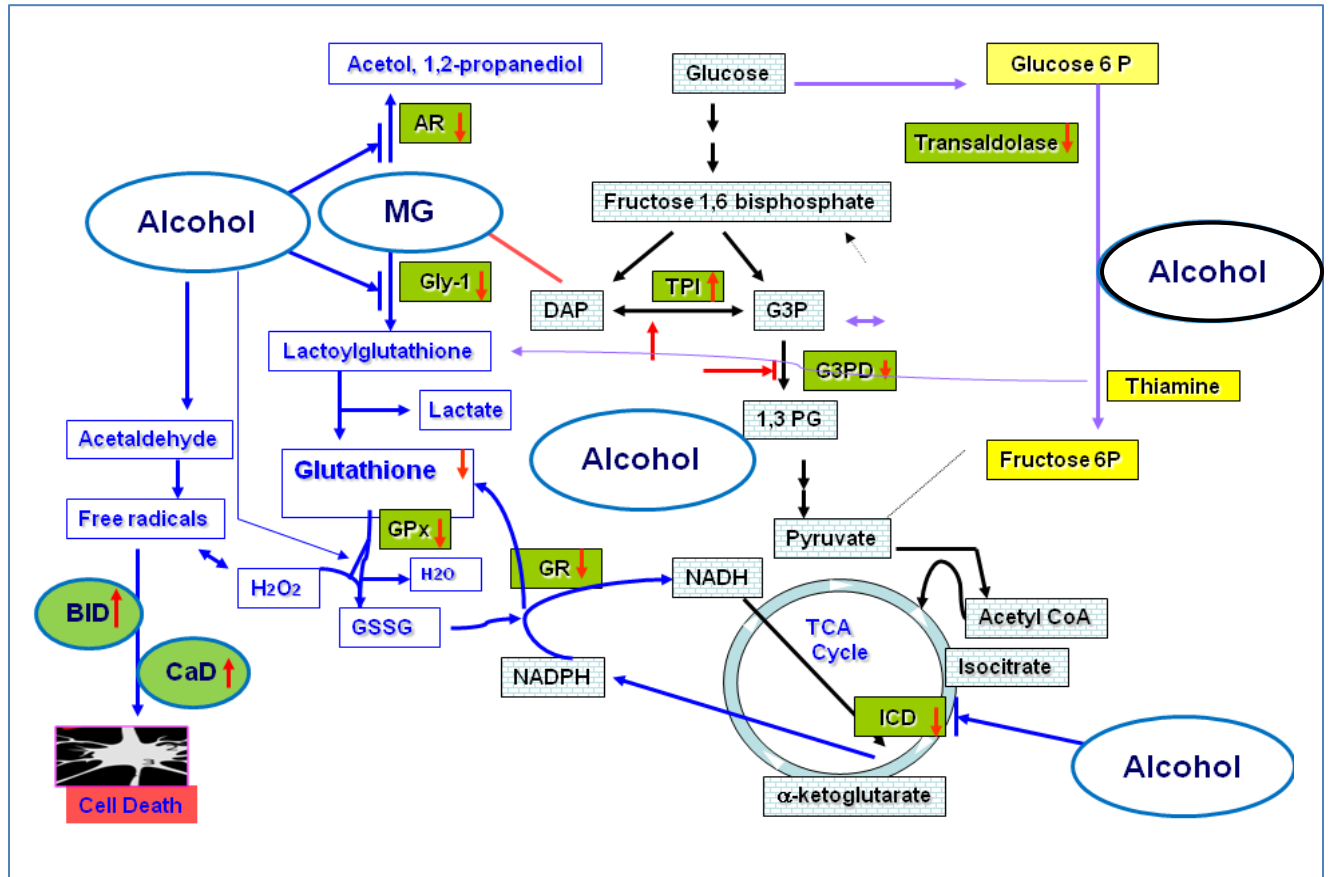

Fig. 1. Hypothetical model for accumulation of toxic carbonyl bioproduct, inhibition of oxidative stress defense system, cascade of thiamine, and apoptosis signaling of alcoholic human brain(glucose 6P, glucose 6 phosphate; fructose 6P, fructose 6 phosphate; TPI, triose phosphate isomerase; 1,3 PG, 1,3 phosphoglyceride; DAP, diacetone phosphate; ICD, isocitrate dehydrogenase; AR, aldose reductase; MG, methylglyoxal; G3P, glycerol-3-phosphate; G3PD, glycerol-3-phosphate dehydrogenase; Gly-1, glyoxalase-1; GPx, glutathione peroxidase; GR, glutathione reductase; CaD, cathepsin D, BID, apoptotic death agonist; TCA cycle, tricarboxylic acid cycle; GSSG, glutathione disulfide; NADH, nicotinamide adenine dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate.

CHAPTER 6

These are references for the introduction and some parts of general discussion

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CHAPTER 7

Supportive Document: Candidate's Published Articles Related to the Study

Mohammed Abul Kashem's scientific contributions:

Publications (23), Citation 535

- **Article: Differential protein expression in the corpus callosum (splenium) of human alcoholics: A proteomics study**

Mohammed Abul Kashem · Gabriel James · Clive Harper · Peter Wilce · Izuru Matsumoto
Abstract: It is widely accepted that the chronic use of alcohol induces metabolic abnormalities and neuronal damage in the brain, which can lead to cognitive dysfunction. Neuroimaging studies reveal that alcohol-induced brain damage is region specific and prominent damage has been observed in both gray and white matter of the prefrontal cortex, and a wide range of white matter structures including the corpus callosum. Molecular mechanisms underlying these structural changes are largely unknown. Using...Show More
Article · Jan 2007 · Neurochemistry International

Chapter: Environmental Proteomics: Extraction and Identification of Protein in Soil

Zakaria Solaiman · Mohammed Abul Kashem · Izuru Matsumoto
Full-text available · Chapter · Jul 2007

Article: CNS proteomes in alcohol and drug abuse and dependence

Izuru Matsumoto · Kimberley Alexander-Kaufman · Takeshi Iwazaki· Mohammed Abul Kashem · Haruka Matsuda-Matsumoto
Abstract: Drugs of abuse, including alcohol, can induce dependency formation and/or brain damage in brain regions important for cognition. 'High-throughput' approaches, such as cDNA microarray and proteomics, allow the analysis of global expression profiles of genes and proteins. These technologies have recently been applied to human brain tissue from patients with psychiatric illnesses, including substance abuse/dependence and appropriate animal models to help understand the causes and secondary... Show More
Full-text available · Article · Sep 2007 · Expert Review of Proteomics

Article: Proteomic analysis of hearts from frataxin knockout mice: Marked rearrangement of energy metabolism, a response to cellular stress and altered expression of proteins involved in cell structure, motility and metabolism

Robert Sutak · Xiangcong Xu · Megan Whitnall · Mohammed Abul Kashem · Des R Richardson

Abstract: A frequent cause of death in Friedreich's ataxia patients is cardiomyopathy, but the molecular alterations underlying this condition are unknown. We performed 2-DE to characterize the changes in protein expression of hearts using the muscle creatine kinase frataxin conditional knockout (KO) mouse. Pronounced changes in protein expression profile were observed in 9 week-old KO mice with severe cardiomyopathy. In contrast, only several proteins showed altered expression in asymptomatic 4...Show More

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Article: Adolescent Rats Find Repeated Δ9-THC Less Aversive Than Adult Rats but Display Greater Residual Cognitive Deficits and Changes in Hippocampal Protein Expression Following Exposure

Heidi R Quinn · Izuru Matsumoto · Paul D Callaghan · Leonora E Long · Iain S McGregor

Abstract: The current study examined whether adolescent rats are more vulnerable than adult rats to the lasting adverse effects of cannabinoid exposure on brain and behavior. Male Wistar rats were repeatedly exposed to Delta-9-tetrahydrocannabinol (Delta(9)-THC, 5 mg/kg i.p.) in a place-conditioning paradigm during either the adolescent (post-natal day 28+) or adult (post-natal day 60+) developmental stages. Adult rats avoided a Delta(9)-THC-paired environment after either four or eight pairings and... Show More

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Article: Kashem MA, Harper C, Matsumoto I. Differential protein expression in the corpus callosum (genu) of human alcoholics. Neurochem Int 53: 1-11

Mohammed Abul Kashem · Clive Harper · Izuru Matsumoto

Abstract: Ethanol is an addictive drug that deteriorates different neuronal pathways in the CNS, leading to the induction of cognitive dysfunction. Neuroimaging analyses revealed that alcohol-induced brain damage appears to be region-specific and major dysmorphology has been observed in the prefrontal cortex and the white matter (WM) particularly in the corpus callosum (CC). Recent diffusion tensor imaging (DTI) analysis indicated that microstructural degradation was prominent in the genu followed by... Show More

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Article: Hargreaves GA, Quinn H, Kashem MA, Matsumoto I, McGregor IS. Proteomic analysis demonstrates adolescent vulnerability to lasting hippocampal changes following chronic alcohol consumption. Alcohol Clin Exp Res 33: 86-94

Garth A Hargreaves · Heidi Quinn · Mohammed A Kashem · Izuru Matsumoto · Iain S McGregor

Abstract: Excessive teenage alcohol consumption is of great concern because alcohol may adversely alter the developmental trajectory of the brain. The aim of the present study was to assess whether chronic intermittent alcohol intake during the adolescent period alters hippocampal protein expression to a greater extent than during adulthood. Adolescent [postnatal day (PND) 27] and adult (PND 55) male Wistar rats were given 8 hours daily access to beer (4.44% ethanol v/v) in addition to ad libitum food... Show More

Article: Comparative proteomics in the corpus callosal sub-regions of postmortem human brain

Mohammed A Kashem · Ranjana Sarker · H Des Etages · Rita Machaalani · Izuru Matsumoto

Abstract: The corpus callosum (CC) is a single anatomical region with homologous cytoarchitecture and divided into four sub-regions such as the rostrum, the genu, the body and the splenium. Neuroimaging analysis revealed that susceptibility to clinical neurological diseases of these sub-regions is variable, indicating biochemical and physiological heterogenecity. To understand the biochemical make up of these regions, we compared the protein expression of these three sub-regional areas [the genu, the...Show More

Article: Differential protein expression in the corpus callosum (body) of human alcoholic brain

Mohammed Abul Kashem · Huyghues Des Etages · Natasa Kopitar-Jerala · Iain S McGregor · Izuru Matsumoto

Abstract: Neuroimage analysis in alcoholic corpus callosum (CC) suggests that microstructural abnormalities are higher in the genu followed by the body and the splenium. Molecular mechanisms underlying these dysmorphologys are still unclear. Protein expression was performed using the CC body samples [(nine controls, seven uncomplicated, and six complicated (with liver cirrhosis) alcoholics] through proteomics approach. Thirty-nine protein spots in uncomplicated and 60 in complicated alcoholics were... Show More

Article: Effects of typical (haloperidol) and atypical (risperidone) antipsychotic agents on protein expression in rat neural stem cells

Mohammed A. Kashem · Rahnuma Ummehany · Wataru Ukai · Eri Hashimoto · Izuru Matsumoto

Abstract: Neural stem cells (NSCs) play a crucial role in the development and maturation of the central nervous system. Recently studies suggest that antipsychotic drugs regulate the activities of NSCs. However, the molecular mechanisms underlying antipsychotic-induced changes of the activity of NSCs, particularly protein expression, are still unknown. We studied the growth and protein expression in haloperidol (HD) and risperidone (RS) treated rat NSCs. The treatment with RS (3 μM) or HD (3 μM) had...Show More

Article: A long hangover from party drugs: Residual proteomic changes in the hippocampus of rats 8 weeks after ??-hydroxybutyrate (GHB), 3,4-methylenedioxymethamphetamine (MDMA) or their combination

Petra S van Nieuwenhuijzen · Mohammed A Kashem · Izuru Matsumoto · Glenn E Hunt · Iain S McGregor

Abstract: 3,4-Methylenedioxymethamphetamine (MDMA) and gamma-hydroxybutyrate (GHB) are popular party drugs that are used for their euphoric and prosocial effects, and sometimes in combination. Both drugs increase markers of oxidative stress in the hippocampus and can cause lasting impairments in hippocampal-dependent forms of memory. To gain further information on the biochemical mechanisms underlying these effects, the current study examined residual changes in hippocampal protein expression measured... Show More

Conference Paper: STUDIES OF ADOLESCENT BINGE DRINKING AND ITS CONSEQUENCES USING THE BEER MODEL IN RATS

I. S. McGregor · G. A. Hargreaves · M. A. Kashem · M. Bowen · P. D. Callaghan

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Article: FEMALE NEUREGULIN 1 HYPOMORPHIC MICE DISPLAY WILD TYPE-LIKE RESPONSES TO THC BUT DISPLAY SUBTLE STARTLE AND LOCOMOTOR HABITUATION DEFICITS

Jarrah Spencer · Mohammed Kashem · Leonora Long · Iain McGregor · Jonathon Arnold
Article · Sep 2010 · Australian and New Zealand Journal of Psychiatry

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Article: Antipsychotic Induced Alteration of Growth and Proteome of Rat Neural Stem Cells

Eakhlas Uddin Ahmed · Selina Ahmed · Wataru Ukai · Izuru Matsumoto · Mohammed Abul Kashem

Abstract: Neural stem cells (NSCs) play a crucial role in the development and maturation of the central nervous system and therefore have the potential to target by therapeutic agents for a wide variety of diseases including neurodegenerative and neuropsychiatric illnesses. It has been suggested that antipsychotic drugs have significant effects on NSC activities. However, the molecular mechanisms underlying antipsychotic-induced changes of NSC activities, particularly growth and protein expression,... Show More

Article · Apr 2012 · Neurochemical Research

Article: Metabolism, Compartmentation, Transport and Production of Acetate in the Cortical Brain Tissue Slice

Caroline Rae · Aurélie D Fekete · Mohammed A Kashem · Fatima A Nasrallah · Stefan Bröer

Abstract: Acetate is a two carbon intermediate in metabolism. It is an accepted marker of astrocytic metabolism, and a substrate for production of metabolites such as glutamine, glutamate and GABA. However, anomalies exist in the current explanations of compartmentation and metabolism of acetate. Here, we investigated these anomalies by examining transport, production and metabolism of acetate. Acetate is a good substrate for the neuronal monocarboxylate transporter MCT2 (K(M) = 2.58 ± 0.8) and the... Show More

Full-text available · Article · Aug 2012 · Neurochemical Research

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Article: Long-term daily access to alcohol alters dopamine-related synthesis and signaling proteins in the rat striatum

Mohammed Abul Kashem · Selina Ahmed · Ranjana Sarker · Eakhlas U Ahmed · Iain S McGregor

Abstract: Chronic alcohol exposure can adversely affect neuronal morphology, synaptic architecture and associated neuroplasticity. However, the effects of moderate levels of long-term alcohol intake on the brain are a matter of

debate. The current study used 2-DE (two-dimensional gel electrophoresis) proteomics to examine proteomic changes in the striatum of male Wistar rats after 8months of continuous access to a standard off-the-shelf beer in their home cages. Alcohol intake under group-housed... Show More

Article · Sep 2012 · Neurochemistry International

Article: Novel molecular changes induced by Nrg1 hypomorphism and Nrg1-cannabinoid interaction in adolescence: A hippocampal proteomic study in mice

Jarrah R Spencer · Keturah M E Darbyshire · Aurelie A Boucher · Mohammed A Kashem · Jonathon Carl Arnold

Abstract: Neuregulin 1 () is linked to an increased risk of developing schizophrenia and cannabis dependence. Mice that are hypomorphic for (HET mice) display schizophrenia-relevant behavioral phenotypes and aberrant expression of serotonin and glutamate receptors. HET mice also display idiosyncratic responses to the main psychoactive constituent of cannabis, Δ-tetrahydrocannabinol (THC). To gain traction on the molecular pathways disrupted by hypomorphism and -cannabinoid interactions we conducted a... Show More

Full-text available · Article · Feb 2013 · Frontiers in Cellular Neuroscience

Article: Hippocampal protein expression is differentially affected by chronic paroxetine treatment in adolescent and adult rats: A possible mechanism of "paradoxical" antidepressant responses in young persons

Emily Aspasia Karanges · Mohammed A Kashem · Ranjana Sarker · Eakhlas U Ahmed · Iain S McGregor

Abstract: Selective serotonin reuptake inhibitors (SSRIs) are commonly recognized as the pharmacological treatment of choice for patients with depressive disorders, yet their use in adolescent populations has come under scrutiny following reports of minimal efficacy and an increased risk of suicidal ideation and behavior in this age group. The biological mechanisms underlying these effects are largely unknown. Accordingly, the current study examined changes in hippocampal protein expression following...Show More

Full-text available · Article · Jul 2013 · Frontiers in Pharmacology

Article: Neuroadaptations in the Striatal Proteome of the Rat Following Prolonged Excessive Sucrose Intake

Selina Ahmed · Mohammed Abul Kashem · Ranjana Sarker · Eakhlas U Ahmed · Iain S McGregor

Abstract: Obesity is a contemporary health problem of rapidly increasing prevalence. One possible cause of obesity is loss of control over consumption of highly palatable foodstuffs, perhaps mirroring the processes involved in drug addiction. Accordingly, the striatum may be a key neural substrate involved in both food and drug craving. We hypothesised here that prolonged exposure to 10 % sucrose solution might cause neuroadaptations in the striatum that are analogous to those previously reported... Show More

Article · Mar 2014 · Neurochemical Research

Article: Ethanol, not detectably metabolized in brain, significantly reduces brain metabolism, probably via action at specific GABA(A) receptors and has measureable metabolic effects at very low concentrations

Caroline D Rae · Joanne E Davidson · Anthony D Maher · Benjamin D Rowlands · Vladimir J Balcar

Abstract: Ethanol is a known neuromodulatory agent with reported actions at a range of neurotransmitter receptors. Here, we measured the effect of alcohol on metabolism of [3-(13) C]pyruvate in the adult Guinea pig brain cortical tissue slice and compared the outcomes to those from a library of ligands active in the GABAergic system as well as studying the metabolic fate of [1,2-(13) C]ethanol. Analyses of metabolic profile clusters suggests that the significant reductions in metabolism induced by... Show More

Article: GLAST but not least-distribution, function, genetics and epigenetics of L-glutamate transport in brain-focus on GLAST/EAAT1

Omar Šerý · Nilufa Sultana · Mohammed Abul Kashem · David V Pow · Vladimir J Balcar

Abstract: Synaptically released L-glutamate, the most important excitatory neurotransmitter in the CNS, is removed from extracellular space by fast and efficient transport mediated by several transporters; the most abundant ones are EAAT1/GLAST and EAAT2/GLT1. The review first summarizes their location, functions and basic characteristics. We then look at genetics and epigenetics of EAAT1/GLAST and EAAT2/GLT1 and perform in silico analyses of their promoter regions. There is one CpG island in SLC1A2... Show More

Article: Metabolomics of Neurotransmitters and Related Metabolites in Post-Mortem Tissue from the Dorsal and Ventral Striatum of Alcoholic Human Brain

Mohammed Abul Kashem · Selina Ahmed · Nilufa Sultana · Eakhlas U Ahmed · Vladimir J Balcar

Abstract: We report on changes in neurotransmitter metabolome and protein expression in the striatum of humans exposed to heavy long-term consumption of alcohol. Extracts from post mortem striatal tissue (dorsal striatum; DS comprising caudate nucleus; CN and putamen; P and ventral striatum; VS constituted by nucleus accumbens; NAc) were analysed by high performance liquid chromatography coupled with tandem mass spectrometry. Proteomics was studied in CN by two-dimensional gel electrophoresis followed... Show More

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Article: Could ethanol-induced alterations in the expression of glutamate transporters in testes contribute to the effect of paternal drinking on the risk of abnormalities in the offspring?

Mohammed Abul Kashem · Aven Lee · David V. Pow · Omar Šerý · Vladimir J. Balcar

Abstract: It has been known that a preconception paternal alcoholism impacts adversely on the offspring but the mechanism of the effect is uncertain. Several findings suggest that there are signalling systems in testis that are analogous to those known to be altered by alcoholism in brain. We propose that chronic alcohol affects these systems in a manner similar to that in brain. Specifically, we hypothesise that excessive alcohol may disturb glutamatergic-like signalling in testis by increasing... Show More

Article: Exposure of rat neural stem cells to ethanol affects cell numbers and alters expression of 28 proteins. Kashem. M, A, Sultana, N., Balcar, V.J. 2018**.** Neurochem. Res. In Press.